Journal of Applied Sciences Research, 3(6): 485-494, 2007

Anatomical and Ultrastructural Changes in Citrus Leaves Infected with *Citrus psorosis virus* Egyptian Isolate (CPsV-EG)

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Abstract: *Citrus psorosis ophiovirus* (CPsV), is considered to be of the most serious and determental virus pathogen's citrus species trees. CPsV-EG was isolated from natural infected citrus grapefruit (*C. paradisi* Macf.) at ARC. CPsV-EG was detected on the basis of biological indexing which gave oak leaf pattern (OLP) on Dweet tangor and serological assay by DAS-ELISA using Mab specific CPsV. Young grapefruit leaves of both healthy and CPsV-EG infected plants were studied to figure out the effect on histological and ultrastructural changes. Light micrographs of semithin sections figured out several histological changes. In general CPsV-EG-infection clearly affected the upper epidermis where composed of non-tabular parenchyma cells covered by a thin layer of cuticle. Crystal idioblast (CI) containing cells are lacking in the palisade layer and protrude into the epidermis. The oil glands are lacking compared with healthy leaf. Secondary growth occurs in midvein and major lateral veins in smaller veinlets. The vein endings consist of a single trachoid strand of elongated parenchyma cells enclosed by the bundle sheath compared with healthy ones. The ultrastructure of infected leaves showed important variations. Infected cells developed the presence's large number of abnormal chloroplasts, mitochondria and cellular abnormality described so far is the presence of hypertrophied nuclei. Cells of CPsV-EG infected citrus plants show deformation elongated and curved mitochondria. Also, show nucleus with several dark stained bodies, displaced toward nucleus periphery along nuclear envelope and sometimes nucleolus appeared as abnormal shaped. Inclusion bodies as intercellular structures produced as result of virus infection. They might also contain virus particles.

Keywords: *Citrus psorosis virus*, infection, citrus leaves, Electron Microscope.

INTRODUCTION

Egypt stands at present, among the seventh largest citrus producing countries in the world with a total production of 2.887.599, represented about 40.1% from the total production of fruit crops, represented about 21.8% from the total production of citrus crops. The fruited cultivated area of citrus is 327.838 Fed. The exported yield about 630.000 Ton., represented about 21.8% from the total production of citrus crops. The main citrus variety in Egypt is the orange among the different citrus spp, represented about 60.4%, then Mandarin 27.5%, then Lemon 10.9%. While the other citrus spp such as sweet Lemon, Grapefruit and Sour orange represented 1.2%. Among graft transmissible diseases that have been reported from Egypt, as well as in the rest of the Mediterranean countries, *Citrus tristeza disease, Citrus psorosis disease* and viroid disease are of the most serious diseases and remain the most spread diseases in the country[29,24,16,14]. Psorosis disease was caused by *Citrus psorosis ophiovirus*[7]. In infected trees a scaly bark symptoms on the trunk, staining of interior wood of branch and gummy as well as shortened leaf internodes and mottling patterns on leaves. Poor fruit quality and decreased yield were recorded[24].

The present work was carried out to detection and isolation of CPsV Egyptian isolate in a commercial farm and variety reveal the effect of CPsV-EG infection on ultrastructure of citrus leaf cells.

MATERIALS AND METHODS

Source of Virus Isolate: Citrus trees samples (Grapefruit) grown in farm of Agricultural Research Center (ARC). These citrus trees carrying different

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Fig. 1: Naturally infected citrus trees with CPsV-EG showing bark scalling upper grafting zone above the bud union.

external symptoms like that caused by CPsV {Trees were visually inspected for leaf (oak leaf pattern, mottling, ringspot, chlorosis, vein enation, crinkly), bark (bark scaling, gummies, concavities) and fruit symptoms (acorn-shaped and ringspot)} were obtained (Fig 1). These infected citrus trees were used for CPsV isolation.

Detection of CPsV-EGa:

DAS-ELISA: (Double antibody sandwich Enzyme-linked immuonosorbent assay) was used for CPsV-EG detection in shoots of infected trees, using specific monoclonal antibody for CPsV-EG (Agritest, Italy) according to[5].

Biological Indexing: Grapefruit sample was indexed by graft inoculation with two blind buds, using specific woody indicator plant for CPsV-EG (Dweet tangor) grafted on Volkameriana lemon as rootstock. The grafted plants were kept under insect proof in greenhouse indexing at 24-27ºC (max. day) / 18-21ºC (min. night) and recorded the symptoms development for 1 month according to[24].

Grapefruit sample was examined for the presence of *Citrus tristeza virus* (CTV) using direct tissue blot immuno-assay (DTBIA) according to[18], *Spiroplasma citri* using Diene's stain according to El-Dougdoug *et al.,*[10] and *Citrus excortis viroid* (CEVd) using direct tissue blot hybridization according to Sambrook *et al.,*[26].

Isolation and Propagation of CpsV-EG: Tree (infected grapefruit) was visually inspected for mottling and chlorosis as well as ringspot on leaves and scaly bark on trunk of tree. Which it gave positive results with specific monoclonal antibodies (+ve DAS-ELISA was used as a CPsV-EG source) and biological indexing.

The virus was isolated and propagated by graft inoculation with eye buds from infected Grapefruit on Sour orange. The inoculated plants were kept under greenhouse conditions (24-27ºC/16 light day) and confirmed by DAS-ELISA.

Detection of Inclusion Bodies in Epidermal Strips: Crystalline viral inclusions were detected using the epidermal strips obtained from the lower surface of infected leaves of grapefruit (25 days post inoculation). The strips were removed using forceps, then mounted in distilled water and viewed under the light microscope, magnification of 400X[4].

Tissue Processing and Photomicrography: Small leaf blade segments (about 1 mm) were cut from five different leaves showing typical external symptoms of CPsV-EG infection as well as healthy ones. The selected tissue samples were fixed and processed for electron microscopy[19] as follows: Samples were fixed for 4 hrs in 0.08 M cacodylate buffer pH 7.4 containing 5% gluterapdehyde and 4 % paraformaldehyde. The fixed specimens were washed three times at half hour intervals with 0.1 M cacodylate buffer pH 7.4, 3 % sucrose. These samples were post fixed in 1 % osmium tetroxide dissolved in a solution of 0.1 M cacodylate buffer pH 7.4 and 2 % sucrose. Following three hours wash in 0.1 M cacodylate, 3 % sucrose pH 7.4, samples were dehydrated in ascending concentration of alcohol series, sequentially followed by propylene oxide, then propylene oxide plus epoxy resin (1:1 V/V) and finally embedded in epoxy resin. Thin sections were cut, then stained with uranyl acetate and lead citrate and viewed with a JOEL JM 100-C electron microscope (Electron Microscope Unit, Al-Azhar University, Cairo).

RESULTS AND DISCUSSIONS

Results:

Detection and Isolation of CPsV-EG: CPsV-EG (Egyptian isolate) was detected in infected citrus trees, cultivar grapefruit which exhibited bark scaling and gum symptoms by DAS-ELISA using Mab specific CPsV. CPsV-EG was also detected by indexing on healthy citrus Dweet tangor seedlings which gave oak leaf pattern on Dweet for 25-30 days post-inoculation (Fig 2).

On the other hand, these trees gave -ve results with CTV using specific Mab for CTV, -ve results with CEVd using specific probe of CEVd and -ve results with *Spiroplasma citri* using Diene's stain.

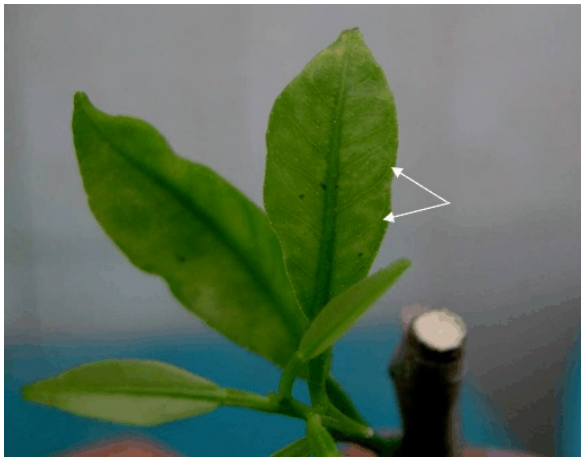

Fig. 2: Citrus Dweet tangor seedlings inoculated with CPsV-EG by grafting transmission showing oak leaf pattern (OLP).

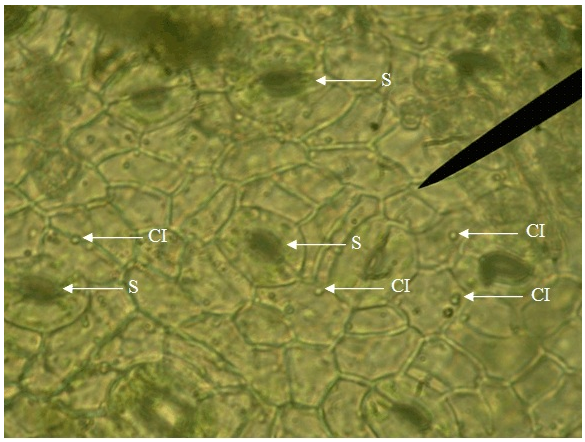

Fig. 3: Crystalline inclusions (CI) and closed stomata (S) induced by CPsV-EG (25 days post inocultion) in infected leaves of *C. paradisi* Macf. (Magnification = 400X)

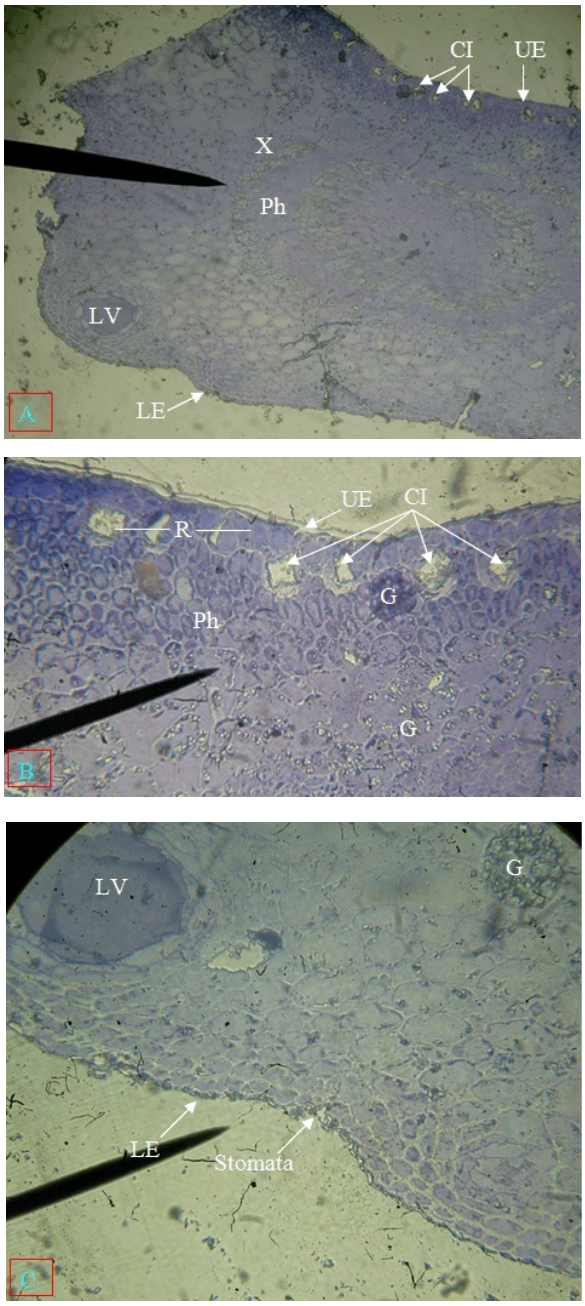

Fig. 4: Light micrographs of healthy citrus grapefruit leaf showing upper (UE) and lower epidermis (LE); Hypodermis (R), mesophyll tissue (L); lateral vein (LV); oil gland (G) and crystal idioblast (CI).

CPsV-EG was isolated from selected infected citrus trees by grafting on healthy citrus Dweet seedlings. The CPsV-EG was propagated on healthy citrus seedlings Sour orange by grafting inoculated. These results were confirmed by DAS-ELISA against

CPsV monoclonal antibodies. These plants were used and maintenance host for the CPsV-EG isolate.

Detection of Inclusion Bodies in Epidermal Strips: Crystalline inclusions (CI) were readily observed in the cytoplasm of epidermal strips of CPsV-EG infected upper leaves of grapefruit 25 days post inoculation (Fig 3). The virus affects the stomata (S) which appeared close due to lignification.

Anatomical Features: In light micrograph of semithin sections healthy citrus mature leaf cv. Grapefruit (*C. paradisi* Macf.), the lamina is nearly flat (Fig 4-A).

Upper and lower epidermal cells are barrel shaped. The upper epidermis layer is slightly larger than that of the lower epidermal cells. The upper epidermis is composed of tabular parenchyma cells covered by a thick layer of cuticle (Fig 4-A,B). Stomata are lacking. Large, calcium-oxalate (Crystal idioblast (CI)) containing cells occur in the palisade layer and protrude into the epidermis (Fig 4-A,B). Thin-walled epidermal cells covering the oil glands (G) are arranged so that three or more cells occur at the center of the cover and other epidermal cells are concentrically arranged around them (Fig 4-B,C).

The lower epidermis is composed of tabular parenchyma cells interspersed with stomata as shown in Fig (4-C). Epidermal cells covering the oil glands (Fig 4-C) are arranged like those of the upper epidermis.

The mesophyll: The palisade parenchyma cells are cylindrical and tightly packed into two or three layers (Fig 4-A,B,C). In leaves with tree layers, the cells of the third layer are shorter than the first and second layers and there is air space between them.

The spongy mesophyll is approximately eight layers thick and contains a large amount of intercellular space (Fig 4-B,C). The spongy cells are nearly spherical and tightly packed. The number of tightly packed cell layers is influenced by growing conditions. The leaves of field-grown trees have several such layers and those of greenhouse trees only one (Fig 4-C).

The structure of the vascular bundles are encased in bundle-sheath cells that somewhat resemble spongy parenchyma cells (Fig 4-B,C). Secondary growth occurs in the midvein and major lateral veins, but not in the smaller veinlets. The vein endings may consist of a more one tracheid or a uniserrate strand of elongated parenchyma cells enclosed by the bundle sheath.

In light micrographs of CPsV infected citrus mature leaf cv. grapefruit, the subepidermal parenchyma (Hypodermis) consists of one layer of

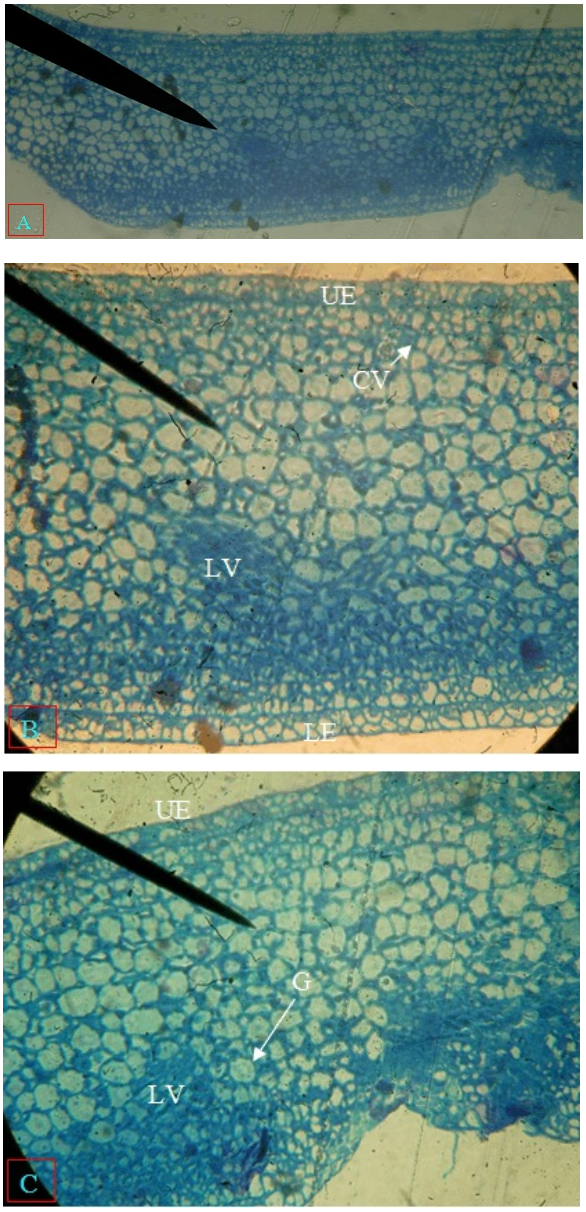

Fig. 5: Light micrographs of infected citrus grapefruit leaf with CPsV-EG showing:

(A) Deformed infected blade and unequal thickening and small upper (UE) and lower epidermis (LE) cells (40X).

(B) Batches of dark stained parenchyma and chlorenchyma cells which involved to several cavities (CV) and appearance of small sized wavy walled subepidermal parenchyma cells (400X).

(C) Appearance of dark-stained fiber batches and small size of lateral vein (LV) in mesophyll. No appearance oil gland (G) and crystal idioblast (CI) (400X).

compacted cells greater than healthy ones (Fig 5-A,B,C). The upper and lower epidermal cells layer are similar than that healthy ones (Fig 5-A,B,C).

The upper epidermis is composed of non-tabular parenchyma cells covered by a thin layer of cuticle (Fig 5-A,B,C). Stomata are found. Crystal idioblast (CI) containing cells are lacking in the palisade layer and protrude into the epidermis (Fig 5-A,B,C). The oil glands are lacking and so that two or three cells occur at the center of the cover and other epidermal cells are not concentrically arranged around them (Fig 5-B,C).

The epidermis is deformed and composed of non tabular parenchyma cells interspersed with stomata as shown in Fig (5-C). The palisade parenchyma cells are not cylindrical and tightness packed into more layers (Fig 5-B,C). In leaves with two layers, the cells of the second layer are shorter than the first and there is no air space between them.

The spongy mesophyll is approximately six layers thick and not contains of intercellular (Fig 5-B,C).

Secondary growth occurs in midvein and major lateral veins in smaller veinlets. The vein endings consist of a single trachoid strand of elongated parenchyma cells enclosed by the bundle sheath.

Ultrastructural Features: The ultrathin sectioning technique was carried out on CPsV-EG infected grapefruit leaves as well as healthy ones. This study aim to the CPsV-EG effects on host cells throw transmission electron microscope.

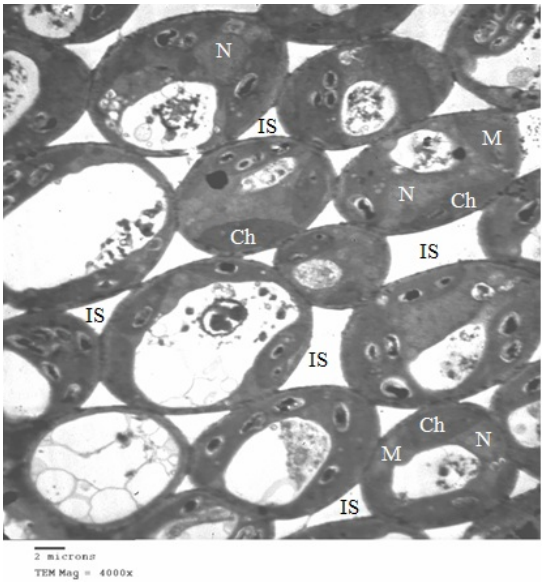

Fig. 6: Ultra-micrograph section of healthy grapefruit leaf showing healthy mesophyll tissue.

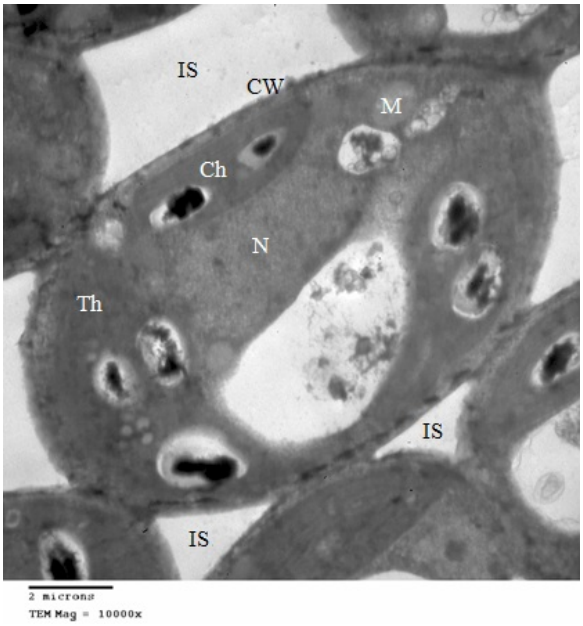

Fig. 7: Ultra-micrograph section of healthy grapefruit leaf showing healthy mesophyll cell.

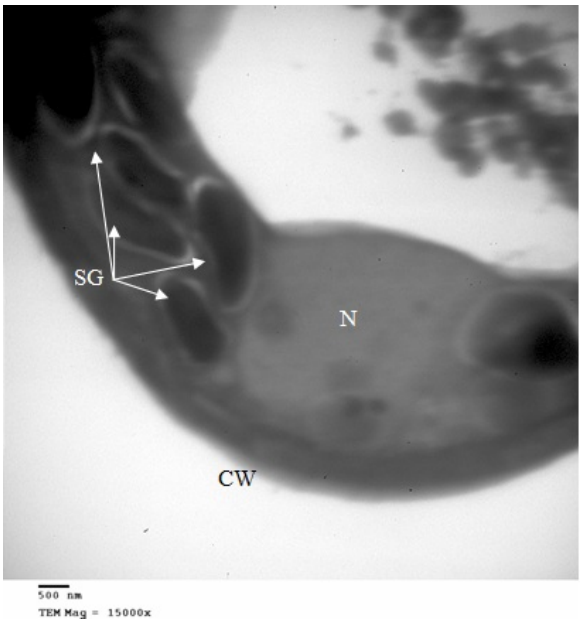

Fig. 8: Ultra-micrograph section of healthy grapefruit leaf showing magnified chloroplastid shows bright starch grains (SG) and nucleus (N).

Healthy leaf cells: In the present study, examination of ultrathin sections of healthy grapefruit mature leaves, showed mesophyll cells with relative large intercellular spaces (IS). The mesophyll cells are nearly rounded chlorenchyma with uniformly thin cell wall and contained nucleus (N), chloroplasts (Ch) and

mitochondria (M) (Fig 6,7). The chloroplast in healthy cells typical lens-shaped and bounded by an envelope, enclosing the stroma. Grana discs (Gr) arranged in regular rows and connected to thylakoids (Th). The mitochondria are slightly ovoid and bounded by a smooth envelope enclosing matrix. The mitochondrial matrix contains electron transparent area, and the nucleoids. Some mitochondria matrix contain electro dense regions (Fig 7). The vascular bundles composed of phloem and xylem tissues. The phloem tissue differentiated into normal sieve tube and companion cells (Fig 9). Xylem vessels appear nearly rounded (Fig 9).

CPsV-EG Infected Leaf Cells: The grapefruit leaf infected with CPsV-EG sometimes revealed the presence of completely destroyed cells (Fig 10,11). The ultrathin sections of CPsV-EG infected leaf, showed mesophyll cells with relative small intercellular spaces. The mesophyll tissue is large and low number of cells are lacking chlorenchyma with thin cell wall and contained deformed nucleus, chloroplasts and mitochondria compared with healthy mesophyll tissue (Fig 10,11). The chloroplasts alterations in grapefruit CPsV-EG infected cells were compared with healthy ones. Cells of infected leaf showed slightly elongated chloroplastids with irregular rows of grana which decreased in number (Fig 10,11). Another features of alteration are appearance of plastoglobules in unusual numbers within grana and stroma of chloroplast and appearance of destructed regions in chloroplastids which do not organized into grana and thylakoid system (Fig 12). Different degrees of degenerative changes in chloroplastids and starch grains were observed like clumbed, partially destructed chloroplastids and starch grains which reduced in size and numbers (Fig 12).

Cells of CPsV-EG infected leaf show degenerated mitochondrion with destructed envelope. Another feature of mitochondrial alteration is appearance of cylindrical inclusions within mitochondria (Fig 11,12). The mitochondria are slightly round and bounded by a smooth envelope non enclosing matrix. The mitochondrial matrix no contains electron transparent area and the nucleoids.

Cells of CPsV-EG infected leaf show small and destructed nucleus with several dark stained botches and sometimes nucleolus appeared deformed shape (Fig 11).

Examination of ultrathin sections of grapefruit leaf infected with CPsV-EG revealed the presence of completely destroyed cells (Fig 11). The cell wall

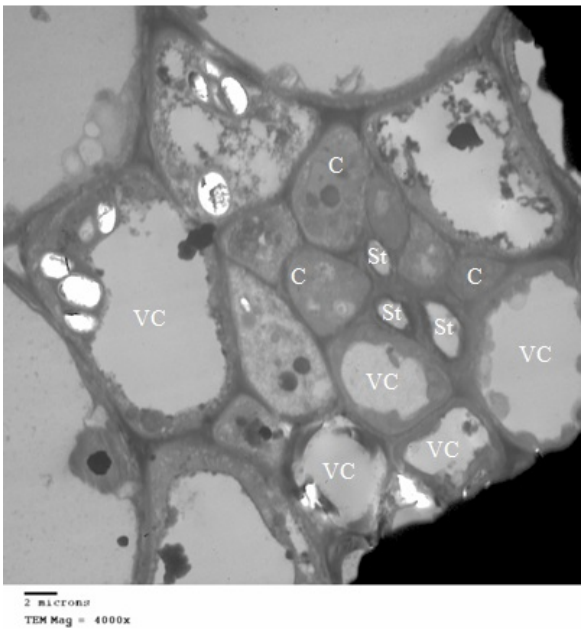

Fig. 9: Ultra-micrograph section of healthy grapefruit leaf showing magnified lateral vein shows vacuoles (VC), sieve tubes (St) and companion cells (C).

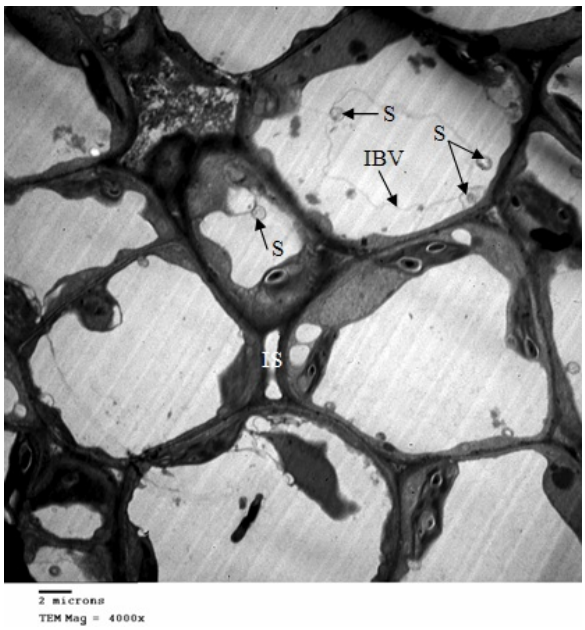

Fig. 10: Ultra-micrograph section of CPsV-EG-infected grapefruit leaf cells showing slightly elongated chloroplastids with irregular rows of grana, simple (S) inclusion bodies and inclusion bodies vacuole (IBV).

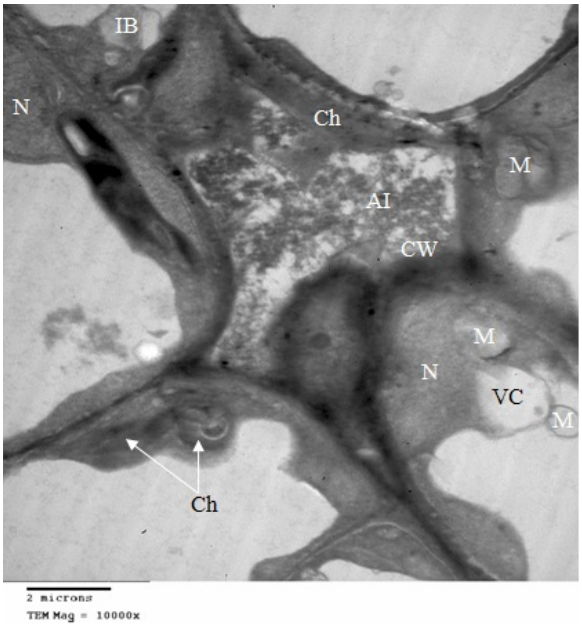

Fig. 11: Ultra-micrograph section of CpsV-EG-infected grapefruit leaf cells showing disorganized chromoplastids (Ch) and amorphous inclusions (AI) as well as thinning and breaking of cell wall (CW).

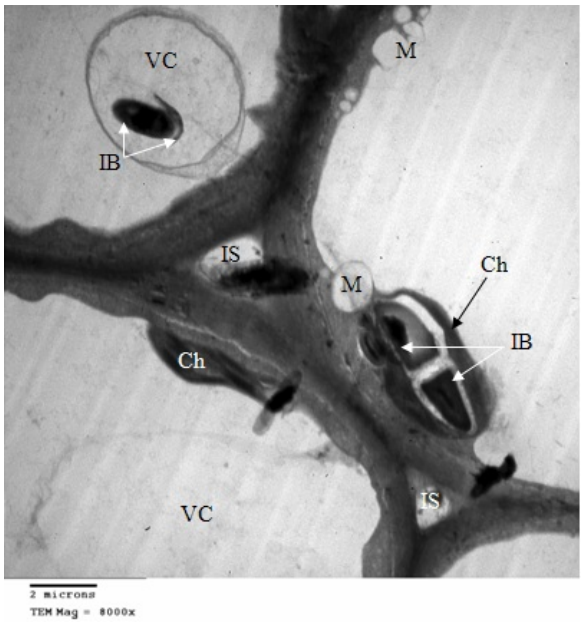

Fig. 12: Ultra-micrograph section of CpsV-EG-infected grapefruit leaf cells showing reduced and deformed chloroplastids.

is thinned and destructed and no lignification (Fig 11). Deformation of the cell wall and increasing in rate of lignifcation due to small size of intercellular spaces (IS) between mesophyll cells (Fig 10,12,14) compared with healthy ones (Fig 6,7).

Inclusion bodies are intracellular structures produced as a result of viral infection. They may contain virus particles (V), virus related materials or ordinary cell constituents in a normal or degenerating condition (Fig 11,15). Ultrathin sections in infected cells revealed the presence of structures consisting of multiple membranes, frequently associated with an internal core either simple or compound (Fig 10,11,12) and usually appeared along plasmamembrane into vacuoles (Fig 10,12). These multimembrane bodies appeared as tubules in their longitudinal section (Fig 12). Another type of inclusions is appeared inside the chloroplastids and vacuoles (Fig 12) and cytoplasm (Fig 14) as a electron dense dark angular inclusion bodies. Also amorphous inclusions (AI) which aggregate of single small rounded particles appeared in infected cells (Fig 11) within cytoplasm.

The vascular bundles composed of phloem and xylem tissues. The phloem tissue contained much less active sieve elements and destructed companion cells (Fig 15). The xylem vessels attained less diameter as compared with those healthy ones and also they contained several spherical dark stained-bodies which precipitated on vessels walls (Fig 13).

Discussion: *Citrus prorosis ophiovirus* Egyptian isolate (CPsV-EG) was isolated from naturally infected citrus exhibited bark scaling and gum symptoms at Agric. Res. Cent. Giza-Egypt. CPsV-EG was detected on the basis of biological assay which gave oak leaf pattern on Dweet and serological assay using specific Mab specific CPsV by DAS-ELISA[2,6]. The virus CPsV is considered to be of the most serious and determental virus pathogen's citrus species trees[1,23]. It is a strong limitation for germplasm and propagation materials movement.

The available information about incidence, isolation and identification of CPsV in Egypt as well as cytopathic effects on citrus leaves disease severity is not studied yet. Therefore, this study was carried out on citrus cv. grapefruit is cultivated in orchards and nurseries in Egypt.

Some authors[9,10] mentioned that, more one viroid, phytoplasma and 5 viruses affecting the genus *Citrus* have been described. The CPsV induced distinct symptoms in commercial cultivars. Because virus and virus like agents infected many citrus cultivars in commercial farms, the obtained samples which using for CPsV-EG isolation were examined serologically

by immunoprinting, Diene's stain and tissue print hybridization for the presence of *Citrus tristeza virus* (CTV), *Spiroplasma citri* and *Citrus excortis viroid* (CEVd). The results showed that the tissues of grapefruit samples gave negative results using specific monoclonal antibodies of CTV and *Citrus exocortis viroid* using tissue blot hybridization. These results indicate the samples were virus and virus like agents tested and infected with CPsV-EG only[14].

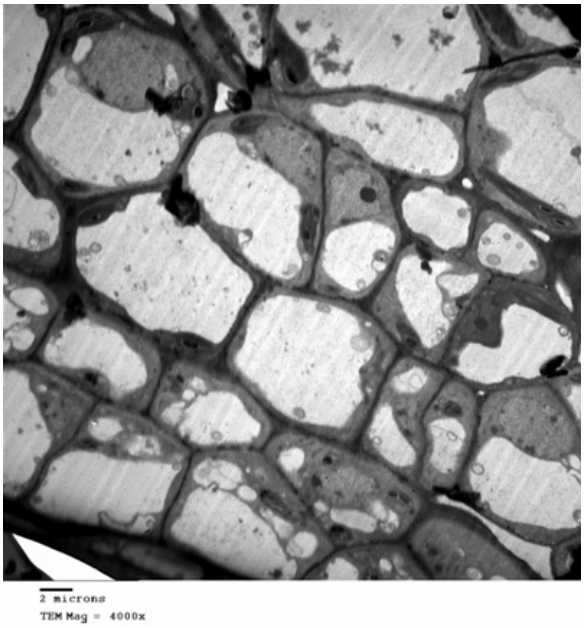

Fig. 13: Ultra-micrograph section of CPsV-EG-infected grapefruit leaf cells showing xylem vessels attained less diameter and contained several spherical dark stained-bodies.

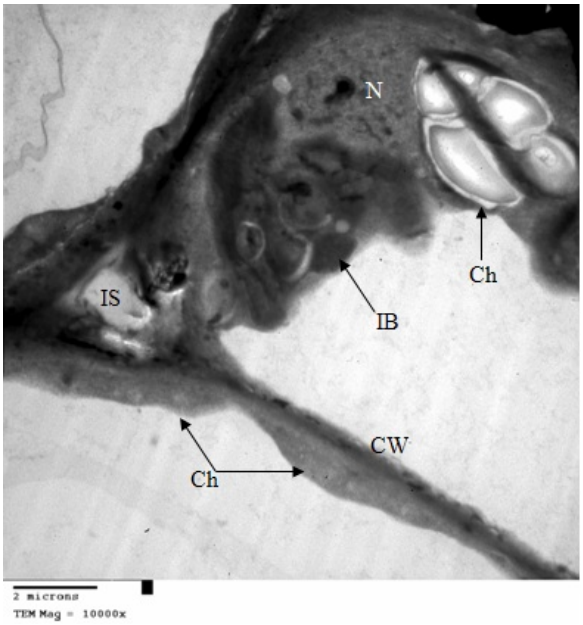

Fig. 14: Ultra-micrograph section of CpsV-EG-infected grapefruit leaf cells showing cytoplasmic inclusions.

The Egyptian isolate of CPsV was detected on citrus trees which are extensively cultivated in open field. Citrus trees were showing. bark scalling and gum symptoms were observed on trunk and the branches on the 2 branches on the 2 sides of trees[17,22,28]. In greenhouse and laboratory, it was detected biologically indexing on citrus cv. Dweet[20,21,25] and gave positive with specific monoclonal antibodies[6]. Applying hybridization assay, immunoprinting used specific monoclonal antibodies for CTV and Diene's stain to confirm the presence of CPsV-EG isolate as single infection was in agreement with that reported by[10,2,6,14].

Light micrographs of CPsV-EG infected citrus mature leaf cv. Grapefruit showed that the upper epidermis is composed of non-tabular parenchyma cells covered by a thin layer of cuticle. Crystal idioblast (CI) containing cells are lacking in the palisade layer and protrude into the epidermis. The oil glands are lacking compared with healthy leaf. Secondary growth occurs in midvein and major lateral veins in smaller veinlets. The vein endings consist of a single trachoid strand of elongated parenchyma cells enclosed by the bundle sheath. In harmony with the present results, CPsV-EG infection clearly affected the conductive tissues. It decreased phloem tissue thickness, fibers; xylem tissue thickness and vessel diameter. The glands in both number and diameter were reduced. As for leaf mesophyll cells infection diminished palisade layers. Such tissue elements showed almost cuboidal shape with few chloroplast content. All mentioned anatomical changes can be attributed to different indirect affects occurring in the host plant may be due to the lower auxin level resulting from virus infection[3], as a result of viral infection and not attributed to the virus it self[11].

The *Citrus prorosis ophiovirus* that infected citrus cells showed that main cytopathic changes were the presence's large number of abnormal chloroplasts, mitochondria and cellular abnormality described so far is the presence of hypertrophied nuclei[8]. The chloroplast alterations in citrus leaves cells infected with CPsV-EG were studied. Cells of infected plants showed destructed regions in chloroplast which does not organized into grana and thylakoid system. These results were in accordance with obtained by[27,11,15]. Who studied the cytological changes in the chloroplast which occurred in the infected cells as a

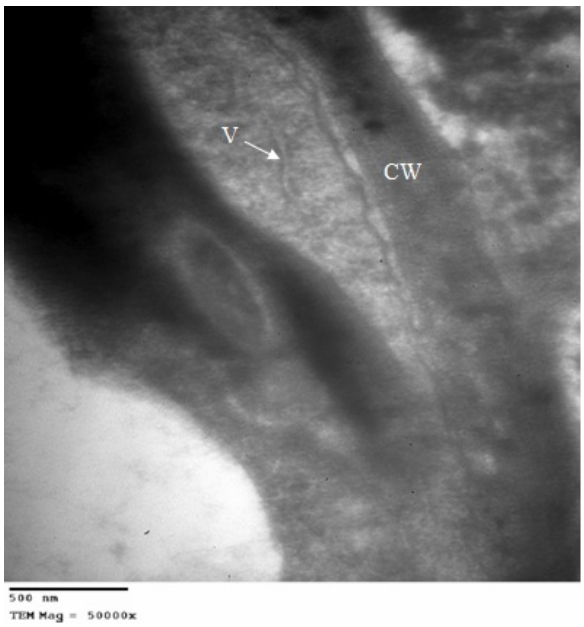

Fig. 15: Ultra-micrograph section of CPsV-EG-infected grapefruit leaf cells showing virus particles (V).

result of infection with CPsV, CEVd. Different degrees of degenerated changes in chloroplast aggregated, partially destructed and reduced in size and number. These abnormalities effects were in harmony with those obtained by[12,15,14]. Who reported that virus and viroid infection sometimes have unusual aggregated of chloroplast in infected cells.

Cells of CPsV-EG infected citrus plants show deformation elongated and curved mitochondria. There is another degenerated mitochondrion with distracted envelope. Another feature of mitochondrial alteration is appearance of cylindrical inclusions with mitochondria. These abnormalities were in harmony with those obtained by[8,27,13].

Cells of CPsV-EG infected citrus plants show nucleus with several dark stained bodies, displaced toward nucleus periphery along nuclear envelope and sometimes nucleolus appeared as abnormal shaped. Those abnormalities were obtained by[8,27,13,15].

Inclusion bodies as intercellular structures produced as result of virus infection. They might also contain virus particles. Ultrathin sections of infected citrus leaf cells revealed the presence of structures consisting of multiple membranes either simple or compound and usually appeared along plasmamembrane produced into vacuoles and vesicles formed invigilation of the plasma lemma and/or tonoplast. These Para mural bodies were of different size and multi vesicular bodies as well as hypertrophic growth in plasma membrane or in tonoplast and the onset gross symptoms and were not found in uninfected cells. The occurrence of multi membrane bodies in the infected cells was also found by[12,15].

The biological properties reported in this study confirmed the identification of virus isolate CPsV-EG as a ophioviridae, i.e. symptoms in open field, biological indexing, serological reaction, and cytopathic effects.

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