Egyptian J. Virol. 1, 343-353 (2004)

# Histo-cytopathological Effects of *Cucumber Mosaic Cucumovirus* on Squash leaves

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An isolate of *Cucumber mosaic Ccucumovirus* (CMV) infected cucumber plants was detected by DAS-ELISA and confirmed by inoculation of *Chenopodium amaranticolor* . CMV-isolate was propagated on squash plants var. Eskandarani. After 15 days, systemic symptoms appeared in the newly formed leaves in the form of vein banding, severe mosaic, yellowing, malformation and leaves wilting. Histopathological studies of CMV-infected squash leaves showed changes such as compact mesophyll, disintegrated and less lignified metaxylem , slight upward blistering of leaf blade , closed stomata , short palisade and rounded spongy cells as well as vascular elements of the midrib formed in two patches , the upper patch completely divided in two parts . Scanning electron microscopy showed alteration in epidermal cells elongated, deformed and disconnect hairs, decrease of opened stomata, destroyed and forming gaint cells. The midrib cells of infected leaves showing clear elongations as well as deformation. Transmission electron microscopy illustrated highly thickened with clear secondary deposition of cell wall , the cytoplasm showed many vacuoles , several rounded hallow center inclusions and aggregates of virus particles , chloroplasts showed weak differentiated thylakoid system, rounded osmophilic globules and large deformed starch grains . Mitochondria appeared deformed without cristae. The nucleus appeared deformed with clear accumulation of marginal nuclear material. Multimembrane body and endoplasmic reticulum fragments also appeared.

## INTRODUCTON

Squash plants are one of the predominant cucurbit crops used as a source of food by the majority of people in EGYPT, which are good sources of carotenes, sugars, food fibers, potassium and vitamins B and C.

Cucurbit crops were and still suffering a great deal of losses in their growth yields and quantity .The most causative agents are viral infections specially *Cucumber mosaic virus* (CMV).

CMV has a wide range of hosts and causes many diseases and severe economic losses in vegetable, forage, ornamental and fruit crops worldwide (Staniulius *et al.*, 2000). In present investigation field samples were collected from cucumber plants suspected of being infected with a virus.

The present investigation aimed to:

1) Isolation and identification of CMV from naturally infected cucumber plants obtained from virology lab. , Faculty of Agriculture, Ain Shams University.

2)Histopathology and cytopathology were studied to determine what tissue , cell and cells organalles showed abnormalities were caused by CMV infection.

## MATERIALS AND METHODS

Cucumber plants (*Cucumis sativus* var. Bremo) showing viral

symptoms in the form of net mosaic, blisters, crinkle, yellowing and malformation were collected from the farm of Faculty of Agriculture, Ain Shams University and used as virus source.

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) technique was applied as described by Clark and Adams (1977) for CMV detection in collected samples using an ELISA kit (completely ready for use) that was supplied by SANOFI (Sante Animale, Paris, France).

**Histopathology of CMV-infected *Cucurbita pepo* leaves and stems using light microscopy**

The purpose of the present investigation was to determine what anatomical abnormalities which may occur in tissue of *Cucurbita pepo* infected with CMV showing mosaic symptoms. Permanent slides were made in young full expanded leaves showing mosaic and also in stem of the same plant.

***1.The preparation of permanent slides:***

The technique used for preparation of permanent slides from microtome sections were mainly those used by Johansen (1940), Sass (1951) and Purvis *et al.* (1964) with special modifications.

**Cytopathology of CMV-infected *Cucurbita pepo* leaves by using electron microscopy**

***1.Ultrathin sections (Transmission electron microscopy):***

Small pieces of CMV infected and healthy squash leaves were cut., fixed for 5 hours with 2% glutaraldhyde in 0.1 M phosphate buffer, pH 6.8 and then , post-fixed in cold buffered 1% Osmium tetraoxide for 2 hours , rnsed three times in 0.1 M phosphate buffer , pH 6.8 . Then, kept in uranyl acetate for 3 hours and followed by rinsing in distilled water. After fixation, the tissue pieces were dehydrated in a graded series of ethanol solutions and embedded in a mixture of methacrylat-stiyrol (Stockem and Komnick, 1970). Ultrathin sections were cut on a Reichert ultramicrotome equipped with glass knives and were stained with lead citrate for 2-5 minutes at room temperature (Reynolds, 1963). Infected and healthy sections were examined with a Jeol 100-C electron microscope at E.M. Section, The Regional Center for Mycology and Biotechnology, Faculty of Science, Al-AzharUniv.

***2. Scanning electron microscope examination:***

Healthy *Cucurbita pepo* and CMV-infected leaves were prepared for scanning electron microscopy. Leaf specimens were cut. Fixed in 2.5 % glutaraldhyde for 24 hours at 4°C. Post-fixed in 1 % Osmium tetraoxide for 1 hour at room temperature (Harley and Fergusen, 1990). The specimens were then dehydrated with ascending concentration of acetone till dried critical point and finally sputter coated with gold. The examination measurements and photographing were done through a Jeol scanning electron microscope (JSM-T330A) equipped with image recorded and processing system (Semafore) center lab in Faculty of Agriculture-Ain-Shams University.

## RESULTS AND DISCUSSION

Samples of Cucumber leaves showing viral symptoms were reacted positively agains CMV specific

antiserum . These samples were mechanically inoculated on indicator hosts: *Chenopodium amaranticolor*, *Nicotiana glutinosa* and *Zinnia elegans*. Results showed necrotic local lesions appeared on Ch. *amaranticolor* and Z. *elegans* while mosaic symptoms appeared on *N. glutinosa*. CMV isolate was biologically purified onto healthy *Ch. Amaranticolor* plant. After 2-4 days circular chlorotic local lesions (1-2mm diameter) appeared which are then turned into red spots (with necrotic center and chlorotic margin) . Single local lesion was separated and used in the inoculation of new healthy *Ch. amaranticolor* plant . The same type of local lesions appeared . These local lesions were used to inoculate healthy squash plants (*Cucurbita pepo* cv. Eskandarani ). After 10 days systemic symptoms appeared in the newly formed leaves in the form of vein clearing , severe mosaic , malformation ( Fig. 1 ) . yellowing and then wilting of the plant .

**Histopathology of CMV-infected *Cucurbita pepo* leaves and stems using light microscopy**

***Healthy leaf:***

Lamina of healthy leaf is nearly flat with the midrib slightly bulging from the upper and lower sides (Fig. 2.1H) . Upper and lower epidermal cells are widely barrel shaped. The upper epidermal are clearly larger than that of the lower epidermal cells. Hairs are rare while there are few stomata. The mesophyll consists of one layer of compact palisade cells towards the upper side and isodiametric loosely arranged towards the lower side (Fig.2.2H). Vascular elements are found in two patches, xylem vessels of the lower patch are arranged in rows with the protoxylem directed upwards and the metaxylem downwards, vessels appear nearly rounded. Phloem cells are found above and below the lower patch of xylem as outer and inner phloem . Phloem cells appear only below the upper patch . they are differentiated into sieve tubes , companion cells and phloem parenchyma

***Infected leaf:***

The blade of infected leaf shows slight upward blistering: the midrib shows clear bulging from the upper and lower sides. Many multicellular hairs appear from the upper epidermal cells (Fig. 2.1I). The upper and lower epidermal cells are nearly of the same size and appear covered with thick cuticle. Hairs are present and there are many closed stomata. The mesophyll consists of one layer of compact palisade cells shorter than that of healthy ones, while spongy tissue appeared rounded and compact with few air spaces. In general, mesophyll tissue appeared rich in chloroplasts (Fig. 2.2I).

Vascular elements are found in two patches, xylem and phloem of the lower patch appeared normal with somewhat less lignified and disintegrated metaxylem . The upper patch appeared completely divided into two parts, with clear reduction in number of xylem vessels, the phloem is found in amphiphloic manner.

***Healthy stem:***

Sections of healthy stems of *Cucurbita pepo* show that the ground tissue is differentiated into narrow cortex formed of one third collenchyma layer and two third parenchyma layer and wide pith formed of nearly rounded parenchyma cells .In the center there is a clear hollow. There are two rings of bicollateral vascular bundles embedded

in pith cells. The xylem of the vascular bundle clearly show regular arrangement. The outer and inner phloem appear clearly formed of sieve tube, companion cells and phloem parenchyma .(Fig . 2.3.H)

*Infected stem:*

Sections of infected stem show that the cortex appear normal with collenchyma and parenchyma. Cells of pith appear collapsed with strongly waved cell wall. The bicollateral vascular bundles appear with few and irregularly arranged xylem vessels. Outer and inner phloem cells appear somewhat wider collapsed and contain heavy tannins. (Fig. 2.3I)

**Cytopathology of CMV-infected *Cucurbita pepo* leaves by using electron microscopy**

*1.Ultrathin sections (Transmission electron microscopy):*

To study the effect of virus of infection on host cells of CMV-infected *Cucurbita pepo* leaves, ultrathin sections were made and examined by transmission electron microscope.

The results showed the highly thickening of the cell wall with secondary depositions appeared clearly (Figs. 4). The cytoplasm appeared containing many small vacuoles (Fig. 5B), several rounded hollow center inclusions and aggregates of virus particles (Fig. 4), clear numerous scrolls appeared in the cytoplasm (Figs. 6).

The chloroplasts of infected host cells showed weak differentiated thylakoid system (Fig. 5A).

The mitochondria of infected host cells appeared clearly deformed, elongated or rounded in shape and without cristae (Figs. 5B, 6A). The nucleus of the infected host cells appeared deformed (Fig. 7), showed clear segregation of nuclear material of the nucleolus into marginal granular accumulation. The nuclear membrane showed a small protrusion near a very clear multimembrane body. Normal endoplasmic reticulum and ribosomes appeared around the nucleus (Fig. 7).

*2.Scanning electron microscope examination:*

To study the effect of virus infection on host epidermal tissue cells of CMV-infected Cucurbita pepo leaves , scanning electron microscopy was carried out for healthy and infected specimens .

The results showed that the epidermal cells of the healthy leaves were turgid and normal in shape with wavy walls. Many simple, multicellular and glandular hairs appeared. The stomata were numerous and appeared closed or slightly opened (Figs. 8 a, b).

The epidermal cells and hairs of healthy leaf midrib appeared turgid and normal (Fig. 9A).

The epidermal cells of the infected leaves appeared shrinked with clearly broken hairs and widely opened stomata (Figs. 9B). The cells of the midrib of the infected leaves appeared more elongated with clearly broken and deformed hairs (Fig. 9B).

## DISCUSSION

*Cucumber mosaic virus* has been reported world wide in cucurbits. In the present study, CMV was isolated from *Cucumis sativus* var. Bremo as reported by many investigators (Magid, 1991 and Polak, 1999). Infected plants showed viral symptoms in the form of net mosaic, blister, crinkle, malformation and yellowing. The samples were collected from the farm of Faculty of Agriculture,

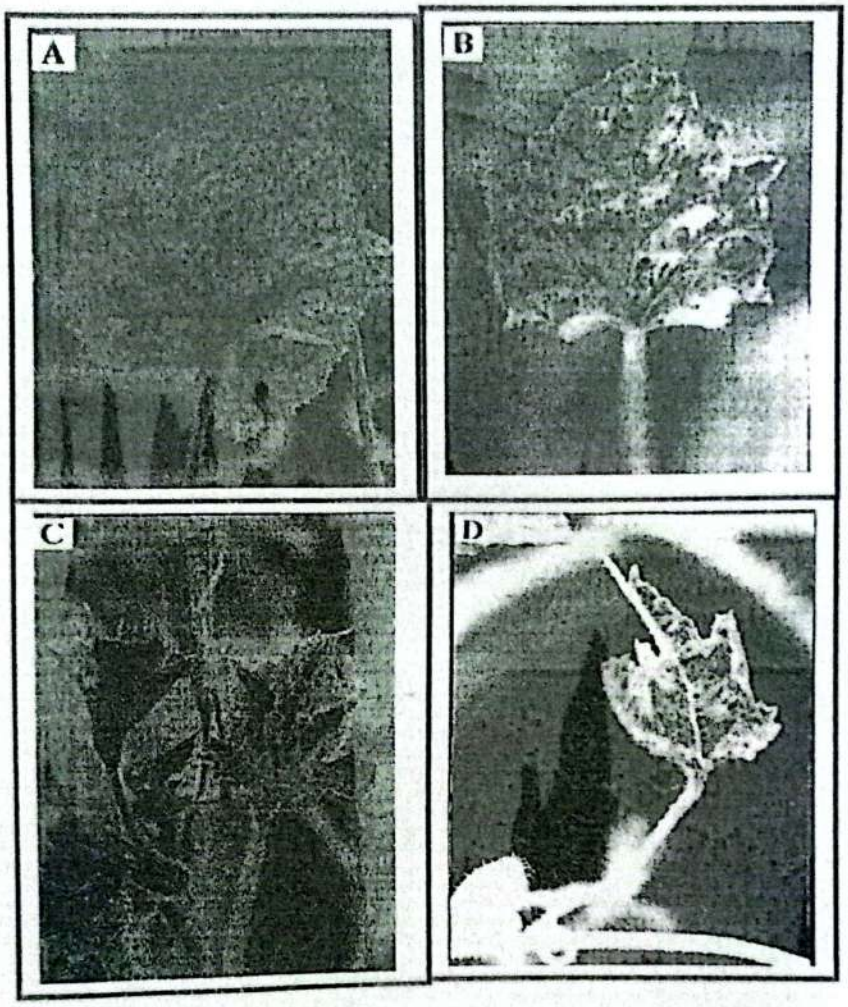


Fig. (1): CMV- isolate induced symptoms on squash plants A. Mild mosaic. B. Severe mosaic. C. Malformation and reduction of leaf blade. D. Elongation and twisting of leaf petiole around the stem and extension of the midrib.

Fig. (2): Transection (1) healthy and infected midrib of leaf (Magn.X40) , (2) healthy and infected mesophyll tissue (Magn. X100), (3) healthy and infected stem vascular bundle (Magn. X400). H.healthy , I: infected

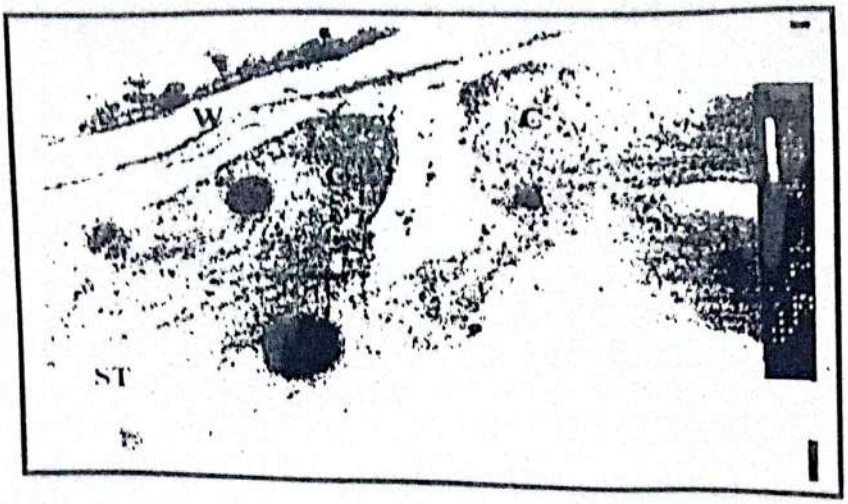


Fig. (3): A chloroplast of infected cell showing osmophilic globules , large starch grains and undifferentiated thylakoid system (Magn. X 30.000). C: chloroplast , O : osmophilic bodies. Th:thylakoid and St : starch grain .

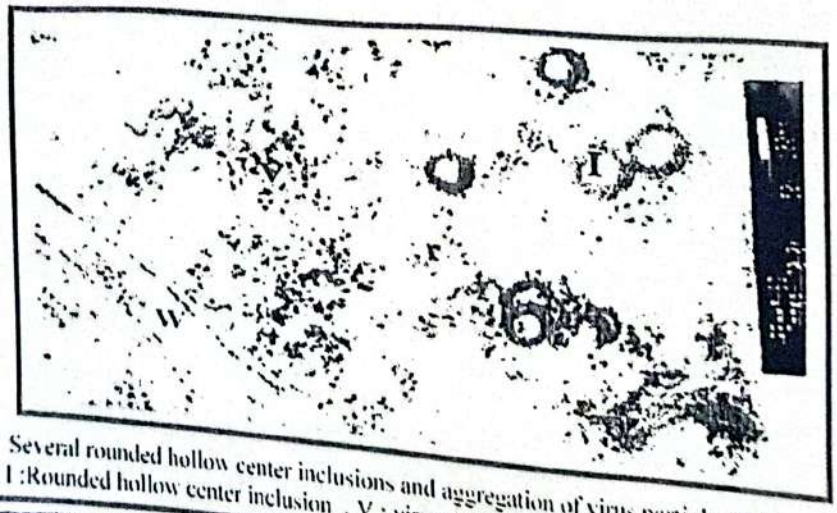


Fig. (4): Several rounded hollow center inclusions and aggregation of virus particles (Magn. X 50.000). I :Rounded hollow center inclusion , V : virus particles and W :cell wall.

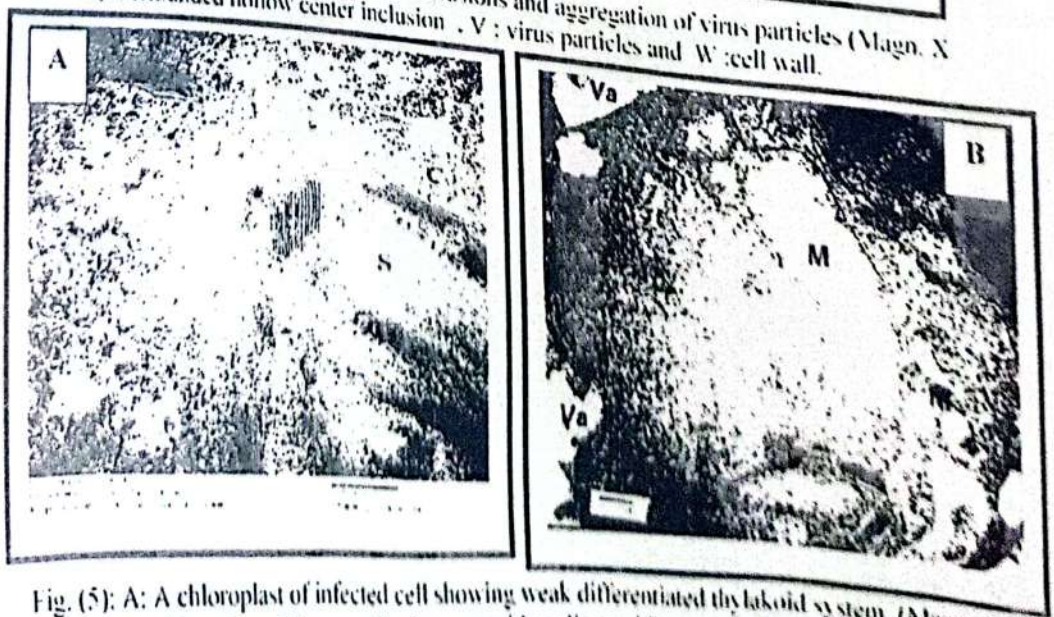


Fig. (5): A: A chloroplast of infected cell showing weak differentiated thylakoid system (Magn. X 40.000). C: chloroplast. GR: grana II : intergranal lamellae and S: stroma. B: Infected cell showing elongated , deformed mitochondria and many small vacuoles (Magn. X 20.000). M : mitochondrion and Va : vacuole

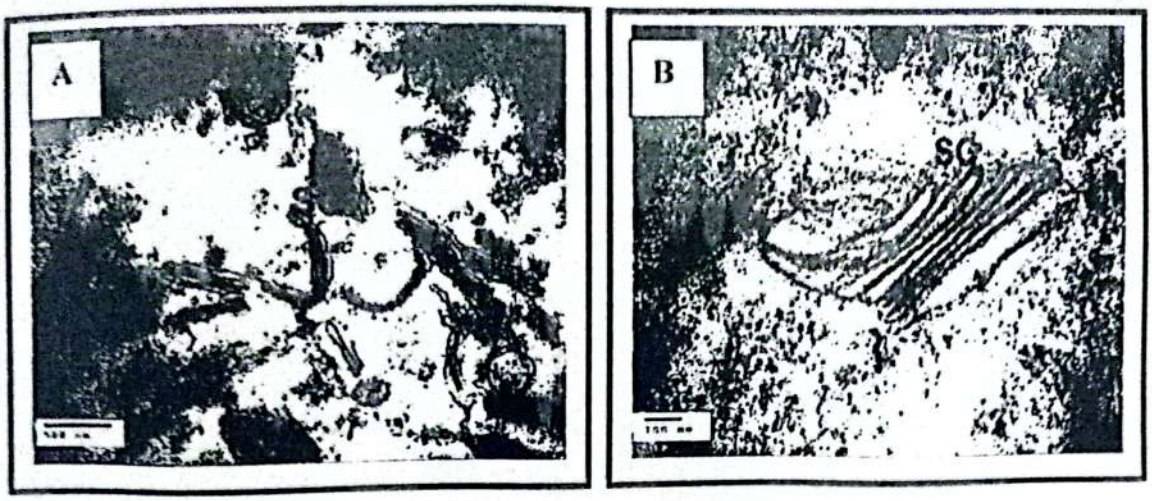


Fig. (6). A:Part of a cytoplasm containing numerous scrolls. (Magn. X 30.000). M: mitochondrion and SC : scroll . B: Magnified scrolls. (Magn. X 80.000). SC : scroll

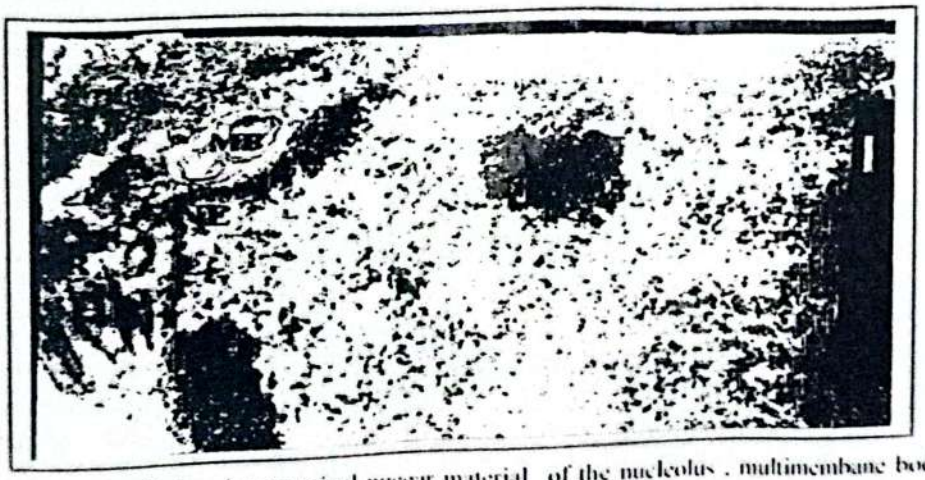


Fig. (7): Infected cell showing marginal nucear material of the nucleolus , multimembane body and endoplasmic reticulum (Magn. X 25.000).
ER : endoplasmic reticulum , MB : multimembane body , N : nucleus , n : nucleolus , NE : nuclear envelope and NM : nuclematerial .

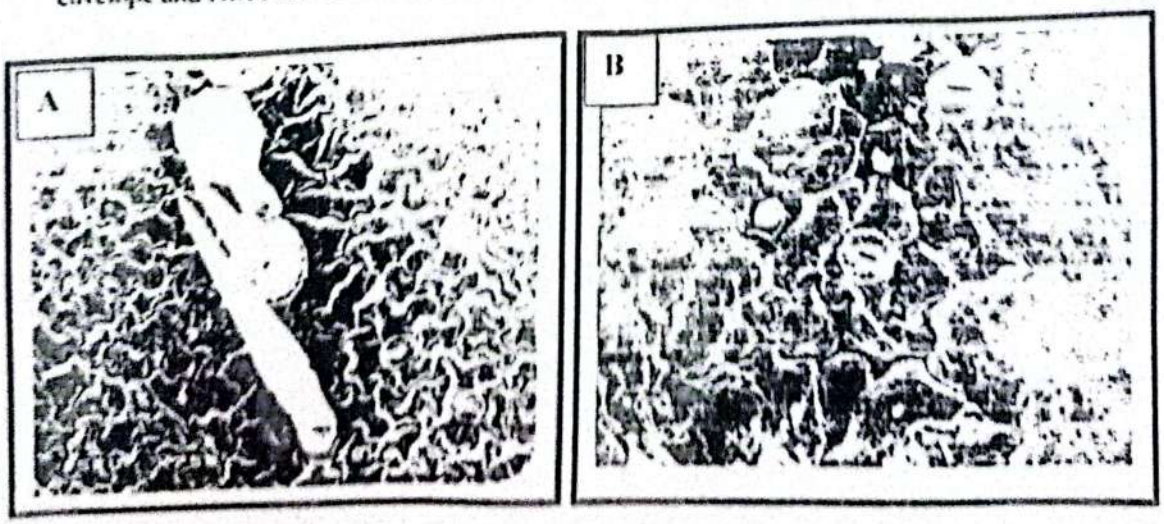


Fig. (8): .A: Epidermal cells of healthy leaf showing multicellular , glandular hairs and closed or slightly opened stomata .B: Epidermal cells of infected leaf showing shrinkage , broken hairs and opned stomata.

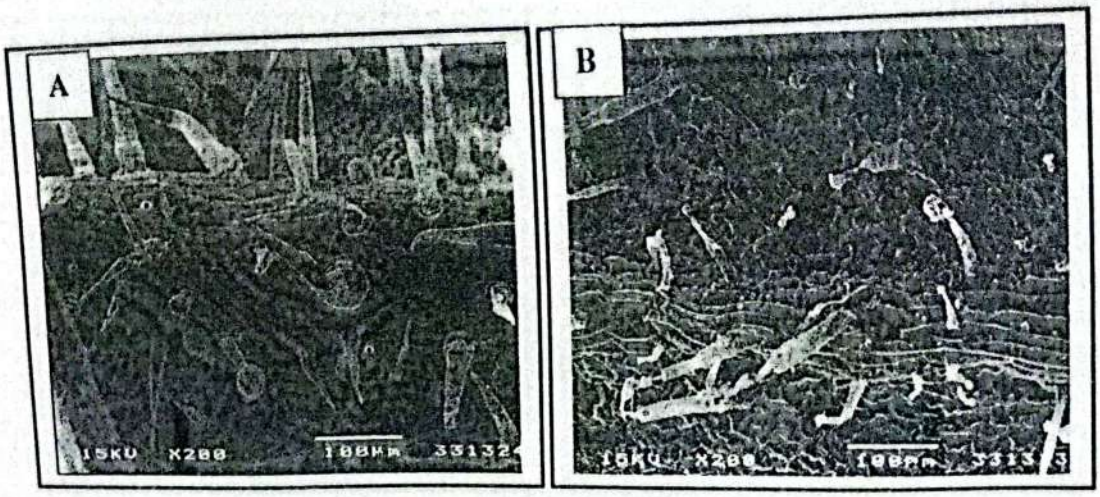


Fig. (9): A: Normal turgid epidermal cells and normal hairs of healthy leaf midrib . B : Midrib cells of infected leaf showing clear elongation and broken hairs.

Ain Shams University. Using DAS-ELISA technique our results showed positive reaction with 10 samples, which exhibited net mosaic, mottling, blisters and epinasty. The result was in accordance with that obtained by Tsorlianis *et al.* (2000) and Tessitori *et al.* (2002). *Cucurbita pepo* used as a virus propagative host, systemic symptoms produced in the form of net mosaic, mild mosaic, mosaic mottling, malformation then wilting of whole plant. Our results were in agreement with Smith (1957) and Kyriakopoulou and Bem (19 The histopathological studies of *Cucurbita pepo* leaves infected with CMV revealed many alterations. The leaf blade showed slight upward blistering. This result was in agreement with that obtained by Nasser (2001). The midrib appeared clearly bulged from the upper and lower sides. Many multicellular hairs appeared from the upper epidermal cells. These results were in accordance with that observed by El-Shamy *et al.* (2000) . The mesophyll consists of compact palisade cells shorter than that of healthy ones, while spongy tissue appeared rounded and compact with few air spaces, also mesophyll tissue appeared rich in chloroplast. This is in agreement with that proved previously by El-Shamy et *al.* (2000) and Sayed *et al.* (2001). Vascular elements appeared in two patches, upper patch appeared completely divided into two parts. Xylem vessels appeared clearly reduced in number and metaxylem appeared less lignified and disintegrated. These results were similar to that obtained by Dubey and Bhardwaj (1982) and Sayed (2002). The histopathological studies of *Cucurbita pepo* stems infected with CMV showed that cortex appeared normal with collenchyma and parenchyma cells while the pith cells appeared collapsed with strongly waved cell wall. These results were in accordance with that observed by Eskarous *et al.* (1991) and were contradictory to that found by Dubey and Bhardwaj (1982). The bicollateral vascular bundles appear with few and irregularly arranged xylem vessels. Outer and inner phloem cells appear somewhat wider collapsed and contain heavy tannin. These results were similar to that obtained by El-Dougdoug *et al.* (1993) who stated that the infected plants had active sieve elements in phloem tissue The cytopathological studies of *Cucurbita pepo* leaves infected with CMV showed

rounded hollow center inclusions. These inclusions produced as a result of virus multiplication. These inclusions are novel structures not previously reported in Association with CMV infection. Electron microscopy either transmission or scanning can assist the rapid identification and characterization of many plant viruses. Some individual viruses can be distinguished by the inclusions they induce (Edwardson & Christie, 1978 and Abdel-Ghaffar *et al.*, 2003). Alteration of cell organelles induced by CMV infection was studied and these alterations represented by were found in cell wall, cytoplasm, chloroplasts, mitochondria and nucleus. The results revealed the presence of multimembrane bodies similar to that described by Tu and Hiruk (1971) Aggregates of virus particles within the cytoplasm were observed. These finding were in agreement with that obtained by Iizuka and Yuonki (1975) and Sanger *et al.* (1998) Many small vacuoles within cytoplasm were also observed in CMV-infected leaves . our results were in agreement with that obtained by Martelli and Russo (1985) and Sayed *et al.*(2001) Deformed chloroplasts were observed in the form of malformation, weak differentiated thylakoid system , and large deformed starch grains in CMV-infected leaves. These results were in accordance with that obtained by El-Kewey(1991) and Sayed (2002) .
Mitochondria of infected host cells appeared clearly deformed, elongated or rounded in shape and without cristae. The nucleus appeared clearly deformed with segregation of nuclear material to a marginal position. These results were in agreement with that obtained by Kim and Fulton (1984) Electron micrographs obtained by scanning electron microscopic examination of healthy and CMV-infected squash leaves showed a great virus effect on epidermal cells , trichomes , as well as stomata. Scanning electron microscopy of virus infected leaves was not reported . The only reported literature were on infected some fungal leaves. Although direct evidence as to the role of cuticle and epidermis in resistance is limited , it is nevertheless obvious that both are of major importance in preventing introduction of the virus into the plant . Successful infection is correlated with the number of ectodesmata in the outer walls of epidermal cells , probably because ectodesmata serve as infection sites ( Lobenstien *et al.*, 1995) .

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