Histo-Pathological Changes in Leaves Cells of Squash Plants infected with *Squash leaf curl begomovirus* (SqLCV)

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Abstract: *Squash leaf curl virus* (SqLCV) was isolated from squash plants cultivated in Fayoum Governorate. Field inspection of squash leaf curl viral disease was determined according to visual symptoms. Squash plants naturally diseased with SqLCV showed systemic viral symptoms of leaf curling, leaf crinkle, vein banding, fruit malformation and stunting. Virus was confirmed using PCR. *Bemisia tabaci* insect was able to transmit SqLCV from infected to healthy squash seedlings. The resultant purified virus preparation gave a UV spectrum typical of nucleoprotein. Maximum and minimum absorbance was recorded at 257 and 245 nm, respectively. The purified virus yield obtained in this study was 1.1 mg/ml/100g leaf tissues using the extraction coefficient of 7.7. Light microscopy was used to recognize the effect of *Squash leaf curl virus* (SqLCV) on the anatomy structure of different organelles such as stem, leaf petiole and leaf blade. The results obtained showed that: Infection of squash plants by *Squash leaf curl virus* (SqLCV) led to a decrease in stem section diameter by (10.9%), this decrease was due to the decrease in average diameter of cavity by (33.3%). While, other stem components measurements were showed an increase in its measurements; infection of squash plants by SqLCV decreased the section diameter of leaf petiole by (24.4%), this decrease resulted from the decrease in average thickness of ground tissue by 25% and decrease of average diameter of cavity by 17.9%. While, the other leaf petiole components was less affected; squash leaves were greatly affected as a result of the infection *Squash leaf curl virus* (SqLCV). This infection was led to an increase in midvein dimension by (22.2% x 28.0%). This increase resulted from the increase in midvein vascular bundle dimension by (127.3 x 76.9%). Electron microscopy was used to recognize the internal changes on internal organelles due to infection of *Squash leaf curl virus* (SqLCV). The results obtained showed that, severe damage in chloroplasts including thylakoids in grana; uneven thickening in the cell walls resulted from developing the curly symptoms in squash leaves; severe damage in mitochondria; aggregates of cytoplasm; nucleus becomes swelled.
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Key words: *Squash leaf curl virus*; SqLCV; Histology; Electron microscopy; light microscopy

1. Introduction

Squash leaf curl virus (SqLCV) belongs to genus begomovirus of family *Geminiviridae*. SqLCV is a severe viral disease of squash (*Cucurbita pepo* L.). In Egypt SqLCV was found to occur naturally in *Malva parviflora* (Al-Musa *et al.* 2008). Out Egypt, *Squash leaf curl geminivirus* (SqLCV) was first observed in squash in California during 1977 to 1978 (Flock and Mayhew, 1981). The virus was first purified and characterized by Cohen *et al.* (1983).

SqLCV virus is transmitted efficiently by whitefly, *Bemesia tabaci* (Cohen *et al.* 1983 and Mc Creight, 1984). The genome organization of SqLCV is similar to those of other begomoviruses, with five open reading frames (ORFs) encoded by DNA A, and two ORFs encoded by DNA B (Lazarowitz and Lazdins, 1991). Homologous to ORFs of other begomoviruses, and perform similar functions. DNA A encodes the coat protein (AR_1), the replication initiator protein (AL_1), the transcriptional activating protein (AL_2), a replication enhancer protein (AL_3), and a potential protein of unknown function (AL_4). Specificity of replication is determined by AL_1 protein-origin interactions (Sunter and Bisaro, 1991 and 1992; Lazarowitz *et al.* 1992 and Padidam *et al.* 1995), whereas the gene products of the AL_2 and AL_3 ORFs are functionally intercha-ngeable among different begomoviruses.DNA B encodes two proteins (BL_1 and BR_1) required for systemic movement. Both movement proteins are determinants of viral host range (Ingham *et al.* 1995).

Squash leaf curl virus (SqLCV) belongs to genus begomovirus of family *Geminiviridae*. Begomoviruses, whitefly-transmitted geminiviruses (Genus *Begomoviruses*, Family: *Geminiviridae*), are among the most widespread and damaging plant viruses worldwide (Brown, 2001; Brown *et al.*, 1995 and Varma, 2003). Begomoviruses, restricted to dicots, cause economic losses in vegetable crops in the sub tropics and tropics

(Brown, 1994; 2000; Brown and Bird, 1992) where the whitefly vector *Bemisia tabaci* occurs (Brown, 2001). The morphology of geminivirus particles is unique and they are characterized by twin icosaheaderal capsid approximately 20×30 nm in size encapsidating a single molecule of covalently closed circular single stranded DNA (ssDNA) genomes of 2500 to 3000 bp. that replicate in the nuclei of the infected cells via a double stranded DNA (dsDNA) intermediate (Harrison and Robinson, 1999; Varma and Malathi, 2003). Geminiviruses have unique, twinned icosahedral particles which encapsidate circular single-stranded DNA. Their genomes are composed of either one or two DNA segments (Pooma *et al.* 1996). This investigation was carried out to achieve ultrathin sectioning using electron microscopy to recognize the internal changes induced by virus, and abnormalities caused by SqLCV using light microscopy.

2. Materials and Methods

2.1. Virus isolate

Infected squash samples (*Cucurbita pepo* cv. Eskandarani) were used for virus isolation on squash cv. Eskandarani seedlings by insect transmission (virus free white flies, *Bemisia tabaci*) in persistant manner. The inoculated plants were kept in insect-proof cages. After 3-6 weeks, the new symptoms appeared similar to the original symptoms on the collected plants and confirmed using PCR.

2.2. Insect transmission

Non-viruliferous whiteflies, *Bemisia tabaci* maintained on cabbage in an insect-proof cage, were sucked into the bottle and allowed to feed for 24 h on virus infected squash plants. Viruliferous whiteflies then were transferred to healthy young squash seedlings for 48 h. (30 insects/ plant). Whiteflies were killed by spraying with insecticide (Actelic 1.5 ml/L.) and plants were maintained in a growth chamber at 28 to 30°C. Plants showing severe stunting, vein banding and vein clearing and leaf curling were used as a virus source for this study (Isakeit *et al*. 1994).

2.3. Virus purification

Purification was carried out using technique as described by Goodman and Bird, (1978). Partially purified suspension was examined by Electron Microscopy Unit, Faculty of Agriculture, Cairo University using negative staining technique as described by Bozzola and Russell (1999).

2.4. Histo-pathological studies

2.4.1. Light microscopy

For anatomical study, samples of squash (*Cucurbita pepo* cv. Eskandarani) were collected at the ages of 4 weeks including leaf blade, leaf petiole and stem. Samples were killed and fixed in F.A.A. solution (10 ml formalin + 5 ml glacial acetic acid + 50 ml ethyl alcohol 95% + 35 ml distilled water) for 72 hours, then dehydrated and cleared in n-butyl alcohol series, and embedded in paraffin wax of 56-58ºC. Cross sections of 20µ thick were cut, using a rotary microtom, adhesived on slides by "Haupt's adhesive" then stained with the crystal violet–erythrosin combination, cleared in carbol xylene and mounted in Canada balsam (Sass, 1961).

2.4.2. Electron microscopy (ultrathin sections)

The effect of squash leaf curl virus (SqLCV) infection on the cell components of *Cucurbita pepo* cv. Eskandarani was studied. Ultra-histopathological changes due to virus infection were studied using E.M. according to Hanschke and Schauer (1996) in Electron Microscopy lab, Faculty of Agriculture, Cairo University.

3. Results and Discussion

Squash leaf curl virus (SqLCV) was isolated on healthy squash plants cv. Eskandrani from naturally diseased squash (*Cucurbita pepo* cv. Eskandrani) exhibited viral symptoms, using whitefly (*Bemisia tabaci*) transmission. After four weeks, symptoms of leaf curling, leaf crinkle and vein banding were produced. These plants were used to propagate virus on healthy squash plants cv. Eskandrani and were kept under greenhouse conditions for different studies. The isolated virus was identified as SqLCV using PCR (Figures 1&2).

3.1. Insect transmission

Bemisia tabaci insects were able to transmit SqLCV from infected to healthy squash seedlings as in Table (1). Whitefly-transmitted geminiviruses are the most important constraint to the production of vegetable crops in many countries in the world. According to (Jones, 2003) virus species are transmitted by whiteflies, 90 % of these viruses belong to the *Begomovirus* genus. Data presented here demonstrate the transmission of SqLCV on cucurbits in Egypt. The introduction of this virus to Egypt might have occurred through transplant movement between Egypt and other countries or through viruliferous *B. tabaci* that moved from infected cucurbits fields in neighbouring countries. SqLCV was easily transmitted by *Bemisia tabaci*, of the family *Aleyrodidae*, order *Homoptera*, in a persistent manner. Many investigators reported similar results (Maruthi *et al*. 2007; Ghanim *et al.* 2007; El-Dougdoug *et al*. 2009; Ali-Shtayeh *et al.* 2010 and Helmi, 2010).

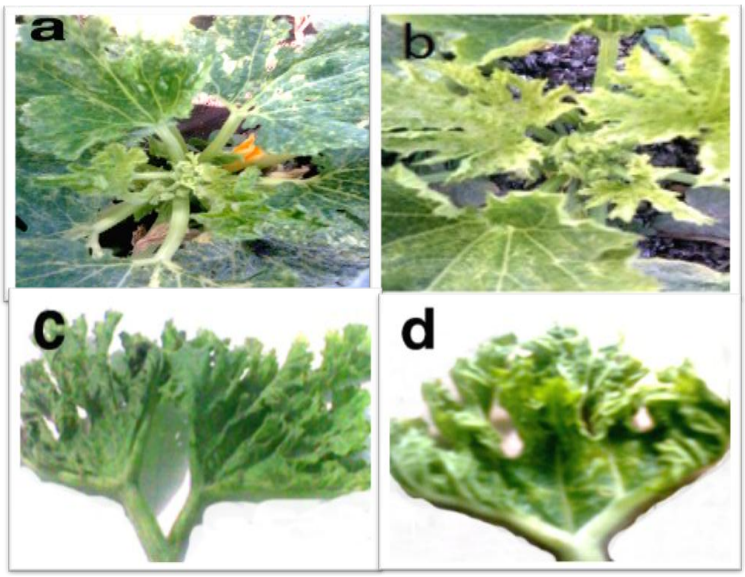

Figure 1. Symptoms of SqLCV on squash leaves (*Cucurbita pepo* cv. Eskandrani) under field conditions showing; (a) and (b) leaf curling and stunting, (c) and (d) leaf crinkle, leaf narrowing and chlorosis at different times of plant age.

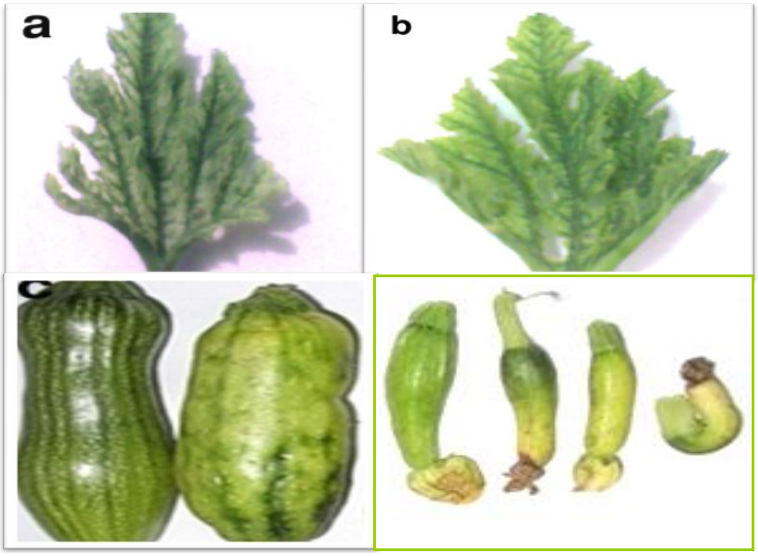

Figure 2. Symptoms of SqLCV on squash plants under field conditions showing; (a) and (b) vein banding, (c) and (d) fruits malformation at different times of plant age.

Table 1. Transmission of SqLCV by *B. tabaci*.

Transmission mode	Symptoms	Incubation period (Weeks)	A/B	Virus transmission efficiency (%)
Whitefly (*B. tabaci)*	leaf curling , leaf crinkle, vein banding	3-4	20/24	83.33%
	Sever stunting	4-5		

A/B: Number of infected plants / total number of inoculated plants.

3.2. Purification

Virus purification is undertaken with objectives of obtaining virus preparation of both high quality and good yield. The resultant purified virus preparation gave a UV spectrum typical of nucleoprotein. Maximum and minimum absorbances were recorded at 257 and 245 nm, respectively. The A_{260}/A_{280} and Amax/Amin ratios were 1.7 and 1.1, respectively as illustrated in Fig. (3). The purified virus yield obtained in this study was 1.1 mg/ml/100g leaf tissues using the extraction coefficient of 7.7 with a spectronic 2000 spectrophotometer. Electron microscopic examination of partially purified preparation of SqLCV revealed the presence of isometric particles, with paired *Geminivirus*, (dimmers), when negatively stained with uranyl acetate. The dimension of single particle is 22nm and paired particle ranged from 20 X 30 nm to 24 x 30 nm (Fig. 4). Squash leaf curl virus was partially purified using a modification of the procedure of Goodman and Bird (1978).The purified virus yield was the same those obtained by 1.95 mg/ml/100g Goodman and Bird (1978) who used differential centrifugation to purify SqLCV and other *Geminivirus*. On the other hand, this yield was rather higher than those obtained by Cohen *et al*. (1983) which they obtained 1.5 mg/ml/100g tissue. It might be due to the different methods used in purification and virus strains. The use of purified viral DNA for PCR amplification provides several advantages. It eliminates the time consuming steps of total nucleic acid isolation and purification, avoids possible inhibitory effects of co isolated impurities on PCR amplification, and allows detection of viruses that occur in low titers (Rampersad and Umaharan, 2003). The examination with the electron microscopy of the isolated virus particle revealed the presence of isometric and pentagonal in shape, with single and paired Gemini virus, (monomers and dimmers) with dimension of 22 nm and 20 X 30 nm respectively, when negatively stained with 2 % uranyl acetate pH 7.0. These results were similar with that obtained by (Cohen *et al*. 1983; Brown and Nelson, 1989; Al-Shahwan *et al*. 2002; Farag *et al*. 2005; and El-Dougdoug *et al*. 2009).

3.3. Histo-pathological studies

3.3.1. Light microscopy

3.3.1.1. Stem

Data in Table (2) and Fig. (5) showed that, infection of squash plants by *Squash leaf curl virus* (SqLCV) led to a decrease in stem section diameter by (10.9%), this decrease was due to the decrease in average diameter of cavity by (33.3%). While, other stem components were showed an increase in its measurements. Cortex thickness was increased mainly due to the increase in its cell number. Also, there was an increase in average thickness of vascular bundles as shown in its length and width. Similarly, average of pith diameter was increase due to the increase in its average cell diameter, while its number of layer was less affected.

3.3.1.2. Leaf petiole

Data in Table (3) and Fig. (6) indicated that, infection of squash plants by SqLCV decreased the section diameter of leaf petiole by (24.4%), this decrease resulted from the decrease in average thickness of ground tissue by 25% and decrease of average diameter of cavity by 17.9%. while, the other leaf petiole components was less affected.

3.3.1.3. Leaf blade

Data in Table (4) and Fig.(7) revealed that, squash leaves were greatly affected as a result of the infection by *Squash leaf curl virus* (SqLCV). This infection was led to an increase in midvein dimension by (22.2% x 28.0%) this increase resulted from the increase in midvein vascular bundle dimension by (127.3 x 76.9%). The increase in midvein vascular bundle dimension was accompanied with an increase in the diameter of xylem vessels, while their number was less affected. While the infection of squash leaves by SqLCV showed a greatly decreased in leaf blade thickness, this decrease was due to the decrease in both spongy and palisade tissues, but the former was more affective than later. Similar results were obtained by Dubey and Bhardwaj, (1982); El-Hammady *et al.* (1983); Eskarous *et al.* (1984); Buchter *et al.* (1987); Roberts, (1989); Tzeng *et al*. (1993); Singh and Rathi (1996); Ashraf *et al.* (1999); Reddy *et al.* (2006); Prestes *et al.* (2009). Gevorkyan *et al.* (1976) and Burdonov (1978) showed that, infection with virus caused a reduction in the width of cells in the palisade parenchyma. The leaf blade is reduced in thickness. Kaminska and Zawadzka (1977) reported that, in trees infected by apple rubbery wood virus, the xylem was unevenly and poorly lignified. Cells were much smaller, vessels fewer and xylem rays larger. Buzhoryanu (1984) reported that, in virus-infected tobacco leaves there was a reduction in lamina thickness due to a contraction of cells, particularly the palisade layer and the parenchyma, and a reduction in the intercellular spaces. El-Dougdoug *et al.* (1993) evaluated the effect of *Citrus exocortis viroid* (CEVd) infection on the histology of young orange (*Citrus sinensis*) leaves. Light microscopy investigation of the leaf petiole and cross sections of the leaf blade showed several histological changes. In general, CEVd-infection affected the conductive tissues. Infected phloem tissues showed less active sieve elements, and phloem radial thickness and secondary phloem fibers were reduced. The thickness of xylem tissue and vessel diameter was also reduced, as was the number and diameter of glands. Infection reduced the palisade layers. Also, Sofy *et al.* (2007) evaluated the effect of *Citrus*

psorosis virus (CPsV) infection on the histology of young orange (*Citrus sinensis*) leaves.

Vigliocco *et al.* (1993) studied histology of leaves of maize infected by maize rough dwarf fijivirus (MRDV). It appeared that vascular bundles of the 2nd and 3rd order were first affected with accumulation of dense granular contents in some phloem cells, initiation of hyperplasia extending towards the abaxial epidermis and subsequent differentiation of xylem, phloem, and parenchymatous elements in the proliferating cellular mass. This cellular mass extending beyond the leaf epidermis constitutes an enation, a characteristic symptom of infection by MRDV. Ishak and El-Deeb (2004) reported that, the most important changes due to seetpotato chlorotic stunt virus (SPCSV) infection were confined to the vein region. In general, almost all the anatomical characters of the midrib investigated by light microscopy were increased. However, a reduction was observed in the diameter of xylem vessels and phloem area as well as the thickness of the leaf blades. Kunkalikar *et al*. (2007) showed that, papaya ring spot virus brings about histological and histochemical changes in papaya upon infection. In diseased leaves, palisade cells were markedly distorted. The spongy cells lost their normal round shape with complete disintegration.

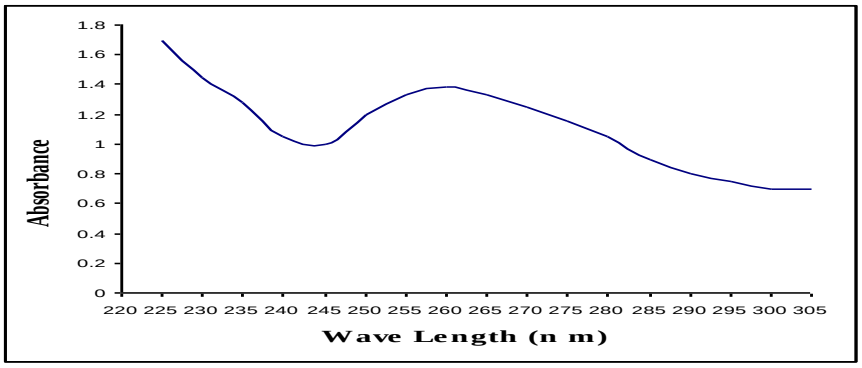

Figure 3. UV absorption spectrum of the purified SqLCV.

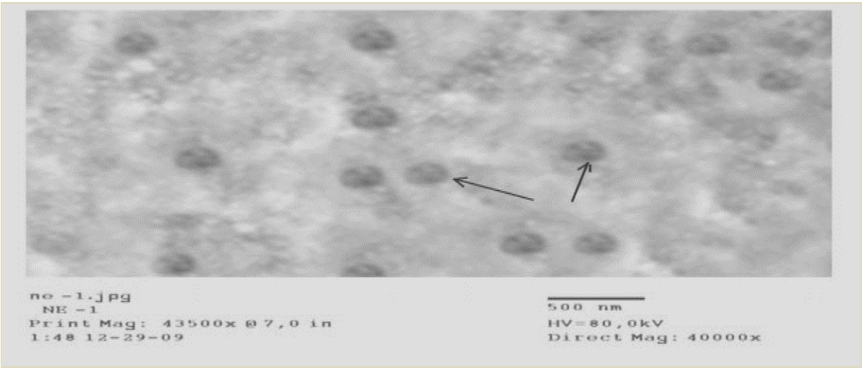

Figure 4. Electron micrographs showing the partially purified squash leaf curl gemivirus negatively stained with 2 % Uranyl acetate, bar represents 500 nm.

Table 2. Effect of infection by SqLCV on squash stem structure.

Characters	Section diameter µ	Cortex thickness µ	Average cortical cell No.	Average cortical cell diameter µ	Length of vascular bundle µ	Width of vascular bundle µ	Modularly ray width µ	No. xylem vessels /bundle	Average diameter of xylem vessels µ	Average diameter of pith µ	Average pith cell No.	Average pith cell diameter. µ	Average Thickness of cavity µ
Healthy	6875	640	15	120	625	625	650	15	48	400	7	50	3937
Diseased	6125	700	20	100	925	845	450	14	45	450	8	75	2625

Table 3. Effect of infection by SqLCV on squash leaf petiole structure.

Characters	Section diameter µ	Av. Diameter of ground tissue µ	Thickness of vascular bundle µ	Av. diameter of xylem vessels µ	No. xylem vessels /bundle	Av. Thickness of cavity µ
Healthy	4300	100	180	65	11	2437
Diseased	3250	75	250	60	7	2000

Table 4. Effect of infection by SqLCV on squash leaf blade structure.

Characters	Midvien		Median vb		Average Mx vessels diameter µ	No. of Mx vessels in median vb	Blade thickness µ	Palisade thickness µ	Spongy thickness µ
	Width µ	Length µ	Width µ	Length µ					
Healthy	1800	2050	550	650	45	40	150	50	75
Diseased	2200	2625	1250	1050	60	30	100	25	50

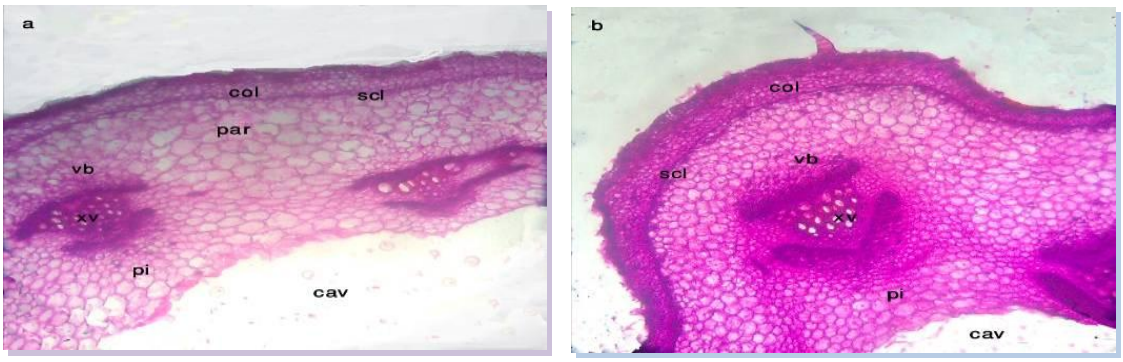

Figure 5. Transection of squash stem: a) Healthy b) Diseased
(col = colenchyma, scl = sclerenchyma, par = parenchyma, vb = vascular bundle, xv= xylem vessels, pi = pith and cav= cavity).

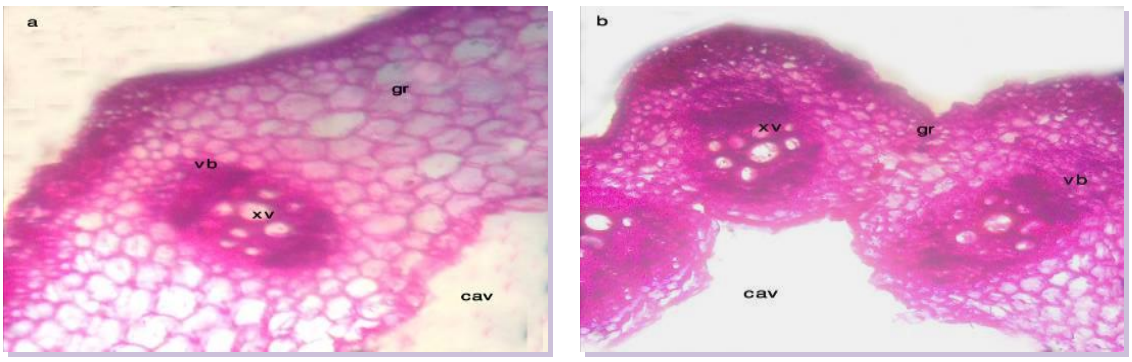

Figure 6. Transection of squash leaf petiole: a) Healthy b) Diseased
(gr = ground tissue, vb = vascular bundle, xv = xylem vessels and cav = cavity).

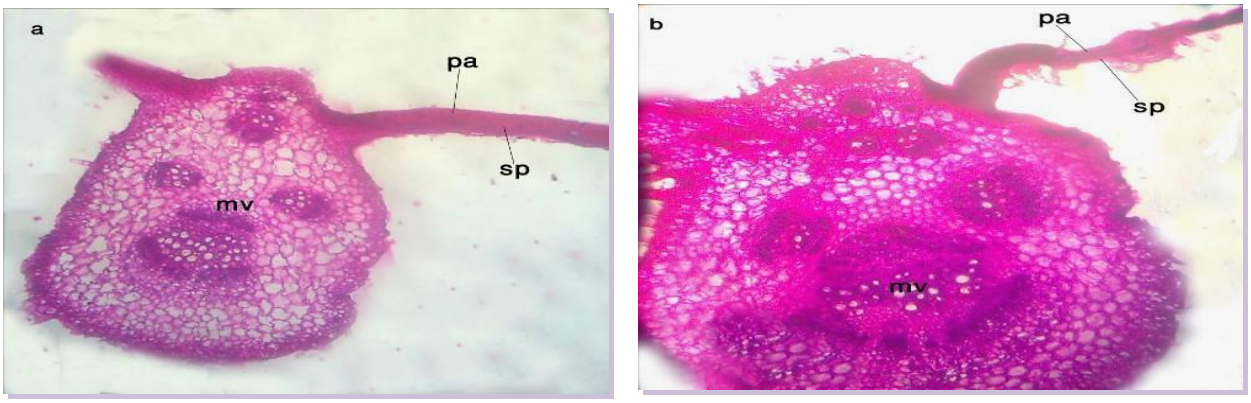

Figure 7. Transection of squash leaf: a) Healthy b) Diseased
(mv = midvien, pa = palisade tissue and sp = spongy tissue).

3.3.2. Electron Microscopy (E.M.)

This experiment was carried out to recognize the internal changes of organelles because of squash leaf curl virus (SqLCV) resulted in significant pathological changes of the infected plants. Infection of the leaves from SqLCV-infected squash plants by electron microscopy resulted in many pathological changes in cell wall, cytoplasm, nucleus, chloroplast and mitochondria. As shown in Fig. (8), uneven thickening in the cell wall resulted from developing the curly symptoms in squash leaves. The infection of SqLCV caused the formation of aggregates of cytoplasm as in Fig. (9). Regarding nucleus, the infection of SqLCV led to the disruption of nucleus membrane. Nucleus becomes swelled Fig.(10). Regarding chloroplast, the infection of SqLCV led to severe damage in chloroplasts including thylakoids in grana Fig. (11). Data shown in Fig.(12) indicated that, severe damage in mitochondria was occurred. The internal cristae were disrupted. Even though electron microscopy is limited to well-equipped laboratories, its use for virus detection can be expanded to less-developed laboratories if samples can be sent through the mail for analysis at center having this capacity. Electron microscopy has two great advantages, namely, the speed with which the results can be obtained, and the convincing, if not unequivocal, nature of visual evidence. This experiment was carried out to recognize the internal changes induced by SqLCV. In cytoplasm abnormal shaped organelles were observed in the cytoplasm. In addition to many of virus-specific vesicles. These vesicles may be the sites of viral RNA replication. Chloroplasts became distorted and misshapen. In addition degradation of chloroplasts was observed. Mitochondrion was affected due to virus infection which caused the formation of vacuole-like vesicles in irregular shape in mitochondria, and disarranged. In addition to the mitochondria was malformed and its outer membrane was ruptured. There was an uneven thickening in the cell walls resulted from developing the curly symptoms in squash leaves. Nucleus also affected with virus infection which caused rupture of its membrane and degeneration of the nucleus. Similar results were obtained by different investigators (Kim *et al.* 1986; Roberts, 1989; Pinner *et al.* 1993; Abdel-Salam *et al.* 1998 and Zhang-ZhongKai *et al.* 2003). Electron microscopy of ultrathin sections in infected leaves showed several anatomical deviations in the ultrastructure of some organelles such as chloroplasts associated with alteration and severe damage in thylakoids and grana. Abnormal building up of oily inclusions and empty vacuoles in the chloroplast stromas were observed. Uneven thickenings in the cell walls of the phloem tissues were found in all samples showed the characteristic external symptoms compared with healthy plant tissues. It has been found that uneven thickening in the cell walls resulted in developing the curly symptoms in tomato leaves. It was also concluded from this study that in response to virus infection, severe damage in chloroplasts including thylakoids in grana resulted in 44-79% reduction in chlorophyll "a and b" causing the yellowing symptoms and crop losses (Montasser, 2011).

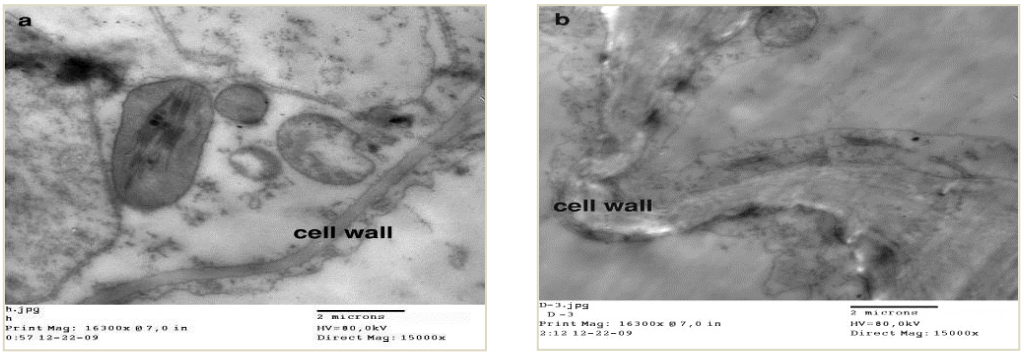

Figure 8. Electron micrograph of a thin section in squash leaf cell infected with SqLCV showing the effect of SqLCV on cell wall. a) Healthy b) Diseased

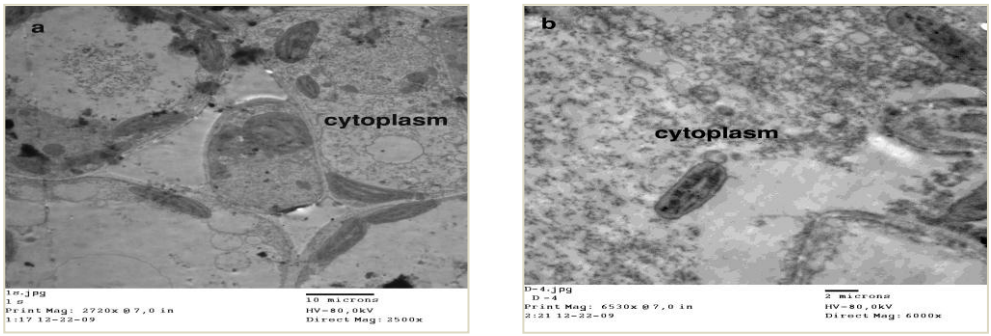

Figure 9. Electron micrograph of a thin section in squash leaf cell infected with SqLCV showing the effect of SqLCV on cytoplasm. a) Healthy b) Diseased

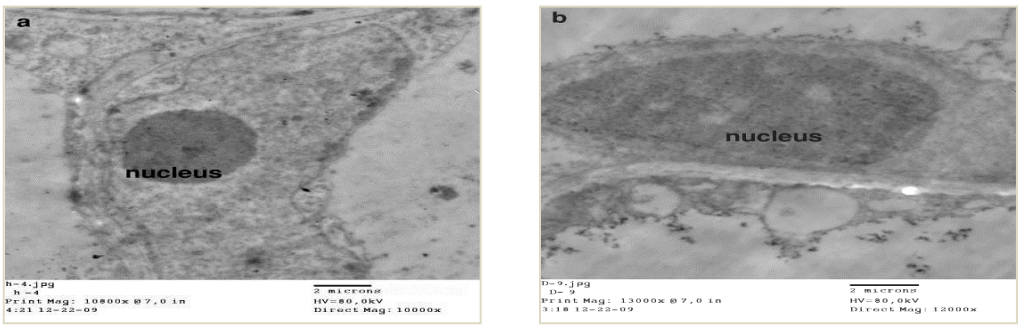

Figure 10. Electron micrograph of a thin section in squash leaf cell infected with SqLCV showing the effect of SqLCV on nucleus. a) Healthy b) Diseased

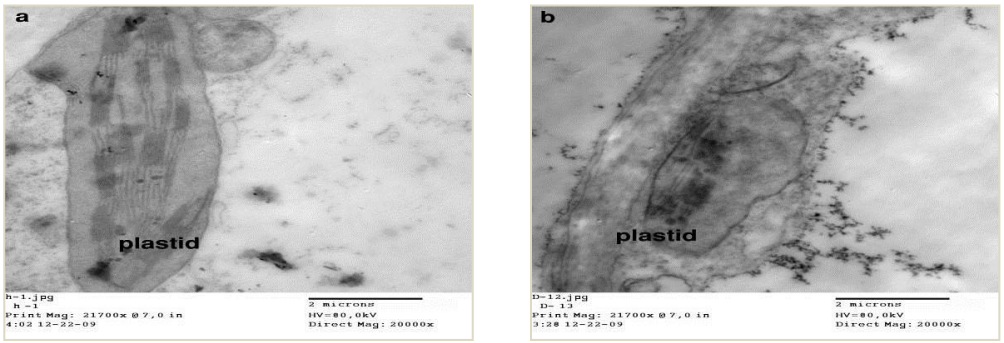

Figure 11. Electron micrograph of a thin section in squash leaf cell infected with SqLCV showing the effect of SqLCV on chloroplast. a) Healthy b) Diseased

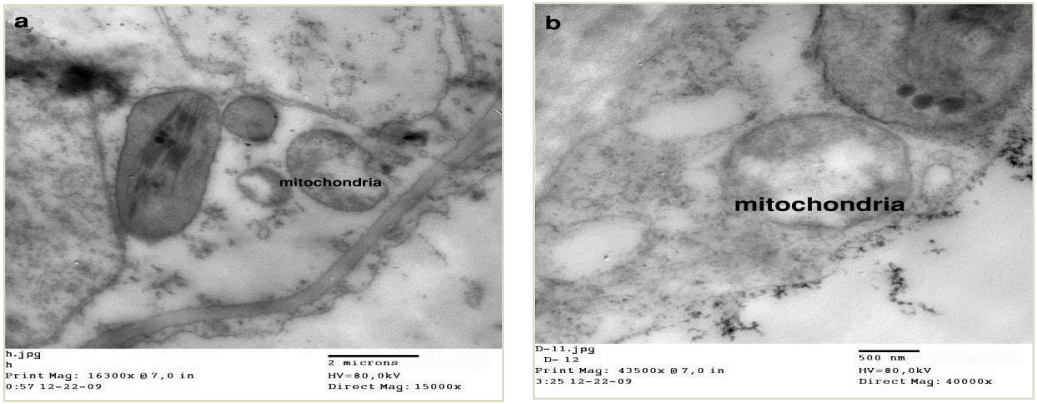

Figure 12. Electron micrograph of a thin section in squash leaf cell infected with SqLCV showing the effect of SqLCV on mitochondria. a) Healthy b) Diseased

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