International Journal of Agricultural Technology 2014 Vol. 10(5):1213-1229
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

A Dramatic Physiological and Anatomical Changes of Tomato Plants Infecting with *Tomato Yellow Leaf Curl Germinivirus*

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Khalil, R. R., Bassiouny, F. M., El-Dougdoug, K. A., Abo-Elmaty, S. and Yousef, M. S**.** (2014). A dramatic physiological and anatomical changes of tomato plants infecting with *tomato yellow leaf curl germinivirus*. International Journal of Agricultural Technology 10(5):1213-1229.

Abstract This study was to investigate the changes of physiological and anatomical in tomato plants infected with *tomato yellow leaf curl virus* (TYLCV) isolate. it was found the growth characteristic, cell membrane stability index, photosynthetic pigment, carbohydrate contents and related to mineral ions contents of tomato plants. As were decreased, when compared with healthy plants. While electrical leakage increased than healthy plants. The mesophyll cells of infected tomato plants appeared relatively small or without intercellular spaces the palisade have lacking chlorenchyma with thin cell walls and chlorenchyma cells contained several cavities. Also the upper epidermis is composed of tubular paranchyma cells covered by thin layer of cuticle, when compared with healthy plants. infected cells developed the presence of large number of abnormal vascular bundles, cell wall, cytoplasm membrane, chloroplast, mitochondria and nuclei. the chloroplast showed slightly elongated with irregular rows of grana destructed regions in chloroplastids which does not organize into grana and thylakoid system. Deformation elongated and curved mitochondria. Nucleus with several dark stained bodies destructed.

Keywords: TYLCV, growth, Chlorophyll, carbohydrate, membrane stability, ultrathin sections, epidermal cell, tomato plants.

Introduction

Tomato *(Lycopersicon esculentum Mill., Solanum lycopersicon* L.*)*, belongs to a large family of plants called the Solanaceae. Egypt ranks fifth in the world for tomato production (FAO, 2010). Tomato is susceptible to many viruses and considerable yield losses and diminished fruit quality can occur due to single or multiple viral infections. The power of growth; decrease of yield and quality of tomato were observed under protective and open field cultivation (Shahwan, 2010). Tomato yellow leaf curl disease (TYLCD) is one of the most

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devastating viral diseases of tomato plants *(Solanum lycopersicum L.)* worldwide (Moriones *et al*., 2011).

A dramatic biochemical changes in virus infected plants result in decrease of both quality and quantity of infected crops. Various reports suggest that virus multiplication inside the plant cell alters different biochemical constituents of plants and disrupt the physiological process like photosynthesis, transpiration and respiration of the infected plants which affect the growth and yield (Tajul, 2011). Abiotic stress decreased membrane stability index of *Zea mays* genotypes (Collado *et al.*, 2010). In similar trend, when chickpea plants were subjected to salt stress (100 mM NaCl), this stress was reported to enhance electrolyte leakage and lipid peroxidation in leaves (Sheokand *et al*., 2008).

Plant viruses are changes in chlorophyll which degradation of chlorophyll content in virus infected plants (Bertamini *et al.*, 2003 and Hemida, 2005). The sugars play pivotal roles in the life cycle of plants The sugar induced feedback inhibition of photosynthesis is one example of a sugar regulated process. When carbohydrates accumulate in mature source leaves, repression of genes involved in photosynthesis is observed and as a consequence photosynthesis is reduced (Wobbes and Sjef Smeekens, 2004). 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.

The aim of the present study to investigate the physiological, biochemical and cell ultrastructure changes in tomato plants infecting *tomato yellow leaf curl virus* through determination electrolyte leakage, membrane stability index, photosynthetic pigments, carbohydrate contents, mineral ions and cytopathic effects of tomato plants.

Materials and methods

Source of the virus isolate

Tomato plants (*Lycopersicon esculentum cv.* Castle Rock) showed naturally distinct geminivirus symptoms were collected from Fac. Agri., Ain Shams Univ. farm. Collected samples were tested for the presence of TYLCV serologically by using indirect enzyme immunosorbent assay (indirect ELISA) as described by (Clark and Adams, 1977) using TYLCV specific polyclonal antibody kindly obtained by Prof. Dr.Khalid El-Dougdoug (Virology lab, Fac. Agri, Ain Shams Univ.).

Isolation and propagation of the virus isolate

Plants samples which gave Positive (+ve) ELISA reaction were applied for virus isolation .The virus was isolated and propagated on healthy tomato cv.Castle Rock seedlings cultured under green house condition by (virus free whiteflies, Bemisia tabaci biotype B) in persistent manner, as described by Noha El-Dougdoug (2013). The inoculated plants were kept in insect proof cages under greenhouse condition. After 3-6 weeks, the new symptoms appeared were confirmed with indirect ELISA.

Determination of growth parameters

Samples of tomato plants Castle Rock from each healthy and infected with TYLCV isolate were collected at the vegetative stages (40 days old plants) to determine growth characteristics (shoot and root length (cm), area of leaves per plant, number of leaves per plant , fresh and dry weight of shoot and root (g/plant).

Estimation of electrolyte leakage

The total inorganic ion leakage from the leaves was measured by the method described by Sullivan and Ross (1979). Twenty leaf discs of 2 ml diameter were placed in a boiling tube containing 10 ml deionized water. The tubes were heated at 45 ℃ (ECa) and 55 ℃ (ECb) for 30 min each in a water bath and the electrical conductivity (EC) was measured with a conductivity meter. Subsequently, the contents were boiled at 100 ℃ for 10 min and the EC was again recorded (ECc). Electrolyte leakage was calculated with the formula:

$$\text{Electrolyte leakage (\%)} = \frac{\text{ECb} - \text{ECa}}{\text{ECc}} \times 100$$

Estimation of membrane stability index

The membrane stability index (MSI) was estimated by placing 200 mg of leaves in 10 ml double distilled water in two sets. One set was heated at 40 ℃ for 30 min in a water bath and the electrical conductivity (C1) was measured. The second set was boiled at 100 ℃ in a boiling water bath for 10 min and the conductivity (C2) was measured; both conductivities were measured using a conductivity meter. The MSI was calculated using the formula described by Premchandra *et al.* (1990) and modified by Sairam (1994): MSI = [1 – (C1/C2)] × 100

Determination of photosynthetic pigments

The contents of the photosynthetic pigments chlorophyll *a* (chl a), chlorophyll *b* (chl b)and carotenoids in fresh leaves were determined using the spectrophotometric method recommended by Metzner *et al.,* (1965) and described by Hassanein *et al.* (2009). The pigments (as µg/ml) was calculated using the following equations:

Chl *a* = 10.3 E663 − 0.918 E644
Chl *b* = 19.7 E644 – 3.87 E663
Carotenoids = 4.2 E452.5 – (0.0264 chl *a* + 0.4260 chl *b*)
Finally, the pigment contents were expressed as µg g−1 dry weight (DW) of leaves.

Determination of carbohydrate content

Soluble sugar was extracted from air –dried leaf tissue with 80% ethanol. One gram of the dried tissues was homogenized with 80% ethanol then put in a boiling water bath for 15 minutes. After cooling, the extract was filtered and the filtrate was oven dried at 60 ℃ then dissolved in a known volume of water to be ready for soluble sugars determination (Homme, *et al*., 1992). The soluble sugars were determined by the anthrone sulfuric acid method described by Scott and Melvin (1956). Polysaccharide content was determined in the dry residue left after extraction of soluble sugars. A known weight of dried material was added to 10 ml 1.5N sulphuric acid in sugar tube with air reflux and heated at 100 ℃ in a water bath for 6 hours (Hodge and Hofreiter, 1962). The hydrolysate was made up to a known volume to be ready for polysaccharide determination by the method of anthrone sulphuric acid reagent. Total carbohydrates content was calculated as the sum of the amounts of soluble sugars and polysaccharides in the same sample. All data were calculated as mg 100 g^{-1} DW of leaves.

Determination of Certain Minerals

Inorganic cations Na^{+} , K^{+} , Mg^{+2} and P^{+3} ions were extracted from dried plant material according to Chapman and Pratt (1978). Sodium and potassium were estimated by flame emission technique as adopted by Ranganna (1977). Magnesium and calcium were determined simultaneously by ICP spectroscopy according to the method of Soltanapour (1985).

Ultrastructure changes

The effect of TYLCV on anatomical structure of tomato leaves were studied according to Johansen (1940) and Corgan and Widmoyer (1971). The changes created in tomato leaf cells which infected with TYLCV isolate were investigated with JOEL JM 100S electron microscope (Electron Microscope Unit. The Regional Center of Micology and Biotechnology (RCMB), Al-Azhar University Cairo) as described by Momma and Takahashi (1982).

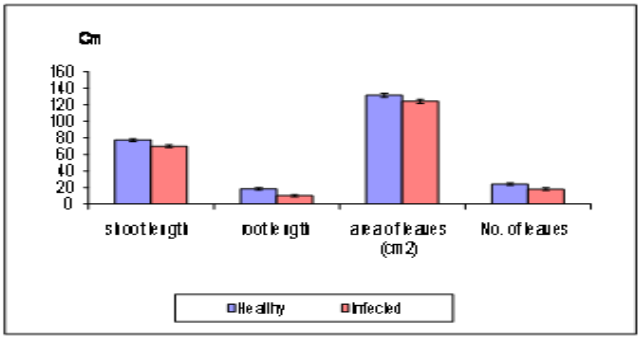
Statistical analysis

The experiment was set up in a completely randomized design. The mean values of growth parameters were calculated from five replicates and all other mean values in the study were calculated from three replicates. All data were analyzed statistically by one-way ANOVA using the Statistical Package for Social Science (SPSS) program. The bars in all figures represent standard deviations of the replicates from the means.

Results

Growth parameters

The effects of *tomato yellow leaf curl virus* (TYLCV) on plant growth, expressed as shoot length, root length, area of leaves per plant, number of leaves per plant, fresh and dry weights of shoots and roots of tomato plants were significantly reduced in response to infected plants (Fig.1), the reduction was observed in plant subjected to TYLCV was estimated by 19.89% in shoot length, 60.1% in root length, 34.82% in area of leaves , 59.37% number of leaves per plant, 50.16 % and 55.57% in fresh and dry weights of shoots respectively, and 63.5% and 74.33% in fresh and dry weights of roots respectively, as compared with those of healthy plants.



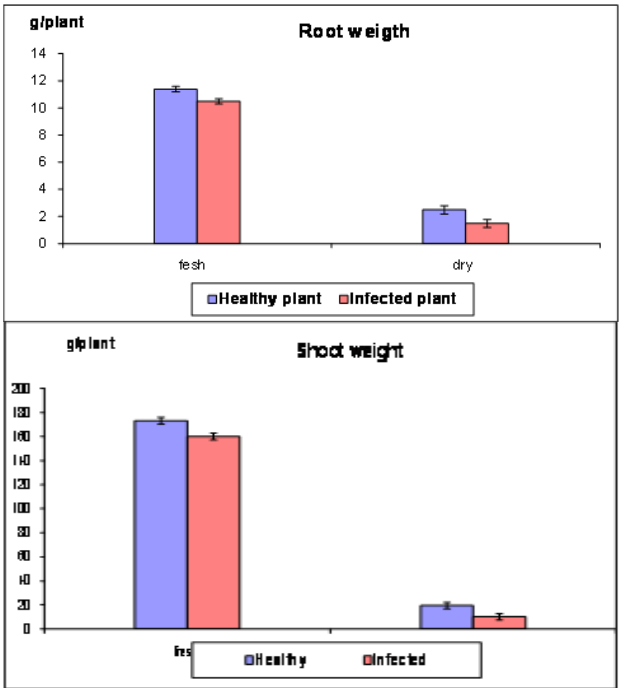


Fig. 1. Histograms showing the effect of tomato yellow leaf curl virus TYLCV on shoot and root length, area of leaves, number of leaves per plant fresh and dry weights of shoot and roots of tomato plants. Each value is the mean of five replicates. Error bars represent the standard deviation.

Electrolyte leakage and membrane stability index

Electrical leakage and membrane stability index enables to assess the injury of cell membrane. As shown in Fig. 2 infected tomato plant with tomato yellow leaf curl virus caused increases in electrical leakage about 7.45% over in uninfected plants and decreases in membrane stability index about 13.42%, when compared with healthy plants.

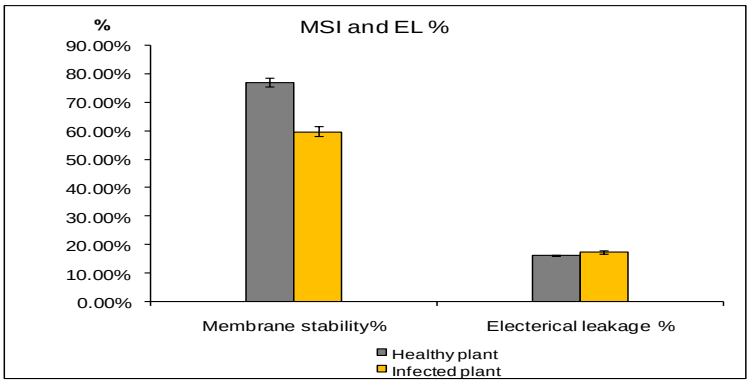


Fig. 2. Histograms showing the effect of tomato yellow leaf curl virus on membrane stability index % and electrical leakage % of tomato plant. Each value is a mean of 3 replicates.

Changes in photosynthetic pigment contents

Data represented in fig. 3 revealed that , chlorophyll a , chlorophyll b , carotenoids and total pigment contents were significantly reduced by infection with tomato yellow leaf curl virus as compared with control healthy plant and this reduction recorded as in chlorophyll a 56.44%, chlorophyll b 75.10%, Carotenoids 73.78% and total pigments 65.26%. The results show also that the total contents of chlorophylls (a+b) decreased 62.75% with infection by TYLCV. This changes was associated with increase in the value of chl. a/chl. b. 45.67%, all these when compared with healthy plants.

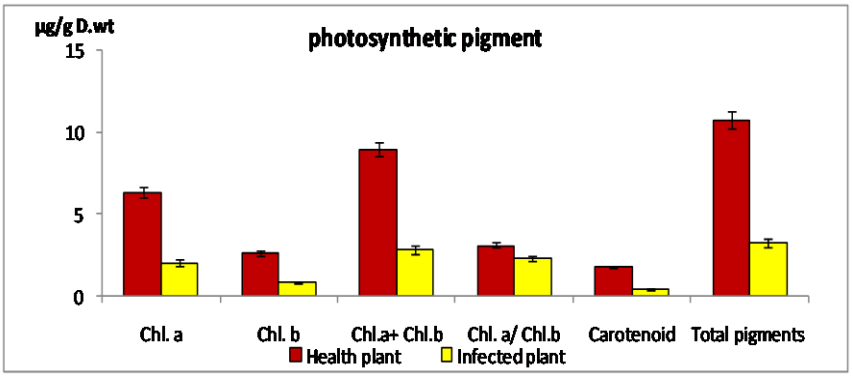
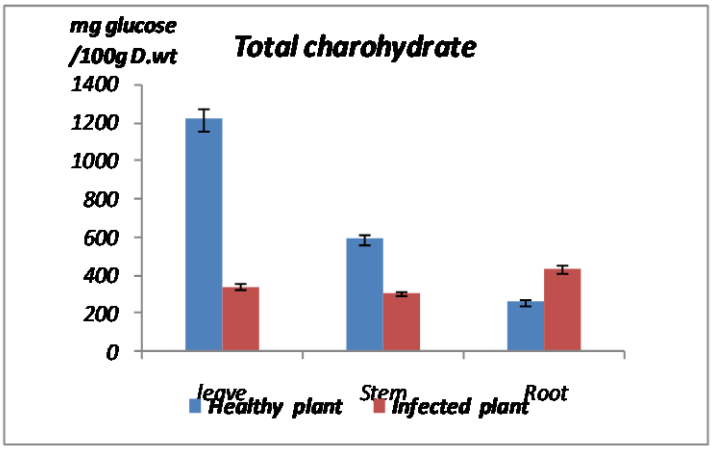
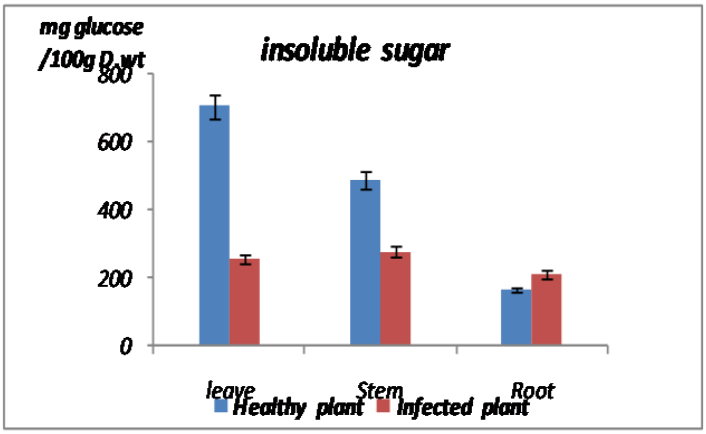


Fig. 3. Histograms showing the effect of tomato yellow leaf curl virus on photosynthetic pigments of tomato plant leaves. Each value expressed as µg/g D.wt. Each value is a mean of 3 replicates.

Changes in carbohydrate contents

The pattern of changes in the amount of various carbohydrate fractions in leaves , stem and root of tomato plant subjected to infection stress with tomato yellow leaf curl virus are demonstrated in fig. 4. The data clearly show that, the applied infection with the virus caused markedly decreased in soluble sugar, insoluble sugar and total carbohydrates contents in leaves and stem. While detected increases in soluble, insoluble and total carbohydrate contents of roots as compared with uninfected tomato plants. The magnitude of the decreases in total carbohydrate contents in infected plants was estimated by 771.84% in leaves and 48.07% in stems and increase by 67.44% in roots when compared with reference healthy plants.





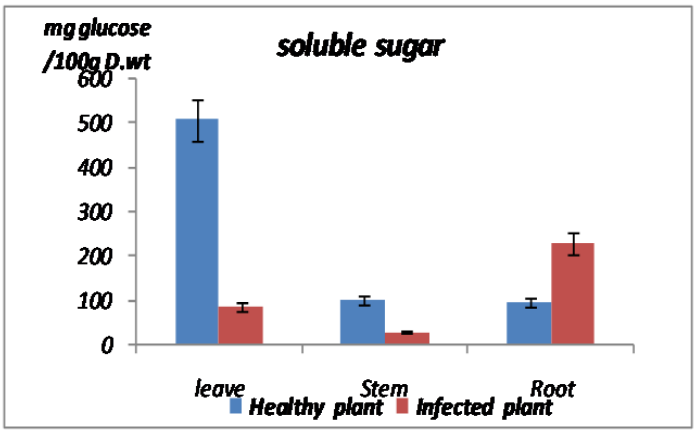
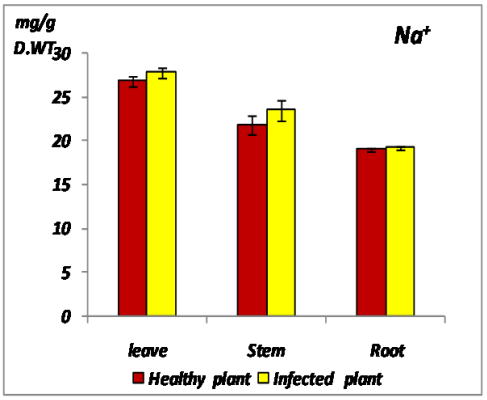
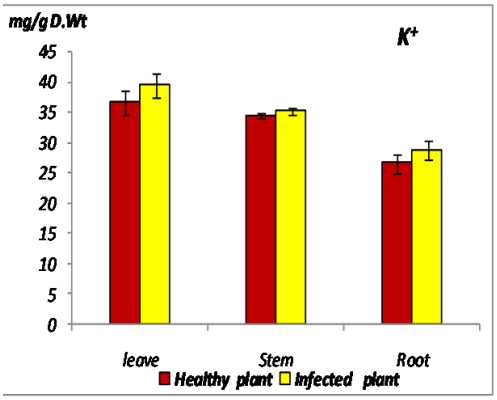


Fig. 4. Effect of tomato yellow leaf curl virus on carbohydrate contents of leave, stem and root of tomato plants. Values are expressed as mg glucose /100g D.wt. Each value is a mean of 3 replicates.

Inorganic cations contents

The results obtained in fig.5. showed that changes in the contents of inorganic cations (Na^{+},K^{+},Mg^{++} and P^{+++}) in both healthy and infected tomato plants by TYLCV virus. Sodium and potassium contents showed increased by subjected tomato plants to TYLCV virus this observed in leaves, stem and root system as compared with healthy tomato plants. Also P^{+++} increased by infected tomato plant with TYLCVin leaves and root but decreased in stem part. While, The magnesium contents gradually decrease with infected tomato plants in leaves, stem and root system as compared to healthy plants





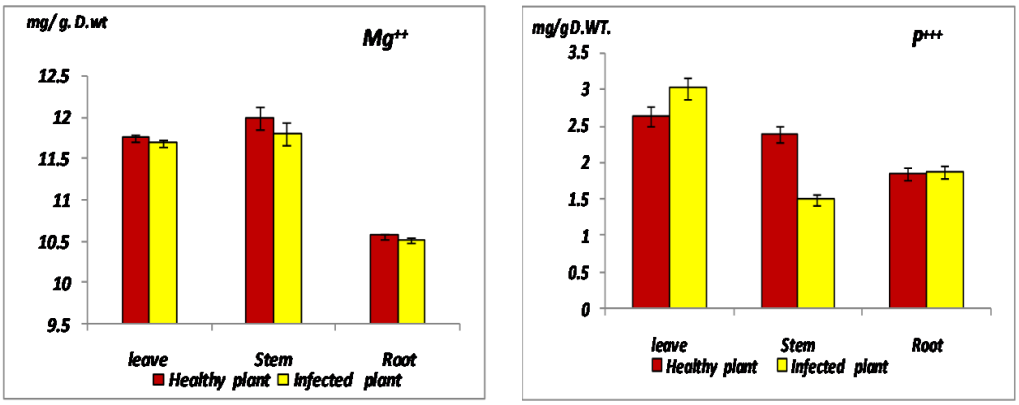


Fig. 5. Effect of tomato yellow leaf curl virus on Na^{+}, K^{+}, $Mg^{+}$and P^{+++} on leave, stem and root of tomato plant (mg/ g. D.wt.) . Each value is a mean of 3 replicates.

Cytopathic effects

Tomato yellow leaf curl virus reacted with different degree with tomato plants as main host .It was caused leaf curl, leaf narrow, leaf yellowing and plant stunting (Fig. 6) . The react was studied thought cytopathic changes in leaf tissue and cells.

Healthy leaves

In light micrograph of section in healthy tomato leaves shows the nearly flat lamina (fig 7 A and B). The upper and lower epidermal cells were barrel formed .The upper cell was slightly larger than that of the lower epidermal cells. Thin walled epidermis cells covering the hairs were arranged so that, numerous cells occurred at them.

Fig. 6. (A) Healthy leaves of tomato plants and (B) Leaves of tomato plants. inoculated with TYLCV isolate showing leaf curl, leaf narrow, leaf yellowing and plant stunting.

The upper epidermis is composed of tubular parenchyma cells. The mesophyll cells (palisade parenchyma) were cylindrical and tightly packed into two or theselayer. The spongy parenchyma contains a large into two or three layer. The spongy parenchyma contains large number of intercellular spaces (fig 7 A and B). In light micrograph section of infected leaves rugosity were concave lamina (fig 7A and B).The upper and lower epidermis were compacted and smaller cells compared to healthy ones (fig 7A and B). AS well as they were not tubular parenchyma cells. Stomata were many in lower epidermis .The hairs were large and arranged so that contain the two to three or multicellular. The mesophyll cells differentiated into many layers and no intercellular spaces (fig 7A and B).

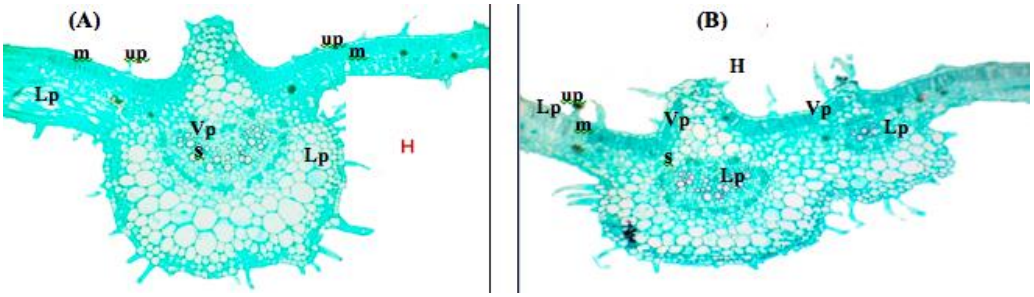


Fig. 7. Light micrograph of tomato leaves cross section , (A) healthy Leaf and (B)infected leaf showing different changes in cells and tissues of 30 days post infection . H: hairs; Vp: vascular bundle; Up: upper epidermis; S: spongy; Lp: lower epidermis; M : mesophyll

Utrastructure changes

The ultrathin sections of TYLCV infected tomato leaves revealed some completely destroyed cells. The mesophyll cells showed relatively small or without intercellular spaces (fig. 9); palisade tissues have a low number of cells are lacking chlorenchyma with thin cell walls (fig. 9) compared with healthy ones (fig. 8). Also the cells contained deformed nuclus chloroplasts mitochondria and destroyed cell membrane as well as small or without vacuole (Fig. 9) compared with healthy mesophyll tissue (fig. 8). The chloroplast alterations in infected cells which showed slightly elongated chloroplastids with irregular rows of grana which decreased in number (fig. 9). The destructed regions in chloroplastids which does not organize into grana and thylakoid system (fig .9). Degenerated mitochondria with destructed envelope (fig. 9) the mitochondria are slight, rounded and bounded by a smooth envelope non-enclosing matrix compared with healthy mitochondria (fig .9). As well as, destructed nucleus with several dark stained bodies and sometimes nucleolus appeared in a deformed shape (fig. 9).

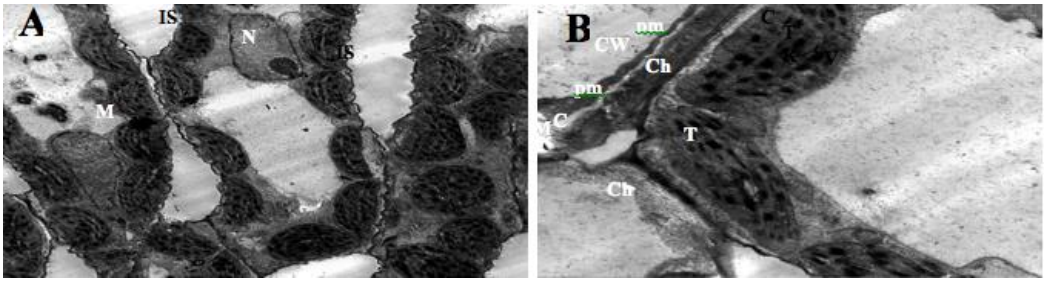


Fig. 8. Ultra micrograph section of healthy mesophyll (A) mesophyll tissue and (B)cells showing normal organal cell. C: cytoplasm Ch: chloroplast CW: cell Wall M: mitochondria Th: Thylakoid S: Stomata X: xylem Pm: plasma membrane T : Tonoplast membrane V: vacuoles N : nucleus VS : vessles Ep : epidermis.

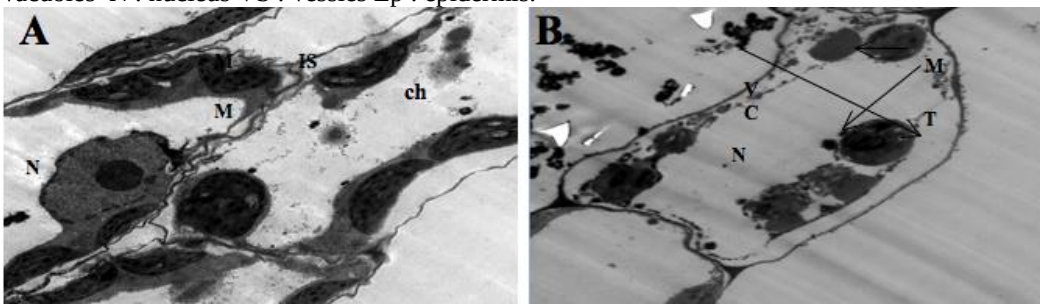


Fig. 9. Ultra micrograph section of TLYCV infected tomato leaf cells showing (A) Deformation of mesophyll tissue and (B) mesophyll cell Gathered protoplasm.

Discussion

In the present work tomato yellow leaf curl virus significantly influenced growth parameters and metabolic activities of tomato plant. In this respect, most growth parameters as height of shoot, root length, number of leaves per plant, area of leaves per plant, fresh and dry weights of shoots and roots of tomato plant were significantly reduced with proceeding virus infection. Our results are in agreement with those obtained by Pozarlar *et al*., (2013). they reported that tobacco mosaic virus (TMV) infection caused decreases in shoot and root length , fresh and dry masses of shoot and root, leaves number and leave area in Ergenekon, Kumsal plants.

The biochemical alterations of cellular constituents are reported to be directly related to morphological deviation of virus infected plants and the extent of vegetable loss is largely determined by visible symptoms (Levy and Marco, 1982). In this respect Bàdàràu *et al*., (2012) found that, water stress is one of the most important environmental factors that limits the growth, yield and quality of potato crops. Potato plants are very susceptible to water deficit, which causes a severe reduction in leaf area, fresh weight and stolon development.

Membrane damage could indirectly be evaluated by measuring solute leakage (Electrolyte leakage) from cells (Ekmekei *et al.,* 2007) and membrane stability index (Ali *et al.,* 2008., Bassuany, *et al*., 2014). It clear from the obtained results that, infection with TLYCV caused increase in electrolyte leakage and decrease in membrane stability index of tomato plant as compared with healthy plants.

Virus-infected tomato plants generally have reduced photosynthetic levels in comparison to their non infected counterparts, primarily due to a reduction in photosystem II (Bertamini *et al*., 2004). In the present work the reduction in photosynthetic pigments may be attributed to the infected action of virus on biosynthesis of pigments, increasing their degradation and/or maintaining damage of the chloroplast thylakoid could be a result from the mineral deficiency. In this respect El-Sawy (2009) found that the reduction in plant pigments concentrations may be due to decrease in absorption of some ions as Mg and Fe which were involved in chlorophyll biosynthesis under stress conditions . The observed reduction in Mg^{++} contents, which is needed for chlorophyll synthesis in infected tomato plants, in the present work, reinforced the view that TYLCV decreased chlorophyll biosynthesis. Moreover , the decrease in chlorophyll contents in infected tomato plants.

Carbohydrates which represent one of the main organic constituents of the dry matter, derived from photosynthesis, were found to be affected by infected stress, the obtained results showed that, infected tomato plant by TYLCV caused markedly decreases in soluble sugar, insoluble sugar and total carbohydrate contents in stem and leaves and these fraction increase in root system, when compared with reference control. Hemida (2005) found that bean yellow mosaic virus infected *Phaseolus vulgaris* plants caused the pigmentation and carbohydrates were parallel to each other, where they were decreased with time. Thus, in *Phaseolus vulgaris* synthesis of carbohydrates was completely associated with photosynthetic apparatus. Similar results obtained by Radwan *et al.* (2007) they reported that the content of chlorophyll pigments, soluble , insoluble sugars and total carbohydrates decrease by zucchini yellow mosaic virus (ZYMV) in *cucurbita pepo* leaves of infected plants.

It is worth to mention that, The reduction in soluble, insoluble and total carbohydrates of infected tomato plants by TYLCV virus concomitantly with decreased in growth rate and reduction in the leaf photosynthetic pigments and this occur either due to decreases in photosynthetic activity and/or increased in respiration (Singh 1973).

Concerning mineral ions content, the obtained results showed that TYLCV stress induced noticeable decrease in Mg^{+} ions in Leaves, stem and root system of TYLCV infected tomato plants when compared with healthy

plants. The data revealed also an increase in the contents of potassium and sodium ions in leaves, stem and root and another increases in phosphorus ion contents in leaves and roots although its decrease in stem. The decrease in total phosphorus percentage in diseased plant parts over healthy plant indicates that, in the infected plant the virus synthesis was at the expense of available phosphorus present in the plant (Singh and Mall 1974). Holden and Tracey (1948) also found decrease in total phosphorus content per plant in TMV infected tobacco plant. Increase of sodium level in tomato plants under stress condition is a defensive mechanism for plants in stress condition by controlling of osmotic pressure in cells, and absorption of water and nutrient solute from the soil.

The microtone and semithin sections of TYLCV infected tomato plants or castle rock leaves showed that, the upper and lower epidermal cells were nearly of the same size but the lower epidermal cells were deformed, the sub-epidermal paranchyma consists of one layer of compacted cells greater than these in healthy ones. The mesophyll tissue appeared rich in chloroplasts and batch of dark stained paranchyma and chlorenchyma cells contained several cavities. The upper epidermis is composed of tubular paranchyma cells covered by thin layer of cuticle compared with healthy ones. The results are in agreement with Eman shanwan (2010).

TYLCV infected tomato cells showed that, other cytoplasmic changes were the presence of large number of abnormal chloroplasts, mitochondria, nucleus and cellular abnormality described so far , is the presence of hyper trophied nuclei (Diener,1971).The chloroplast alternation in tomato leaf cells infected with TYLCV, showed destructed regions in chloroplast which does not organized into grana and thylakoid system. These results were in accordance with these obtained by Harvi (1980) and El-Dougdoug *et al* .(1993,1998 and 2002) they showed the cytological changes in the chloroplast which occurred in the infected cells as a result of infection with potato spindll tuber viroid (PSTVd) citrus exocortis viroid (CEVd) citrus cachexia viroid (Ccavd)and mango malformed caused (Viriod – like RNA).

Conclusion

From the results obtained in the present investigation, it could be concluded that, TYLCV infected tomato plants causes reduction in growth, photosynthetic pigments, carbohydrate contents, Mg^{++} ions and Increase of sodium level in plants at stress condition which consider a defensive mechanism that plants in stress condition by controlling of osmotic pressure in cells, and absorption of water and nutrient solute from the soil.

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(Received 19 May 2014; accepted 31 August 2014)