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MOLECULAR IDENTIFICATION OF SOME ISOLATES OF *TOMATO YELLOW LEAF CURL VIRUS* (TYLCV) AND THEIR CYTOLOGICAL EFFECTS IN INFECTING TOMATO (*SOLANUM LYCOPERSICUM* L.) PLANTS

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Abstract

The study was aimed to isolate and diagnose three different isolates (6, 7, and 13) of *Tomato yellow leaf curl virus* (TYLCV) using the polymerase chain reaction (PCR) technique. The TYLCV isolates were obtained from fields located in the provinces of Najaf and Karbala in Iraq. The nucleotide sequences of the PCR-amplified products (of approximately 789 base pairs (bp)) were determined to identify the genetic similarities and differences among the TYLCV isolates. The nucleotide sequences of the virus isolates were searched using Basic Local Alignment Search Tool (BLAST) that showed that all the isolates belonged to TYLCV. The whitefly (*Bemisia tabaci*) transmission of the TYLCV isolates 6 and 7 to the two cultivars (Marmande and Moneymaker) of tomato showed different effects in the temporal appreance and severity of the symptoms. The isolate 7 was more severe in symptom production than the isolate 6. The cytological effects of TYLCV-infected tomato plants were also recorded by examining the leaves and stems sections of the virus-infected and healthy tomato plants using the light microscope. The results revealed various histological abnormalities in the leaves and stems of the TYLCV-infected plants.

Key words : *Tomato yellow leaf curl virus,* cytological, tomato.

Introduction

Tomato (*Solanum lycopersicom* L.) is one of the important vegetable crops that ranks the second worldwide after the potato crop (Ajlan *et al.,* 2007 and FAOSTAT, 2015). Tomato fruits have high nutritional value containing some important minerals such as calcium, iron, phosphorus and some vitamins such as A and C, as well as fibers, protein, citric acid and sugars (Naika *et al.,* 2005; Ssekeyewa, 2006). They is also rich in carotenoids, especially lycopene, which is one of the strongest natural antioxidants that have an important role in preventing the growth of cancer cells such as lung cancer and prostate cancer in addition to delay the aging of body cells, in addition to its importance in protecting the heart from some diseases (Rao and 2000 and Leonardi *et al.,* 2000).

The expansion of the cultivation of this crop in greenhouses throughout the year led to the emergence of many pathogens, such as *Tomato yellow leaf curl virus* (TYLCV), restricted for the production due to the availability of suitable conditions for the spread and development of the disease during the year (Barone and Frusciante, 2007). TYLCV is one of the most common viruses in the world, including Iraq, causing economic losses of up to 100% in many economic crops, including tomato. YLCV is highly efficiently transmitted by the whitefly (*Bemisia tabaci*) in a persistent, non-propagative circulative manner (Pakkianathan *et al.,* 2015). The symptoms of the disease appear in the form of yellowing of the edges of the leaves and wrapped inward with severe wrinkling and dwarfism (El-Dougdoug, 2006). There are many techniques have been used to diagnose

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plant viruses, including polymerase chain reaction (PCR) (Cai *et al.*, 2014).

Therefore, the aim of this study was to isolate and diagnose different isolates of TYLCV using the PCR technology and to determine the nucleotide sequences of the PCR-amplified products in order to identify the genetic similarities and differences among the TYLCV isolates collected from some farms located in Najaf and Karbala provinces in Iraq. The cytological variations in the TYLCV-infected tomato plants were also investigated in the current studies.

Materials and Methods

Source of TYLCV isolates

Tomato plants, which exhibited viral infection symptoms of severe dwarfism, wrinkling and yellowing in the leaves of infected plants, were collected from some desert farms in Najaf and Karbala governorates during the agricultural season 2017-2018. All plants were planted separately according to the collection area in 21 x 21 cm plastic pots and grown in the greenhouse of the Faculty of Agriculture at the University of Karbala after being placed in wooden boxes with dimensions of 60 x 40 x 40 and covered with muslin cloth. Healthy tomato plants with 4-5 true leaves were placed in the same boxes every 2-3 weeks for transmitting by *B. tabaci* insects to maintain the virus isolates.

Diagnosis of TYLCV isolates using the Polymerase chain reaction (PCR)

Total genomic DNA was extracted from fresh leaves sampled from the tomato plants using the Geneaid Plant Mini Kit® (Cat. No: GP100), following the manufacturer's instructions. The quality and quantity of each extracted DNA sample was measured by a UV Spectrophotometer (Thermo Scientific, Germany). The quantified DNA samples were then stored at -20ºC, until use.

The DNA samples were PCR-amplified with the primer pair CP-F (-GAATTCATGTCGAAGCGWCCA) and CP-R (GAATTCTTAATTTK RTAYTGAAT CATAGAA) (Kim *et al.*, 2011). PCR amplification was performed using *Taq* DNA polymerase (Roche, Cat. No. 11 146 173 001) in a final volume of 20µl PCR reaction-mixture containing 1µl of each primer (10pmol), 2µ1 of 10X PCR buffer, 2µl dNTPs (2 mM), 3µl template DNA (30ng/µ1), and 1unit of *Taq* DNA polymerase. Thereafter, the sample volume was adjusted to 20µl with the nuclease-free water.

PCR amplification was performed using the following cycling parameters: initial denaturation at 94ºC for 1 min followed by 35 cycles each consisting of final denaturation at 94°C for 30 sec, annealing temperature at 62ºC for 30 sec, initial extension for 1 min, and final extension at 72°C for 5 min. PCR-amplified products were separately resolved on a 1% agarose gel for 140 min at 80V, 400mA and visualized with ethidium bromide staining under UV illumination. The gel images were captured using the gel documentation system (Vilber Lourmat, Taiwan).

Collection, rearing and identification of whiteflies

A number of adult whiteflies for TYLCV transmission were collected, using a hand aspirator, from the eggplant fields at the College of Agriculture, University of Kerbala, Iraq. The whiteflies were reared on healthy eggplant plants placed in 120 x 50 x 50 cm wooden boxes covered with two layers of muslin cloth. To confirm that the whiteflies were free of the virus, young and healthy tomato plants (cultivar Moneymaker) were placed in the wooden box that contained the healthy eggplant plants and whiteflies. The old eggplant plants were replenished with newly grown plants every 2-3 weeks.

The genomic DNA was extracted from five individual whitefly adults from each collected sample using the DNA extraction kit (Favorgen® Biotech, Cat. No: FAPGK 001) following the steps recommended by the manufacturer. PCR was done to amplify mitochondrial cytochrome oxidase I (mtCOI) gene using the primer pair CO1-F (TTGATTTTTGGTCATCCAGAAGT) and CO1-R (TCCAATGCACTAAT CTGCCATATTA;Frohlich *et al.*,1999). The thermocycling conditions were: 1 cycle of preheating for 5min at 95 C followed by 35 cycles each for 30sec at 94^0C, 45 sec at 52^0C and 1min at 72^0C, then followed by an extension at 72^0C for 5min. Amplified PCR products were checked by agarose gel electrophoresis and visualized by ethidium bromide staining under UV illumination. The size of the PCR product was checked by comparison with that of a 1Kbp DNA ladder marker (Promega, Cat. No. G5718).

Transmission of TYLCV by whiteflies

The vector transmission studies of the TYLCV isolates, collected from different areas in the Najaf and Kerbala provinces, were carried out using the whiteflies. The 300 adult non-viruliferous whiteflies, previously reared on the healthy plants of eggplant, were shifted by a hand aspirator to the tomato plants (cultivar Super Marmande) infected with a certain isolate of TYLCV and placed in 40 x 40 x 50 cm wooden boxes covered with two layers of muslin cloth. These whiteflies were allowed an acquisition access period of 48 hours. Afterwards, four tomato plants with 4-true leaves each were inoculated with 50 insects/ plant. After a 48-hour inoculation access period, all plants were sprayed with the insecticide Levo

2.4 SL (Oxymatrine 2.4 SL, Sineria©, Holland). After two hours of insecticide spray, the plants were moved into a shed made of two layers of muslin cloth and placed inside a greenhouse. A control treatment was carried out following the same steps described above except that the white flies were fed on healthy tomato plants (cultivar Marmande) grown in another shed in the same greenhouse. The plants were monitored daily to record the onset and development of pathological symptoms expressed by the individual viral isolates. All the laboratory and greenhouse studies were conducted in the Plant Virology Laboratory of Plant Protection Department at the College of Agriculture, University of Kerbala, Iraq.

Nucleotide sequence analysis of the TYLCV isolates

The nucleotide sequences of the PCR products (amplicons) amplified from the individual TYLCV isolates were generated using the forward and reverse primers used for PCR amplification, by the Microgen Biotechnology Company (South Korea). The identification of the studied viral isolates and the degree of similarity and difference in their nucleotide sequence from the reported TYLCV isolates was done by searching in the database of the National Biotechnology Information Center (NBIC) using Basic Local Alignment Search Tool (BLAST) software (Zheng *et al.,* 2000). The nucleotide sequences of the amplicons derived from the TYLCV isolates and the whiteflies were used to draw the phylogenetic tree using MEGA6 software (Tamura *et al.,* 2013).

The effects of TYLCV-infection on the anatomical structure of tomato leaves and stems

To determine the effect of TYLCV virus on the cellular and histological structure of the infected plant, a plant with PCR-confirmed TYLCV infection was examined in this study. The anatomical studies were conducted using a sharp and sterile scalpel and samples of leaf blade and stem of healthy and infected tomato plants with the same age and variety. The excised sections were cut into very thin slices under an optical microscope. The slices were placed on glass slides and were bleached to remove the chlorophyll pigment. The bleached slices were stained with a drop of 1% Safranin solution. The slides were examined to record the anatomical changes in the virus infected and non-infected tissues.

Results and Discussion

Molecular identification of different TYLCV isolates

The PCR amplification of TYLCV from the infected tomato plants using the degenerate primer pair CP-R and CP-F produced the amplicons of the expected size (~789 base pair (bp) from all the virus-infected tomato plants. The BLAST search and analysis of the nucleotide sequences generated from the amplicons produced from the virus-infected plants showed that all the viral isolates belonged to TYLCV. The results also revealed clear differences in the sequence of nitrogenous bases of the coat protein between some of these investigated viral isolates and the already reported TYLCV isolates registered in the NCBI database.

The TYLCV isolate 13 collected from a tomato plant gathered from the desert Farm of Kerbala showed a genetic similarity of about 100% in the sequence of nitrogenous bases with the isolate collected from the tomato plant gathered from a desert Farm of Najaf province. It was also observed that the similarities between the sequence of nitrogenous bases of the coat protein of Najaf isolate 6 and 7 and Kerbala isolate 13 ranged from 96-99%. It was also found from the neighbor-joining tree that isolates 6 and 7 appeared in separate clades from the clade of the other isolates in this study due to the genetic divergence between these isolates as shown in Fig. 1. Therefore, these isolates were selected in order to identify the effect of the genetic difference between these isolates on the nature and severity of the symptoms caused by their infection.

Results also revealed clear differences between the

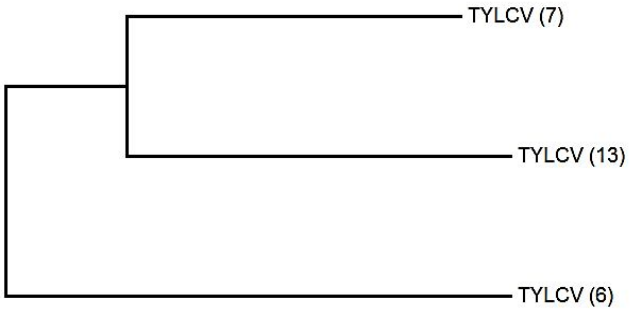


Fig. 1: Neighbor-joining tree showing the genetic relationship between Najaf TYLCV isolates 6 and 7 and Kerbala's TYLCV isolate 13.

nucleotide sequence of the coat protein of isolates 6 and 7 and the isolates previously deposited in the NCBI database. By comparing the sequences of nitrogenous bases of the coat protein of these isolates, differences in several regions were observed as shown in Fig. 2 and Fig. 3.

The neighbor-joining tree also showed that TYLCV isolate 6 appeared in the same clade with another TYLCV isolate (MF429946.1) which was previously isolated from Iraq and registered in the NCBI database. The similarity

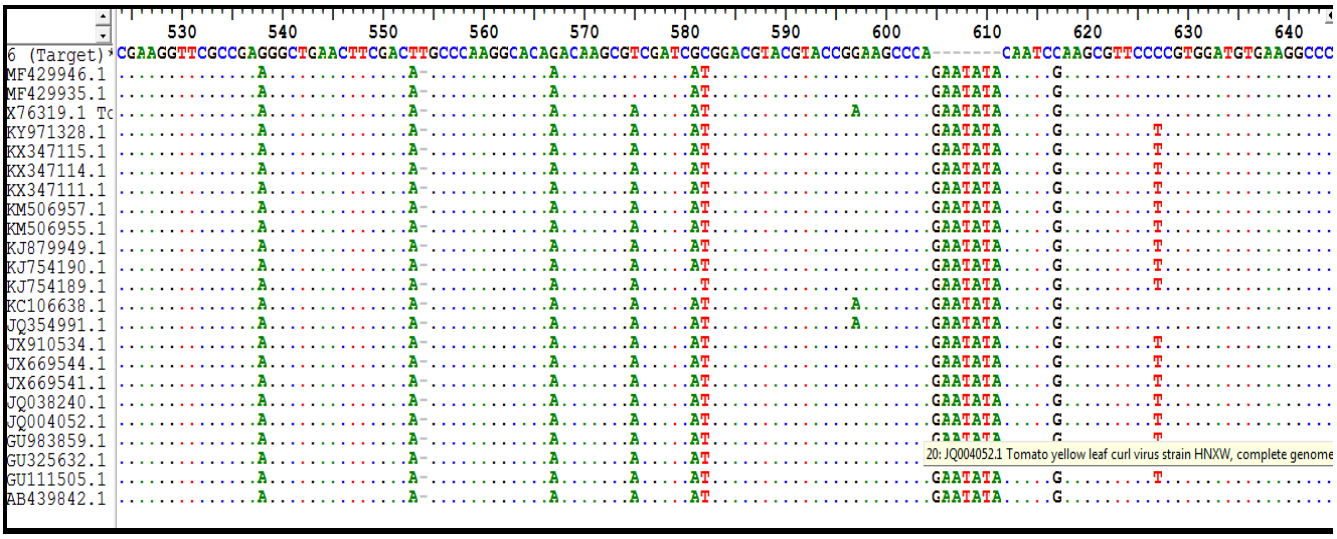


Fig. 2: Nucleotide sequence alignment of the studied TYLCV isolate 6 and other TYLCV isolates registered in the NCBI database. Identical bases are represented by dotted lines.

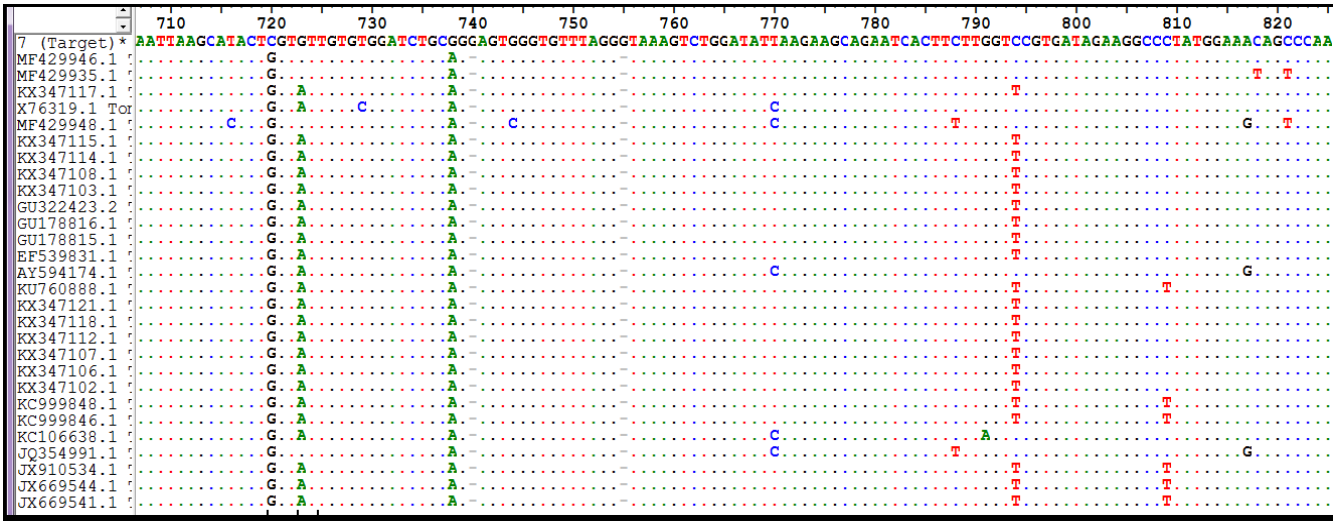


Fig. 3: Nucleotide sequence alignment of the studied TYLCV isolate 7 and other TYLCV isolates registered in the NCBI database. Identical bases are represented by dotted lines.

between the two isolates was found to be about 99% as shown in Fig. 4.

However, it is obvious from Fig. 5 that TYLCV isolate 7 appeared in a separate clade from the other TYLCV isolates registered in the NCBI database. This isolate was genetically closer to the TYLCV isolate (MF429946.1) in the NCBI database, with a similarity of 99% (see Figure 5). The other TYLCV isolates showed a similarity of 97%-98% with the TYLCV isolate 7.

Symptomatological observations for the TYLCV isolates 6 and 7

The results of inoculation of two different cultivars of tomato plants (*i.e.*, Super Marmande and Moneymaker) by the viruliferous whiteflies carrying either TYLCV isolate 6 or 7; showed that these isolates had varying effects on the onset of symptoms as well as their type and severity. For instance, inoculation of tomato plants of cultivar Super Marmande with the virus isolate 6 led to the delayed emergence of the symptoms (22 days post inoculation (dpi)). The symptoms appeared in the form of obvious yellowing of the veins of the upper leaves of the plants and were less severe in the middle and lower leaves of the same plant. Other symptoms such as small leaf size, short internodes and plant dwarfing were also observed as shown in Fig. 6. However, in case of inoculation to the Moneymaker tomato plants, the symptoms were early in appearence (18 dpi) in the upper leaves of the inoculated plants. Firstly the expression was as a slight crease in the edges of the top leaves, which gradually extended to the bottom leaves to include all the leaves of the plant. Generally, the symptoms were less severe than those seen on the tomato variety Super

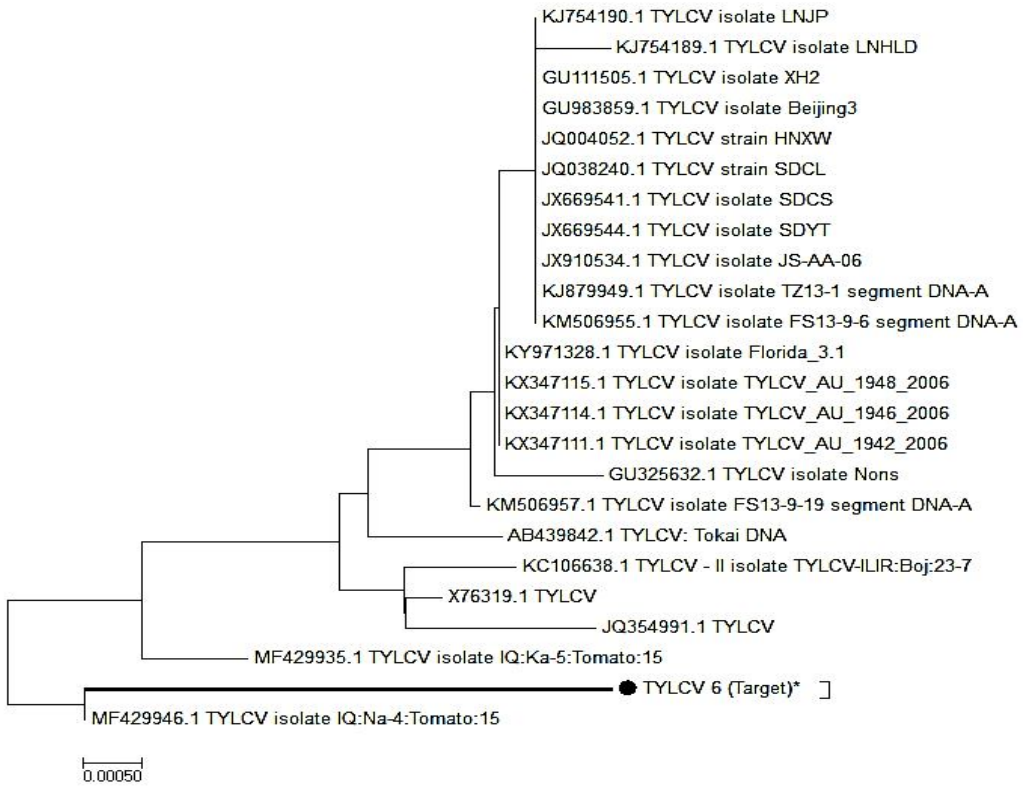
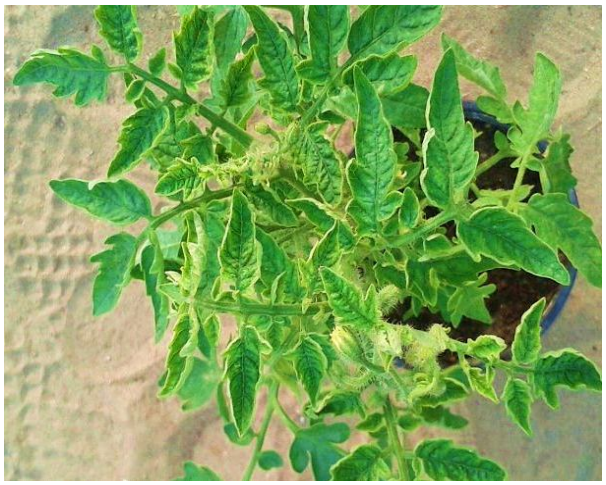


Fig. 4: Neighbor-joining tree showing the genetic relationship between Najaf TYLCV isolate 6 and other TYLCV isolates registered in the NCBI database.

Fig. 5: Neighbor-joining tree showing the genetic relationship between Najaf TYLCV isolate 7 and other TYLCV isolates in the NCBI.



Tomato plant (cultivar Super Marmande) infected with TYLCV isolate 6

Tomato plant (cultivar Moneymaker) infected with TYLCV isolate 6.

Tomato plant (cultivar Super Marmande) infected with TYLCV isolate 7

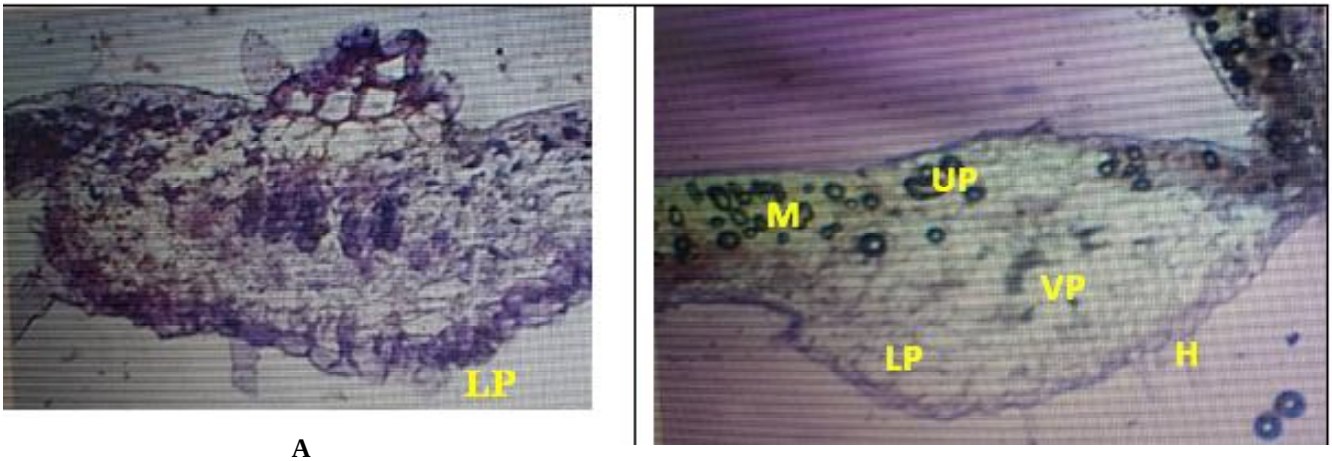
Tomato plant (cultivar Moneymaker) infected with TYLCV isolate 7.

tomato plant (cultivar Super Marmande)-uninfected with TYLCV.

tomato plant (cultivar Moneymaker)-uninfected with TYLCV.

Fig. 6: TYLCV-induced symptoms in the cultivars Super Marmande and Moneymaker of tomato at 60 dpi resulting from the infection of TYLCV isolates 6 and 7.

Fig. 7: PCR-Amplified DNA of TYLCV from infected Super Marmande and Moneymaker tomato plants. 1, 2, 3, and 4: PCR products from Super Marmande and Moneymaker tomato plants infected with TYLCV isolate 6. 5, 6, 7, and 8: PCR products from Super Marmande and Moneymaker tomato plants infected with TYLCV isolate 7. NC: Negative control without addition of DNA to PCR reaction mix. +PCR: PCR product from a tomato plant infected with TYLCV. M: 1Kbp DNA ladder marker with base pair sizes listed on the left of the figure.



A
Cross section of a leaf from a healthy tomato plant.

B
Cross section of a leaf from a tomato plant infected with TYLCV.

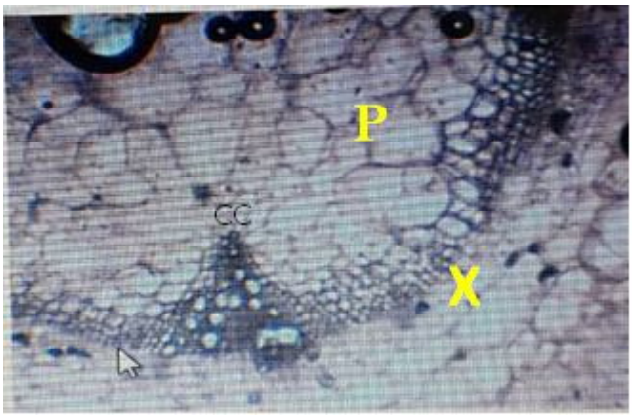
Fig. 8: Cross section of a healthy tomato leaf (A) and TYLCV-infected tomato leaf (B) using an optical microscope at a magnification of 400X.

Marmande when inoculated with the same viral isolate (*i.e.*, isolate 6).

It was also observed that inoculation of Super Marmande tomato plants with TYLCV isolate 7 caused the early emergence of the symptoms (15 days after inoculation (dpi)). The symptoms were represented by yellow and folded edges of the upper leaves of the infected plant. Later, the symptoms developed to include all the leaves of the plant with a clear yellow inter-veinal discoloration of the affected leaves. Furthermore, the symptoms included short internodes, plant dwarfing, and reduced size and number of the leaves which affected negatively on flowering of the infected plant compared to the uninfected plants. It was also obvious that the symptoms caused by infection with the TYLCV isolate 7 were more severe than those resulting from TYLCV isolate 6 as shown in Fig. 6. The presence of TYLCV in these infected and symptomatic plants was confirmed through PCR amplification of TYLCV, using the primer pair (CP-F and CP-R) targeting the TYLCV coat protein as depicted in Fig. 7.

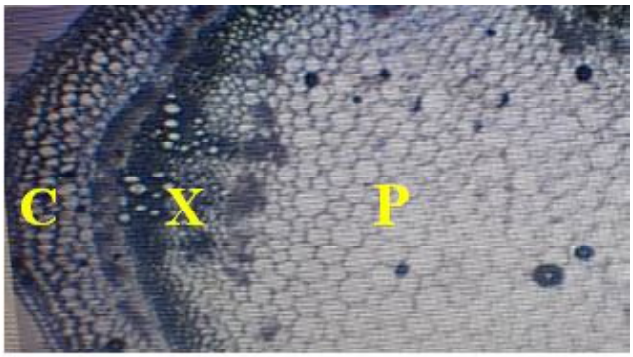
The effects of TYLCV-infection on the anatomical structure of tomato leaves and stems

The microscopic examination of cross sections of the leaves from the TYLCV infected tomato plants showed the presence of atrophy or weakness in the growth of tissue cells of the leaf. In the infected plant (B), there was a clear reduction in the number of xylem and phloem vessels which were almost unable to be seen compared to those in the uninfected plant (A). The results also showed that the capillaries (H) in the leaves of the uninfected plant (A) were bigger and wider than those observed in the infected plant (B). A significant effect was also observed in the upper (UP) and lower (LP) epidermal cells and other tissues in the leaf (M) as shown in Fig. 8.



A

Cross section of a stem from a healthy tomato.



B

Cross section of a stem from tomato plant infected with TYLCV.

Fig. 9: Cross section of a healthy tomato stem (A) and TYLCV-infected tomato stem (B) using an optical microscope at a magnification of 400X.

Microscopic examination of some cross sections of the stem of a tomato plant infected with the virus, revealed significant atrophy of the xylem vessels (X). Besides, the vessels were scattered compared to uninfected plants in which the vessels were aligned and arranged. Pith and cortex cells were found to be smaller in infected plants than in the healthy plants as shown in Fig. 9.

Similar internal symptoms were recorded in the plants infected with other plant viruses such as in the case of citrus trees infected with *Citrus psorosis ophiovirus* (CPsV) and potato plants infected with *Alfalfa mosaic virus* (AMV) that led to the atrophy and change in the size and shape of the leaves of infected plants as well as reduction in the number and size of xylem and phloem vessels as compared to the healthy plants (Sofy *et al.*, 2007 and El-Abhar, *et al.*, 2018). Badr, *et al.*, (2014) mentioned that *Bean common mosaic virus* has a significant effect on leaf cells as well as the vessels in the stem and leaves.

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