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Characterization and Molecular Detection of *Tomato spotted wilt Tospovirus* Infecting Tomato in Egypt

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Tomato spotted wilt virus was originally isolated from naturally infected tomato fruits. The virus infected a wide host range showing different symptoms varied from local necrosis, systemic necrosis, systemic mosaic, yellowing, bronzing or stunting. The virus transmitted by mechanical means as well as by *Thrips tabaci*. Its TIP was between 45-50 °C, DEP was 10^{-3} and LIV was between 5-6 hrs. Local lesion technique for biological purification and electron microscopic analysis confirmed the production of mature, infectious virus particles, underlining the conclusion that a full infection cycle was completed in this system. Both the structural viral proteins nucleoprotein (N) and the envelope glycoproteins G1 and G2 and the nonstructural viral proteins NSs and NSm were accumulated to amounts sufficient for detection and cytopathological analysis. Electron micrograph of the virus showed that it is quasi-spherical and its diameter ranges from 80 to 100 nm. The virus caused thickening of the cell wall and changes in the chloroplast structure. Virus identification was confirmed by dot-blot immunoassays. RT-PCR assays using primers complementary to the nucleocapsid protein gene (NPs) were used to detect two isolates of TSWV from *Lycopersicon esculentum* and *Physalis peruviana* plants. Total nucleic acids were reverse transcribed using Retrotool reverse transcriptase enzyme and the PCR reactions were performed for 30 min in a capillary thermal-cycler. RFLP analysis of the PCR products was performed using *Mse*I restriction enzyme. The results showed a dimorphic restriction digestion profile that was suitable for identifying the two isolates. The method described here is rapid, reliable and highly recommended to be used in such virological and pathological studies

INTRODUCTION

Tospoviruses are "emerging" viruses not only in the sense of their increasing economic importance as pathogens on a worldwide basis, but also in that our understanding of the complexity of this new viral taxon only began to emerge during the last decade. However, diseases attributed to *Tomato spotted wilt virus* (TSWV), the type species for the *Tospovirus*, was first reported in Australia about 1915. Prior to 1990, TSWV was considered a monotypic group of plant viruses (Moyer, 1999). Tospoviruses are the only viruses in that family that infect plants. The genus *Tospovirus* contains the plant-infecting members of the family *Bunyaviridae*, a large group of enveloped viruses with tripartite genomes of negative and ambisense RNA (Elliott, 1990).

Tomato spotted wilt virus (TSWV), the type member of this genus (German *et al.* 1992), replicates in both plant hosts and thrips (*Thysanoptera: Thripidae*), its exclusive insect vector (Ullman *et al.*, 1993; and Wijkamp *et al.*, 1993). During the 1980's TSWV caused significant losses on peanuts, tobacco and tomatoes in the Southeastern United States (Moyer, 1999). It has also caused significant losses on these crops in Eastern Europe and South

America. In recent years, *Tomato spotted wilt virus* has caused crop losses on a wide variety of greenhouse vegetable and ornamental plants across the United States and Canada (Zitter and Daughtrey, 1989). Vegetatively propagated ornamentals are the most likely source for a TSWV infestation since the virus is not seed borne (Zitter and Daughtrey, 1989). Four species are considered the most important vectors because of their wide distribution and the overlapping host ranges of these species and TSWV; *Frankliniella occidentalis* (western flower thrips); *F. schultzei, F. fusca* (tobacco thrips); *and Thrips tabaci* (onion thrips) (Moyer, 2000). The western flower thrips is the chief TSWV vector in greenhouse settings around the world (Quintanilla, 1980; Davis *et al.*, 1990; and Peters *et al.*, 1991) *T. tabaci is* widely distributed in tropical, warm, and cool temperate areas around the world. *F. occidentalis* and *F. fusca* both occur in the United States. Mexico, and as far north as Canada.

The host range of TSWV includes more than 400 plant species, both monocots and dicots, in 50 families (Cho *et al.* 1987 and Peters *et al.* 1991). Virus spread is rapid in a greenhouse with a western flower thrips population. Some plants develop symptoms within 5 days of feeding by infected thrips. The natural occurrence of TSWV in the field is not well documented in Egypt; However, it is a serious problem in many southern states (Law and Moyer, 1990; German *et al.*, 1992).

TSWV virions contain two glycosylated membrane proteins (G1 and G2), a putative RNA-dependent-RNA polymerase (RdRp) protein (L) and the nucleocapsid protein (N), which encapsidates the small (S), medium (M) and large (L) genomic RNAs (Moya *et al.*, 2000).

Due to the wide host range of the virus and vector and lack of effective management strategies which might lead to major worldwide economic losses, an early and accurate detection of infected plants is discussed in this study as critical steps for disease control.

MATERIALS AND METHODS

Virus Isolation and identification

Tomato fruits showing symptoms suspected to be due to TSWV infection were collected from the farm of the Faculty of Agriculture, Ain shams University, Kalubia Governorate and used to inoculate *Nicotiana rustica* L. plants. The infected leaves (lyophilized) were kindly provided by Microbiology Department, Faculty of Agriculture, Ain shams University. The virus was maintained in *Nicotiana rustica* L. plants in greenhouse at 25-35 °C. Typical ringspot symptoms of TSWV appeared on *Nicotiana rustica* L. grown in the greenhouse, Agricultural Research Center (ARC), were mechanically transferred onto *Lycopersicon esculentum* Mil plants. The virus was purified biologically through two consecutive passages onto the local lesion host, *Petunia hybrida* Vilm, followed by one passage on *Nicotiana rustica* L. The resulting local concentric ringspots were singly back inoculated onto *Nicotiana rustica*. Finally, the systemic necrosis appears on the newly formed leaves were mechanically transferred onto *Lycopersicon esculentum* Mill plants for virus propagation. The virus was identified on the basis of host range studies, symptomatology, mode of transmission and stability in the infectious sap. Virus identification

was insured by serological tests, electron microscopy and some molecular studies.

In addition, two isolates of TSWV infecting *Lycopersicon esculentum* Mill and *Physalis peruviana* Ryd6 were identified by restriction digestion of RT-PCR products.

Identification of the present virus was carried out as follows:

I: Biological studies

A. Transmission

1-Mechanical transmission

About 15 tomato plants were mechanically inoculated with the virus under study and five plants were maintained as control without inoculation. To test the possibility of mechanical transmission of TSWV, infected *Lycopersicon esculentum* Mill plants were washed and frozen for 24 hours. "Solvent 4" pH 7.2 buffer (0.1 M Na_2HPO_4-NaH_2PO_4, pH 8.3, 0.02 M of Na_2SO_3 and 0.02 M EDTA) as described by Sayed *et al.* (2003) was used for mechanical transmission studies. The extract was filtered through cheesecloth and the tested tomato plants of 40-50 day old dusted with 600-mesh carborundum were inoculated with infectious leaf extract diluted with the buffer (1:1, v/v).

2-Insect transmission (Thrips transmission)

Virus-free colony of specific vector (*Thrips tabaci*) raised on onion plants were used in transmission tests. The differential ability of larval and adult *Thrips tabaci* to acquire and transmit TSWV was tested by allowing 1-day-old adults to feed for 24 hr on 15 infected tomato plants placed in isolated cage covered with organza cloth. The cage was kept outside the greenhouse during the time of the experiment. Insects were removed and transferred for 48 hr onto 10 healthy tomato plants using about 30 insects per plant. Plants were kept under glass lantern covered from above with cheesecloth tight with rubber band. The plants were maintained in the isolated cage for symptoms development. The virus free insects were maintained on five healthy tomato plants as control.

B: Host range

Ten seedlings from each of 24 different host plants belonging to 11 families were mechanically inoculated with sap extracted from infected *Lycopersicon esculentum* Mill leaves and maintained in the isolated cage for early symptoms development. The symptoms produced and the incubation periods of the virus in the host were studied. Non-inoculated plants were left as a control. Plants showed no symptoms were checked by back inoculation to the indicator host plant or by DBIA and / or ELISA using TSWV polyclonal antiserum (TSWV-AS) (Kindly provided by Prof. Dr. H.S. Savithri, Dept of Biochemistry, Indian Institute of science, India.

C: Virus stability

Stability in infectious sap

Dilution end point (DEP), thermal inactivation point (TIP), and longevity (LIV) of the virus isolate were determined according to Noordam (1973).

II. Serological studies

a) Dot blotting immunobinding assay (DBIA)

DBIA test, described by de Ávila *et al.* (1990), was used to detect the

virus presence in tested hosts as well as in determining serologic relationships between its isolates.

b) Direct ELISA

Direct ELISA test was performed as described by Clark and Adams (1977) to test the presence of TSWV in mechanically infected host plants.

III. Electron Microscopy

1- Partial Purification of the Virus

About 100 gm of virus-diseased leaves of *N. rustica* L. of 21-30 day old mechanically inoculated with TSWV and showing typical symptoms were macerated in a mortar with an equal volume of solvent 4 pH 7.2 buffer (0.1 M Na_2HPO_4-NaH_2PO_4, pH 8.3, 0.02 M of Na_2SO_3 and 0.02 M EDTA). The extract was filtered through cheesecloth and centrifuged for 20-30 min at 3000-5000 rpm in SiGmA 2K15 centrifuge. The supernatant was concentrated by centrifugation at 30000 rpm for 120 min. The pellets were resuspended in an amount of 0.01M Na_2SO3 equal to 1/10 of the original weight of tissue (Black *et al.*, 1963).

2- Electron Microscopic Examination

Electron microscopic examination of the partially purified suspension of the TSWV were negatively stained with 2% phosphtungestic acid (PTA), pH 7.0, as described by Walkey (1991). Ultrathin sections were made in parts of *Nicotiana rustica* leaves showing light green to yellow areas and dark green areas. The sections were prepared following the method of Lin and Langenberg (1983). Examination was carried out using electron microscope (Philips E.M.401 in the EM unit of Ain Shams University Specialized Hospital) at magnifications of X7000 to X36000.

IV. Molecular studies

1-Primer selection

Molecular characterization was done using RT-PCR. Using primers 5' *TTAAGCAAGTTCTGTGAG* 3' and 5' *ATGTCTAAGGTTAAGCTC* 3' specific to the nucleocapsid (N) protein gene of TSWV, the N protein gene was amplified by RT-PCR (Jain *et al.*, 1998, and Pappu *et al.*, 1998). The oligonucleotide primers were from Metabion GmbH (Lena-Christ-Strasse 44, D-82125 Martinsried / Deutscland).

2-Extraction of total nucleic acid

Total RNA was extracted from symptomatic leaves of *Lycopersicon esculentum, Pelargonium sp., Nicotiana rustica and Datura metel* using *High Pure RNA Tissue Kit* (Roch Molecular Biochemicals) Cat No. 2033674.

3-RT-PCR

A modification of the method of Boonham *et al.* (2001) was used for synthesizing complementary DNA strand by adding 7 µl of total nucleic acids primed with 50 pmol of forward primer TSWV1 *(5'-ATGTCTAAGGTTAAGCTC 3')* in a total volume 20 µl containing 4 µl of 5x RT buffer (Biotools, Biotechnological & Medical Laboratories, S.A. Madrid, Spain), 1 µl of 10 mM dNTPs, 1 µl of enhancing buffer, and 1.5 µl of Retrotools Reverse Transcriptase (Biotools, Biotechnological & Medical Laboratories, S.A. Madrid, Spain). The reaction was performed at 70 °C for 45-60 min. For PCR, 50 pmole of each amplification primer TSW1 (forward primer) *(5' ATGTCTAAGGTTAAGCTC-3'* and

TSW2 (reverse primer) *(5' TTAAGCAAGTTCTGTGAG-3')* (Jain *et al*., 1998) were selected based on TSWV nucleocapsid (N) protein gene, located on the S-RNA (Jan *et al*., 2000). 5 µl of each cDNA reaction, and 5 U/µl of High Expand Fidelity DNA polymerase (Roche) were used in a 5x Standard DNA buffer containing 20 mM Tris HCl, pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2$ SO_4, 2 mM $MnCl_2$, 0.1% Triton X-100 and 10 µg/ml of nuclease-free BSA. The amplification reaction was carried out in a total volume of 50 µl using the capillary PCR system from IDAHO technology and using 0.2 ml micro Amp PCR tubes. Hard denaturation of the DNA was performed at 95 °C for 1 min followed by 35 cycles of amplification with denaturation at 94 °C for 15 sec, annealing at 45 °C for 30 sec, and extension at 72 °C for 15 sec. A single tailing cycle of long extension at 72 °C for 45 sec was carried out in order to ensure flush ends on the DNA molecules. The PCR products were analyzed by electrophoresis onto 1.0% agarose gels and the size of the DNA fragments were determined in accordance with molecular weight markers. A fragment of 777 bp was amplified and cloned directly into pGEM-T-Easy cloning vector (Promega, Madison, WI).

4-RFLP analysis of RT-PCR products

Ten µl of amplified products were restricted with 5 units of *MSe*I restriction endonuclease (Life Technologies Inc.) according to Finetti *et al.* (2002). Reactions were done following manufacturer's instructions (Roche) and digested products were analysed by electrophoresis in 2% agarose gel in TAE.

RESULTS AND DISCUSSION

1-Biological studies

A-Transmission studies

1-Mechanical transmission

TSWV was transmitted easily to tomato seedlings in the greenhouse. Symptoms appeared 10-21 days post inoculation. Inoculated plants showed chlorotic and necrotic ringspots followed by systemic mosaic yellowing, bronzing or stunting on the successive leaves (Fig.1).

2-Insect transmission (Thrips transmission)

Thrips tabaci insects were able to transmit TSWV from infected to healthy tomato seedlings. Infected seedlings showed ringspot symptoms after one month from inoculation. The results were verified with DBIA analysis.

B- Host range

Based on the physical properties, biological properties, serology, and molecular detection, the virus under study is, indeed, an Egyptian isolate of *Tomato spotted wilt virus* (TSWV). During our examination for host range (Table 1) and Fig. (1), it was found that TSWV had a wide host range. Different host plant species, varieties and cultivars were tested for symptoms expression. However, 16 hosts were selected to be tested using DBIA, and ELISA testes and listed in Table (1). The induced symptoms on the tested hosts ranged between mosaic, mottling, chlorotic spots, necrotic lesions, necrotic ring spots, concentric necrotic rings, yellowing or bronzing of leaves, and leaf drop or wilting. Leaf distortion and plant stunting were also observed. This is in accordance with De Angelis

et al. (1994) and Adam and Kegler (1994) who reported that *Tomato spotted wilt virus* has one of the widest host ranges of any known plant virus. Hean (1940) and Best (1968) stated that visible local symptoms usually consisted of chlorotic or necrotic lesions. Systemic symptoms were generally more severe, including mosaic, mottling, chlorotic or necrotic ring spots, leaf deformation and stunting. These statements are in accordance with our findings (Fig.1, and Table1). Symptom expression may be variable on the same host according to the age of infected plants, the level of nutrition and particularly with environmental conditions, especially temperature. In some hosts symptoms of infection may disappear under hot or cold conditions even though the virus is still present in the host.

Table (1): Host range of *Tomato spotted wilt virus* (Tospovirus) tested by mechanical inoculation.

Test plant	*Family*	*Common name*	Observed Symptoms	Serological tests DBIA	ELISA
Antirrhinum majus	Scrophulariaceae	Great Snapdragon, Dragon's mouth	NLL, NR	+	+
Apium graveolens L.	Apiaceae	Celery	NLL	+	+
Arachis hypogaea L.	Fabaceae	Earth nut, Ground nut, Pea nut	CR	+	+
Capsicum annuum L. cv. California Wander	Solanaceae	Red pepper, Guinea pepper	CR	+	+
Chenopodium murale L.	Chenopodiaceae	Wall goose-foot	NLL	NT	NT
Convolvulus arvensis L.	Convolvulceae	Lesser birdweed, Corn blind, Corn lily	NLL, NR	NT	NT
Datura metel L.	Solanaceae	Downy thorn-apple, Metel	NLL, CN	+	+
Datura stramonium L.	Solanaceae	Thorn-apple	NR	+	+
Eruca sativa	Brassicaceae	Rocket	NLL	NT	NT
Cucurbita pepo L.	Cuccurbitaceae	Vegetable-marrow	SYM	-	-
Lactuca sativa L.	Compositae	Lettuce	LD	+	+
Lupinus termis L.	Leguminosae	Egyptian lupine	NLL, NR	NT	NT
Lycopersicon esculentum Mill. Cv. Money Maker	Solanaceae	Tomato	BR, R, ST, B	+	+
Medicago sativa L.	Fabaceae (Papilionaceae)	Lucerne, Alfalfa	NLL, NR	+	+
Nicotiana glutinosa L.	*Solanaceae*	Tobacco	NLL, CN, SN	+	+
Nicotiana rustica L.	Solanaceae	Wild tobacco	NLL, NR, SN	+	+
Nicotiana tabacum cv. *kentuckey*	Solanaceae	Tobacco	NLL, CN	NT	NT
Nicotiana tabacum cv. *white barley*	Solanaceae	Tobacco	NLL, CN	+	+
Pelargonium sp	Geraniaceae	Geranium	CLL, YW	+	+
Petunia hybrida Vilm.	Solanaceae	Garden Petumia	NLL, CN	+	+
Phaseolus vulgaris L. cv. Giza 6	Fabaceae	French bean	NLL, NR	NT	NT
Physalis peruviana L.	Solanaceae	Cape gooseberry, "Harankash"	M., M.	+	+
Pisum sativum L. cv. Little Marvel	Fabaceae	Garden pea	NLL, NR	NT	NT
Vicia faba L. cv. Giza 2	Fabaceae	Horse (Field) bean	NLL	NT	NT

B= blistering, BR= bronzing, CL= chlorosis, M, M= mosaic and mottling, CN= concentric necrotic rings, CR= chlorotic ring spot, NLL= necrotic local lesion, NR= necrotic rings, R= rolling, SN= systemic necrosis, ST= stunting, LD= leaf discoloration, SYM= symptomless infection.

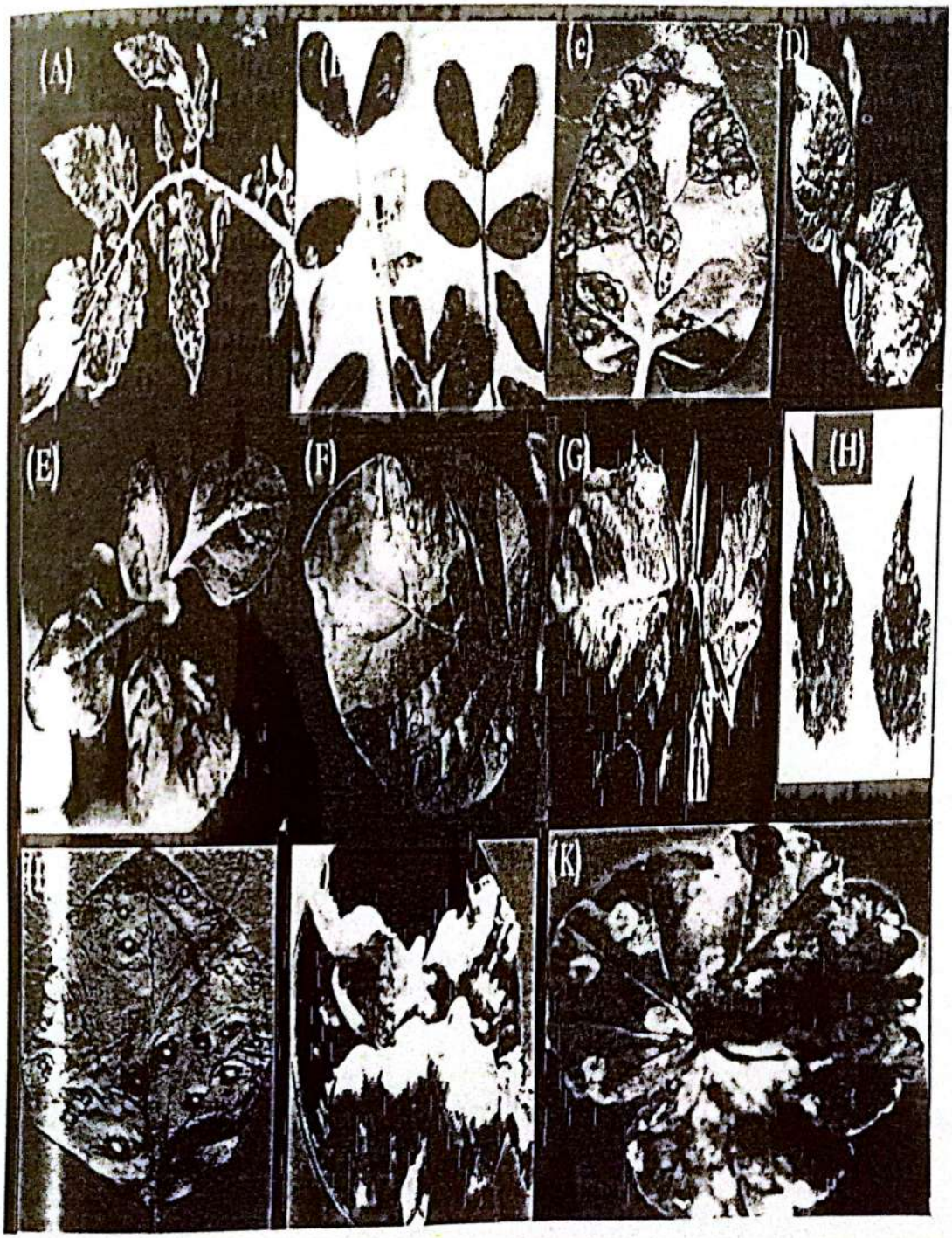

Fig.1. (A): *Lycopersicon esculentum* plants showing thickening of young leaves especially the veins and the newly formed leaves start bronzing accompanied by wrinkling of leaves. (B): *Arachis hypogaea* leaves showing chlorotic ring spot. (C): *Nicotiana glutinosa* leaf showing local lesions which gradually increase in size forming spots of concentric necrotic zones. (D) *Datura metel* leaf showing necrotic local lesions developed into concentric necrotic rings. (E): *Petunia x hybrida* plant showing necrotic local lesions. (F): Nicotiana tabacum cv. Kentuckey leaves showing necrotic local lesions (G): *Datura stramonium* leaves showing necrotic rings. (H): *Physalis peruviana* showing mosaic and mottling. (I): *Nicotiana tabacum cv. White Barley* showing necrotic local lesions. (J): *Lactuca sativa* plant showing light marginal wilting and leaf discoloration, the plants grow from one side. (K): *Pelargonium sp.* Leaf showing chlorotic local lesions.

C- Virus stability

Our results indicated that the virus is sap transmissible. Its thermal inactivation point is 45-50 °C; the dilution endpoint is 10^{-3} and the virus was completely inactivated after incubation for 5-6 hours at room temperature (Table 2). Our results are in accordance with Best (1946); Da Graca *et al.* (1985); Adam and Kegler (1994) and Alkhazindar (1999).

Table (2): Stability of TSWV in crude sap.

TIP	DEP	LIV
45-50 °C	10^{-3}	5-6 hrs

II- Serological studies

DBIA results showed cross reactivities between TSWV-AS (India) upon reaction with serially diluted crude sap preparations of TSWV from 16-tested host (Fig. 2).

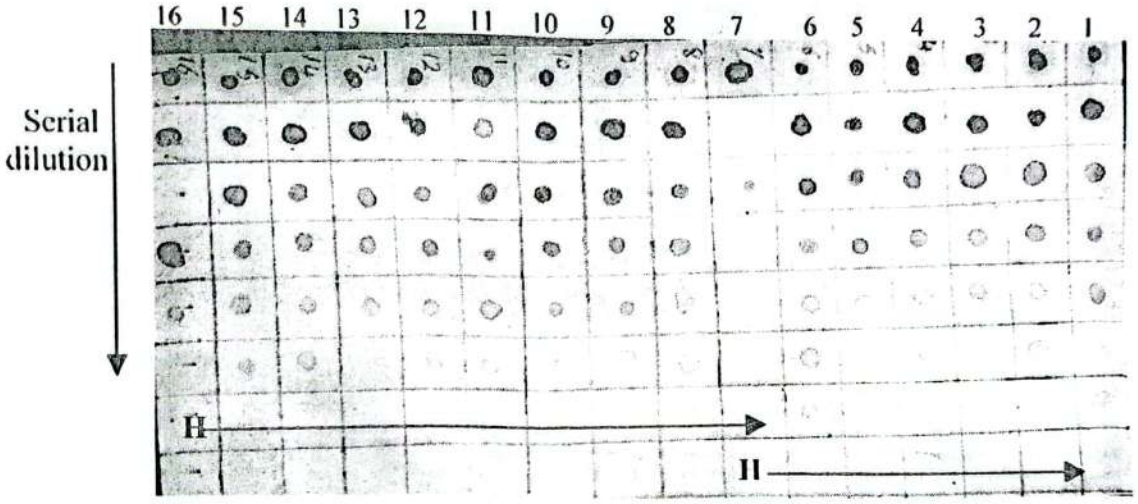

Fig. (2): DBIA showing the cross reactivities between TSWV-AS (India) upon reaction with crud sap preparations serially diluted from 16 different host ranges. Including *Lycopersicon esculentum* (1), *Pelaronium sp*, (2) and *Physalis peruviana* (3) H: Healthy plants showing No signals.

III- Electron microscopy

Electron microscopic examination of negatively stained partially purified preparations of the virus under test with 2% PTA (Fig. 3) revealed the presence of quasi-spherical particles ranging from 80-100 nm in diameter. The result is in agreement with Black *et al.* (1963); Mohamed and Randles (1972); Honda *et al.* (1989) Adam *et al.* (1990) and De Angelis *et al.* (1994).Electron microscopic examination of TSWV-diseased mesophyll of *Nicotiana rustica* revealed that virus-induced inclusion consists of elongated rigid filamentous material and/or large inclusions of elongated tubular structures arranged in loose bundles and appear in the cytoplasm between some clusters of virus particles and groups of dense masses. The NSs protein accumulates in long tubular structures that may associate as loose bundles. The origin of the filamentous structures or fibrous inclusions is unknown but they did not seem to be derived

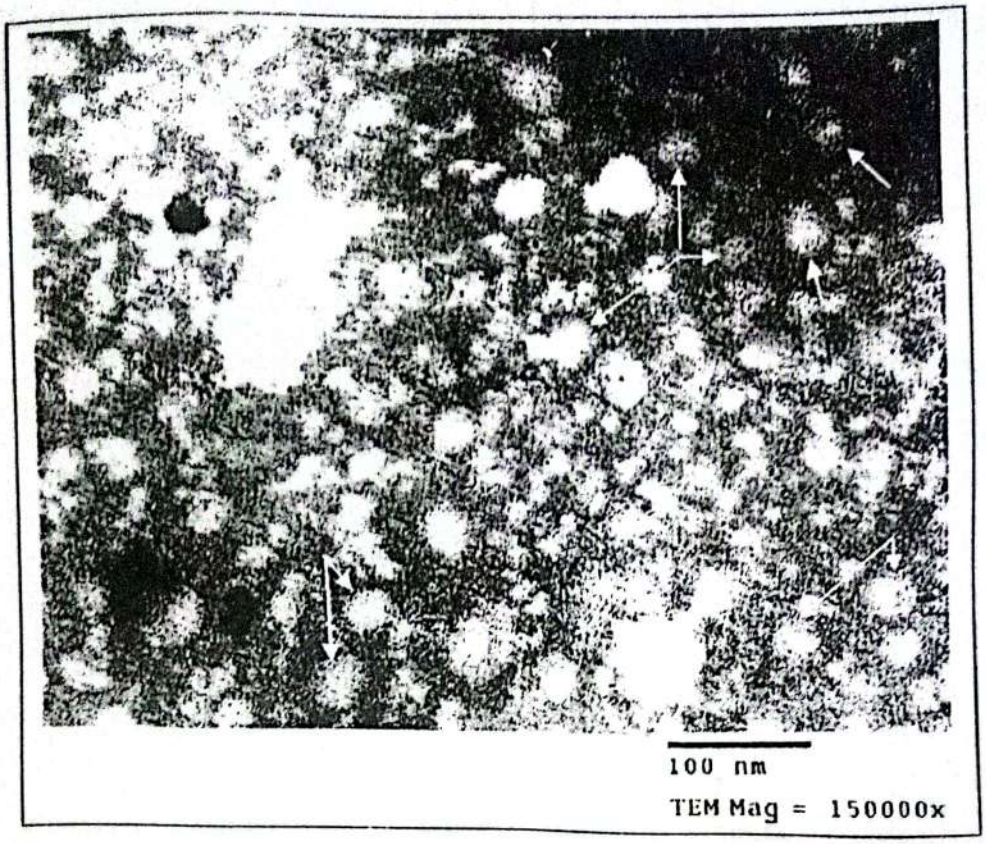

Fig. (3). Electron micrograph of partially purified virus preparation stained with phosphtungestic acid (PTA). Magnification power (X) = 150000

from Golgi apparatus, endoplasmic reticulum or other unusual cell membranes (Milne 1970). NSm protein is detected in granular electron-dense inclusions. Also excess N protein is observed as electron-dense, granular material in the cytoplasm (Fig. 4). These masses have rugged outline and are usually somewhat larger in size than the inner diameter of the virus particles. It was designated as viroplasm which appear in the cytoplasm. Its structure, composition and function are unknown but it may consist of ribonucleoprotein and is probably involved in the early stages of forming virus particles (Milne 1970). In 1971, Ie stated that the dense masses have been shown to be proteinaceous. The presence of the dense masses in the infected cells (Fig. 4) constitutes an additional reliable characteristic of TSWV infection (Peters *et al.* 1991). In agreement with our findings of the previously mentioned two types of inclusions had been found by Kitajima (1965). Francki and Grivell (1970). Milne (1970). Law and Moyer (1990). Peters *et al.*(1991). De Avila *et al.* (1991). Urban *et al.* (1991). Kitajima *et al.* (1992). Adam and Kegler (1994). Rudzinska and Kaminska (1997). during their studies on *Tomato spotted wilt virus* infected many different hosts other than tomato plants. Mitochondria and nuclei were nearly absent from the cells. Generalized plasmolysis was evident in most of the cells with thickened cell wall (Fig. 5) Ultrastructural electron microscopic observations of TSWV-diseased mesophyll showed the accumulation of *Tomato spotted wilt virus* particles in few aggregates in the cytoplasm (Fig. 6)

Virus particles observed in ultrathin sections of infected cells are usually similar in structure to those found in partially purified preparations (Kitajima, 1965, Milne, 1970; Francki

and Grivell, 1970; Ie, 1971; Paliwal, 1976 and De Avila *et al.* 1991). The infected chloroplasts appeared numerous in numbers with membrane damage, degeneration of the grana and disorganization of the internal lamellae

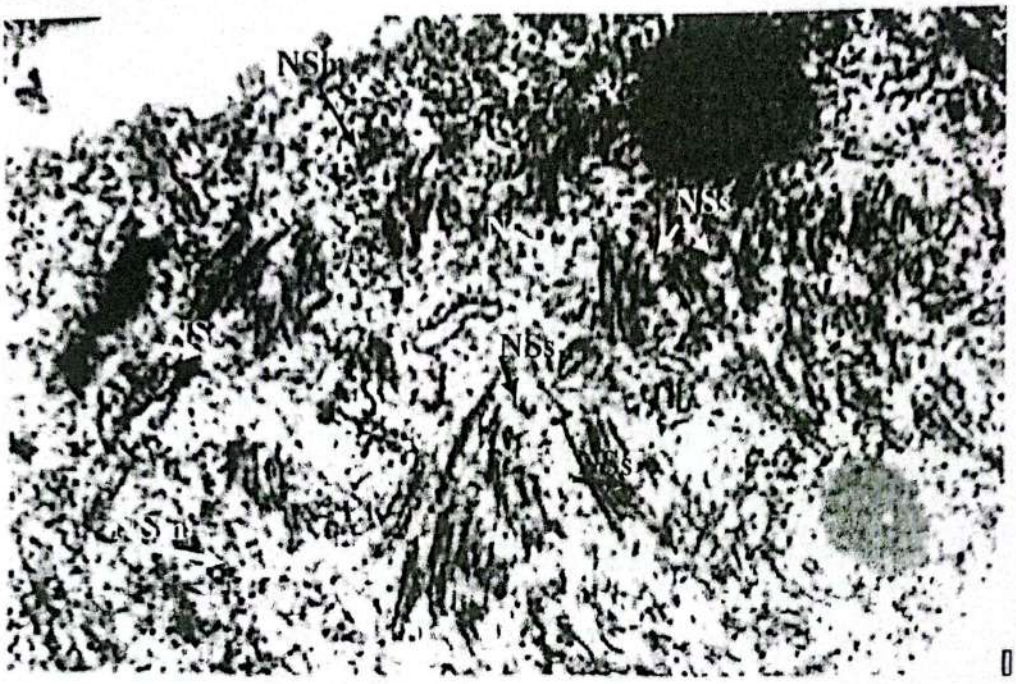

Fig. (4): Ultrathin section of *Nicotiana rustica* leaf tissue infected with TSWV. The NSs protein accumulates in long tubular structures that may associate as loose bundles. NSm protein is detected in granular electron-dense inclusions. Also excess N protein is observed as electron-dense, granular material in the cytoplasm. X= 30,000

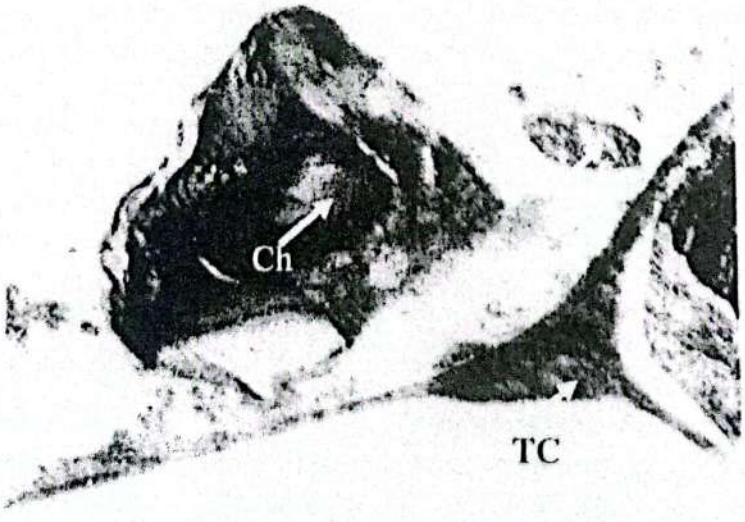

Fig. (5): Ultrathin section in mesophyll of *Nicotiana rustica* leaf showing degenerated chloroplast (Ch) and thickened cell wall (TCW). X= 17,000

were also observed. They were deformed and swollen with reduced lamellar system (Fig.7). Mitochondria were nearly absent from the cells Nuclei also might be absent from such cells. Generalized plasmolysis was evident in most of the cells. Our results were in agreement with that reported by Tu *et al.* (1968), Ehara and Misawa (1975), Kolesnik *et al.* (1987), Koiwa *et al.* (1991), Soweha *et al.* (1992), Zhou *et al.*(1992), and White and Sehgal (1993).

TSWV has singly enveloped particles end up in large membrane envelopes as a result of self-fusion of doubled enveloped virion (DEV) (which have two Golgi-derived membranes), or fusion with endoplasmic reticulum (ER) membranes but some had hollow cores and dense envelopes or dense cores with hollow envelopes (Milne, 1970). These envelopes surrounding singly enveloped virus particles (SEV), clusters must consequently consist of both Golgi and ER derived membranes and is probably involved in the early stages of forming virus particles (Fig. 8). De Avila *et al.* (1991) studied the cytopathology of the TSWV; it was similar to that of our TSWV isolates. Virus particles occur in clusters between membranes. Viroplasm withdense masses were found close to the clusters in the cytoplasm. Milne (1970) reported that the paired paralleled membrane (PPM) may be produced de novo since they did not seem to resemble any of the cellular membrane structures. Goldbach *et al* (1999) proposed a model for the maturation of *Tomato spotted wilt virus* (TSWV) particles with a protoplast infection system, in which the chronology of different maturation events could be determined. Our results obtained were in accordance with that obtained by Goldbach *et al* (1999) indicating that the site of

Fig. (6): Ultrathin section in mesophyll of *Nicotiana rustica* leaf showing aggregates of virus particles (V). X= 17,000

Fig. (7): Electron micrograph of *Nicotiana rustica* infected with TSWV showing large intercellular spaces (ICS) between cells of the sponge mesophyll tissue. Chloroplast (Ch) are swollen and numerous. X= 12,000 ,

TSWV particle morphogenesis was determined to be the Golgi system in which the viral glycoproteins G1 and G2 accumulate in the Golgi prior to a process of wrapping, by which the viral nucleocapsids obtain a double membrane (Fig. 8). In a later stage of the maturation, these doubly enveloped particles fuse to each other and to the endoplasmic reticulum to form singly enveloped particles clustered in membranes.

IV- Molecular studies

Our objective of this study was to characterize TSWV isolates occurring in Egypt. The nucleocapsid (N) protein gene was isolated by reverse transcription-polymerase chain reaction (RT-PCR) from total nucleic acid extracts from TSWV-infected plants. The primer pair specific to the N gene of the TSWV-L isolate was used. The two primers TSW1 (5' ATGTCTAAGGTTAAGCTC 3' and TSW2 5' TTAAGCAAGTTCTGTGAG 3') gave a PCR product of about 777 bp from the majority of the samples tested (*Pelargonium sp.*, *Physalis* peruviana and Tomato) (Fig. 9). Differentiation of TSWV isolates between Lycopersiconesculentum and Physalis peruviana was based on restriction enzyme digestions of the amplification products of reverse transcribed negative sense viral RNA. The primer pair TSWV1 and TSWV2 (Jain et al., 1998), facilitated amplification of 777 bp specific PCR product from both Tomato and Physalis peruviana as predicted (Pappu *et al.*, 1998).

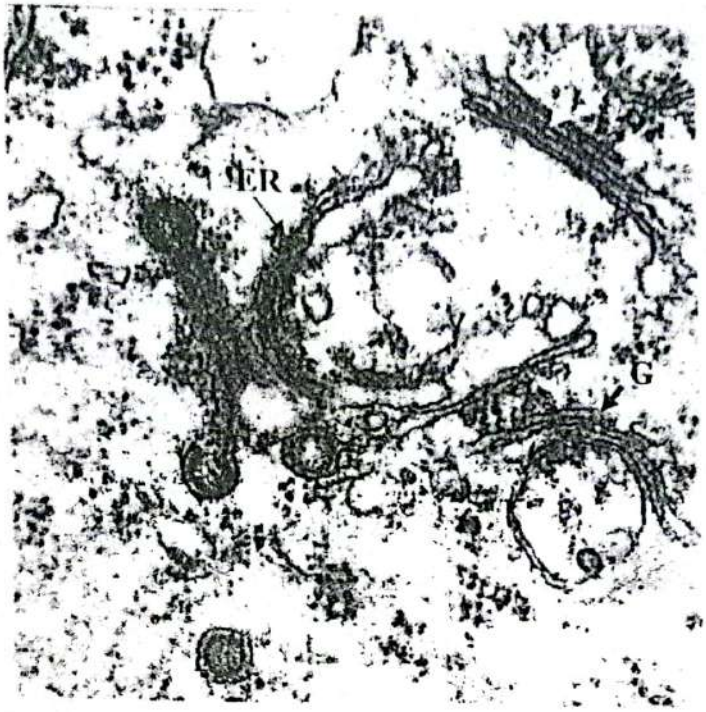

Fig. (8): Ultrathin section in mesophyll of *Nicotiana rustic* showing curving and wrapping of Golgi stacks (G) to form enveloped virus particles (V) with double membrane (PPM). X= 36,000

By comparing this predicted PCR product with the others amplified from *Pelargonium sp.* and *Datura metel*, it was shown that there were great differences in the size of the amplified products (Fig 9) which means that they might be considered as different isolates of TSWV.

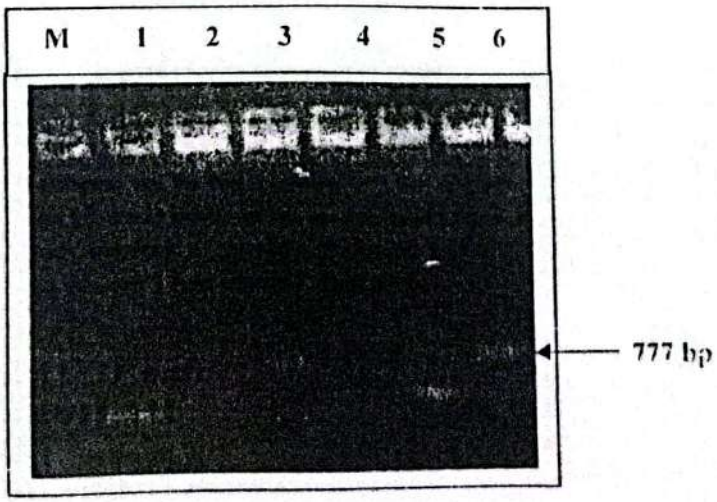

Fig. (9): 0.7% Agarose gel electrophoresis showing the amplified RT-PCR products of TSWV/NPs gene using TSWV1 and TSWV2 primers. Total RNAs extracted from *Datura metel* (lanes 1 and 2), *Lycopersicon esculentum* (~777 bp) (lane 3), Lane 4: Negative control (No RNA template). *Pelargonium sp.* (lane 5) and *Physalis peruviana* (~777 bp) (lane 6), M: DNA Molecular weight Marker XVI (Roche, Applied Science).

RFLP analysis results of the PCR products obtained from the two virus isolates (the virus under study which isolated from *Tomato* and the other one, *Physalis peruviana* isolate, kindly provided from Dr. Maha Al-Khazendar, Faculty of science, Cairo University) indicated that two or more *Mse*I restriction sites were present in the NP fragment of tomato isolate whereas two *Mse*I restriction sites were present in the same fragment of *Physalis peruviana* isolate. In fact, after digestion with *Mse*I, the 777 bp amplified product (Fig. 10, lane 1) of tomato and *Physalis peruviana* isolates gave two distinct restriction patterns, *i.e.* three fragments of approx. 480, 120 and 70 bp for tomato isolate (Fig. 10, lane 2), and two fragments of approx. 550 and 120 bp, for *Physalis peruviana* isolate (Fig. 10, lane 3). Our results were in accordance with that obtained by Finetti Sialer *et al.* (2002). We have shown that these two isolates can be readily identified by targeting NP sequences flanking *Mse*I restriction sites with RT-PCR/ RFLP.

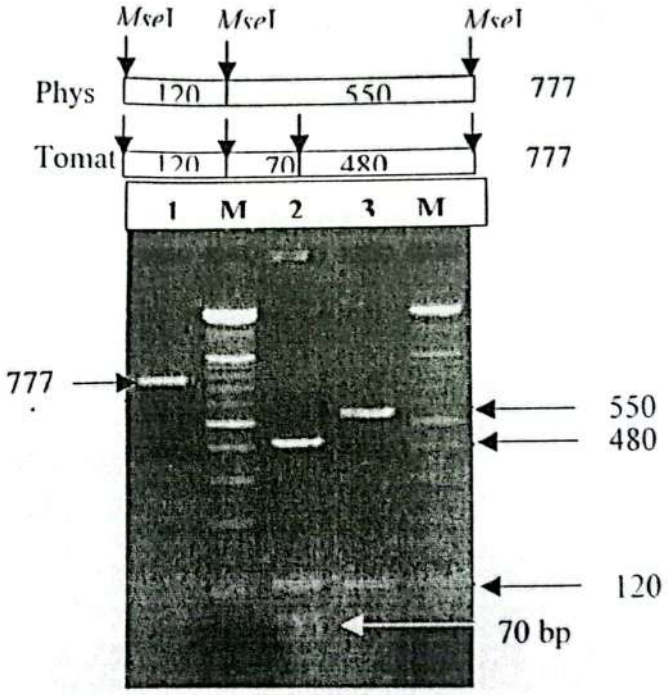

Fig. (10): Electrophoretic analysis in 2% agarose gel of an amplified product (lane1) and *Mse*I restriction patterns obtained from *Lycoperscon esculentum* (lane 2) and *Physalis peruviana* (lane 3) TSWV isolates. M: 100 bp ladder (Roche). Above, a cartoon model showing *Mse*I dimorphic restriction digestion profile performed on both Tomato and *Physalis peruviana*.

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