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RESEARCH

Characterization of a novel asymptomatic isolate of *Tomato spotted wilt virus*, Infects chrysanthemum plants in Egypt

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ABSTRACT

Background*:* A latent infection by *Tomato spotted wilt virus* (TSWV) in traded chrysanthemum plants at the local level constitutes a severe threat to the Egyptian farming.
Methods: The TSWV isolate in asymptomatic infected chrysanthemum plants was detected using double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). Host range studies were carried out by mechanical inoculation of biologically purified isolate on a set of different vegetable, ornamental and herbaceous plants. RT-PCR was used to amplify the full length nucleocapsid protein (N) gene of TSWV; the amplified 777 bp fragment was molecularly cloned and sequenced. The phylogenetic tree was constructed using the DNAMAN software.
Results: The obtained results demonstrated that, this isolate of TSWV causes asymptomatic infection for chrysanthemum plants in different locations in Giza governorate. The ELISA was less efficient than the RT-PCR for the diagnoses of the latent infection. RT-PCR accurately identified 26% more positive samples without symptoms than did the DAS-ELISA test. Moreover, RT-PCR was used to confirm TSWV infection related to the development of local lesions and systemic symptoms from mechanical inoculations. The nucleotide sequence of the TSWV-N gene has been deposited in the GenBank with accession number KY569403. Phylogenetic analysis showed that the fragment sequence shared variable levels of similarity and 93.82% max identity with that of N-gene sequences from Japan (AB038341), Pakistan (KX121046), South Korea (AB910533) and Turkey (KM407603).
Conclusion*:* The relatively low levels of sequence identity (91.7% to 93.8%) to N-gene demonstrated the release of a new isolate of TSWV in Egypt. Moreover, the study showed the susceptibility of onion and sweet potato to TSWV-isolate suggesting the chance of spread of viral infection on cultivated crops in the future. The information generated in this study will be useful for alleviating problems associated with non-specific symptoms and can be used to aid efficient management strategies directed against TSWV.

Keywords: Chrysanthemums; Latent infection; *Tospovirus*; *Tomato spotted wilt virus*; Nucleocapsid protein; N-gene; DAS-ELISA; RT-PCR; Cloning.

BACKGROUND

Chrysanthemum is a flowering plant and popularly known as areola, within the family *Asteraceae* and genus *Chrysanthemum*, has a wide range of floral colors and shapes making it one of the best desirable florists and affordable for landscaping in Egypt. Under Egyptian conditions, chrysanthemum plants like any other plants are threatened by various pathogens mostly caused by plant viruses. To date, nine viruses and two viroids are known to infect chrysanthemums (Cho *et al*., 2013).

Tomato spotted wilt virus (TSWV) is one of the most plant viruses infecting chrysanthemums plants as the type member of the genus *Tospovirus* (Thrips-transmitted) belonging to family *Bunyaviridae*. The genome of TSWV consists of three single-stranded linear RNA segments which according to their sizes have been denoted small (S, 2.9 kb) RNA, medium

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(M, 4.8 kb) and large (L, 8.9 kb) RNA. The L RNA is of negative sense, whereas the M and S RNAs are ambisense, i.e., the RNA encodes one protein in the messenger sense or viral sense (V) and another protein in the viral complimentary sense (Vc). The virion is quasi-spherical, enveloped particles of 80-120 nm in diameter and composed of four structural proteins: a putative RNA-dependent-RNA polymerase (RdRp) protein, nucleocapsid (N) protein and two viral coded glycoproteins (G_N, amino terminal and G_C, carboxy-terminal), embedded in the viral envelope that protrude through the surface of the virus particle as spikes, in addition to two nonstructural proteins (NSm and NSs). Nucleocapsid (N) of TSWV is the main structural protein that plays key role in assembling genomic RNA into ribonucleoprotein (RNP), which serves as a template for both viral gene transcription and genome replication. These characteristics place TSWV in the virus family *Bunyaviridae,* but the genome organization places TSWV in its own genus, where it is the type member. Genome sequence data, such as the sequences of nucleocapsid gene (N), specificity of thrips vector and host range represent the main keys in classification criteria of a *tospovirus* at the species level (Li *et al*., 2015; Zhang *et al*., 2016).

TSWV infects more than 1000 plant species in more than 90 families, and produces a wide range of foliar symptoms. Symptoms are influenced by the type and strain of a virus, type and variety of host plant, and environmental factors such as temperature (Pappu *et al*., 2009; Kulshrestha *et al*., 2013). Chlorotic spots on top leaves followed by wilting and development of severe necrosis on leaves were the most common symptoms of TSWV-infected plants in most fields (Chung *et al*., 2006; Morshedi *et al*., 2013; Stankovic *et al*., 2013). Although chrysanthemum plants can be severely affected by TSWV, symptoms may develop quite slowly or may not all occur at the same time and some infected chrysanthemum cultivars can remain as symptomless carriers for up to 10 weeks or few months (Balukiewicz *et al*., 2005; Morshedi *et al*., 2013).

In the present study, TSWV has been detected using serological and molecular tests in naturally infected chrysanthemum plants showing severe symptoms on leaves as well as asymptomatic infected plants. Therefore, the initial objective of this work was to isolate TSWV from asymptomatic infected plants and then identify the virus on the basis of biological properties among the most common or other hosts if possible after inoculation. Phylogenetic analysis was then conducted to gain information about the similar identity of the TSWV-isolate that reported in this study with available TSWV sequences from other parts of the world.

MATERIALS AND METHODS

Plant samples collection

Twenty-five samples of different chrysanthemums plants (*Chrysanthemum* spp.) with symptoms resembling those caused by *tospovirus* infection and 20 samples not linked with these symptoms were collected from public botanical gardens in the Giza governorate at the same time. Additionally, 30 pots of multi-bloomed chrysanthemums plants without viral symptoms were purchased from commercial orchards during 2016. A part of each kind of chrysanthemum samples was stored in plastic bags (one sample per bag) for appropriately labeling with the area collected. Samples were then taken to diagnostic labs for testing and to provide accurate identification of virus isolate.

Viral serology and detection

Plant extracts were prepared by grinding 0.1 g of the above samples separately with sterile pestle and mortar in one ml of 0.1 M phosphate buffer saline (PBS), pH 7.4. Double antibody sandwich (DAS)-ELISA (Enzyme-linked immunosorbent assay) was applied according to method described by Clark and Adams (1977) using two commercial specific antisera to

SVNV (Agdia, USA) and TSWV (Bioreba, Switzerland) as well as several sets of specific antisera to CMV, IYSV and TMV which were previously prepared by Virus and Phytoplasma Res. Dept. at the Agriculture Research Centre, Giza, Egypt. Absorbance readings were measured with a microplate reader (Dynatech MR 7000) at 405 nm. Negative controls were included in each DAS-ELISA assay from healthy chrysanthemum plants that had tested by RT-PCR (Reverse transcription-polymerase chain reaction) as described below. Samples were considered to be positive when the value of absorbance was more than twice the average value of the negative control. ELISA absorbance data from tested samples were recorded and showed the absorbance values only to TSWV. These data were compared with RT-PCR, in order to evaluate the accuracy of a screening test.

Biological properties of TSWV

Asymptomatic infected plants (ELISA-positive samples) were selected and then the inoculum was prepared in 0.1M phosphate buffer, pH 7.0 containing 0.2% sodium sulfite and 0.01M 2-mercaptoethanol at a weight/volume ratio of 1:10 (Mandal *et al*., 2008). Under controlled conditions, the virus isolate was biologically purified through a single local lesion technique repeated three times on *Nicotiana tabacum* cv. White Burley (Margaria *et al*., 2014). The virus was mechanically transmitted to *Gomphrena globosa* for virus propagation (Ie, 1970) and used as a source of virus in the following experiments. The inoculations were performed to ten plants of various chrysanthemum species (Negative PCR) and to three plant of each indicator species listed in Table 3. The plants were inoculated by rubbing the leaf surfaces using cotton swab and celite powder as an abrasive. A number of plants were left without inoculation for comparative evaluations. The inoculated plants were monitored daily for a period of 28 days' post inoculation. RT-PCR was performed to verify the presence of TSWV in leaves of the inoculated plants.

Molecular detection and characterization of TSWV

Extraction of total nucleic acids

Total RNAs from all symptomatic and asymptomatic chrysanthemum tissues as well as inoculated leaves of each host plant were extracted using Gene jet™ plant RNA purification mini kit (Thermo Scientific, USA) according to manufacturer's manual and the purified RNA used as a template for RT-PCR amplifications.

RT-PCR and Primer design

The TSWV nucleocapsid (N) gene complete codon encoded by the S-RNA segment (De Haan *et al*., 1990) was amplified by RT-PCR according to the method described by Gilman (1988). RT-PCR was conducted using Verso™ 1-step RT-PCR system, obtained from Thermo Fisher Scientific Co. (Waltham, MA, USA), according to the manufacturer's instructions. A specific primer pairs TSWV-Nf and TSWV-Nr were designed and used for full-length amplification of the N gene in one step RT-PCR. The forward primer sequence was: 5′-ATGTTTAAGGTTAAGCTCA-3′ while the reverse primer sequence was: 5′-AGCAAGTTCTGTCAGTTTTG-3′. The RT-PCR reaction was optimized to be performed in a final volume of 50 µl. The final concentrations of the reaction components were: 25 µl of 1-Step PCR Master Mix, 200 nM of each forward and reverse primer, 1 µl Verso enzyme mix, 2.5 µl RT-Enhancer and 3 ng of template (RNA). Amplification was performed in an automated T-Gradient Biometra (Germany) thermal cycle. The RT reaction started with incubation at 50°C for 15 min, followed by denaturation at 95°C for 2min. The PCR reaction was performed by 35 cycles starting with denaturation at 95°C for 1min, primer annealing at 47°C for 1minute and extension at 72°C for 1 min, finally an additional extension step at 72°C for 10 minutes was performed. RT-PCR products were mixed with 6x loading buffer, stained with gel star (Lonza,

USA) and analyzed by electrophoresis on 1% agarose gels then visualized by UV illumination using Gel Documentation System (Gel Doc 2000, Bio-Rad, USA). A 100 bp DNA ladder (Invitrogen, CA, USA) was used.

Cloning, DNA sequencing and sequence analysis

To confirm the identity of the amplified fragment, the purified RT-PCR products were cloned directly into the cloning vector pCR4-TOPO according to the manufacturer's instructions (Invitrogen, CA, USA). The DNA construct consists of the plasmid vector pCR4-TOPO ligated with the insert fragment for the amplified (N) gene was used to transform TOP10 competent cells according to manufacturer's instructions (Invitrogen, CA, USA). Plasmid DNA construct was purified from the transformed bacterial cells using the QIAprep Spin Miniprep Kit (Qiagen, Germany). Sequencing of amplicons was performed using the universal M13 primers. The identity of cDNA fragment was compared and verified by database searches using the online BLAST at the National Center for Biotechnology Information homepage (https://www.ncbi.nlm.nih.gov/). Phylogenetic analysis was carried out by means of the DNAMAN software package (Lynnon BioSoft, Vaudreuil-Dorion, Quebec, Canada).

RESULTS

Visible and invisible viral symptoms

The observed collected samples from both public botanical gardens and commercial orchards in Giza governorate were diverged. Thirty-three percent (25 out of 75) of all the collected samples showed pathological symptoms due to a virus infection and 67% (50 out of 75) of the samples initially appears to be healthy according to the results of DAS-ELISA. The most prevalent samples included mosaic, necrotic local lesion, tip necrosis, stunting and flower distortion (Fig.1).

Fig. 1: Set of naturally infected chrysanthemum samples with typical symptoms of *Tomato spotted wilt virus*. A and B): Flowers distorted and tip necrosis on leaves. C): Mosaic, necrotic local lesion and stunting. D): Commercial infected plants that seem to be healthy and free of virus.

Definitive diagnosis of viral infection

To investigate the viral infection, chrysanthemum samples with/without necrotic local lesion or tip necrosis were tested by DAS-ELISA and RT-PCR. The results of DAS-ELISA confirmed the presence of TSWV in some of the symptom-bearing samples and non-symptomatic plants using TSWV specific antiserum whereas, it was tested negatively with CMV, IYSV, TMV and SVNV antisera .To ensure that plants were initially infected with TSWV, an amplified fragment of 777 bp was detected after agarose electrophoresis of RT-PCR products when total RNA isolated from naturally infected leaves as well as viral RNA accumulation in asymptomatic infected leaves were used. No fragments were amplified from the RNA extracted from the comparable healthy leaves or some symptomless plants with negative DAS-ELISA results (Fig.2 does not display all of the data).

Data demonstrated in Table (1) reveal that, in the case of non-symptomatic plants the technique of RT-PCR was found to be more sensitive than DAS-ELISA to detect TSWV in chrysanthemum leaves, whereas15 and 28 out of 50 samples were positive for TSWV by DAS-ELISA and RT-PCR, respectively.

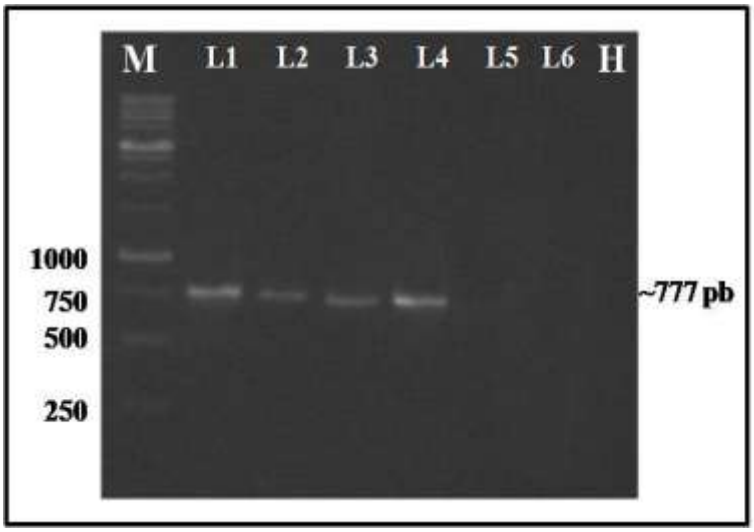


Fig. 2: Agarose gel electrophoresis patterns of RT-PCR products of *Tomato spotted wilt virus*. M: 1 kb DNA ladder. L1: Sample of the naturally infected chrysanthemum plants. L2: Asymptomatic sample collected from gardens. L3: Asymptomatic sample of commercially available plants. L4: Asymptomatic chrysanthemum sample that showed negative results when tested with ELISA. L5 and 6: Negative samples by means of RT-PCR. H: Healthy control plant.

Table (1): Comparison of ELISA and RT-PCR assays for the detection of TSWV in asymptomatic leaf samples.

Location	No. of tested plants	ELISA		RT-PCR	
		Infected	Healthy	Infected	Healthy
P-botanical gardens	20	6	14	11	9
Com-orchards	30	9	21	17	13

P: Public. Com: Commercial.

Biological properties

The virus under investigation was isolated from asymptomatic infected chrysanthemum plants as well as gave positive results with TSWV specific antiserum. After biological purification through single local lesions on *Nicotiana tabacum* (cv. White Burley), the resulting virus was propagated in *Gomphrena globosa* causing necrotic local lesions on the inoculated leaves and systemic mottle on the upper leaves after 4-28 days' post inoculation.

Results in Table (2) and Figures 3, 4, 5 and 6; show the reaction of TSWV with several tested hosts. Total 15 species belonging to nine families were assessed as sources of virus based on both symptoms expression (Figs. 3, 4 and 5, some data not shown) and the use of molecular detection (Fig. 6). Inoculated plants showed a great variability in symptoms expression, such as necrotic and chlorotic local lesion, necrotic spot, mosaic, mottling, vein necrosis, leaf and flowering deformation.

Table 2: Host range and symptomatology of TSWV isolate.

Plant			Common name	Symptoms
Family	**Species[(a)]**	**Cultivar**		
Amaranthaceae	*Gomphrena globosa*		Globe amaranth	L,NS,Mo
Amaryllidaceae	*Allium cepa*	Giza20	Onion	CHLL,S
Apocynaceae	*Catharanthus roseus*	Rosea	Periwinkle	SM
Asteraceae	*Chrysanthemum sp.*	Hybrid[(b)]	Chrysanthemum	NLL,S,FW
Chenopodiaceae	*Chenopodium Album*		Goosefoot	CHLL
	C. quinoa		Quinoa	NLL
Cucurbitaceae	*Cucumis sativus*	Beit Alpha	Cucumber	CHLL
Convolvulaceae	*Ipomoea batatas*	Mabrouka	Sweet potato	SM,NR,MN
Fabaceae	*Vicia faba*	Giza 429	Broad bean	SM
Solanaceae	*Capsicum annuum*	California Wonder	Pepper	MM,NS, VN,MN
	Datura stramonium		Jimson Weed	CHLL,NS
	Nicotiana tabacum	White Burley	Tobacco	NLL,LD
	Petunia hybrid		Petunia	NLL
	Solanum lycopersicum	Castel Rock	Tomato	CHLL,NS
	Solanum tuberosum	Spunta	Potato	NS

(a) Host plant species were chosen to represent a range of numerous varieties to the botanical families widely grown in Egypt. **(b)** An unspecified (or unknown) species. The evaluation was verified through symptoms expression and by molecular assay. **CHLL**: Chlorotic local lesion. **FW**: Flower wilt. **L:** local lesions. **LD**: Leaf deformation. **Mo:** Mottle**. MM**: Mild mosaic. **MN**: Marginal necrosis. **NLL**: Necrotic local lesion. **NR**: Necrotic region. **NS**: Necrotic spot. **S**: Systemic symptoms. **SM**: Systemic mosaic. **VN**: Vein necrosis.

Fig. 3: Inoculation of the virus isolate (TSWV-KY569403) on some selected host plants. A): Three successive serial passages of the virus on *N. tabacum* cv. White Burley. B): The response of *C. album*, *C. quinoa* or *S. lycopersicum* plant for the mechanical inoculation (from left to right). C): Symptoms of mild mosaic followed by systemic leaf deformation, necrotic spots, vein necrosis and marginal leaf necrosis in pepper leaves after inoculation.

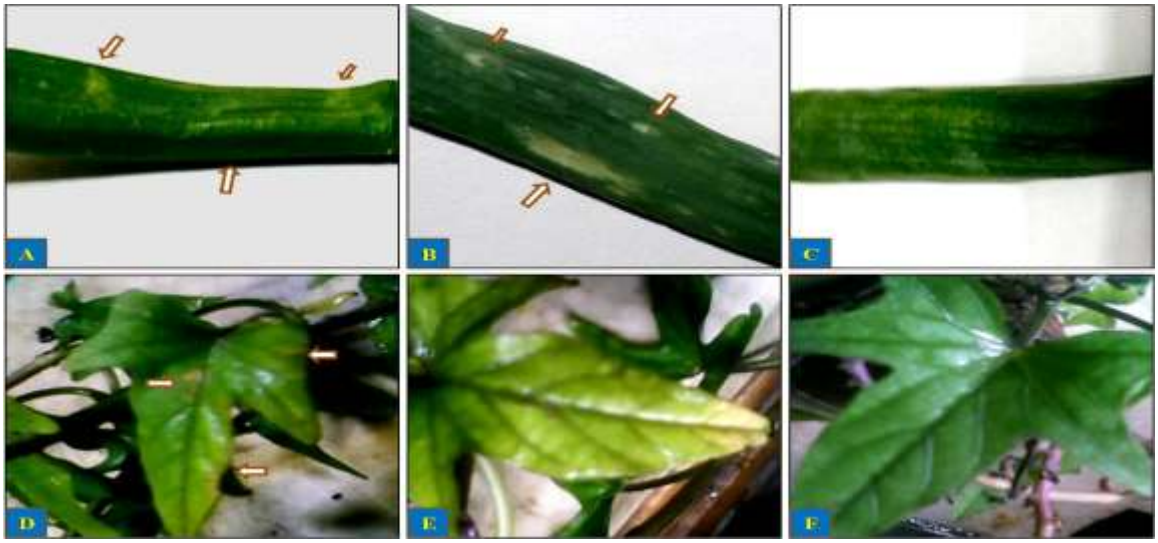


Fig. 4: Symptoms of TSWV-isolate on onion or sweet potato that was positive in RT-PCR assay. A and B): Local and systemic symptoms of TSWV in Onion, as indicated by arrows compared with healthy ones (C). Images (D and E): TSWV-inoculated sweet potato leaf showing necrotic regions, marginal necrosis (arrows) and systemic mosaic compared with healthy ones (F).

Fig. 5: Photos show the progress of systemic symptoms of chrysanthemum plants after mechanical inoculation with TSWV-isolate. A: Necrotic local lesions. B: Collapsing of stem with wilting and falling of the flowers.

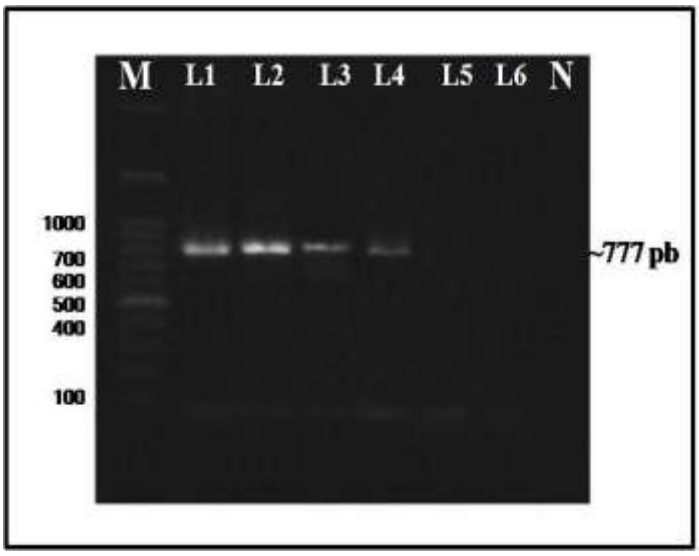


Fig. 6: Agarose gel electrophoresis patterns of RT-PCR products of *Tomato spotted wilt virus* from mechanically inoculated host plants. M: 1kb DNA ladder. L1, L2, L3 and L4: Inoculated leaves of potato, periwinkle, onion and sweet potato, respectively. L 5 and 6: Healthy leaves of onion and sweet potato, respectively. N: Negative control without template DNA.

Molecular cloning and phylogenetic analysis

Following RT-PCR, the resulting amplicons were cloned into pCR4-TOPO cloning vector. Recombinant clones were extracted and digested using the *EcoRI* restriction enzyme. The electrophoresis analysis for the digested clones showed a clear fragment at the expected size of the nucleocapsid (N) protein gene of TSWV (777bp) as well as a characteristic band at the size of the digested TOPO vector (4000bp) that shown in Figure (7). The positive clones, whose fragments were correctly inserted, were selected for DNA sequencing. The nucleotide sequence of the amplified PCR fragment, that represents the full sequence of N gene for the TSWV isolate, was submitted to the GenBank by assigned number KY569403 to TSWV isolated from infected chrysanthemum plants without symptoms. The nucleotide sequence was compared with those of twenty isolates from different geographical regions. The details of the isolates used in the phylogenetic analysis are presented in Table (3) and Figure (8). Comparing the N-gene sequence obtained in the present study with N-gene sequences from the GenBank database demonstrated a relatively low similarity ranged from 91.7% to 93.8%. The highest identity (93.82%) was reported when the Egyptian isolate was compared with sequences from Japan (Tospo-C isolate, AB038341), Pakistan (ADPK1 isolate, KX121046), South Korea (AT1 isolate, AB910533) and Turkey (Antalya isolate, KM407603). The sequence identity matrix showed that these four sequences are extremely homologous in the range of 96-98% among themselves (Fig.9). Moreover, comparison of nucleotide sequence of TSWV isolate from Egypt showed close identity (93.5%) with sequences of other three isolates from China (KC294570), Italy (FR693048) and South Korea (KJ139671), and these three isolates presented similar level of homology (97%) whereas, all 20 isolates used in analysis presented sequences with very high levels of similarity (95.4-99.7%) to each other (Fig.9).

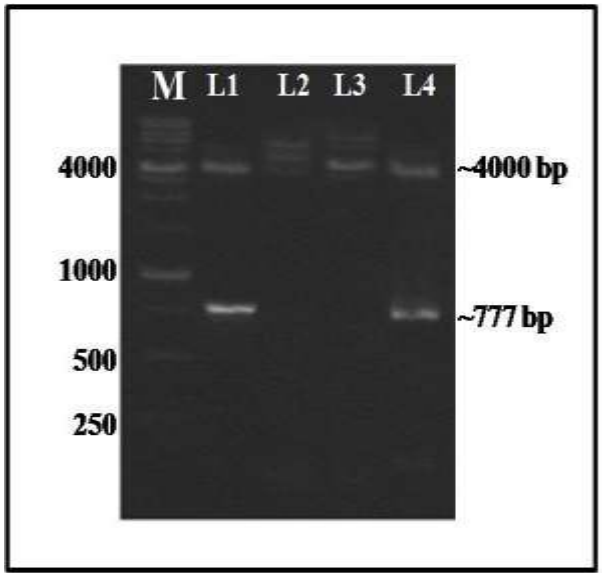


Fig. 7: Electroplate agarose gel showing restriction analysis for TOPO/TSWV-N clones. L1 and L4: Positive clones showed the release of the TSWV-N gene at the distinct band ~777 bp and the digested TOPO vector at ~4000 bp after digestion of plasmids using *EcoRI*. L2 and L3: Negative clones after *EcoRI* digestion. M: 1 kb molecular weight DNA ladder.

Table (3): Percentage of nucleotide sequence identities (%) between TSWV-Egyptian isolate and other TSWV-isolates available in the GenBank.

Isolate name	Original host plant	Geographical origin	Accession No.	Identity %[a]
Tospo-C	Chrysanthemums	Japan	AB038341	**93.82**
MAF02	Tagetes	New Zealand	KC494482	93.31
YN-2	Iris	China	KC294570	93.56
AT1	Brugmansia	South Korea	AB910533	**93.82**
SW	Tomato	South Korea	KJ139671	93.56
LE 98/527	Tomato	Germany	AJ297611	93.31
CHM6	Chrysanthemums	Italy	FR693048	93.54
Is-141	Calceolaria	Montenegro	GU339506	93.44
ADPK1	Capsicum	Pakistan	KX121046	**93.82**
1439	Capsicum	Virginia-Australia	AY818321	92.92
Antalya	Capsicum	Turkey	KM407603	**93.82**
LC	Tomato	Spain	X94550	92.28
DAR76606	Chickpea	Australia	AY611529	92.24
B	Basil	Poland	KU308366	93.05
CIE43	Endive	France	FR693049	92.79
BS97	NP[b]	Bulgaria	AJ297610	93.05
A5-mum	Chrysanthemums	Zimbabwe	KX192330	93.05
VE-223	Tomato	Brazil	GU369726	92.15
Tomato-Tift	Tomato	USA	HQ406983	91.76
TwPep1	Capsicum	Taiwan	GU222651	92.54

[a]Highest identity values are indicated in bold. [b]The host name of this isolate is not published (NP).

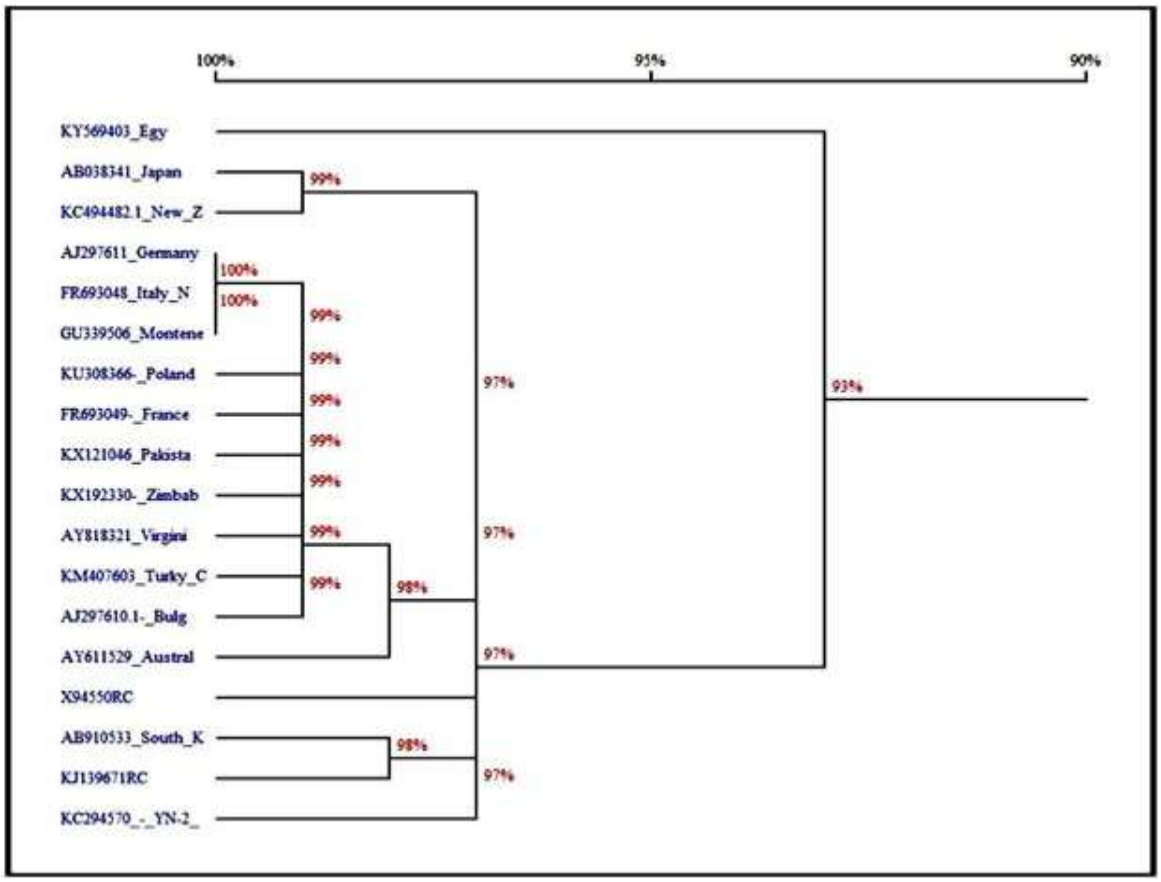
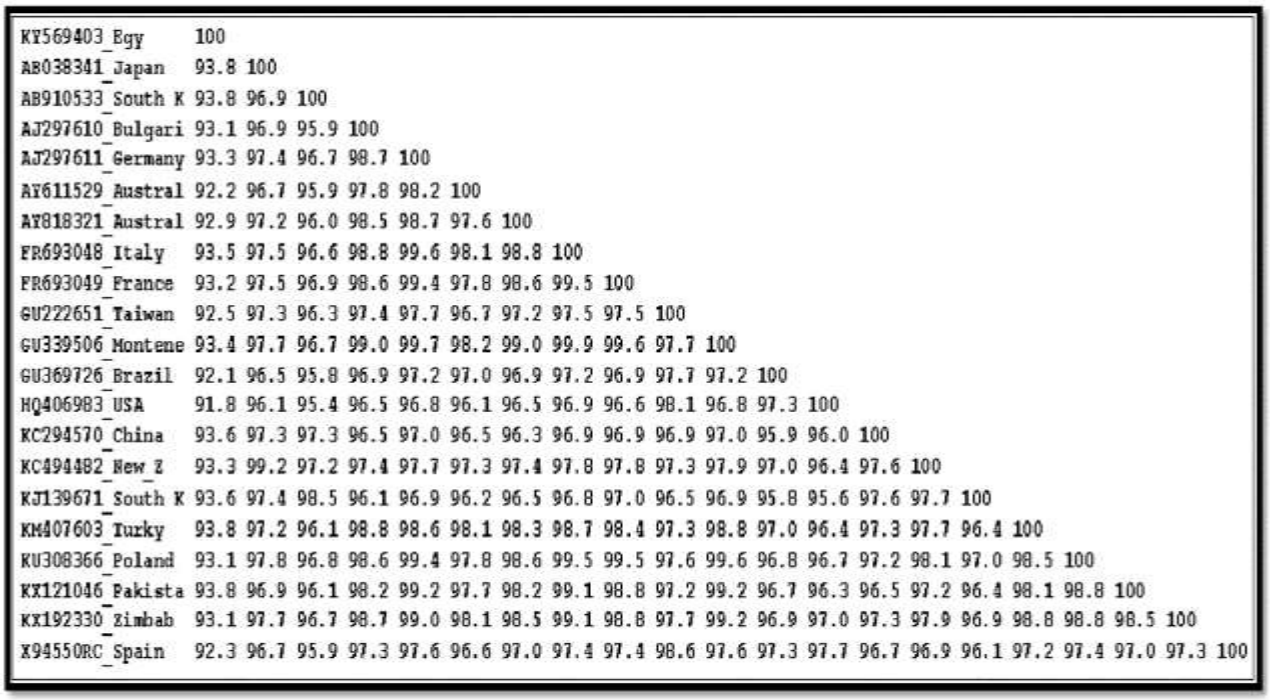


Fig. 8: The phylogenetic tree based on multiple sequence alignments for the nucleocapsid (N) gene of TSWV-Egyptian isolate and other isolates available in the GenBank.



KY569403_Egy	100																				
AB038341_Japan	93.8	100																			
AB910533_South K	93.8	96.9	100																		
AJ297610_Bulgari	93.1	96.9	95.9	100																	
AJ297611_Germany	93.3	97.4	96.7	98.7	100																
AY611529_Austral	92.2	96.7	95.9	97.8	98.2	100															
AY818321_Austral	92.9	97.2	96.0	98.5	98.7	97.6	100														
FR693048_Italy	93.5	97.5	96.6	98.8	99.6	98.1	98.8	100													
FR693049_France	93.2	97.5	96.9	98.6	99.4	97.8	98.6	99.5	100												
GU222651_Taiwan	92.5	97.3	96.3	97.4	97.7	96.7	97.2	97.5	97.5	100											
GU339506_Montene	93.4	97.7	96.7	99.0	99.7	98.2	99.0	99.9	99.6	97.7	100										
GU369726_Brazil	92.1	96.5	95.8	96.9	97.2	97.0	96.9	97.2	96.9	97.7	97.2	100									
HQ406983_USA	91.8	96.1	95.4	96.5	96.8	96.1	96.5	96.9	96.6	98.1	96.8	97.3	100								
KC294570_China	93.6	97.3	97.3	96.5	97.0	96.5	96.3	96.9	96.9	96.9	97.0	95.9	96.0	100							
KC494482_New_Z	93.3	99.2	97.2	97.4	97.7	97.3	97.4	97.8	97.8	97.3	97.9	97.0	96.4	97.6	100						
KJ139671_South K	93.6	97.4	98.5	96.1	96.9	96.2	96.5	96.8	97.0	96.5	96.9	95.8	95.6	97.6	97.7	100					
KM407603_Turky	93.8	97.2	96.1	98.8	98.6	98.1	98.3	98.7	98.4	97.3	98.8	97.0	96.4	97.3	97.7	96.4	100				
KU308366_Poland	93.1	97.8	96.8	98.6	99.4	97.8	98.6	99.5	99.5	97.6	99.6	96.8	96.7	97.2	98.1	97.0	98.5	100			
KX121046_Pakista	93.8	96.9	96.1	98.2	99.2	97.7	98.2	99.1	98.8	97.2	99.2	96.7	96.3	96.5	97.2	96.4	98.1	98.8	100		
KX192330_Zimbab	93.1	97.7	96.7	98.7	99.0	98.1	98.5	99.1	98.8	97.7	99.2	96.9	97.0	97.3	97.9	96.9	98.8	98.8	98.5	100	
X94550RC_Spain	92.3	96.7	95.9	97.3	97.6	96.6	97.0	97.4	97.4	98.6	97.6	97.3	97.7	96.7	96.9	96.1	97.2	97.4	97.0	97.3	100

Fig. 9: Homology matrix for 21 isolates of TSWV encoding the N protein and relationships among themselves.

DISCUSSION

The present study dealt with the characterization of latent isolate of *Tomato spotted wilt virus* in chrysanthemum plants based on serological and molecular tests as well as host range reaction. Although, the serological methods are simple and easy to perform, false-negative results can also occur because of the instability and the variable distribution of the virus in chrysanthemum plants (Delaure *et al*., 2008). A number of studies are reporting that ELISA was less sensitive than PCR for detecting TSWV in field-grown chrysanthemum in Japan (Matsuura *et al*., 2002; 2004), and among 22 plant species in Australia (Kulshrestha *et al*., 2013). Similarly, Vasquez and Angarita (1999) found that ELISA kits were not sensitive enough to be used against two Colombian isolates of *tospoviruses* in chrysanthemum. Also, according to the results reported by Dang *et al*. (2009) ELISA and RT-PCR were differed for detecting TSWV in 17 peanut root samples. So, the highly difference in the present findings (26%) between ELISA and PCR methods support the general conclusion that RT-PCR is a more sensitive method than serological studies for the diagnosis of TSWV infection, especially with the loss of early signs or symptoms of the disease.

During the course of mechanical inoculation to a range of experimental hosts, TSWV-isolate severity was visually assessed by developing symptoms as well as detection of TSWV-N protein directly from samples. Several studies confirmed the pattern of symptoms that previously described for different host plants (Balukiewicz *et al*., 2005; Morshedi *et al*., 2013; Salem *et al*., 2012; Berniak, 2016). Previous studies have provided evidence that this virus displays a considerable degree of biological diversity symptoms of TSWV infection differ among hosts and can be variable in a single host species. Also, present evidence of TSWV-N gene in host plants suggesting a possible wide host range used for Egyptian TSWV isolate, and as potentially of epidemiological significance for spread of current isolate to different crops as sweet potato and onion; since the virus has been detected in naturally infected sweet potato (*Ipomoea leucantha*)

in USA (Valverde *et al*., 2007) and onion seed crop in Serbia (Stankovic *et al*., 2012) or sweet onion (Variety: Yellow Granex) in Georgia (Mullis *et al*., 2004). So, additional surveys are needed to obtain a better understanding of TSWV incidence in order to evaluate its impact on the productivity for those crops.

The TSWV-EGY was restricted to a few lesions on the inoculated leaves of *Solanum tuberosum* in spite of the fact that TSWV infection causes severe symptoms on potatoes under field conditions (Pourrahim *et al*., 2001). TSWV transmission mechanically from infected hosts to potato is very difficult or unattainable (Abad *et al*., 2005). In fact, potato is more resistant to mechanical inoculation with TSWV, and successful inoculation is most effective when performed with TSWV isolate from potato rather than from other hosts (German, 2001). In parallel, several studies reported, mechanical inoculation of TSWV and another *Tospovirus* such as SVNV are not very efficient in other hosts such as peanuts and soybeans (Hoffmann *et al*., 1998; Mandal *et al*., 2001; Khatabi, *et al*., 2012). Subsequently, TSWV-isolate was evaluated on ten healthy chrysanthemum plants, particularly related to symptoms which tend to develop slowly with equal degree of severity over time as they appeared in almost every case. This situation appears to be consistent with the finding of other studies that chrysanthemums are susceptible host plants to TSWV and symptoms on infected plants often develop slowly after inoculation (6–8 weeks) or may be restricted to a few leaves (Matteoni and Allen, 1989; Van de Wetering *et al*., 1999; Morshedi *et al*., 2013).

In the present study, the size of the virus-specific purified fragment of nucleocapsid (N) gene from chrysanthemum plants was estimated to be about 777 bp. This result was similar to the previous results reported by Chung *et al*. (2006) and Morshedi *et al*. (2013). Based on the phylogenetic information from molecular data, there are three observations worth mention. Firstly, the Egyptian TSWV isolate was determined both by homology search in nucleotide databases and by comparative analyses of the nucleocapsid genes of several strains of TSWV. Secondly, using of sequence-based molecular analysis provided a clear demonstration of divergence (≥6.18%) between the Egyptian TSWV isolate and the other available isolates. Thirdly, genetic diversity among 21 isolates of TSWV did not correlate with specific host species or geographical region. Therefore, the results have been suggested that these isolates are related and may share common capsid assembly suggesting a distinct Egyptian isolate (KY569403) with unusual sequence which may be probably evolved or mutated from the same ancestor *Tomato spotted wilt Tospovirus* occurring worldwide but in particular from certain countries from Asia and Europe (Japan, Pakistan, South Korea and Turkey). The findings in this study are in agreement with the previously reported studies that the sequence of nucleocapsid gene (N) represent a key step in the generation of rapid and reliable data for detection or identification of *Tospoviruses* where nucleic acid-based tests had been used successfully to diagnose different tospovirus species (approved and tentative) over the last years (Stankovic *et al*., 2012; Dong *et al*. 2013).

In conclusion: *Tomato spotted wilt virus* appeared and spread in Giza governorate correlated with asymptomatic infected plants movement from greenhouses production that already have a problem related with the current TSWV-isolate. Onion and sweet potato are new sensitive indicator plants. The TSWV may increase the risks of natural infection in the future. Symptoms of TSWV vary depending on the host, necrotic spots and leaves deformation are some of the many symptoms exhibited by the current isolated virus. The phylogenetic analysis in correlation with the biological characterizations and symptomatology confirmed the release of new isolate that asymptomatically infects chrysanthemum plants in Egypt.

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