An - Najah National University

Faculty of Graduate Studies

Serological Detection of Tospovirus Infecting Tomato in West Bank-Palestine

By

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Dedication

To the one and only to my mother she was the reason of what I doing today and the reason of all good thing that occurs in my life

She was encouraged me to complete my high study then left me alone in this way.

To my great father thanks a lot for your care and support thanks also for your patients.

To my beloved sister Rahmeh and his husband Emad

To my sweet little sister Leena and his husband Husam

To my lovely brothers Hashem, Mohamed and Basem thanks all for your love, support and lasting respect for my work on a Master thesis.

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All thanks for Hiba and his husband Dr.Moayad Bsharat.

I also thanks my beautiful family for standing with me in each step of my life.

الاقرار

أنا الموقعة أدناه، مقدمة الرسالة التي تحمل عنوان:

Serological Detection of Tospovirus Infecting Tomato in West Bank-Palestine

أقر بأن ما اشتملت عليه هذه الرسالة هي من نتاج جهدي الخاص، باستثناء ما تمت الاشارة اليه حيثما ورد، وأن هذه الرسالة ككل أو أي جزء منها لم يقدم من قبل لنيل درجة أو لقب علمي أو بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's name: **اسم الطالبة:**

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List of Abbreviations

ºC	Degree Celsius
CMV	Cucumber mosaic virus
DAS-ELISA	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay
gm	Grams
KDa	Kilo Dalton
ml	Mill mole
µl	Microliter
MW	Molecular Wight
nm	Nanomolar
PCBS	Palestinian Central Bureau of Statistics
PCR	Polymerase Chain Reaction
PVY	Potato virus Y
RdRp	RNA dependent ENA polymerase
RNPs	Ribonucleocapsid proteins
ss-RNA	Single strand ribonucleic acid
TBSV	Tomato bushy stunt virus
TCMV	Tomato chlorotic mottle virus
TCSV	Tomato chlorotic spot virus
TMV	Tomato mosaic virus
TRMV	Tomato rogues mosaic virus
TSWV	Tomato spotted wilt virus
TYLCV	Tomato yellow leaf curl virus
TYRV	Tomato yellow ring virus
TYSV	Tomato yellow spot virus
X	Concentration

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Abstract

Tomato (*Solanum lycopersicum*) consider as one of the top popular crop in Palestine; is infected with several pathogens where *Tomato Spotted Wilt Virus* (TSWV) is one of the most devastating one, causing serious damages and large economical losses on tomato crops. This study was aimed to reveal the presence and prevalence of the virus in Palestinian territories (West Bank) for the first time. About 232 samples were collected from different governorate of northern of West bank Palestine during the growing season 2017 / 2018 and were tested for TSWV by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). During the field surveys, several symptoms were noticed on tomato plants in the field of Nablus; Tubas; Qalqilia; Tulkarem; Jenin and Jericho. The appeared symptoms were on leaves as leaf yellowing, brown rings or line patterns. The whole plants showed reduction in growth, chlorotic and necrotic spots on both leaves and fruits. These systematic symptoms were considered as the best indication for the existence of (TSWV).By serological test, the presence of TSWV on tomato plants in Palestine was confirmed; where the prevalence of the virus was reached up to 1.27%. This low percent was considered alarming, since the possibility of transmitting the virus through

thrips that commonly widespread in the country. Looking for virus resistant varieties was recommended. This research study was to confirm the presence of this important virus in Palestine for the first time.

Chapter one

Introduction and Literature Review

Chapter one

Introduction and Literature Review

1.1. Introduction

Solanum lycopersicum is the scientific name of tomato, which is the most popular crop in Palestine. According to the (Palestinian Central Bureau of Statistics PCBS) in 2012 tomato was expanded on an area of 7100.32 dunum in Jenin, Tubas, Tulkarem, Nablus and Jericho. Tomato cultivated mainly in greenhouses that were totaled more than 31% and it also cultivated in open field but in less amount.

Tomato is an important crop which provides balanced diet and colorful additions to any meal. On the nationwide, tomatoes produced and consumed the second most other vegetable. The bioavailability of nutrients depends on the processing of tomatoes. Nutrients that are riches in tomatoes are lycopene, beta-carotene, folate, potassium, vitamin C, flavonoids and vitamin E (Willcox et al.,2003).

Area cultivated with tomato crop in Jericho totaled 2376.27 dunums which more than Jenin that have 2058.62 dunums of area cultivated with tomato, Then Tubas which totaled 1578.32, Finally Tulkarem and Nablus that are totaled 725.09 and 362.02 respectively(Table 1) (PCBS, 2012).

Table 1: The area cultivated with tomato in dunums for the five governorates.

Governorate	Tomato area in dunums
Jericho	2376.27
Jenin	2058.62
Tubas	1578.32
Tulkarem	725.09
Nablus	362.02

According to the agricultural department in winter the area cultivated by tomato totaled 1060.90 dunums in Jericho and Al-Aghwar Governorate which's more than the area cultivated in spring, summer and autumn (Table 2).

Table 2: Tomato area in dunums based on the session in Jericho and Al-Aghwar Governorate (2012).

Agricultural session	Area (dunums)
Winter	1060.90
Spring	143.25
Summer	351.00
Autumn	821.12
2376.27	

Tomato area in Jericho and Al-Aghwar governorate classified based on the state of crop into three classes; single, associated and mixed that are totaled 2154.87, 110.40 and 111.00 dunums respectively. The area of tomato in Jericho and Al-Aghwar that's harvested totaled 2112.32 dunums and (Table 3) (PCBS, 2012).

Table 3: Tomato area in dunums based on status of tomato crop in Jericho and Al-Aghwar Governorate (2012).

Status of tomato crop	Area (dunums)
Single	2154.87
Associated	110.40
Mixed	111.00
2376.27	

The agricultural session defer from Jericho to Jenin. The area cultivated by tomato in summer totaled 1043.11 dunums in Jenin Governorate that more than the area cultivated by tomato in spring, autumn and winter which are totaled 407.76, 309.23 and 293.02 dunums respectively (Table 4).

Table 4: Tomato area in dunums based on the agricultural session in Jenin Governorate (2012).

Agricultural Session	Area (dunums)
Winter	293.02
Spring	404.76
Summer	1043.11
Autumn	309.23
Not Stated	5.50
2058.62	

The single tomato based on state of crop has the higher total for area cultivated by tomato in Jenin Governorate (Table 5) (PCBS, 2012).

Table 5: Tomato area in dunums based on the state of tomato crop in Jenin Governorate (2012).

State of tomato crop	Area (dunums)
Single	1904.38
Associated	32.64
Mixed	113.60
Not stated	8.00
2058.62	

Tubas, Tulkarem and Nablus have less area cultivated by tomato than Jericho and Jenin. Tomato area in Tubas and Tulkarem according to the agricultural session totaled 579.24 and 329.02 dunums respectively in summer session that area more than the area in other session. But the agricultural session in Nablus totaled 170.20 dunums in winter (Table 6) (PCBS, 2012).

Table 6: Tomato area in dunums based on agriculture session in Tubas, Tulkarem and Nablus Governorate, 2012.

	Governorate		
Agricultural session	Tubas	Tulkarem	Nablus
Winter	473.82	207.6	170.20
Spring	327.50	102.66	63.84
Summer	579.24	329.02	63.15
Autumn	194.76	83.30	64.83
Not stated	3.00	2,50	____
Total	1578.32	725.09	362.02

Single crop is the status of tomato crop in Tubas, Tulkarem and Nablus that are totaled 1365.82, 681.38 and 331.26 dunums respectively (PCBS, 2012). This mean tomato crop cultivated alone in the field. (Table 7).

Table 7: Tomato area in dunums based on status of crop in Tubas, Tulkarem and Nablus Governorate. (2012).

	Governorate		
State of crop	Tubas	Tulkarem	Nablus
Single	1365.82	681.38	331.26
Associated	12.00	27.11	15.46
Mixed	200.50	16.60	15.30
Total	1578.32	725.09	362.02

1.2. Literature Review

The previous researches on tomato crops by using immunosorbent technique showed that the most dominant viruses infecting tomato in the northern of the West Bank were *Tomato yellow leaf curl virus* (TYLCV). This virus spread from 28-93% in Jenin and Tubas. The second most spread of virus in tomato crops was *Cucumber mosaic virus* (CMV), which ranged from 15-51% in the research area. But other viruses infecting tomato like *Tomato mosaic virus* (TOMV) and *Potato virus Y* (PVY) were found in very few samples of tomato (Sawalha, 2011).

A serious damages and large economical losses on tomato crops occasionally occur by viral infection. The magnitude of damages and economical losses depends on the strain of the virus, the infection time, the variety of tomato and other factors (Amro et al., 2014).

Tomato production in Palestine is not higher enough due to different factors as the inadequacy of management, climate and higher level of pests. But the viral infection is the most important factor that can cause weakness in crop production (Sawalha, 2011).

There are 35 plant families that can be infected by *Tomato spotted wilt virus* (TSWV) including dicots and monocots. The existing of the certain thrips species were needed for the continuous movement of virus. TSWV previously threat only tropical and subtropical crops, but in the current days the virus spread in several regions because of the western flower thrips distribution and the movement of virus infected plant materials.

Transmission of seed is not important for spread of the disease because the virus existing only in seed coat not in the embryo (Sherwood et al., 2009).

1.3. Tomato infecting viruses

More than 40 viral species can cause infection to the tomato plant and fruit (Morals & Anderson, 2001). In tomato the first virus reported was *Tomato spotted wilt virus* TSWV in Iran. One of the viruses that can cause necrotic lesions on the leaves of tomato and yellow ring spots on the fruits is (TYRV) *Tomato yellow ring virus* (Table 8) (Mehraban et al., 2005).

The following viruses was also detected in infecting tomato crop; *Tomato Yellow spot virus* (TYSV), *Tomato chlorotic mottle virus* (TCMV), *Tomato rugose mosaic virus* (TRMV), *Tomato yellow vein streak virus* (TYVSV) and *Tomato bushy stunt virus* (TBSV).

Tomato and other two plants in the northern West Bank of Palestine were found to be natural reservoirs for the virus *Tomato yellow leaf curl virus* (TYLCV), which is detected using the Polymerase Chain Reaction (PCR)(Sawalha, 2009; Amro et al., 2014).

Table 8: The common genus and species in the family *Bunyaviridae*.

Family	Genus	Species		
Bunyaviridae	Phlebovirus	PVFV	TSCV	UUKV
	Tenuivirus	RSV		
	Hantavirus	HTNV	SEOV	PUUV
	Tospovirus	INSV	**TSWV**	PYSV
	Bunyavirus	BUNV	LACV	

The broadest host range in the genus Tospovirus mainly is *Tomato spotted wilt virus* (TSWV). It has the most economical impact of all (Goldbach & Kuo, 1996; Goldbach & Peters, 1994).

Tomato which is infected by TSWV can be identified by the presence of yellow, brown ring or other line pattern on the fruits, reduction in growth in tomato plant and consisted of systemic chlorotic and necrotic spots on leaves or tip dieback. The differences in symptoms depend on the severity of infection (Mehraban et al., 2005;Sherwood et al., 2009). And the severity of infection based on the environmental factor, host cultivar, pathogen strain and stage of host development (Sevik & Arli-Sokmen, 2012).In more than 60 countries all over the world TSWV had been founded (Karavina & Gubba, 2017).

1.4.Tospovirus

The only plant-infecting virus in the family *Bunyaviridae* is the viruses of the genus *Tospovirus*. This virus can cause large damage in the quantity and quality of fruit, vegetable and ornamental plants (German et al., 1992; Pappu et al., 2009).

Some viruses are mainly limited to ornamental plants like *Impatiens necrotic spot virus* (INSV) (Law & Moyer, 1990). But the most Tospovirus like *Peanut yellow spot virus* (PYSV) (Reddy et al., 1991) and *Iris yellow spot virus* (IYSV) (Cortêz et al., 1998) have little virus effect because of the narrow host range.

The viruses of this genus are enclosed in envelope, quasi-spherical and contain three single stranded RNA segments (ssRNA) which is named according to the size (Figure 1). Two components are strongly packaged in each segment of RNA the first one is the nucleocapsid protein and the second is small amounts of the viral RNA-dependent RNA polymerase (RdRp) (Poelwijk et al., 1993). The presence of the inverted complementary repeat sequence at the end part of all segments of Tospoviral RNA will form the infecting ribonucleocapsid proteins (RNPs) (Bhat et al, 2001).

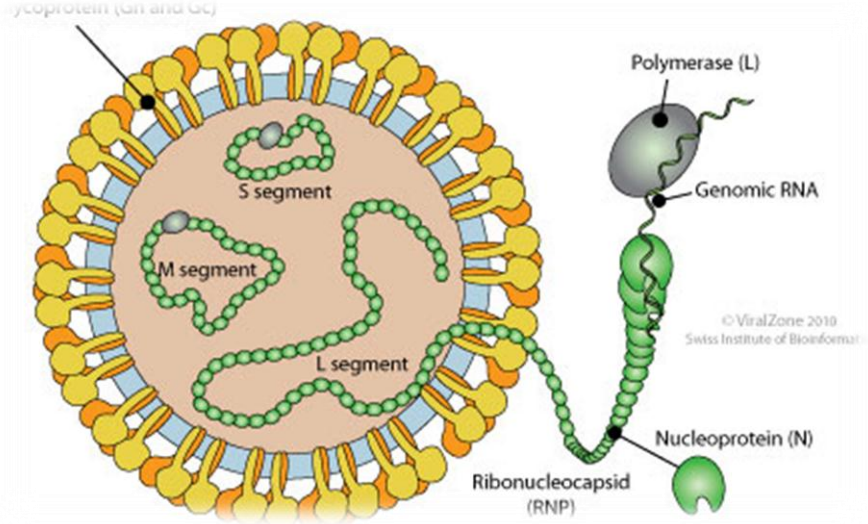


Figure 1:*Bunyaviridae*virion with spherical enveloped. Diameter from 80 to 120nm.

All Tospovirus have ambisense small and medium portion of RNAs. Only the large portion of RNA was being of integral negative polarity. Viral sense for a suppressor of RNA silencing (NSs) encodes by the genomic RNA (Takeda et al., 2002). While the cell-to-cell transmission protein (NSm) encoded by the medium RNA portion in viral sense and the precursor to the glycoprotein one and two in viral sense (Figure 2) (Fukuta

et al., 2004; De Haan et al., 1990; Kormelink et al., 1992). The large RNA portion that also mentioned as L protein encodes the delusive viral (RdRp) (De Haan et al., 1991).

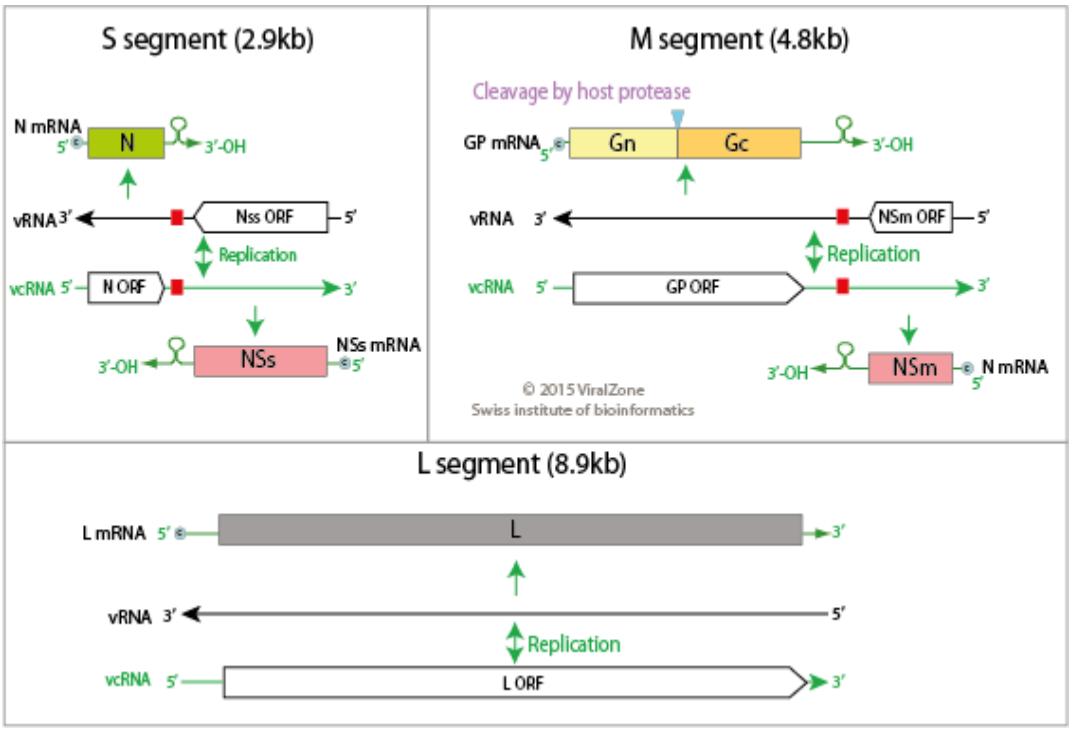


Figure 2: The virus genome of *Tospovirus* spices.

According to the biological and molecular characteristics 14 Tospovirus species have been identified (Table 9). One of them is (TSWV), which is worldwide distributed (Cortes et al., 2001; MaMichael et al., 2002).

Table 9:List of common *Tospovirus* species with the percent of N-protein sequence.

Descriptors				
Serogroup species	**Serological affinity**	**N-protein Sequence**	**Vector specificity**	**Host range**
Tomato spotted wilt virus (TSWV)	+	100%	+	+
Tomato chlorotic spot virus (TCSV)	+	76%	+	+
Groundnut ringspot virus (GRSV)	+	78%	+	+
Impatiens necrotic spot virus (INSV)	+	55%	+	+
Watermelon silver mottle virus (WSMV) including Groundnut bud necrosis virus (GBNV)	+	29%	+	+
Melon spotted wilt virus (MSWV)	+	35%	+	+
Groundnut yellow spot virus (GYSV)	+	?	+	+

1.5. *Tomato Spotted Wilt Virus* (TSWV)

On some occasion (TSWV) cause losses of up to 100% so that is one of the most popular viruses infecting tomato (Rosello et al. 1996).This virus reported firstly in tomato plant (Blancard, 2012).

The farmers scared mostly from TSWV plant virus due to; the complicated nature, the speed of virus transference and the vectors biological activity, the quickly adapt with new variants, and the difficulty in administration for the vectors (Parrella et al. 2003).

The diameter of TSWV virions is 80_120 nm, spherical, enveloped, and the virions surfaces have projections formed of two glycoproteins, G1 and G2 (Figure 1). Virion consists of 5% nucleic acid (RNA), 70% protein, 5% carbohydrate, and 20% lipid. Three negative or ambisensess-RNA species

that form the genome of TSWV, This tree species organized as S (2.9 kb), M (4.8kb) and L (8.9kb). RNAs take pseudocircular or panhandle conformation through the present of partially complementary terminal sequences (Elliott et al., 2000).

The small and medium RNAs encode two proteins in an ambisense arrangement, but the large RNA is monocistronic and negative sense. So that, five protein encoded in the viral genome; a 52-kDa NSs protein is encoded by the virion sense small RNA; 29-kDa structural N protein coat the fragment of RNA genome which lead to encode the nucelocapsids by the complementary sense small RNA; a 34-kDa of NSm protein function in cell-to-cell movement of the virus and the virion sense medium RNA code it; a 127-kDa protein which processed to form glycoprotein G1 and G2 that are function in the formation of the virus surface projection are coded for by the complementary sense medium RNA; and a 330-kDa of RNA polymerase encoded by large RNA. The five proteins enclosed within a membrane bilayer of a host with the large protein (L) totaled 10-20 copies (Figure 3). The misleading RNA dependent RNA polymerase encode by large RNA (Hull, 2002).

The sap of infected plant is the mechanical transporter for the virus TSWV, growers tend to use petunia plant as an indicator to control the transmission of TSWV by thrips(Jenser et at., 2003).

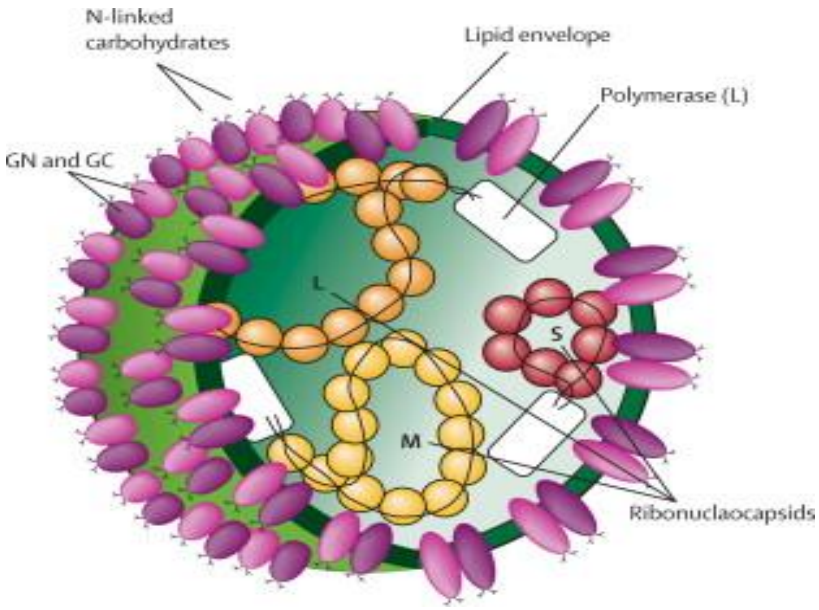


Figure 3: The five protein encoded in the viral genome of the TSWV**.**

1.6. Virus host (tomato) infection

Tospoviruses are sequentially transferred by fixed number of vegetative thrips (Caprile et al., 2009). More than 92 families of plants are infected by over 1300 species (Parrella et al., 2003; EFSA 2012). Thrips are the vector for transmission of a few viruses including TSWV (Figure 4).

Figure 4: *Frankliniella occidentalis* (western flower thrips).

There are four species of thrips: *Frankliniella occidentalis*(western flower thrips); *F. schultzei*, *F fusca* (tobacco thrips) and *Thrips tabaci* (onion thrips), that's defined as the most important vector because of the wide distribution and the relation between species and TSWV (Table 10) (Sherwood et al., 2009).

Table 10: Thrips vector that's reported and their specificity.

Viruses vector	Thrip species
TSWV,TCSV,GRSV,IVSV	*Frankliniella occidentalis*
TSWV,TCSV,GRSV	*F. schultzei*
TSWV,INSV	*F. fusca*
TSWV,TCSV	*F. intonsa*
TSWV	*Thrips tabaci*
TSWV	*T. setosus*
GBNV,WSMV,MSWV	*T. palmi*

1.7Methods for Determination of Plant Virus Infection

1.7.1 Symptomatology

Tomato which is infected by TSWV can be identified by the presence of yellow, brown ring or other line pattern on the fruits (Figure 5), reduction in growth in tomato plant and consisted of systemic chlorotic and necrotic spots on leaves or tip dieback (Figure 6). The differences in symptoms depend on the severity of infection (Mehraban et al., 2005 ; Sherwood et al., 2009). And the severity of infection based on the environmental factor, host cultivar, pathogen strain and stage of host development (Sevik & Arli-Sokmen, 2012).In more than 60 countries all over the world TSWV had been founded (Karavina & Gubba, 2017).

(A)

(B)

(C)

Figure 5: Infection symptoms on tomato fruit (A, B, and C)

(A)

(B)

Figure 6: Infection symptoms on tomato leaves (A and B).

1.7.2. Molecular Method

Real-time (PCR) and reverse transcription-polymerase chain reaction (RT-PCR) (Figure 7), were used in detection of TSWV in *F. occidentalis*, both methods are efficient and sensitive but real-time (PCR) characterized by a higher sensitivity and quantitative analysis of the TSWV (Tsuda et al., 1994; Mason et al., 2003; Boonham et al., 2002; Rotenberg et al., 2009).

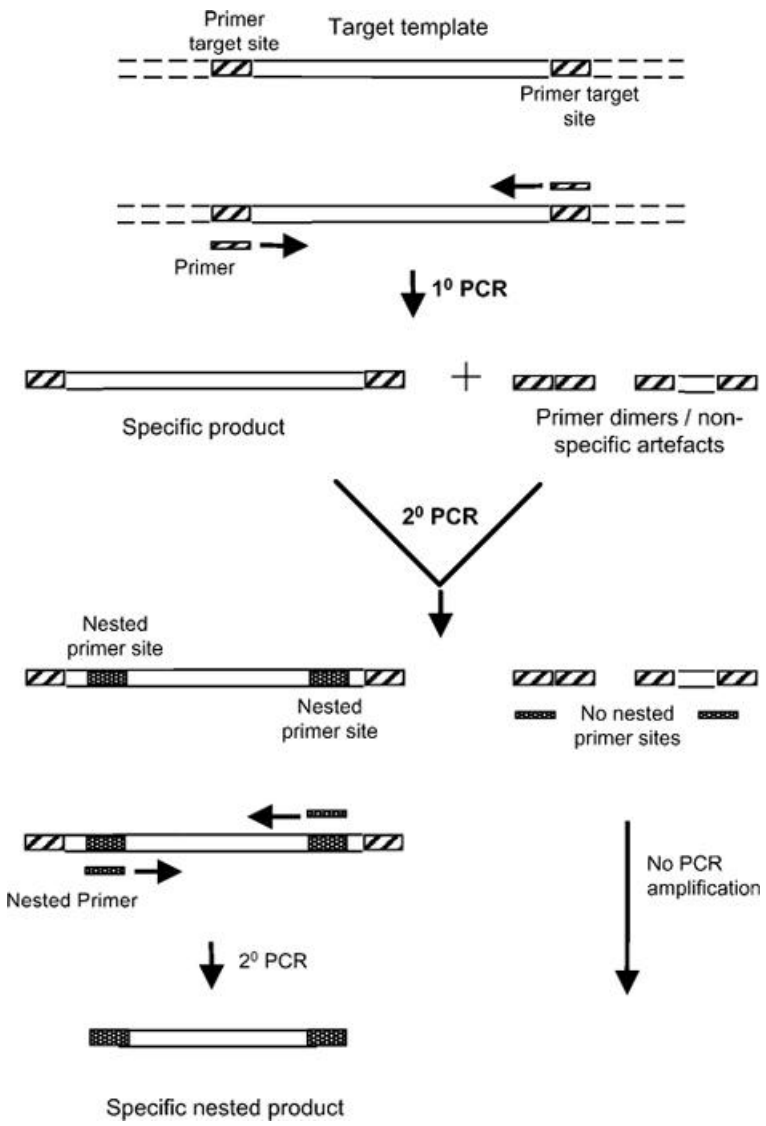
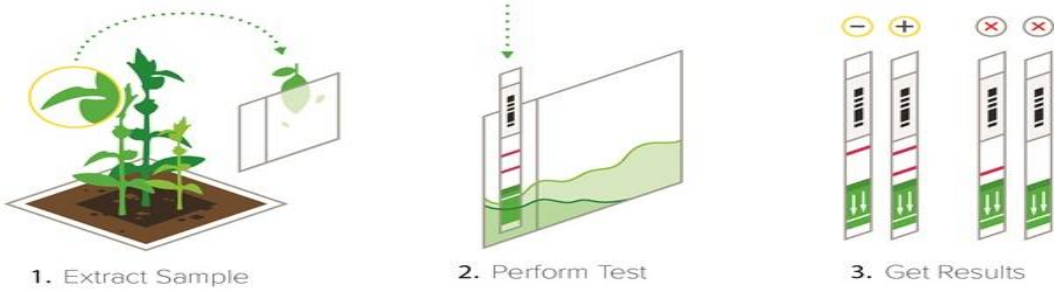


Figure 7: The cycle of (RT-PCR) Reverse Transcription polymerase chain reaction, each cycle of PCR process contain three steps; denaturation, annealing, and extension.

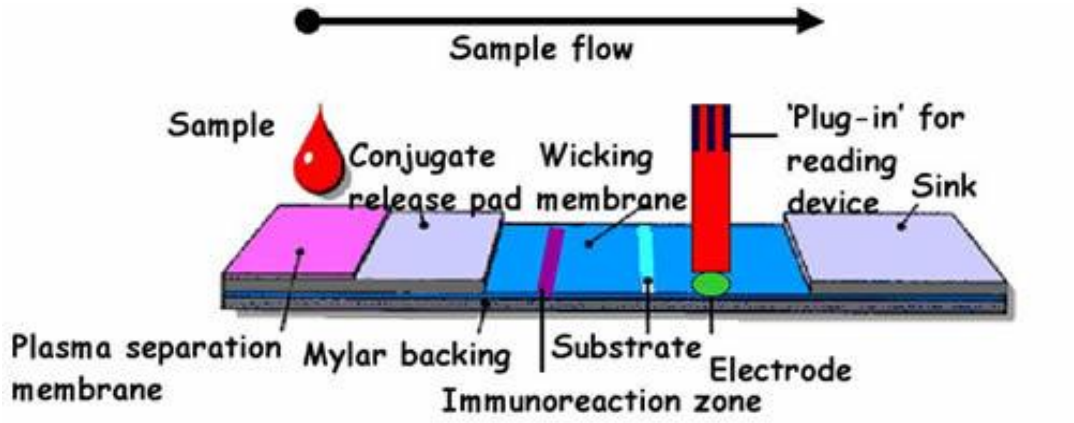
1.7.3. Serological Method

Serological and molecular methods have been used to identify *Tomato spotted wilt viruses* (TSWV). The assay which commonly used to identify Tospoviruses in various thrips vectors like *Frankliniella occidentalis* thrips is Enzyme-Linked immunosorbent (ELISA) (Nagata et al., 2002).

Immunostrips another technique was used for the first time in detection of TSWV in Zimbabwe (Figure 8) it provides an easier to use and quicker detection technique than Enzyme-Linked immunosorbent (ELISA), electron microscope and molecular assay (strange, 2006).



(A)



(B)

Figure 8: Immunostrips technique for detection of TSWV, (A) the main steps of Immunostips, (B) the strip which used for this technique.

ELISA assay used to test large number of samples in short period of time, so that it is simple, flexible, credible, sensitive and economical test. But this test can take several hours in plant tissue extraction. The adhered of antibodies and sample to the microtiter wells need for another incubation period (Figure 9) (Naidu & hughes, 2003; Batool et al., 2011).

In this research study ELISA test was our choice for detection of the virus in tomato fields.

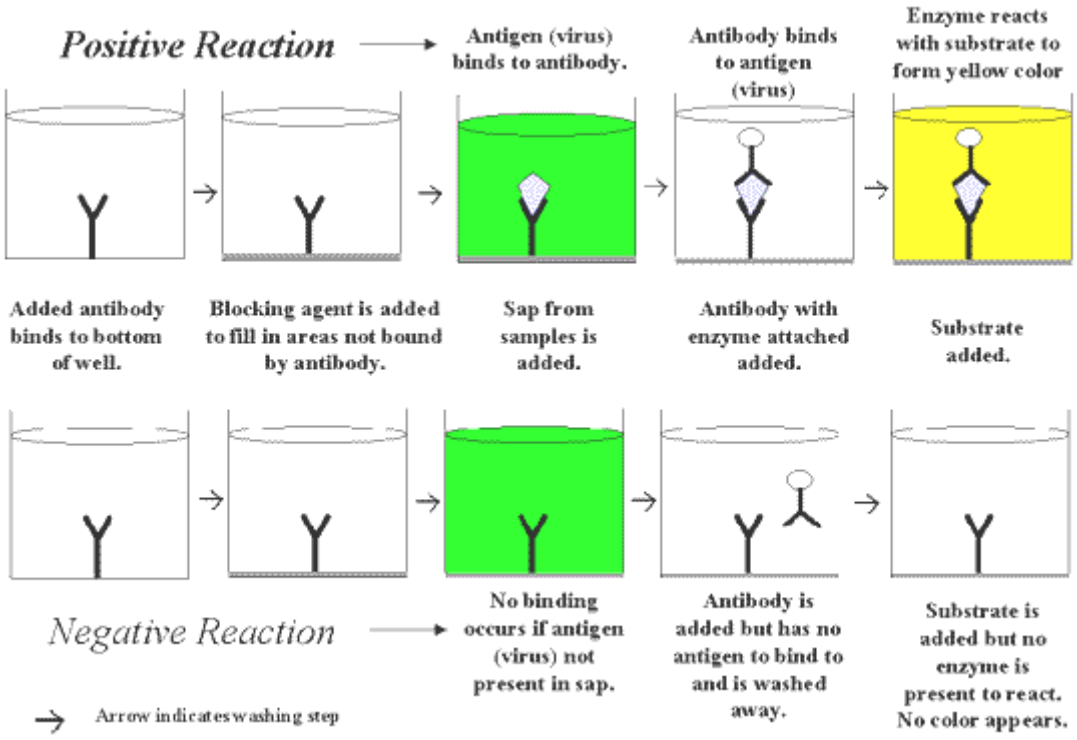


Figure 9: The general steps for DAS-ELISA method.

1.8. Objectives

The main aims of this study were to detect and evaluate the prevalence of tomato spotted wilt virus in the following region of the West Bank (Jericho, Jenin, Tubas, Tulkarem, Nablus, Qalqelia) by using the available serological tools. This would be the first study of the virus status in the country.

Chapter Tow

Materials and Methods

Chapter Tow
Materials and Methods

2. Field Survey and Sample Collection

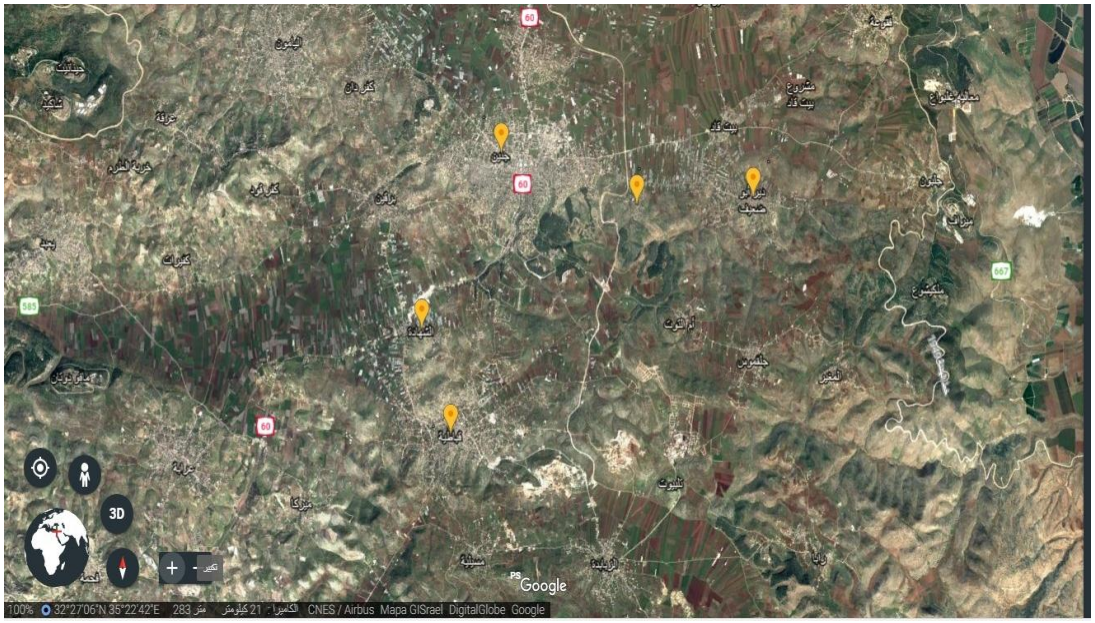
2.1. Location of Sample and Sampling in the Field.

Tomato fields of six districts were studied in the northern region of the west bank (Figure 10) (Jericho, Jenin, Tubas, Tulkarem, Nablus, Qalqelia). About 289 samples of tomato leaves from different fields were collected. Only 235 samples of which; were used in this research (Table 11)**.**

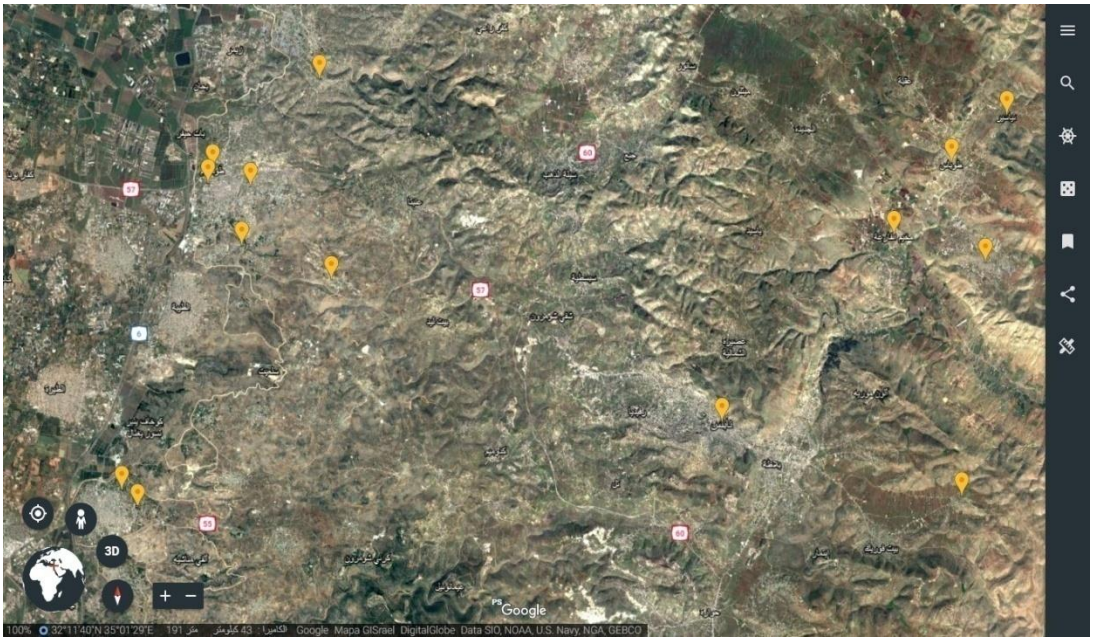
Table 11: The number of tomato samples and greenhouses at each city.

City	Sampling area	Number of samples	Number of greenhouses	Total
Nablus	Bet-Dajan	18	3	51
	Al-Smeet	27	3	
	AL-Bathan	6	2	
Tulkarem	Khadore	4	1	43
	Der-Goson	6	2	
	Thanabeh	21	5	
	Shofeh	12	2	
Tubas	Tamun	15	4	51
	Al-Fara'a	27	4	
	Tyaser	9	3	
Jenin	Ash-Shuhada	6	2	53
	Qabatya	17	5	
	Der Abu Da'ef	27	6	
	Aba-Alsharqya	3	1	
Qalqelia	Al-mdwarah	15	3	33
	Alsaba'	18	3	
Jericho	Al-nweameh	30	4	58
	Aen-Alsultan	21	3	
	Aen-Aldyok	7	2	
				289

Tomato fields of six districts were studied in the northern region of the west bank (Figure 10) (Jericho, Jenin, Tubas, Tulkarem, Nablus, Qalqelia).



(A)



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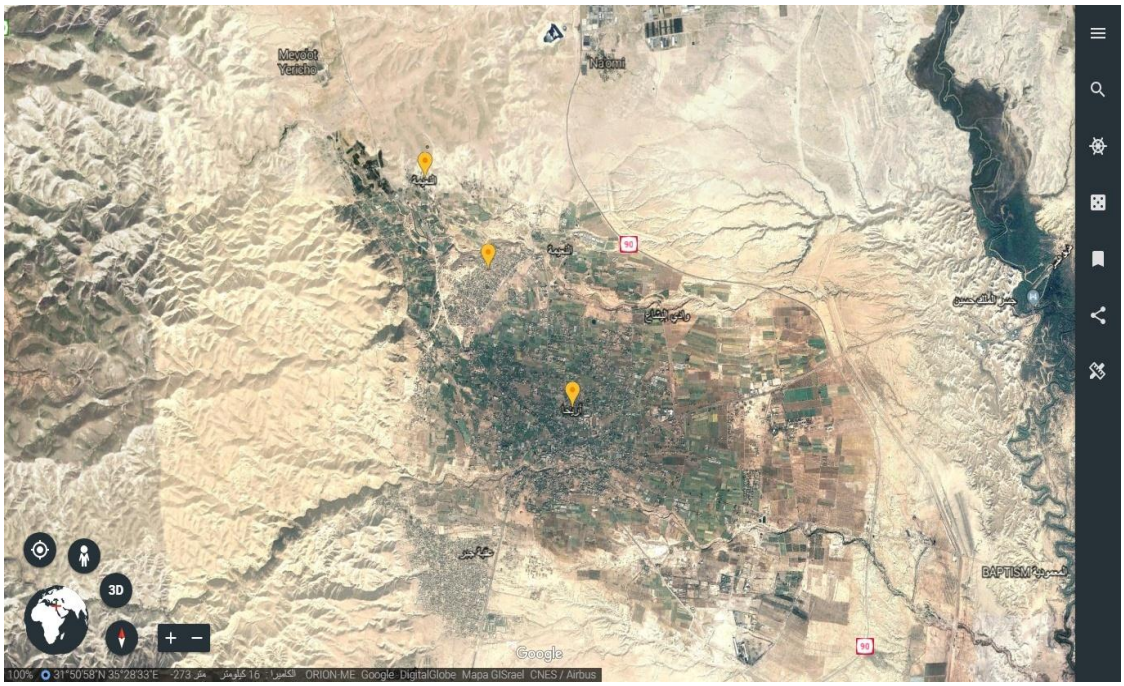

(C)

Figure 10: The six sampling area; (A)Jenin, (B)Tubas, Nablus, Tulkarem, and Qalqelia, (C) Jericho.

The collected samples were taken from about Seventeen varieties (Table 12). Most tomato sample were collected are grown in August and September but in exceptional cases are grown in March or April. The samples were collected during 15/3_ 30/4/2018, almost all samples age between 7-8 months old. The soil where tomato was cultivated in all sampling area was classified as red clay parasites and alkaline soil.

By using sterile gloves and scissors, the samples were collected and stored in plastic bags then transferred to the cold room at 4˚C degree Celsius to be used later in serological test.

Table 12: **The number of tomato varieties at each area sampling**

Tomato Varieties	Number of sample at each city						Total
	Nablus	Tulkarem	Tubas	Jenin	Qalqelia	Jericho	
Cluster (Ikram)	33	9	12	15	9	18	96
Cluster (Nukran)	__	__	__	__	6	3	9
Izmir	18	6	6	13	__	4	47
Cherry	__	__	9	__	__	__	9
Shekram	__	__	9	__	__	__	9
Balahya	__	__	3	__	__	__	3
Princess	__	__	3	__	__	__	3
593	__	7	6	__	__	__	13
554	__	__	__	__	9	__	9
AX64-782F1	__	6	__	__	__	__	6
Granada	__	__	__	12	__	__	12
Gory	__	__	__	10	__	12	22
Izmuna	__	__	3	3	__	__	6
888	__	9	__	__	__	__	9
026	__	6	__	__	__	6	12
Just	__	__	__	__	6	__	6
Mayla	__	__	__	__	3	15	18

2.2.Serological Detection of TSWV by using DAS-ELISA.

The Enzyme-Linked Immuunosorbent Assay (ELISA) test depends on the specificity of the interaction between antigen and antibodies. This test very useful in the detection of viruses and it is usually done in plastic plate containing wells or depressions. The source of antigens was from plant leaf.

Double Antibody Sandwich ELISA (DAS) were used for detection of the virus in tomato samples (Jericho, Jenin, Tubas, Tulkarem, Nablus, Qalqelia).

The basic principle of ELISA based on freezing the movement of antigen onto a solid surface or trapping antigen by specific antibodies; and probing with specific immunoglobulin attached to an enzyme label (Batool et al., 2011).

The addition of a suitable substrate is detectable for the positive reaction in which the enzymes converting the substrate to product that can be visualized by the color.

2.2.1. Required Materials

The followings were the materials used in this study

- ELISA kit specific to TSWV (Agdia)
- Carbonate Coating Buffer (10X)
- PBST Wash Buffer (Phosphate buffered saline powder containing Tween 20)
- ECI Buffer (5X)
- PNP Substrate Buffer (5X)
- PNP Substrate Tablets from Agdia (ACC 00404) (5mg for each tablet)
- Tween 20 (polysorbate 20)
- Alkaline Phosphate Enzyme Conjugate
- Capture Antibody
- General Extract Buffer (sample extraction buffer)
- Distilled or purified water
- 96-well microtiter plates (strip or solid)

- Paper towels
- Micropipette
- Micropipette tips
- Sample grinding device such as; Mortar and pestle
- Airtight container for incubations

2.2.2. Sap Extraction

Tomato leaves from symptomatic and symptomless plants were used for ELISA test. About 0.35-0.4gm of the leaves were grinded in sterile mortar and pistil using the grinding buffer (General extraction buffer provided within the kit as a powder; Tween 20; and distilled water). Plant sap will be stored on ice for immediate use.

2.2.3. The procedure of double antibody sandwich ELISA (DAS).

In the first step a humid box were prepared by lining an airtight container with a wet paper towel, this step for preventing evaporation of samples from the test plate. Carbonate coating buffer with (10X) concentration diluted with distilled water according to the ratio 1:10 provided on the tube. The capture antibody diluted with carbonate coating buffer according to the 1:200 ratio. 100 µl of the prepared capture antibody were loaded into each well of the plate and the plate incubated in a humid box for 4 hours at room temperature or overnight in the refrigerator (4ºC).

Phosphate Buffered Saline with Tween-20 (PBST) wash buffer of 1Xconcentration were diluted by distilled water, 1 gram of PBST buffer were added to the 100 ml of distilled water with continuous stirring. Once the incubation of sample was completed, the plates were washed by using flipping motion to dump the well into a sink. The wells filled completely with 1X PBST, this step were repeated 2 more times to remove all excess liquid. The freshly coated plates were have been used immediately. A composite of up to ten leaves were used to make testing more economical. The ten leaves almost weighted between 0.35 to 0.4 grams.

After the weighting of tomato samples the Agdia's general extraction buffer (GEB) used in order to grind and dilute samples. In this step a sterile mortar and pestle used to grind the tomato leaves. General Extraction Buffer (GEB 1X) diluted with D.W and tween-20 were used to grind tomato leaves. 100 µl of prepared sample was dispensed into sample wells and the plate was placed inside the humid box and incubated overnight in the refrigerator (4ºC).

The next day beginning by filling the plates completely with 1X PBST for washing the plate, repeat 7 times and a quick flipping motion were used to dump the wells into a sink after washing, the frame holed upside down and taped firmly on a folded paper towel to remove all droplet of wash buffer. The test wells should be free of plant tissue. If tissue was still presented, then washing step will be repeated again. ECI buffer, this buffer is most commonly used to dilute enzyme conjugates. The enzyme conjugate always prepared within 10 minutes before used. 5X ECI buffer was diluted by D.W

in the ratio of1:5, then 1 amount of alkaline phosphatase enzyme conjugate diluted with 200amount of diluted ECI buffer before use. After the addition of the enzyme conjugate the solution mixed thoroughly. 100 µl of prepared enzyme conjugate was dispensed per well. The plate was incubated in the humid box for 2 hours at room temperature.

The PNP solution prepared for the final step. Each PNP tablet (ACC 00404) will make 5 ml of PNP solution, at a concentration of 1 mg/ml, about enough for five 8-well strips. About 15 minutes before the end of the above incubation step, 5 ml of room temperature 1X PNP buffer measured for each tablet. Then, without touching the tablets, the PNP tablets were added to the buffer. As the pervious washing steps, the plate was washed 8 times with 1X PBST. X. The wells were inspected by looking for the presence of air bubbles. These were taped firmly on the paper towel to remove any remaining wash buffer and air bubbles. If air bubbles were still present, they were broken with a clean pipette tip. 100 µl of PNP substrate were dispensed into each test well. The plate was incubated for 60 minutes by aluminum foil to protected from direct or intense light. The result was examined by eye and also was measured through the plate ELISA reader at 405 nm.

2.2.4. Buffers formulations (for tomato leaves):

The following Table illustrated the buffers used, their formulations and the amount provided with the Agdia's kit

Carbonate Coating buffer (1X)	Dissolve in distilled water to 1000 ml:		
	Sodium carbonate (anhydrous)	1.59	g
	Sodium bicarbonate	2.93	g
	Sodium azide	0.2	g
	Adjust pH to 9.6 store at 4º C		
PBST buffer (Wash buffer) (1X)	Dissolve in distilled water to 1000 ml:		
	Sodium chloride	8.0	g
	Sodium phosphate, dibasic (anhydrous)	1.15	g
	Potassium phosphate, monobasic (anhydrous)	0.2	g
	Potassium chloride	0.2	g
	Tween-20	0.5	g
	Adjust pH to 7.5		
ECI buffer (1X)	Add to 1000 ml 1X PBST:		
	Bovine serum albumin (BSA)	2.0	g
	Polyvinylpyrrolidone (PVP) MW 24-40000	20.0	g
	sodium azide	0.2	g
	Adjust pH to 7.4 store at 4º C		
PNP buffer (1X)	Dissolve in 800 ml distilled water:		
	Magnesium chloride hexahydrate	0.1	g
	Sodium azide	0.2	g
	Diethanolamine	97.0	ml
	Adjust pH to 9.8 with hydrochloric acid, Adjust final volume to 1000 ml with D.W. store at 4º C.		
General Extraction Buffer (GEB 1X)	Dissolve in 1000 ml of 1X PBST:		
	Sodium sulfite (anhydrous)	1.3	g
	Polyvinylpyrrolidone (PVP) MW 24-40000	20.0	g
	Sodium azide	0.2	g
	Powdered egg (chicken) albumin, Grade II	2.0	g
	Tween-20	20.0	g
	Adjust pH to 7.4 store at 4 º C.		

Chapter Three

Results and Discussion

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Results and Discussion

Tomato form about 32% of the total vegetable production in Palestine which produces annually in 204,000 metric tons (PCBS, 2008).

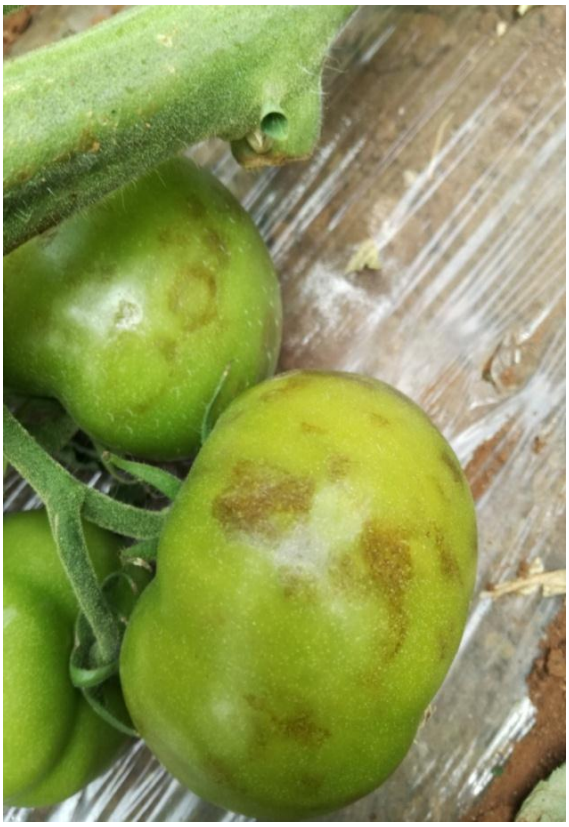
TSWV was considered the largest host-range among plant viruses. In different world regions, this virus was considered as the most scary plant virus by farmers of tomato crop. Preventive and integrated cultural practices such as eradication of weed host; which served as virus reservoirs, combined with vector management strategies, play a crucial role in the control of the virus (Parrela et al., 2003).

3.1 Filed Survey and symptoms evaluation

Viral symptoms were appeared in only one field in Qabatya-Jenin governorate. The infected plants were found exhibiting similar symptoms with little variations. These symptoms characterized by the regular and steady ring spots on tomato fruits and leaves (Figure 11). About 17 samples from 5 different greenhouses were collected (Table 11); four of them were exhibited the virus symptoms. Depending on symptoms evaluation the percent of incidence in that only region was almost about 23.5%; but in respect to all sampling areas, it was equal to 1.72%. This percent of incidence was confirmed later by serological assay. The symptoms were pictured as seen in the (Figure 11).

(A)

(B)



(C)

(D)

(E)

Figure 11: Symptoms of TSWV infection which observed in the field, (A) represent the brown line pattern on tomato veins, (B, C) the brown ring spots on tomato fruits, (D, E) chlorotic, necrotic spots and malformation on tomato leaves.

The results from the field surveys showed (23.5%) of viral symptoms in one region (Qabatya). The symptoms were similar to those of TSWV disease symptoms. These were observed on two growing tomato varieties Granada and cluster (Ikram), the famous tomato greenhouses grown varieties in Palestine.

In TSWV management the most effective preventive strategy was the elimination of weeds; the alternative hosts of the virus (Marchoux et al., 2000).

3.2 Serological Assays

All collected samples were from symptom and symptomless tomato plants. Samples were tested serologically for the presence of TSWV by using Enzyme-Linked Immuunosorbent Assay DAS-ELISA (Agdia Kit). TSWV had been confirmed in only 3 samples out of 232 tested ones. It was noticed that the only positive samples were from Qabatya in Jenin. The three positive samples which were tested by DAS-ELISA were collected from tomato plants showing viral symptoms similar to TSWV infection symptoms.

Depending on the positive results, the incidence percent of TSWV was equal to the positive result in TSWV ELISA test divided by the total tomato samples were tested, as the following equation:

% Incidence TSWV = positive tomato sample in ELISA test ÷ total tomato sample tested ×100%

According to that the percent of incidence of TSWV was about 1.29% in Northern region of the West bank of Palestine.

The existence of TSWV was confirmed by using ELISA kit (Agdia) because the visual test depending on the symptoms of the virus infection (yellow, brown ring or other line pattern on the fruits, reduction in growth in tomato plant and consisted of systemic chlorotic and necrotic spots on leaves or tip dieback) is insufficient to confirm the present of TSWV (Mehraban et al., 2005; Sherwood et al., 2009).

The collected samples were from both symptomatic and symptomless tomato leaves, so that DAS-ELISA important technique to be sure that the plant infected by *Tomato spotted wilt virus.*

The virus infection in most cases developed depending on different factors such as; ecological, environmental factor, and on the virus behavior (the strain of virus and time of infection). Other factors can be the virus development in the plant variety and the age of tomato plant (Parrella et al. 2003).

According to the Ministry of Agriculture (personal information) there was some varieties of tomato plants such as; 554, Just, 026, 593, and Ax64-782fı; were modified to resist viruses. These modified varieties can be cultivated in all seasons of the year. Depending on the statistical

information to the year 2016/2017, agricultural engineerings thinking that the virus which was spread in all governorates in that year was TSWV. Hence it is worth to mention, that such kind of conclusion lead to neglect a large number of tomato fields, due to the undesired symptoms on tomato fruit. The spread of infection also prompt the farmers to change the crop. This study was aimed to reveal the real status and incidence of this virus in the tomato growing fields of Palestine.

All samples were tested by DAS-ELISA which collected from symptoms and symptomless tomato plant. This technique was reliable and effective in detection of TSWV. Three positive samples were detected to confirm the success detection of the virus by this tecniques.

According to a research study in 2012; ten samples of lettuce were reported infected by TSWV in Jordan (Salem et al., 2012, Anfoka et al., 2006). The samples were tested by ELISA, where symptoms had been observed on lettuce crops of the Jordan Valley, reminiscent of those induced by TSWV, which had already been recorded in Jordan on tomato in 2016.

Although, tomato and pepper in Spain was showed to be infected with TSWV; using the naturally resistance genes in the wild *Solanum* and *Capsicum* species plant were found the best strategy to control the disease. A hypersensitive response to TSWV occurs in some accessions of *Capsicum chinense* due to the occurrence of a single dominant resistance Tsw gene that has been flowed in the pepper hybrids, but several factors can alter the expression of this resistance (Moury et al., 1998). In tomato,

the resistance gene was found in *S. peruvianum* and *S. pimpinillefolium*. Trials to use it by most breeding programs were showed that some tomato expressed broad resistance to various TSWV isolates. This resistance, conferred by a single dominant gene, Sw-5, that has been introduced into several Spanish commercial tomato hybrids (Aramburu et al,. 2011).

Chapter Four

Conclusion and Recommendations

Chapter Four

Conclusion and Recommendations

4.1 Conclusion and Recommendations

The result of this study showed the presence of *Tomato spotted wilt virus* (TSWV), despite a little positive result that was obtained from this test compared to the number of samples was tested. This test consider as the first serological test for detection of TSWV in the West Bank- Palestine. The three positive sample were found in Jenin governorate mainly in Qabatya confirm the presence of virus. Also confirm the sensitivity, reliability, and specificity of direct antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) technique in detection of tomato virus (TSWV).

Finally, depending on this study researcher could use the positive result as a base to the molecular test.

Looking for virus resistant varieties was recommended, neglect the field which have this virus and cultivate field by another crop instead tomato because the virus have an incubation period in soil to be sure that virus can't make infection at all.

Use this research study as the first step to make molecular test (by using the positive sample) in order to determine virus molecular characteristic, that helps the researcher to identify the nature of the virus and thus know how to eliminate it.

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جامعة النجاح الوطنية

كلية الدراسات العليا

الكشف السيرولوجي(المصلي) عن فيروس التوبوفايروس الذي يصيب البندورة في الضفة الغربية

إعداد

دالية ذيب هاشم مصري

إشراف

د. رائد الكوني

قدمت هذه الأطروحة استكمالا لمتطلبات الحصول على درجة الماجستير في برنامج العلوم الحياتية، لكلية الدراسات العليا، جامعة النجاح الوطنية، نابلس–فلسطين

2018

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إعداد

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الملخص

يعد نبات البندورة واحدة من أشهر المحاصيل في فلسطين. كما وأنها تصاب بالعديد من الممرضات حيث يعد فيروس الذبول المرقط واحد من أكثر الأمراض المدمرة لمحاصيل الطماطم.

لذلك يسبب حدوث أضرار جسيمة وخسائر اقتصادية كبيرة على محاصيل الطماطم.

تهدف هذه الدراسة الى الكشف عن وجود الفيروس ومدى انتشاره في أراضي الضفة الغربية_ فلسطين لأول مرة.

تم جمع ما يقارب 232عينه من مدن فلسطينية مختلفة شمال الضفة الغربية وذلك خلال الموسم الزراعي 2018/2017 وتم فحص العينات لكشف عن وجود هذا الفيروس من خلال الفحص السيرولوجي المصلي.

من خلال المسوح الميدانية لحقول الطماطم لوحظ وجود عدة أعراض على المحاصيل في حقول نابلس وطوباس وقلقيلية وطولكرم وجنين واريحا.

ظهرت على الأوراق أعراض الاصفرار آو الحلقات البنية أو الخطوط المختلفة، كما أن كامل النبات يحدث عليه تباطؤ في النمو، أيضا ظهرت البقع النخرية أو عديمة اللون على الثمار والأوراق.

وتعد هذه الأعراض المنتظمة والمنهجية أفضل مؤشر على وجود فيروس ذبول وترقط الطماطم.

بواسطة الفحص السيرولوجي المصلي الذي اجري في مختبرات جامعة النجاح اثبت وجود هذا الفيروس في فلسطين.

حيث اظهر هذا البحث أن نسبة انتشار الفيروس تصل الى 1.27% وهذه النسبة المنخفضة تنذر بالخطر وذلك لان الفيروس يمكنه الانتقال والانتشار في البلد على نطاق واسع من خلال الناقل الخاص به وهو الثربس.

نوصي من خلال هذا البحث على تطوير أصناف من الطماطم مقاومة للفيروس.

هذا البحث الدراسي تم لإثبات وجود هذا الفيروس المهم في فلسطين ولأول مرة.