An-Najah National University

Faculty of Graduate Studies

Sanitary Assessment of Viral Diseases Infecting Figs in Northern West Bank -Palestine

By

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This Thesis is Submitted in Partial Fulfillment of the requirements for the Degree of Master in Biological Sciences, Faculty of Graduate Studies, An-Najah National University, Nablus - Palestine.

2015

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Dedication

I dedicate my thesis to my lovely parents (Mohammed and Ferial), my sister (Manal), my brother (Yousef), my dear fiance (Khaleel) and for my best friend Maram Saqer with love and respect.

Acknowledgements

First, I would like to thank my supervisor Dr. Raed Alkowni for his encouragement, patient and help throughout this study.

My thanks for the faculty members of Graduate Studies and Biology department at An-Najah National University for their support during my Master program.

I would like to thank my colleagues Basema Abu-Rumaila, Isra'a Rabaya'a, Maram Saqer, Mays Sarah and Naela Abu-Hatab for supporting during work lab.

Above all, special thanks to my lovely parents for every things.

الإقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

Sanitary assessment of viral diseases infecting figs in northern West Bank-Palestine.

أقر بأن ما شملت عليه هذه الرسالة إنما هو نتاج جهدي الخاص، باستثناء ما تم الإشارة إليه حيثما ورد، وأن هذه الرسالة ككل، وأي جزء منها لم يقدم من قبل لنيل أي درجه أو لقب علمي لدى أي مؤسسة تعليمية أو بحثية أخرى

Declaration

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

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Date: **التاريخ:** ٢٠١٥/٩/٢٠

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List of Abbreviations

Bp	Base pair
cDNA	Complementary DNA
Cm	Centimeter
D.D.H_2O	Double distilled water
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethelendiaminetetraacetic acid
EtOH	Absolute ethanol
FBV-1	*Fig badnavirus-1*
FCV	*Fig cryptic virus*
FMD	Fig mosaic diseases
FMMaV	*Fig mild mottle-associated virus*
FMV	*Fig mosaic virus*
G	Gram
Ha	Hectare
KOAc	Potassium acetate
Ml	Mille liter
Mm	Mille mole
M-MLV	Moloney murine leukemia virus
µl	Micro litter
NaI	Sodium iodide
NaOAc	Sodium acetate
Na2S2O5	Sodium metabisulfite
NLS	Sodium lauryl sarcosine
No.	Number
PCR	Polymerase chain reaction
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
Rpm	Revolution per minute
RT-PCR	Reverse transcriptase – PCR
Taq	*Thermus aquaticus*
Tm	Temperature
TNA	Total nucleic acids
ºC	Degree Celsius

Sanitary assessment of viral diseases infecting figs in northern West Bank- Palestine.

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Abstract

The available data about the sanitary status of crops in Palestine is scarce, due to the lack of diagnostic facilities and equipment, for diseases, lack of specialized technicians, scientists and poor organization of nurseries and farming activities. Fig (*Ficus carica*) is one of the old and the most common fruit tree grown in all the Mediterranean countries, which is among those exposed to diseases and pathogens. Recent investigation indicated that fig mosaic disease was the main pathogenic agent. In this study, field surveys were conducted in the areas of northern region of West Bank- Palestine (Jenin, Tulkarem, Qalqilya, Nablus, Tubas, Salfit and Ramallah) for symptom observation during the two growing seasons of 2014 and 2015. In addition to that, a total of 50 fig samples were collected from the surveyed areas: Jenin (12), Nablus (16), Ramallah (5), Qalqilya (12) and Tulkarem (5 samples), to detect any of *Fig mosaic virus* (FMV), *Fig badnavirus-1* (FBV-1), *Fig cryptic virus* (FCV), and *Fig mild mottle-associated virus* (FMMaV) by using RT-PCR with sets of specific primers. During the field surveys almost two third of visually inspected trees demonstrated one or more of viral symptoms. The symptoms appeared on leaves as well as fruits. The symptoms on leaves were reported as mosaic, mottling; yellowing and deformation. Surprisingly the symptoms appeared

on newly growing leaves, and asymmetrically on trees, suggested the uneven distribution of the virus on the plant. Notably, mite infestations were found on some of those trees inspected as symptomatic, which may indicate they play a role as putative transmissible agents to the virus. Moreover, newly formed figs, were exhibiting yellow ring spots varied in size and strength, explaining the systematic paths of the viral infections and leading to believe of the virus existence and transmissibility within the propagated materials. Necrotic spots were also noticed on infected fig fruit. Molecularly, by using RT-PCR, all inspected viruses except of FMMaV, were detected in tested samples, with overall incidence of (78%). The most spread virus was FMV (60%) followed by FBV-1 (46%). Also were detected about 30% of mixed infection between FMV and FBV-1. Portions of the FMV and FBV genomes were sequenced and showed high similarity with published ones in GenBanks (98-100%). This study is reporting the deterioration of the sanitary status of figs in northern region of West Bank, as indicating by the high incidence of virus- infections in fig yards planted and growing there for many years. This could be considered alarming to fig production and extension in the country due to significant losses caused by the virus. As this study offered insight on the sanitary status of fig in the country, it is also highly recommended to use and produce of healthy fig seedlings and propagating materials.

Key words: fig viruses, RT-PCR, *Ficus carica*, FMD.

Chapter One

Introduction

1.1 Introduction

Agriculture is considered an important department for Palestinians in West Bank and Gaza Strip (WBGS). Also it plays a crucial role in protecting the environment and enhancing biodiversity. Palestine is described by a wide zone of environmental conditions and rich natural biodiversity (FAO, 2009).

Fig (*Ficus carica*) is an old common fruit grown in all the Mediterranean countries. It considered as one of the economically important crops (Aljane *et al*., 2008; Flaishman *et al*., 2008). Figs are deciduous flowering plants in Mulberry family (USDA, 2011; NGA, 2011). Recently, many types of viruses infect fig trees in different countries were detected (Elci *et al*., 2012).

Diseases induced by intracellular infectious agents (viruses, viroids, phytoplasma) represent a major threat, particularly to fruit trees and can stunt growth. The wide geographical incidence of these diseases is a result of the inefficacy of the standard methods used to control plant viruses. Their spread is also due to vectors (any agent that carries and transmits a disease) and from the diffusion of vegetative-reproductive plant material (material that reproduces asexually). It is humans, however, who are mainly responsible for spreading infectious diseases over long distances, through the uncontrolled circulation of plant material and trading stock that may be virus-infected (Alkowni and Srouji, 2009; ARIJ, 1994).

Living organisms are cellular that able to grow and reproduce independently. The simplest creatures living on earth today that satisfy

these criteria are bacteria, but viruses are simpler than that. Viruses are non– cellular form of life. They are obligate intracellular parasites and they exist as inert particles (virions) outside the cell. All viruses have the same basic structure: a core of nucleic acid surrounded by a protein. Viruses occur in virtually every kind of organism that has been investigated for their presence. However, each type of virus can replicate in only a very limited number of cell types, such as enveloped viruses that are usually infected animal cells. Unlike non-enveloped viruses that infect plant cells according to the difference in the structures between them. The suitable cells for a particular virus are collectively referred to as its host range. The size of the host range reflects the histories of co-evolution of the virus and its potential hosts (Peter *et al*., 2001).

1.2 The fig

1.2.1 Botany

The fig tree is a deciduous fruit tree; botanically it is inserted in the order Urticales, family Moracee, and genus *Ficus*. This genus includes about 1000 species, most are native to the tropics or sub-tropics. The most important species for the production of the fruit is *Ficus carica*, with chromosome number 2n = 26 (Morton, 2012; Flaishman *et al*., 2008).

1.2.2 Botanical Characteristics

The average lifespan of fig trees is between 50 and 70 years. The wood is light and soft, easily subject to decay, with little or no use. The roots have high penetration strength, speed of growth and ability to fit into the soil,

even rocky, leading to absorption up water from wet areas relatively distant. Fig trees and fruits contain typical latex secreting cells, producing milky exudates characteristic to all fig cultivars (Crane *et al*., 1950).

Latex is the cytoplasmic fluid of laticiferous tissues that contain the usual-organelles of plant cells such as nucleus, mitochondria and Golgi apparatus. It has been suggested that latex secretion is a defense against mechanical wounding and/or herbivores such as insects, vertebrates, microorganisms, and fungi (John, 1993). Finally, figs' leaves are pubescent, darker at the top, otherwise stinging depending on the cultivar, with a variable number of lobes more or less pronounced and separated from breasts of different depths. (Condit, 1955; Janzen, 1979).

1.2.3 World Market

Statistically, figs are harvested from 427000 ha around the world (FAO, 2009). The Mediterranean has been the most important region of fig production from ancient time, representing more than 82 % of the total world annual production. According to the latest FAO data in 2014, the world's largest producer analyzing the last three years is Turkey, Egypt, Syria and Algeria (FAO, 2014). It can be seen as the world's largest producers are those of the Mediterranean basin (Figure 1-1).

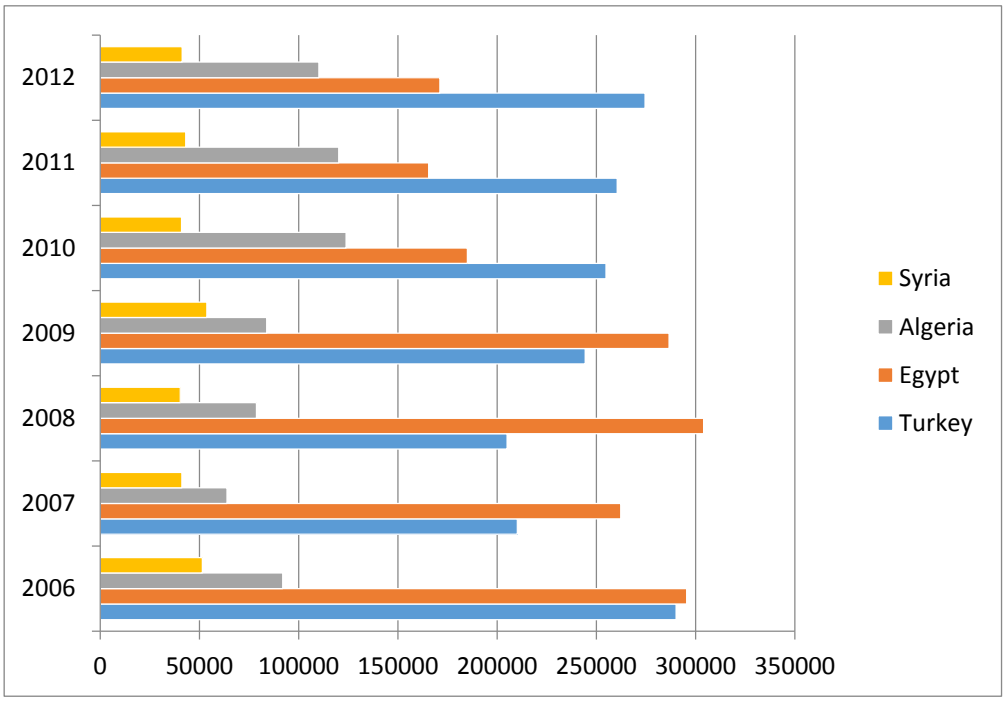


Figure 1-1. Worldwide production of fresh figs in the period 2006-2012, of four Mediterranean countries, which are the main producers in the world (ton/year) (FAO data, 2014).

Palestine is characterized by a wide range of environmental conditions and rich natural biodiversity. The fig trees are grown all over the country in mixture with other fruit trees like olive (Alkowni *et al*., 2015). According to the statistics of the Ministry of Agriculture Center in Ramallah –Balou' (personal communication), it has been monitoring decline in the production of fig in northern West Bank- Palestine. Figure (1-2).

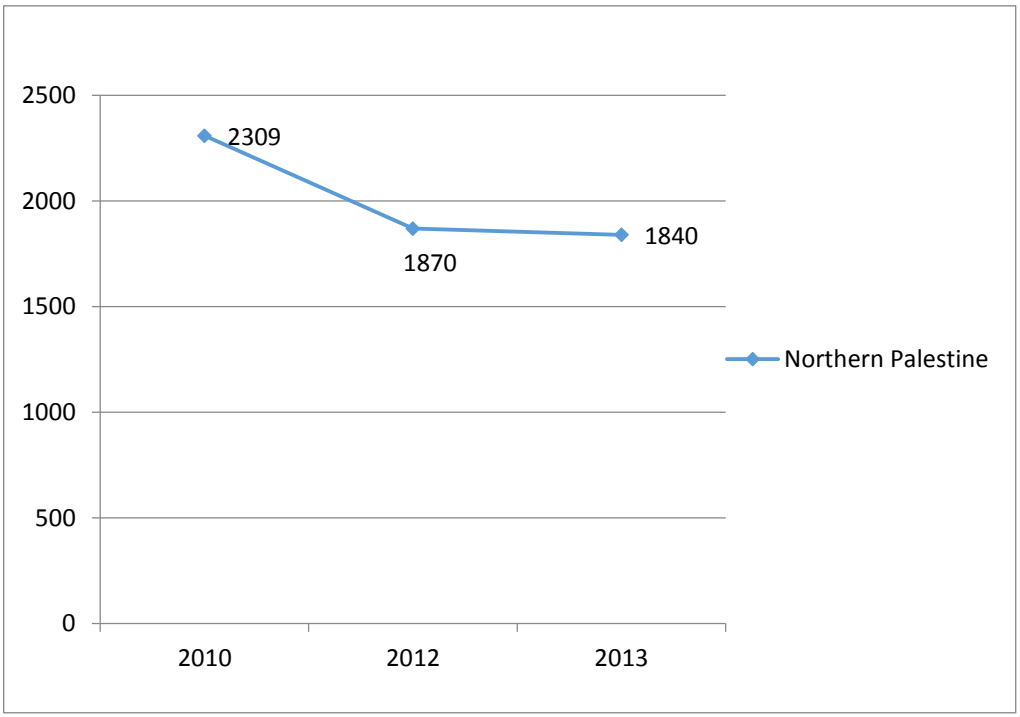


Figure 1-2. Production of figs in northern West Bank- Palestine (ton/ year).

Chapter Two

Litrature Review

2.1 Plant Pathology

Understanding of plant diseases is important to humans, because they cause damage to plants and plant products. Diseases can be caused by pathogens such as fungi, bacteria, and viruses. Fig mosaic disease is transmitted by asexually vegetative propagation and grafting, but not by seed (Condit and Horn, 1933; Blodgett and Gomec, 1967). The natural vector of FMD is the eriophyid mite (*Aceria ficus)* (Flock and Wallace, 1955), with a high percentage of the population (70%) able to transmit the disease (Skara *et al*., 2006).

Mites of two families, *Eriophyidae* and *Tetranychidae*, have been shown to transmit several plant viruses. Virus transmission by eriophyid mites seems to be quite specific, since each of these mites has a restricted host range and it is the only known vector for the virus or viruses that they transmit. Some of the mite-transmitted viruses are stylet borne, while others are circulatory and, of the latter, at least one persists through the molts. The transmission of the pathogen to work of eriophyid mites has been confirmed through the use of electron microscopy and molecular analysis (Caglayan *et al*., 2010). The consistent presence of putative members of the genera *Potyvirus*, *Carlavirus*, *Closterovirus* and *Ampelovirus* in fig plants infected from different countries

(Namba, 1983; Grbelja and Eric, 1983; Elbeaino *et al.,* 2007a, 2007b) currently suggests that the spread of the virus of the fig tree may be involved other types of carriers such as aphids and scale insects.

2.2 Fig Viruses

Recent studies have reported of FMD observation, in leaf tissues fig symptomatic of viral particles of different morphology (Grbelja and Eric, 1983; Serrano *et al*., 2004). Several viruses infecting fig trees have been reported (Table 2-1). *Fig leaf mottle-associated viruses 1* and *2* (Elbeaino *et al*., 2006; Elbeaino *et al*., 2007a, 2007b), *Fig mosaic virus* (Walia *et al*., 2009; Elbeaino *et al.*, 2009a, 2009b), *Fig latent virus 1* (Gattoni *et al*., 2009), *Fig mild mottle-associated virus* (Elbeaino *et al*., 2010), *Arkansas fig closteroviruses 1* and *2* (Elbeaino *et al*., 2011a), *badnavirus-1* (Tzanetakis and Martin, 2010) and *Fig cryptic virus* (Elbeaino *et al*., 2011b).

Table 2-1. List of viruses infecting Fig trees and the year of their identification

Virus	**Year**	**Reference**
Fig leaf mottle-associated virus 1 (FLMaV-1)	2006	Elbeanion et al. 2006
Fig leaf mottle-associated virus 2 (FLMaV-2)	2007	Elbeanion et al. 2007a,b
Fig mosaic virus (FMV)	2009	Walia et al. 2009
Fig latent virus 1 (FLV-1)	2009	Gattoni et al. 2009
Arkansas fig closterovirus-1 (AFCV-1)	2010	Tzanetakis and Marten 2010
Fig mild mottle-associated virus (FMMaV)	2010	Elbeanion et al. 2010
Fig cryptic virus (FCV)	2012	Elbeanion et al. 2011
Fig badnavirus-1 (FBV-1)	2012	Laney et al. 2012

These viruses which infect fig plants can cause problems such as the impact on the productivity of the crop on the wide agricultural areas and viruses may effect on fruiting period of the plant.

In this study, the focus was on some of these fig viruses (Table 2-2) and we will compare our finding with the work on the more prevalent types of viruses in fig with the results of other studies conducted in Turkey, Egypt, Syria and Lebanon.

Table 2-2. Recently identified fig infecting viruses (ICTV, 2014)

Virus name	**Family**	**Genus**
FMV	---	*Emaravirus*
FMMaV	*Closteroviridae*	*Closterovirus*
FBV-1	*Caulimoviridae*	*Badnavirus*
FCV	*Partitiviridae*	*Alphacrpticvirus*

2.2.1 Virus with dsDNA Genome

Family: *Caulimoviridae*: contains of all the plant viruses that replicate by reverse transcription. These viruses have opened circular double-stranded DNA genomes with discontinuities sites in both sides (Bousalem *et al.*, 2008).

Genus: *Badnavirus*: the genome is approximately 7.2-9.2kbp. Semi-persistent transmission is performed by mealy-bugs and leafhoppers, and all stages of life of the mobile carrier can acquire and transmit the virus. Among the viruses belonging to this genus, the DNA of FBV-1 is assumed to be integrated into the genome of the fig (Laney *et al*., 2012).

2.2.2 Virus with (+) ssRNA Genome

Family: *Closteroviridae*: includes two genres with one-partite and bipartite genome; filamentous particles. The genome consists of positive ssRNA, the

transmission is semi-persistent by aphids and other carriers such as whiteflies, coccidian and pseudocode.

Genus: *Closterovirus*: virion contain a single strand of RNA from 14.5 to 19.3 kb in size. The genome is mono-partite, carriers are natural especially aphids. FLMaV and FMMaV are examples on fig (Martelli *et al*., 2002).

2.2.3 Virus with (-) ssRNA Genome

Family: ---

Genus: *Emaravirus*: It have four segments of negative ssRNA of about 7.0, 2.3, 1.6 and 1.4 kb (NCBI, 2012).The newly-detected RNA-5 and RNA-6 contain two further proteins of unknown function (Ishikawa *et al*., 2012).

2.2.4 Virus with dsRNA Genome

Family: *Partitiviridae*: Includes fungi and plants host. The plant virus particles are isometric, bipartite genome consisting of two molecules of linear dsRNA. Infection usually latent and the transmission is by seed and pollen, not for vectors or graft.

Genus: *Alphcryptovirus*: typically contain two dsRNA segments of about 1.5 and 2.0 kbp in length (Nibert *et al*., 2013).

2.3 Principles of Molecular Diagnosis

The tools of diagnosis of viruses that infect fruit tree crops must possess the qualities of versatility and ease of application and with a high sensitivity and specificity, which allow identifying viruses already present in low

concentrations in the tissues of woody plants, especially in the dormant material outside of the growing season.

Molecular techniques are rapidly developing in plant virology, where versatility of use and the reliability of results are mprover. It can be used as a tool for basic research and diagnostic tools. The possibility of *in vitro* enzymatic synthesis of DNA sequences complementary, corresponding to large portions or the entire viral RNA genome, has allowed in recent years to develop methods of molecular investigation which aim to search for specific regions of the viral genome. The ability to explore, for diagnostic and / or identification, the entire viral genome and not just the portion encoding the capsid protein (which represents approximately 10% of the entire genome) is the strength of these techniques against those serological. Other portions of the same genome may, in some moments of the replication cycle of the virus in infected cells, accumulate in moderate amounts in the form of double-stranded RNA encapsidation or RNA replication (Naidu and Hughes, 2001).

The molecular diagnosis has found great possibilities of use especially in the case of a plant virus with a single stranded RNA which represents 75% of plant viruses. It is particularly useful where there are limitations in the use of serological techniques, for example in the diagnosis of:

- unstable viruses.
- poorly immunogenic virus.
- Viruses which it is difficult to obtain purified preparations.

- Viruses with low titer in the natural and not transmissible mechanically.
- RNA satellites.
- Virus strains serologically very close to or indistinguishable.

The techniques of molecular diagnosis are much more sensitive than serological (sensitivity of the order of picograms, 10-12, that is 1000 times more sensitive than serological techniques) for which they are able to detect the presence of the virus even in asymptomatic plants although infected. The nucleotide sequence makes every single nucleic acid strand that can base-pair (hybridize) with another filament only and exclusively in a complementary way. The uniqueness of these relationships (sequence, complementarities, hybridization) enables molecular diagnosis (Codo, 2012).

2.3.1 Gene Amplification (PCR-polymerase chain reaction)

The PCR (Polymerase chain reaction) molecular tool, developed in the 1980s by Kary Mullis, that allows to selectively amplify the DNA sequences related to the virus to be identified (sequence "target"), from a complex mixture of nucleic acids present in the plant sample to be analyzed, by the use of a DNA thermo-stable polymerase (Taq-polymerase) and a pair of specific primers, of two short sequences (primers) that serve as trigger for the reaction. These primers are complementary to the sequences which delimit the DNA fragment that you want to amplify, in our case the virus (which, for RNA viruses, has been

previously reverse transcribed into cDNA). Standard RT-PCR (Chiumenti *et al*., 2013) was used for FMV amplification, adding specific reagents and enzymes, and subjecting the samples to temperature cycles and appropriate variables, one can obtain an exponential increase of the DNA fragment of interest (amplified product) which reaches such concentrations to be detected with a common electrophoresis (visible on a support solid - gel) in the form of colored band with an intercalating agent fluorescent UV is Red gel (Biotium, USA).

2.4 Objectives of the Thesis

Within the frame work of international concern in production of healthy propagated materials as the first step toward food security, viruses and virus-like pathogens were the main problematic pathogens to be considered in any Phytosanitary program (certified healthy plants). For that, sanitary status of crops must be assessed first, with particular emphasis on viral diseases infections. Thus, this research study aimed to assess and report the sanitary status of Fig trees in Northern West Bank; the most fig growing areas of Palestine. For that, the main objectives of this research study were to do:

- Field surveys, to monitor the presence of virus diseases on figs, and the symptoms they cause.
- Molecular detection of some widely spread viruses using RT-PCR techniques.
- Studying portion of genome sequence variations for some of these detected viruses

Chapter Three

Materials and Methods

3.1. Field Surveys and Samples Collection

Several fields which are grown with fig trees in northern state of West Bank- Palestine were subjected to the inspections to detect any viral symptoms. These fig yards were visited during the main growing seasons of the year 2014 and 2015.

Viral symptoms were investigated for several trees randomly selected for each fields and the incidence of infections was reported. In addition to that, the severity of the symptoms had been reported. Some of the fields were visited twice in the two successive growing season and symptoms were inspected and compared.

For molecular assessments, almost 50 samples were collected from main areas know to be planted with figs (Figure 3-1). 3-5 samples were collected randomly from each visited field for later lab tests for the presence of four fig viruses. This number was limited to ensure the best coverage of geographical distribution; origin of material; age of the plants; and cultural methods. The collections were made from both symptomless and symptomatic trees.

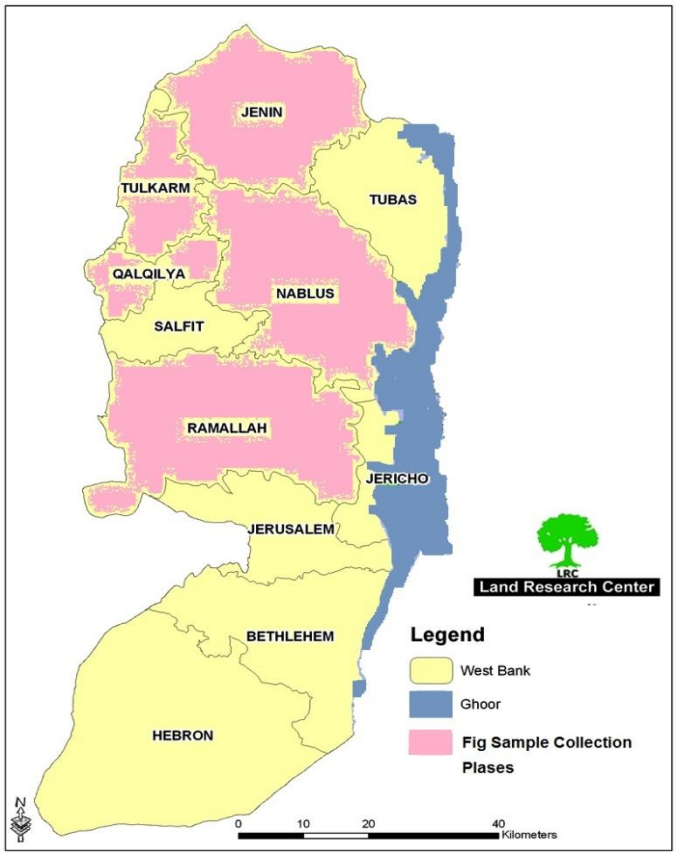


Figure 3-1. Location and distribution of collected samples in northern West Bank-Palestine.

Each sample was composed from several petioles collected from different sites of the tree; grouped and labeled accordingly. These samples were placed in plastic bags and preserved in ice-bag container and transported at the same day to the Research Laboratory of Dr. Raed Alkowni, at the Department of Biology, at Al- Najah National University- Nablus, Palestine, and stored in the refrigerator at 4°C.

3.2 Total RNA Extraction

The total RNA extracted were extracted from approximately 0.1-0.4 g of the petioles, grinded in 1 ml of grinding buffer (4 M guanidine thiocyanate;

2 M of NaOAc, pH 5.2; 25 mM EDTA; 1 M of KOAc; 2.5% PVP-40 and 1% sodium metabisulfite) and were silica-purified (Foissac *et al*., 2001). The mixture was then placed in 1.5 ml micro-tube, vortex and spin centrifuge then left for few minutes on ice. 500 µl of the upper aqueous phase were collected into another micro-tube and 150 µl of NLS to 10%. The mixture was stirred, incubated at room temperature for a few minutes; then samples were placed in heating block at 70° C for 10 minutes with occasional shaking. After that, the samples were cooled for at least 5 minutes on ice and centrifuged at 13,000 rpm for 3 minutes. The extract was transferred into clean tubes joining 500 µl of NaI (6 M), 250 µl of EtOH at 99.97% and 35 µl of silica resuspended well and left at room temperature. After at least 10 minutes, stirring occasionally, 60 seconds centrifugation at 6000 rpm was applied. The supernatant was removed and the tube contains the pellet was dried. Proceed with two successive washes, using the appropriate washing buffer. Finally the pellets were added 150 µl of d.d.H2O, RNase-free, processing samples for 4 minutes at 70 ° C and then in ice. The mixture was centrifuged for three minutes at full speed (13000 rpm) and the supernatant was taken and stored at -20 ° C.

3.3 Reverse Transcription of RNA into cDNA

10 µL of extracted RNA with 1 µl of Random Primers (short randomized hexamers were used at a concentration of 1µg / µl). It was placed for 5 minutes at 95 ° C (denaturation of nucleic acid). The mixture for the synthesis of cDNA consists of:

- 2 µL of M-MLV buffer 10 X,
- 0.5 µL of 10 mM dNTPs,
- 2 µL of 10 mM DTT,
- 1 µL of reverse transcriptase 200 units / µl (enzyme M-MLV),
- 3.5 µL of ddH2O (RNase free water)

The reaction took place in a final volume of 20 µl. The tubes were shaken, followed by brief centrifugation and incubated in a thermostat at 37° C for 2 h. At the end of the reaction, the cDNA was synthesized, cooled in ice and stored at - 20 ° C for later use.

3.4 Gene Amplification

The viruses that were examined are : FMV, FBV-1, FCV and FMMaV. Table 3-1, is listing the names of the viruses associated with their primers used, with relative nucleotide sequence and size of amplicon (the size of the fragment of the target molecule to be amplified).

Table 3-1: identified fig infecting viruses with their accession numbers and primes sequences used for RT-PCR analysis

Virus	Primer sequence 5'-3'		Amplicon size (bp)	References
FMV	F R	CGGTAGCAAATGGAATGAAA AACACTGTTTTTGCGATTGG	302	Elbeaino *et al.* 2009a;& b
FMMaV	F R	AAGGGGAATCTACAAGGGTCG TATTACGCGCTTGAGGATTGC	311	Elbeaino *et al.* 2010
FBV-1	F R	ACCAGACGGAGGGAAGAAAT TCCTTGCCATCGGTTATCTC	474	Tzanetakis and Martin 2010
FCV	F R	TCGGATTGTCTTTGGAGAGG CGCATCCACAGTATCCCATT	353	Elbeaino *et al.* 2011b

The reaction of gene amplification was performed on an aliquot of the cDNA which it had been added to the amplification mixture that containing the nucleotides and the sense and antisense primers for the detection of the segment of the cDNA to be amplified.

Then, 2.5 µl of cDNA were added to the mixture of amplification which is consisting of 5 µl of 5x reaction buffer, 0.5 µl dNTP (10 mM), 0.5 µl sense primer , 0,5 µl antisense primer, 0.2 µl Taq polymerase (5U/µl) (Fermentas®) and sterile water (15.8 µl) up to 25 µl of total volume. The reactions were carried out using 35 cycles of amplification in a thermo-cycler with the following parameters set:

- Denaturation of cDNA molecules: 94 ° C for 2 minutes;
- 30 cycles of amplification with the following segments: 94 ° 30 sec, 55 ° C 30 sec, 72 ° C 45;
- Extension of the final nucleotide chain, through the action of Taq polymerase: 72 ° C for 7 min.

The amplified products were subsequently stored at 4 ° C.

3.5 Agarose Gel Electrophoresis

For the visualization of the amplified products from the enzymatic reaction, 1.2% agarose gel was prepared, depending on the size of the fragments, in 1x TAE for PCR product. The agarose solubility in buffer was added to 1µl GelRed (Biotium, USA) which intercalates into DNA and become fluorescent when excited by ultraviolet rays. That’s why it is used to detect the DNA fragments. The size of the amplified bands was determined by

comparison with a commercial marker of DNA, the 1 kb Ladder Invitrogen®, extent of 5 µl. The run was made at first with a potential difference of 80 V, to facilitate the escape of the samples from the wells in the gel matrix, and continued at 100 V. After the electrophoretic run, the gel was observed and photographed on a trans-illuminator light emitting ultraviolet GelDoc-It (UVP, USA).

3.6 Sequencing and data analysis

The PCR products were cleaned with ChargeSwitch®-Pro PCR Clean-Up Kit (Invitrogen, USA), following the manufacturer's protocol. DNA PCR products were sequenced by dideoxynucleotide chain termination method using 3130 Genetic Analyzer (Applied Biosystem®, Bethlehem University, Palestine). The sequencing PCR reaction was performed with primers used singly in forward and reverse reaction and BigDye® Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystem®, USA).

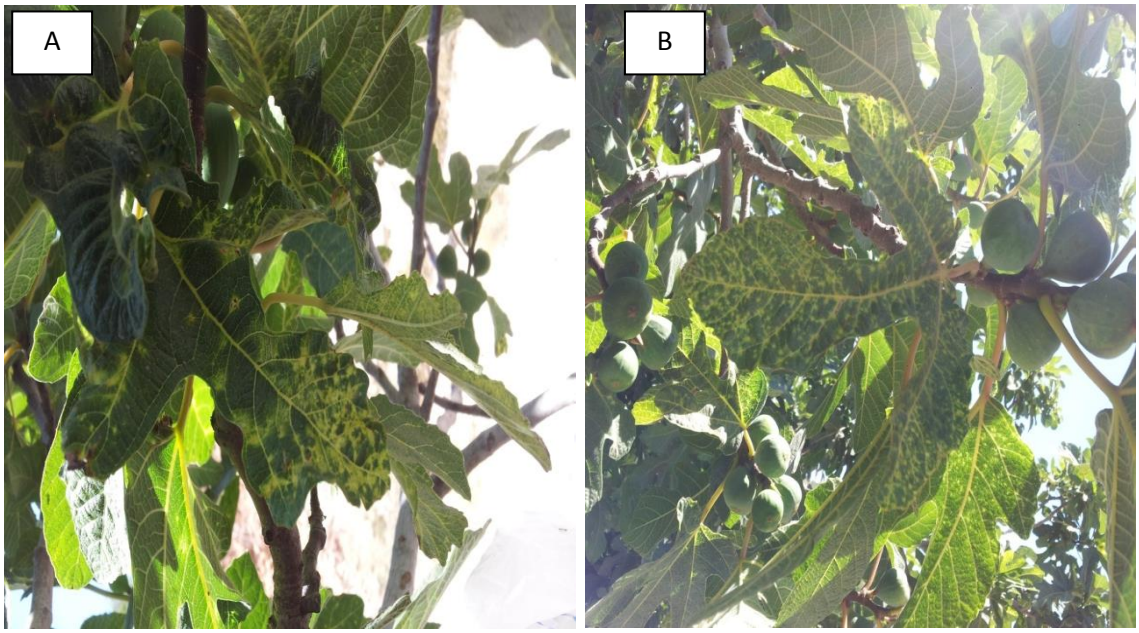
The DNA sequences of 5 samples of FBV, 3 samples of FMV and one sample of FCV were compared with available sequences in NCBI (National Center for Biotechnology Information) using BLAST and multiple alignments were done by using ClustalW.

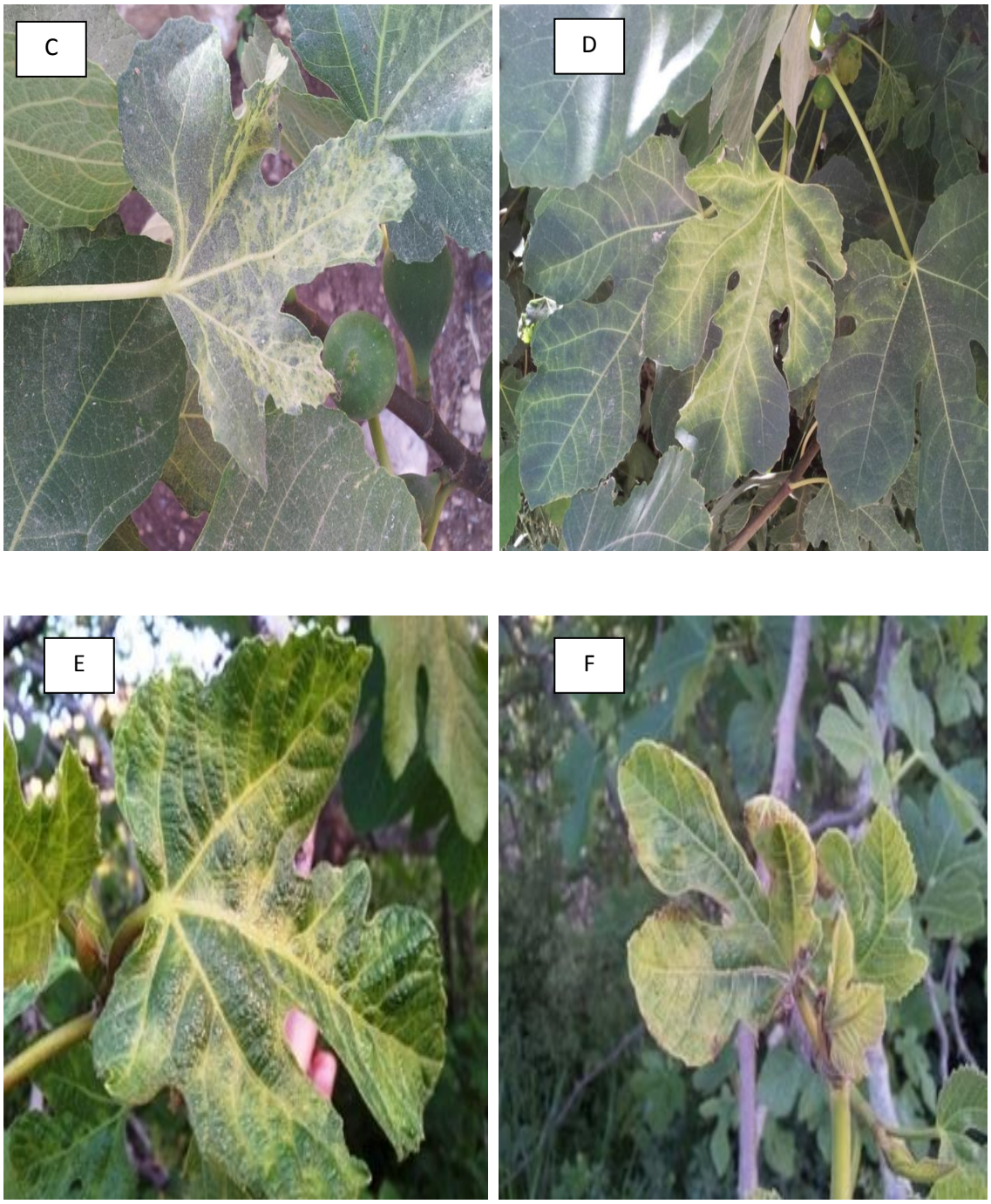
CHAPTER FOUR

RESULTS

4.1 Field surveys and biological identification of virus-associated symptoms in Palestinian fig growing areas

Field surveys inspections to check for any visual viral disease symptoms had been carried out in the fields of figs during the growing seasons of 2014 and 2015, to reveal that viral disease symptoms were rather broad and diverse. Almost two third of visually inspected fig trees were found with one or more viral symptoms. These symptoms were exhibited on leaves and/or fruits. The symptoms on leaves were reported as mosaic; mottling; vein clearing; yellowing and deformation (Figure 4-1).

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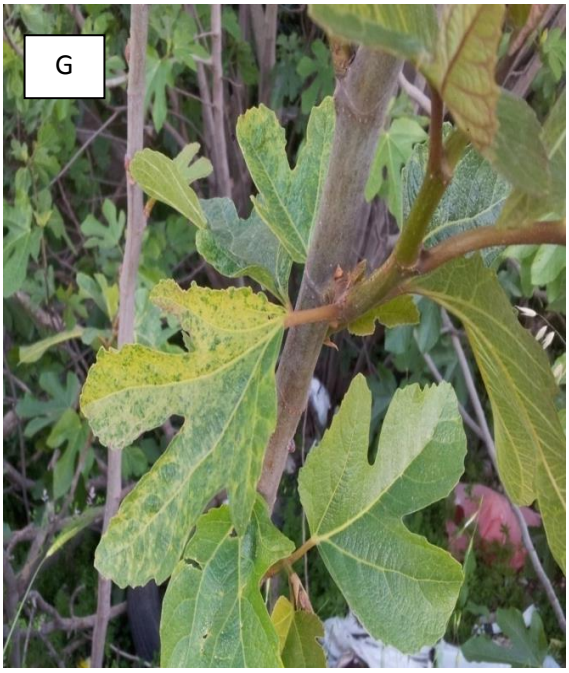
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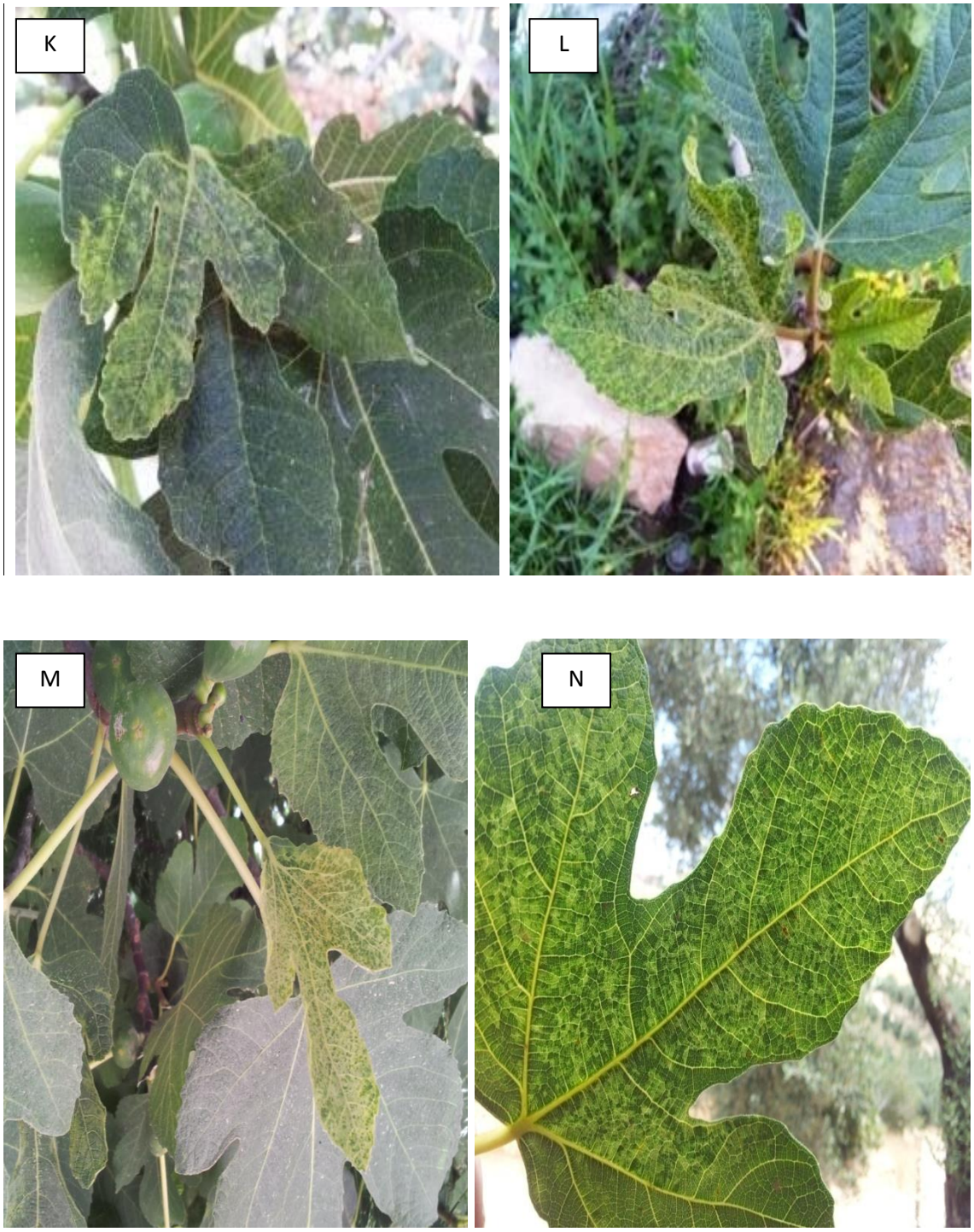

Figure 4-1. Viral disease symptoms were observed in the fig yards which were grown in northern West-Bank of Palestine. Several types of symptoms were noticed on fig leaves, such as mottling (A-B); vein clearing (C-D-E); yellowing (F-G-H); deformation (I-J-K) and mosaic (L-M-N).

Meanwhile the fruits were examined to reveal local lesions, chlorosis, ring spots, and mosaic. It was noticed that the newly formed figs were exhibiting yellow ring spots (Figure 4-2) which were varied in their sizes. The developments of infected fruits were monitored and the necrotic spots were firstly noticed and heavily fruit drops were found later on.

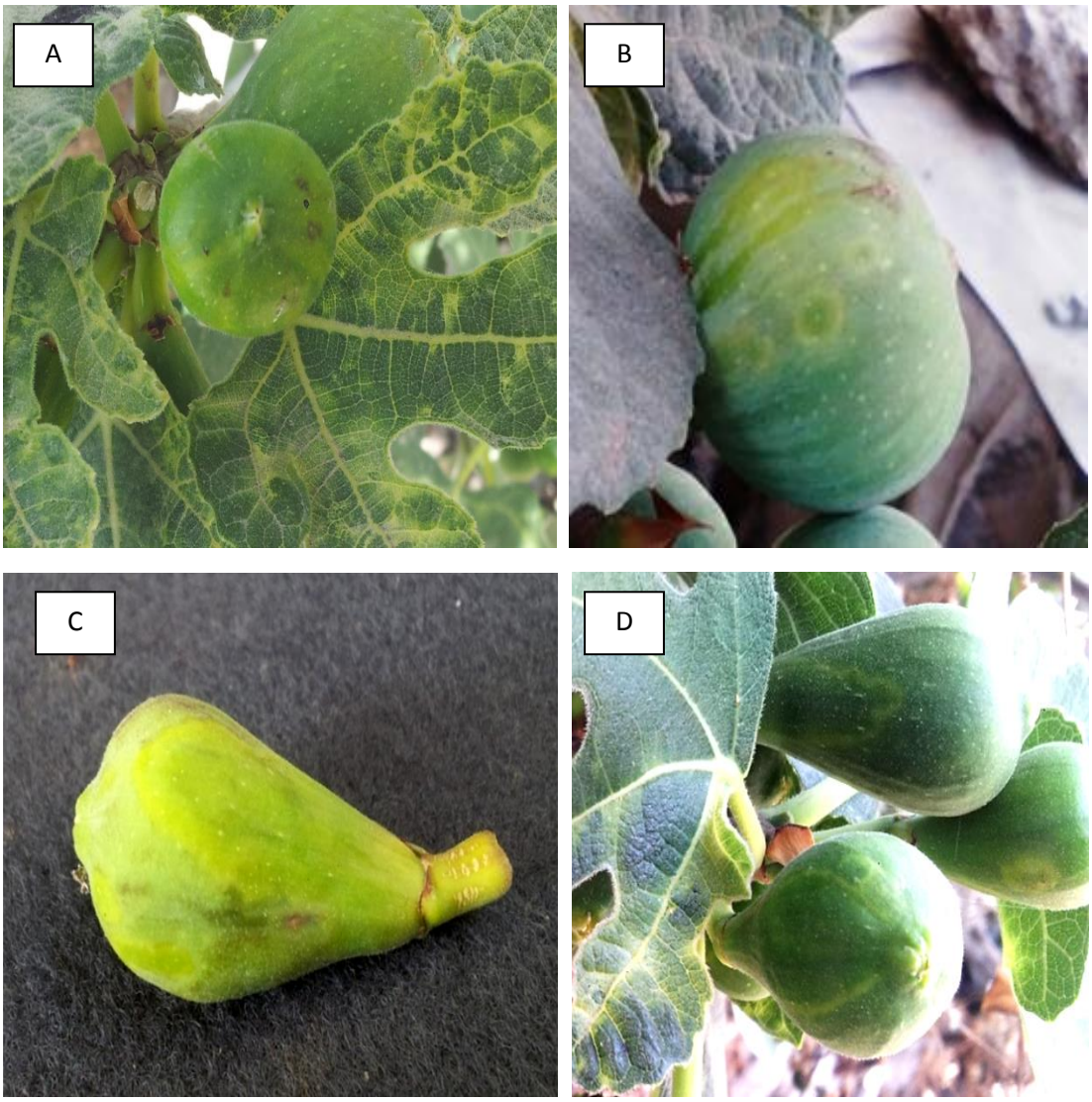

Figure 4-2. Yellow ring spots and necrosis were noticed on the fruit (A-G). These fruits were dropped down later.

4.2 Total RNA Extraction

The extracted total RNA were analyzed in 1.2% agarose gel (Figure 4-3). This was done for all extracted RNAs to ensure their quality extractions.

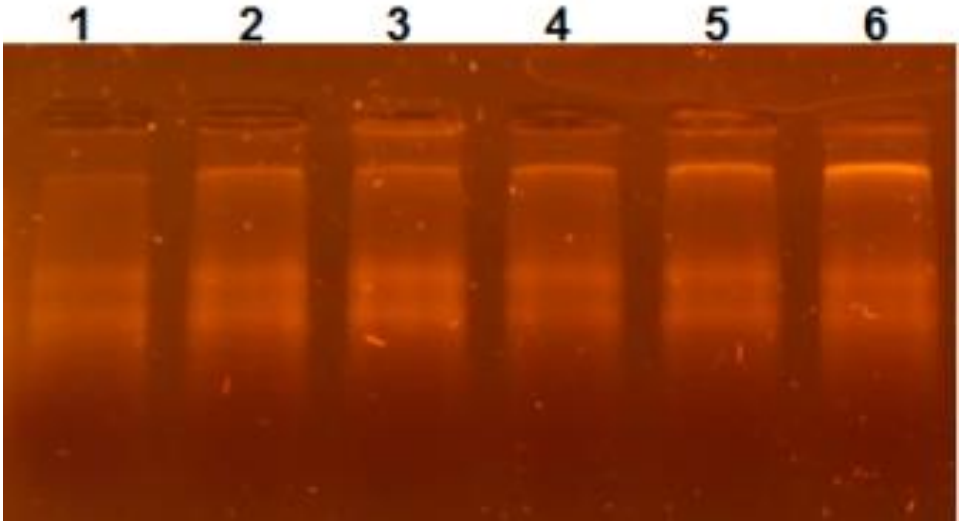

Figure 4-3. Example of electrophoresis of total RNA extraction. 1.2% agarose gel in TBE, colored with GelRed (Biotium). Lane **1-6** represents TNA from different fig leaf samples.

4.3 Detection of FMV by PCR

DNA samples which tested positively for FMV were able to amplify the expected PCR products of a size equal to 302 bp; using specific primer (Elbeaino *et al*, 2009a, b). Results about detection of FMV using PCR in fig samples as presented in Figure 4-4. It showed that 60% of samples were infected with FMV.

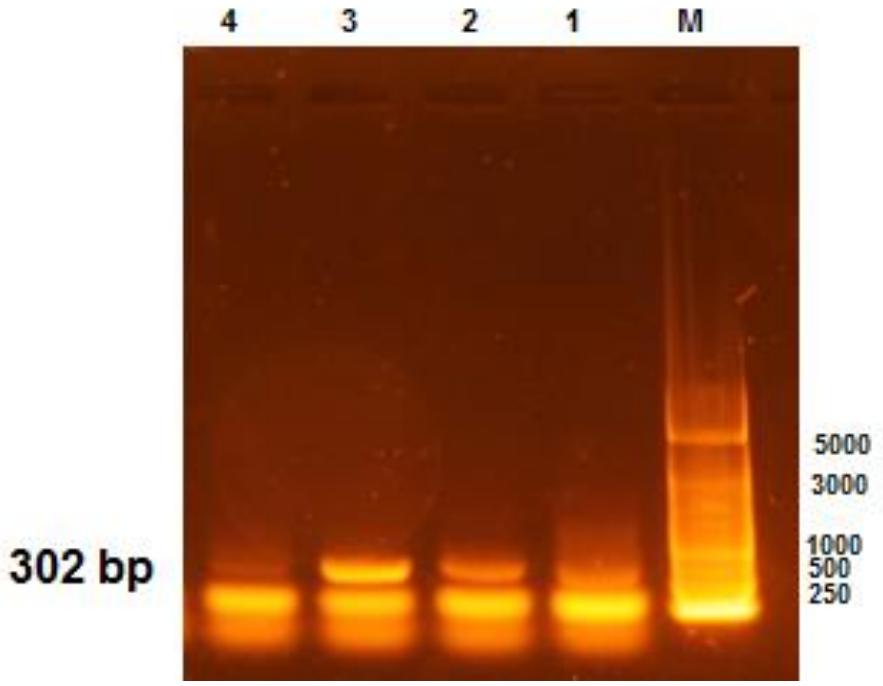

Figure 4-4. PCR results of FMV: Lane **M** contained ladder (1Kb DNA ladder RTU, GeneDireX)), lane **4** represents negative sample, lane **1, 2 and 3** represents FMV positive sample on 302 bp.

4.4 Detection of FBV by PCR

Positive samples were those generated the expected PCR products with size equals to 474 bp. The detection of FBV was done by using PCR with specific primers (Tzanetakis and Martin, 2010) as presented in Figure 4-3. Results of this research showed that 46% of samples were infected with FBV.

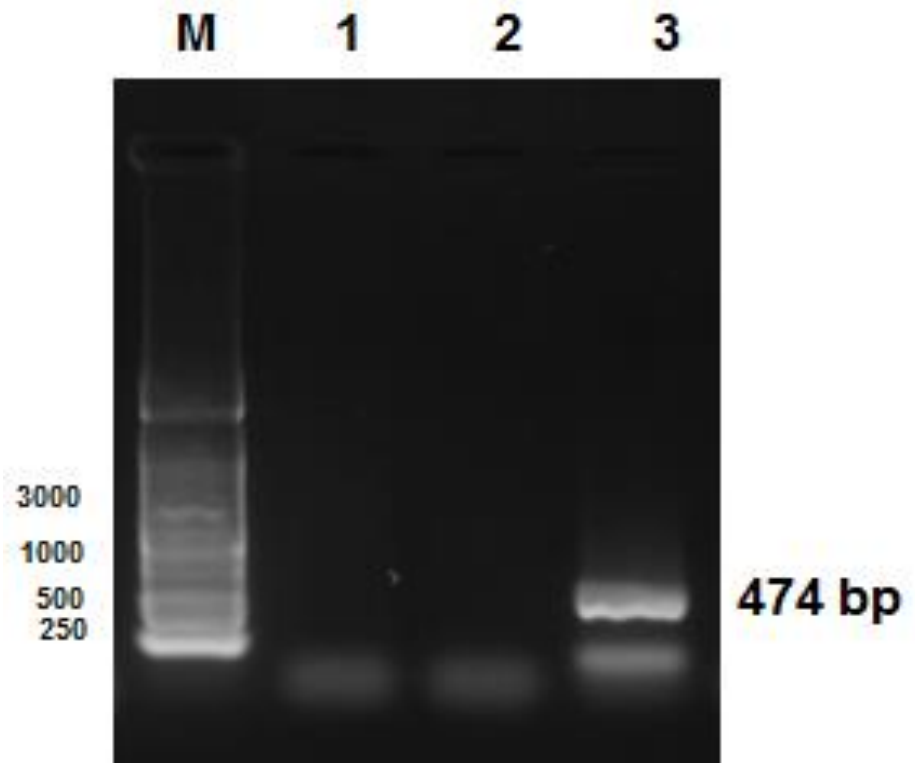

Figure 4-5. PCR results of FBV: Lane **M** contained ladder (1Kb DNA ladder RTU, GeneDireX)), lane **1** and **2** represents negative samples, lane **3** represents FBV positive sample on 474 bp.

4.5 Detection of FCV by PCR

Results of this research showed that only (2%) of fig samples were infected with FCV. Only one sample of fig was detected using specific primers (Elbeaino *et al*., 2011b). The size was equal to 353 bp as presented in (Figure 4-4).

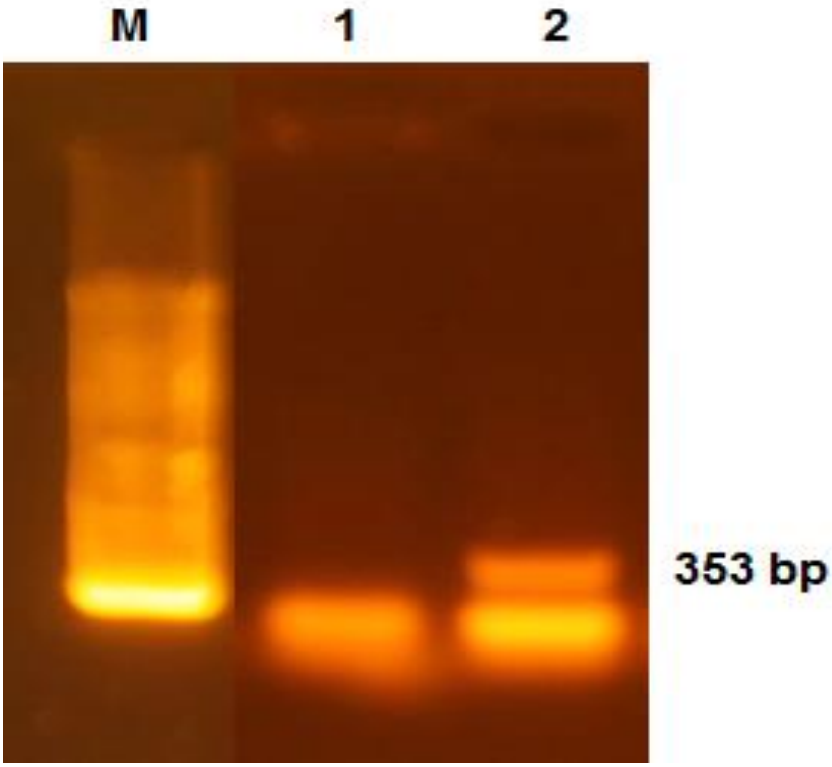

Figure 4-6. PCR results of FCV: Lane **M** contained ladder (1Kb DNA ladder RTU, GeneDireX), lane **1** represents negative sample, lane **2** represents FCV positive sample on 353 bp.

4.6 Results of Gene Amplification

From the analysis of results from assays of molecular diagnosis RT-PCR, relating to each virus, there has been a presence in percentage of 60% of FMV, 46% of FBV and 2% of FCV (Table 4-1)

Table 4.1 Analysis of gene amplification (RT-PCR) tested samples of fig.

Region	Tested	Positive	%	FMV	%	FMMaV	%	FCV	%	FBV	%
Jenin	12	10	83	8	67	0	0	0	0	6	50
Nablus	16	14	87.5	11	68.8	0	0	0	0	9	56.3
Ramallah	5	4	80	3	60	0	0	0		2	40
Qalqilyah	12	8	67	5	42	0	0	1	8	4	33
Tulkarem	5	3	60	3	60	0	0	0	0	2	40
Total	**50**	**39**	**78**	**30**	**60**	**0**	**0**	**1**	**2**	**23**	**46**

4.7 Prevalence of Viruses in Each Province

As regards instead FMMaV, it was not found to be present in any of the samples analyzed. The results showed that the high percentage of FMV was recorded from Nablus 68.8% and Jenin 66.7%, as well as FBV from Nablus 56.3% and Jenin 50% (Figure 4-7).

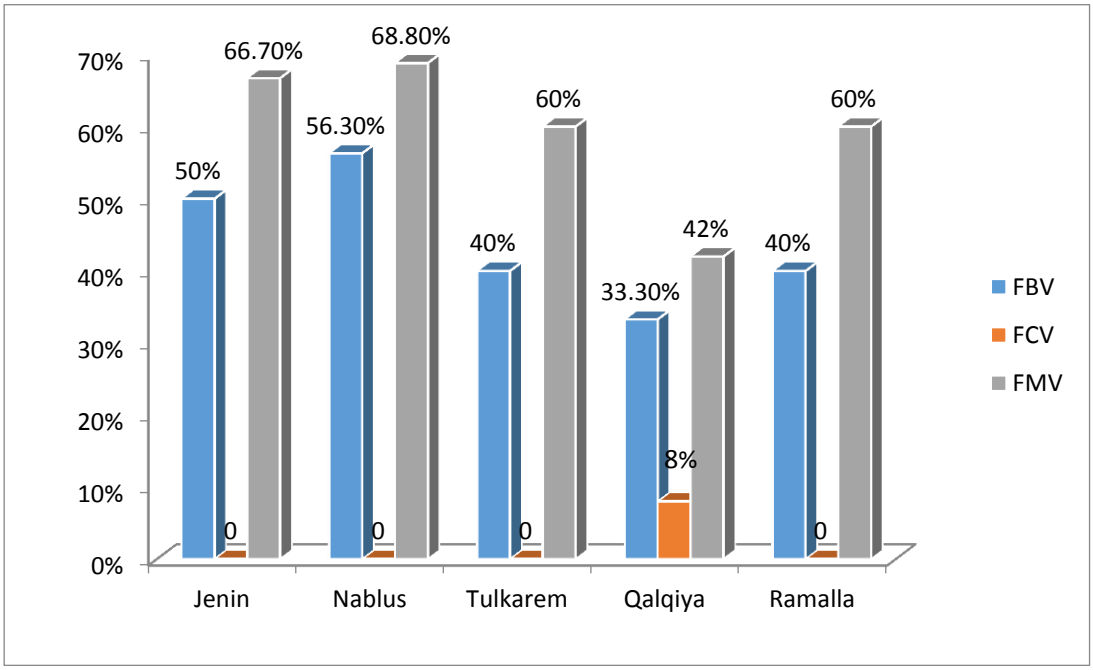

Figure 4-7. Incidence of FMV, FBV and FCV infections in five-growing provinces of northern West Bank- Palestine as determined by RT-PCR assays.

The results show that Nablus province have the most total infected rate with 88% in Nablus (14/16), 83% in Jenin (10/12), 80% in Ramallah (4/5), 67% in Qalqilya (8/12) and 60% Tulkarem (3/5). (Figure 4-8).

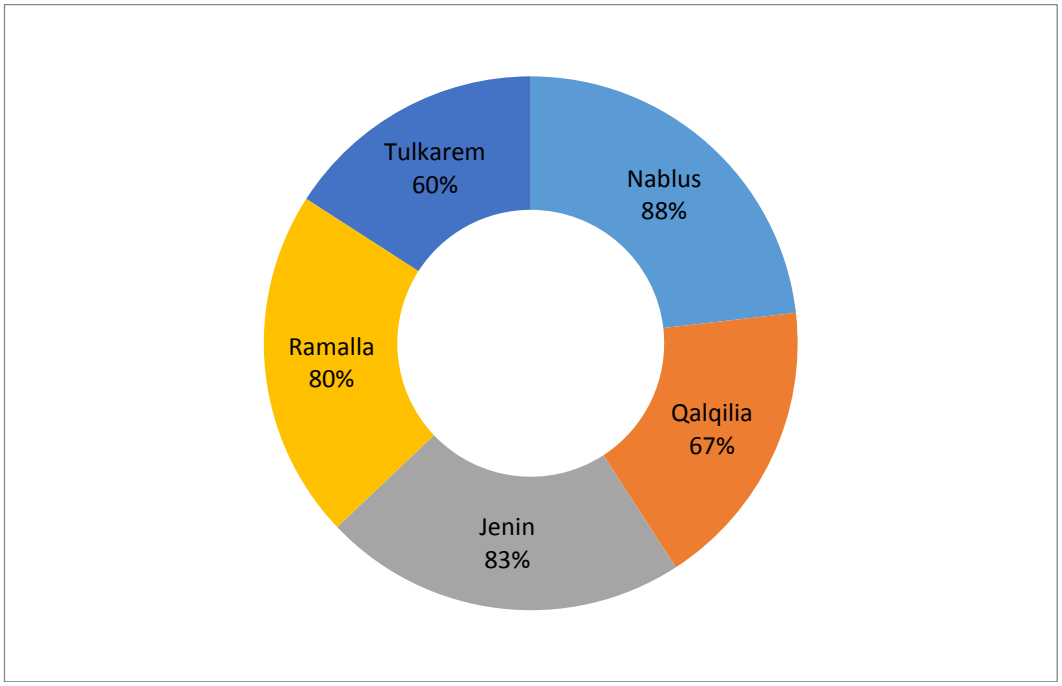

Figure 4-8. Virus incidence on northern West Bank- Palestine fig provinces.

4.8 Sequencing

Depending on the results observed and because of the high percentage of both FMV and FBV, five samples of FBV, four samples of FMV and the only sample of FCV were selected from each province (Appendix A). Results showed that high similarity with published ones in GenBank 98-100% (Appendix B).

Chapter Five

Disscusion, Conclusion and Recommendation

5.1 Discussion

many viruses of fig diseases were reported (Elbeaino *et al*. 2006, 2007a, b; Walia *et al*. 2009; Minafra *et al*. 2012; Gattoni *et al*. 2009; Tzaetakis and Martin 2010), but only FMV was the one of the main incidents of FMD (Elbeaino *et al*. 2009a, b). Symptoms of mosaic were observed on fig trees since 1933 (Condit and Horne 1933), and many different reports of FMD had been published (Bradfute *et al*. 1970; Martelli *et al*. 1993).

In this research, the most common types of plant symptoms produced by systemic virus infections are mosaics, yellowing on leaves (Figure 4-1) and ring spots on fruits (Figure 4-2). Mosaics are characterized by light-green, yellow, or white areas intermingled with the normal green of the leaves or fruit or of lighter–colored areas intermingled with areas of the normal color of flowers or fruit. Ring spots are characterized by the appearance of chlorotic or necrotic rings on the leaves. In many ring spot diseases the symptoms, but not the virus, tend to disappear later on (George, 2005).

Surprisingly, most of mosaic symptoms were noticed on the newly growing leaves of figs in Palestine and asymmetrically on trees, suggested the uneven distribution of the virus on the plant. Notably, mite infestations were found on some of those inspected as symptomatic trees, indicated them as putative transmissible agents to the virus.

Based on previous research from some fig exporting countries, such as Egypt (Elbeshehy and Elbeaino, 2011), Turkey (Elci *et al*., 2012), Tunisia (Elair *et al*., 2014), Lebanon (Elbeaino *et al*., 2012) and Syria (Elbeaino *et al*., 2012); Palestine maintained high incidence of FMV (60%), FBV (46%)

(Figure 5-1). Meanwhile FCV was detected in few samples, showing less incidences compared with other countries.

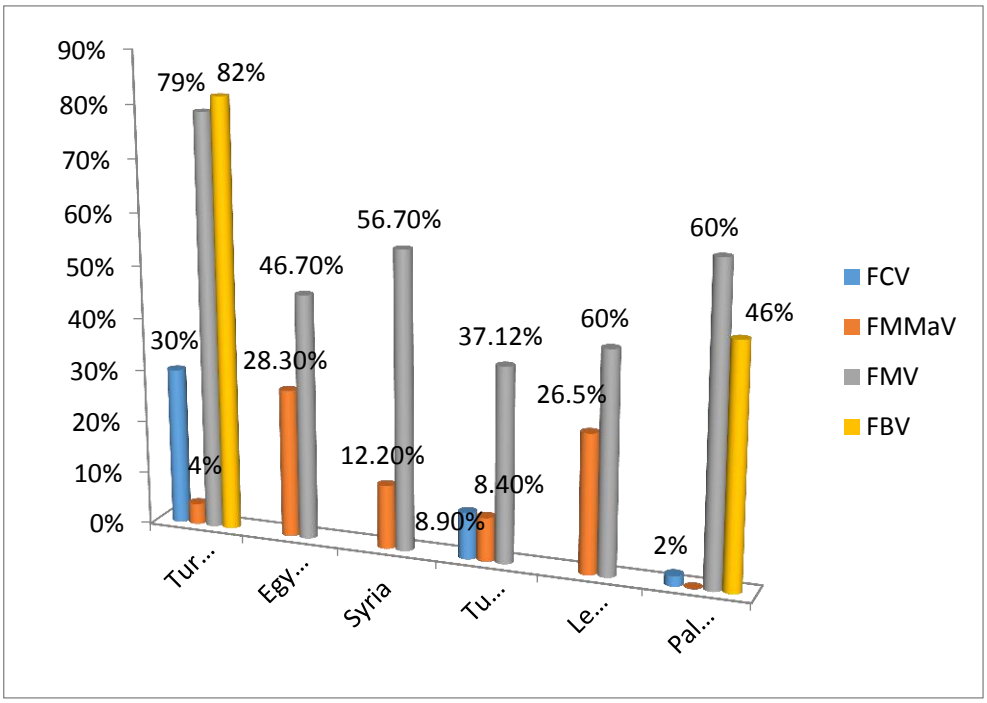

Figure 5-1. Compared with the previous research on viral diseases of figs with northern West Bank- Palestine Results.

Clearly, this study was revealed that the most infected viruses were FMV and FBV are in agreement with a previous survey study of Alkowni research (Alkowni *et al*., 2015). In addition, the fact that FM was recorded in all provinces and field in northern West Bank indicates its seriousness, similarity with a study in Jordan (Al Mughrabi and Anfoka, 2000).

Virus–virus interactions in plants may be of crucial significance for the understanding of viral pathogenesis and evolution and consequently for the development of efficient and stable control strategies (Syller, 2011). In Turkey, complex infection were detected in the most samples and the most

common viruses were FMV+FBV-1 with 90% (Elci *et al.*, 2012) and 52% in Syria (Elbeaino *et al*., 2012). In this research, mixed infections of fig viruses (FMV+FBV-1) were found in the tested cultivars from different provinces with 30%. Hence, most attentions have been paid to virus interactions in multiple infections in infected fig trees.

The results show that Nablus province have the most total infected rate (88%) when it was compared with others (Figure 4-8). This could be referred to most fig nurseries which are located in this province produced by cutting from infected mother trees. Also, pruning tools are used without disinfection between cuttings and may even be shared by different nurseries. The weather conditions in Nablus, with moderate humidity and temperatures, are very favorable for plant propagation, but also favor for the vectors that spread viral diseases. Direct correlation between the geographic distribution and incidence of viral pathogen affecting of agricultural were noticed in this research study. Moreover, due to the different geographic distribution of the fig tree cultivation in the provinces that have been previewed in the research. Depending on the Ministry of Agriculture data in Ramallah- Balou, Till area in Nablus, is the most widely distributed and growing figs (29%). Jaba' - Jenin (10%) , Senjil - Ramallah (6%), Jauos - Qalqilya (5%) and Attel - Tilkarem (2%). So, It is a direct correlation between the geographic distribution and viral pathogen affecting of agricultural crop.

Portions of the FMV and FBV genomes were sequenced to reveal high similarity with published ones in Gen bank (98-100%) (Appendix A and B)

The picture that emerges from the molecular diagnosis of the virus of the fig tree in the numerous accessions of native varieties sampled in this study is quite worrying implications for the quality of fruit production. The almost ubiquitous presence of several viruses, among which the FMV, mirrors the presence of symptoms as extensive mosaic were found. FMV, as known to be transmitted quickly and with high efficiency by means of the mite, it was therefore obviously noticed over large areas of fig plants. However, the majority of the symptoms observed, can't be attributed to a given virus, among those least sought and found, given the abundance of mixed infections of more viruses. The wide range of symptoms described could then be determined by phenotypic synergy due to the association of different viruses, some of which certainly still unknown.

The limited presence of FCV is equally understandable as given to their transmission difficulties. The cryptic virus did not have any known carriers and could be transmitted mostly by seeds or asexual propagation. The constant infection of FBV-1 instead is certainly due to its ancestral co-integration into the genome of fig, and the most likely transmission efficiency through scale insects or other potential insect vectors.

5.2 Conclusion and Recommendations

The outcome of this preliminary work extends the knowledge on the spread of fig viruses in the Mediterranean region, particularly in northern West Bank- Palestine. This is the first report in northern West- Bank fig orchards of FBV, FMV, FCV and FMMaV, talking about the sanitary assessment of

fig viruses and the percentage of appearance on fig trees. Although this assessment was limited to 50 trees, the results obtained clearly indicate how the sanitary status of fig crop has deteriorated in northern West Bank (78% of viral infection). Special worrying is for the incidence of FMV, since this has proved to be the unique virus closely correlated with the FMD (Elbeaino *et al*., 2009a). High incidence of FMV is not surprising considering the way this virus spreads in figs through infected propagating material (cuttings and grafting), and natural vectors (eriophyid mites) (Alkowni *et al.*, 2015). In Palestine there is no information on the presence of *Aceria ficus* (Eriophyidae). However, such presence in the Palestinian orchards would likely aggravate the sanitary status and the level of infections in the surveyed areas. The several FMV-infected samples found in association with most of the mosaic symptoms in the field further confirms what was previously reported regarding the etiology of FMV. In Palestine, fig production is in developing emphasis for more intensive fig cultivation , three thousand tons are produced in almost three thousand and five hundred acres in West Bank in 2010 (PCBS, 2010). Figs like other agriculture crops are infected with viruses. The widespread and severe symptoms were presented on young fig leaves can cause problems such as the impact on the productivity of the crop. It may become more of an agricultural problem with the introduction of intensive cultivation and viruses may effect on fruiting period of the plant. Also, as the results show and based on the geographical distribution, the probability of infection increases with the agricultural area of the crop.

The knowledge we have gained on the incidence of virus diseases of fig in northern West Bank provides information on which a sanitary selection, sanitation and certification programs can be initiated for the production of healthy propagating plant material of fig in this country. In recent years they have added new consisting in particular applications of in-vitro culture of plant tissues: somatic embryogenesis and cryotherapy.

This study is come out with many recommendations regarding to the fig industry. The setting of a regional plan of defense against virus diseases of a particular crop must be based on knowledge of essentially harmful viruses in a given area; the modes of transmission and the epidemiological and ecological conditions favorable to infection. The prevention of viral infections is mainly based on the adoption of the following measures:

- Use of seed and planting material free of viruses.
- Elimination of the possibility of infection.
- Growing species and / or varieties tolerant or resistant to the virus or to the carrier.

Integrating conventional technologies with molecular biology and genetic engineering could enhance desirable characteristics of agricultural crops while reducing the expression of undesirable ones. Using improved conventional breeding in fig, by molecular markers or by the newly introduced genetic engineering technology, could enhance new properties, such as health-promoting compounds.

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Appendixes

Appendex A

Sequences

>FBV-1/J (98%)

TCAATGTTGGTTTGCTTACGAATAAGCCTGTGACGGATTACCTA
GCAAGCAGGGGAGTCCAAGCTTTGCCGGGAAGAAGATACAGAT
CGGAGATGCTACGAGGAAGAAACTGGATCATAAGGCAGCCACA
GATCCAGGCGGCAATGATGCCAAGGAACGTGGAAACAAGGA

>FBV-1/ Q (99%)

TCAGGACTCAATGTTGGATTTGCTTACAGAATAAGCCTGTGACG
GATTACCTAGCAAGCAGGGGAGTCCAAGCTTTGCCGGGAAGAA
GATACAGATCGGAGATGCTACGAGGAAGAAACTGGATCATAAG
GCAGCCACAGATCCAGGCGGCAATGATGCCAAGGAACGTGGAA
ACAAGGA

>FBV-1/T (99%)

ACATGCAATGTTGGATTTGCTTACAGAATAAGCCTGTGACGGAT
TACCTAGCAAGCAGGGGAGTCCAAGCTTTGCCGGGAAGAAGAT
ACAGATCGGAGATGCTACGAGGAAGAAACTGGATCATAAGGCA
GCCACAGATCCAGGCGGCAATGATGCCAAGGAACGTGGAAACA
AGG

>FBV-1/R (98%)

GTTGGTTTGCTTACGAATAAGCTGTGACGGATTACCTAGCAAGC
AGGGGAGTCCAAGCTTTGCCGGGAAGAAGATACAGATCGGAGA
TGCTACGAGGAAGAAACTGGATCATAAGGCAGCCACAGATCCA
GGCGGCAATGATGCCAAGGAACGTGGAAACAAGGAGATTTTTTT
T

>FBV-1/N (99%)

TGTGACGGATTACCTAGCAAGCAGGGGAGTCCAAGCTTTGCCGG
GAAGAAGATACAGATCGGAGATGCTACGAGGAAAAAACTGGAT
CATAAGGCAGCCACAGATCCAGGCGGCAATGATGCCAAGGAAC
GTGGAAACAAGGGTTGGATTTGCTTACAGAATAAGCGGGCAAG
G

>FMV/J (98%)

ATTGCGGTTACGGCGCTGTTTTATTTTATAAAGCGTTAAAAGTTC
CAAGGATACCACCCCTTTGAGAATTCGCCGCTTCGGGATACCAT
TTGTGTTTCCAACAAGATCAACATTAATCTTGCCAGTCTTTGCTC
AACAAGATCAACATTAATCTTGCCAGTCTTTGGTTTCCAACAAG
ATCAACATTAATCTTGCCAGTCTTTAAAAGTTACCAGCCTGCCA
GTTATAAATTTTTGGATCA

>FMV/Q (100%)

TTCGGTATGTGTTTCCAACAAGATTTCCACGCTCAACTTATCCTT
GAATCTGCCAGGGAACCAATTAATTTGGTTTCCAACAAGATCAA
CAAATGGTACCCATTAATCTTGCCAGTCTTTAAAAGTTCAACAA
GATTTGCCAGTTGCCAGTCTTT

>FMV/R (99%)

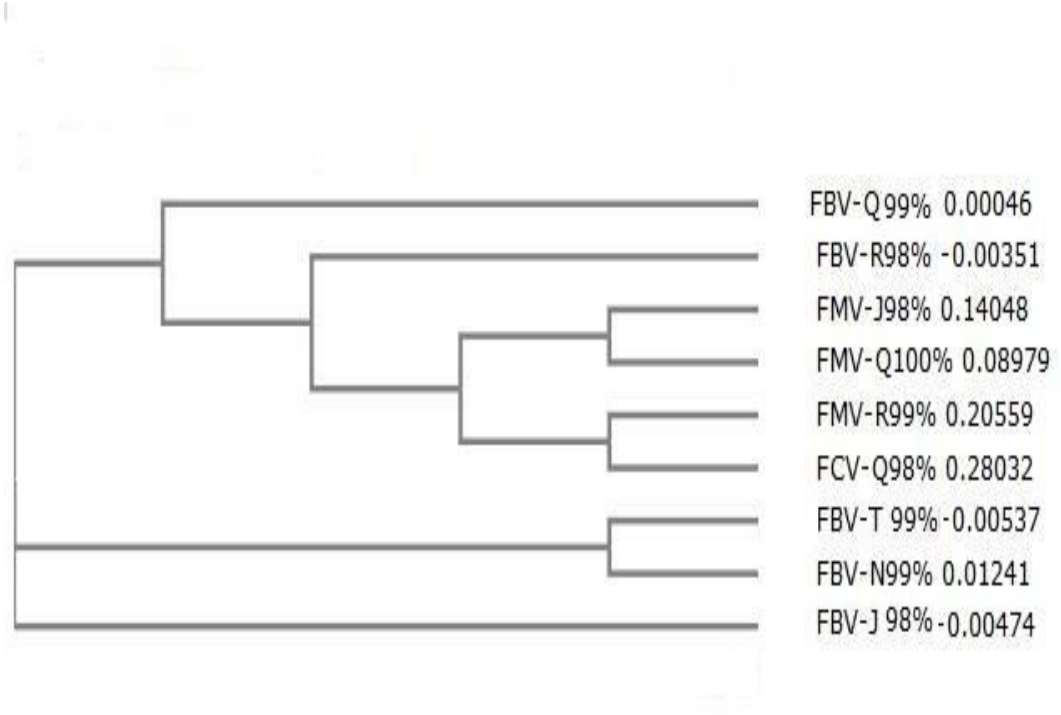
CTGTTATTGTGTTTCCAACAAGATCAACATTAATCTTGCCAGTCT
TTAAAAGTTCTTTCTTACCAGCTTTGATACCATTATCTTGAATCT
GCCAAGGGAACCAGTTATAAATTGCTGGAAATTTGGATCAAATG
GTACCAAATCTATATTG

>FCV/Q (98%)

ATGGAAGCAGGTCTTATAGAGATTGGAAACGACACTTATTGAAG
GAGGTTTTGACCACGTCCCATTGAATCGAGCTCATCAGCCGGCT
ACGGCTATGTTAAAGTTCGGATTGTCTTTCGTTCAAAGGAAATG
CTTAGGTTAACAAATGTGCAGTCTTACTCAGAGAC

Appendix B

Cladogram Tree



Phylogenetic relationship of FMV, FBV and FCV, computed by Clustal W

جامعة النجاح الوطنية

كلية الدراسات العليا

التقييم الصحي للأمراض الفيروسية التي تؤثر على نباتات التين في شمال الضفة الغربية- فلسطين

إعداد

منى محمد يوسف محمود

إشراف

د. رائد الكوني

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

2015

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الملخص

هناك ندرة في البيانات الخاصة عن الوضع الصحي للمحاصيل الزراعية في فلسطين ، وذلك لعدة أسباب منها: عدم توفر معدات لتشخيص الأمراض, نقص الفنيين المتخصصين والعلماء، سوء تنظيم المشاتل والأنشطة الزراعية. شجرة التين (*Ficus carica*) من أقدم أنواع الفواكه والأكثر شيوعا في كل بلدان حوض البحر الأبيض المتوسط، حيث تعتبر من بين أولئك الفواكه التي تتعرض للإصابة بالأمراض والجراثيم المسببة للمرض. أشارت الدراسات الأخيرة أن مرض فسيفساء التين هو المرض الرئيسي المنتشر. في هذا الدراسة أجريت عملية مسح ميدانية في المناطق الشمالية من الضفة الغربية-فلسطين (جنين، طولكرم، قلقيلية، نابلس، طوباس، سلفيت ورام الله) لمراقبة الأعراض وذلك خلال موسمين على التوالي 2014-2015. بالإضافة إلى ذلك، تم جمع 50 عينة من التين وذلك من المناطق التي تم مسحها على النحو التالي: جنين (12)، نابلس (16)، رام الله (5)، قلقيلية (12) وأخيرا طولكرم (5)، للكشف عن أنواع من فيروسات التين مثل *Fig mosaic virus (*FMV*), figbadnavirus-1 (*FBV-1*), Fig cryptic virus* (FCV) و*Fig mild-associated virus* (FMMaV) وذلك باستخدام تقنية الناسخ العكسي- تفاعل البلمرة المتسلسل، بوجود بادئات متخصصة في التفاعل. خلال المسح الميداني وبالاعتماد على الكشف البصري، وجد ما يقرب ثلثين من أشجار التين عليه أعراض فيروسية، فقد ظهرت الأعراض على الأوراق وكذلك على الثمرة. ومن أمثلة أعراض الورق: الفسيفساء، التبقيع، الإصفرار والتشوه. وما أثار الدهشة، ظهور الأعراض الفيروسية على الأوراق حديثة النمو بشكل متزايد والتوزيع الغير متماثل للأعراض على الشجرة. والجدير بالذكر أنه تم العثور على تفشي العناكب على تلك الأشجار المصابة بالأعراض، دالة بذلك على احتمالية كونه الناقل للمرض. إضافة إلى ذلك، تم ملاحظة أوراق حديثة النمو مصابة ببقع صفراء حلقية الشكل تختلف في الحجم

والشدة، دلالة على أن الإصابة الفيروسية أخذت مسلكا منهجيا، ومؤدية إلى الاعتقاد بوجود الفيروس وقدرته الهائلة على الانتقال داخل المادة الحية. وقد تم ملاحظة بقع نخرية على أشجار ثمار التين المصابة. جزيئيا، وباستخدام تقنية الناسخ العكسي-تفاعل البلمرة المتسلسل، تم الكشف في العينات التي تم فحصها عن المعدل العام للإصابة وهو (78%). و كان أكثر أنواع الفيروسات انتشارا هو FMV (60%) يليه FBV-1 (46%). و وجد مزيج من عدوى فيروس FMV و FBV-1 ما يقارب (30%). و قد أظهر تسلسل الجينوم من عينات فيروس FMV و FBV-1 وجود تشابه عالي مع تلك التي نشرت في بنك المعلومات الجيني (98%-100%). في هذه الدراسة، لقد بات جليا التدهور الصحي للتين في المنطقة الشمالية من الضفة الغربية خصوصا بارتفاع عدد حالات العدوى الفيروسية في ساحات التين المزروعة منذ سنوات عديدة. ويعتبر هذا دافعا للقلق و التوجه إلى الإرشاد والتنظيم لما لتلك الفيروسات من خسائر كبيرة على المحصول والإنتاج . كما ألقت هذه الدراسة الضوء على الوضع الصحي للتين في البلاد مع التوجه إلى استخدام أشتال تين صحية خالية من الفيروسات.

الكلمات الرئيسية: فيروسات التين، تقنية الناسخ العكسي- تفاعل البلمرة المتسلسل ، التين، أمراض تبرقش التين.