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Molecular Detection and Identification of Nepoviruses in West Bank-Palestine

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Dedication

To My amazing parents:

The reason of what I reach today

Thanks for continuous care and infinity support

To my great brother Ramzi and his lovely wife Aya

To my beloved brother Hamzah

To My sweet sisters Manar and Reem

For your supporting and encouraging me

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I also thanks a lot my lovely parents for everything.

الاقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل عنوان:

Molecular Detection and Identification of Nepovirus in West Bank-Palestine

الكشف الجزيئي وتحديد وجود النيبو فيروس (Nepoviruses) في الضفة الغربية–فلسطين

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

Declaration

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

اسم الطالب: **Student's Name:**

التوقيع: **Signature:**

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List of Abbreviations

Bp	Base pair
RT-PCR	Reverse transcription – Polymerase Chain Reaction
cDNA	Complementary DNA
dNTPs	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
mM	Millimole
M-MLV	Moloney murine leukemia virus
µl	Microliter
Nep-A	Nepovirus subgroub-A
RNA	Ribonucleic acid
ss-RNA	single-stranded ribonucleic acid
Rpm	Round per minute
spp	species
Taq	*Thermus aquaticus*
EtOH	Ethanol
KOAc	Potassium acetate
NaOAc	Sodium Acitate
GFLV	Grapevine fanleaf virus
PVP	Polyvinylpyrrolidone
s	sense
a	anti-sense
TNA	total nucleic acid
^{o}C	Celsius degree
No.	Number
SOB	Super Optimal Broth
LB	Lysogeny broth
mm	millimeter
ml	milliliter
EDTA	Ethylenediaminetetraacetic acid
TE	Tris-EDTA
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
Tm	Temperature
$d.H_2O$	distilled water

Molecular Detection and identification of Nepoviruses infecting *Grapevine* in West Bank

By
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Abstract

Grapevine (*Vitis vinifera*) which belongs to the family *Vitaceae*, globally, it is the oldest crop, while in Palestine considered the second crop after olives. As the vegetative propagation is still widely used, up to 60 viruses host grapevine. The most severe one is *Grapevine Fanleaf virus* which belongs to the genus Nepovirus and transmitted to the vine mainly by nematodes. This study was aimed to assess the sanitary status of grapevines in Palestine due to the lack of the available current data on its prevalence.

Field surveys were conducted in the main vineyards of the West-Bank in Palestine, 80 samples were collected: Hebron (34), Bethlehem (24), Jenin (11), Nablus (7) and Jericho (4), for symptoms observation during the growing seasons 2015 and 2016 to detect GFLV, using biological and molecular tools at An-Najah National University Biotechnology Labs. The surveys revealed the existence of virus related symptoms as observed (Yellowing and deformation, small leaves, yellow spots, deformed canes, abnormal branching with short internodes length, Yellow mosaic symptoms and malformation). The 25 samples that were tested by mechanical inoculation onto herbaceous plants; *Nicotiana benthamiana* and *Nicotiana accidintalis*, were failed in isolation of GFLV, even though two samples

expressed stunted plant symptom that was appeared only on *N. benthamiana* , which is not indicative for that virus.

By using RT-PCR, none of the tested samples (55) were able to amplify any of GFLV using the virus specific primers, but with a degenerated primers of subgroup A nepoviruses, RT-PCR were able to detect 12 positive sample from different regions Hebron (5), Bethlehem (3), Jericho (2) and Jenin (1) but nothing found in Nablus samples. These results ascertain that nepoviruses were found in our grapevine fields but not of GFLV. That was in accordance of many unreported trials carried in the last decades expecting the absence of GFLV from the Palestinian vines. The results of this study can be as documentary report for the sanitary status of grapevines. The presence of Nepoviruses other than GFLV would open the door for further researches to be conducted for identifying them.

Key words: Nepovirus, RT-PCR, GFLV, Grapevine, indicator plants.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. General background

Agriculture plays a crucial role in the social life of the Palestinian people in West Bank and Gaza Strip. This rule constitutes a clear reflection of agriculture and its role in the Palestinian local economy, that Contributes significantly to gross domestic product, share in the employed labor force, role in trade and role in the economy by virtue of its strong intersectorial linkages (1). In Palestine geographical situation and ecological environment that is unique make viticulture suitable for production high quality grape, its plantation goes back at least 4000 years ago, It grows everywhere in Palestine. Grape contributes of about 12% of the total agricultural production; the average production of grapes in Palestine is over than 80000 tons per year. The highest production comes from the Hebron area reaching 45000 tons, and 15000 from Bethlehem with average cultivated area of about 80 000 donum. According to the Census of Agriculture for a Palestinian Central Statistics at the end of 2010 there are 1,449,262 grape tree in all Palestinian territories, including 1,243,712 in the West Bank and 205,550 in the Gaza Strip (3).

Grapevine (*Vitis vinifera*) which belongs to the family *Vitaceae*, is the oldest fruit crop and also the most economically essential and widely cultivated in the world (2), whereas in Palestine its plantation goes back at least 4000 years, and characterized as one of the most considerable crops that ranks the second among fruit crops grown there after olive in terms of both areas covered as well as economic returns in Palestine.

According to the ministry of Agriculture, its production and plantation concentrated in the southern part of West Bank mostly Hebron governorate (58%) and Bethlehem governorate (27%), and Jenin governorate (6%). Grape contributes of about 12% of the total agricultural production.

There are up to 13 seeded varieties of grapes grown under rainfed conditions in Palestine. Due to the unique geographical environment and ecological region, high quality fruit crops are produced, a few of them are used as table fruit or after manufacturing, in such forms as jams, Dibis (molasses), raisins, Malben (fruit roll), juice, and vinegar. The most important types are; White Grape types: such as Jandali, Zaini, Hamadani, Dabogi, Beiruti (Romani) and Marrawi. Red Grape types: such as Halawani. Black Grape types: such as Shami, Beituni (Baloti), Fahaissi, Shoyoukhi (Darawishi) and Motartash. Other types of seedless white grape are lately introduced in Jericho and fundamentally cultivated under irrigation comprises Berlait and Superior (69).

1.2. Grapevine viruses and virus like diseases

Perennial and fruit crops constant infection lead to reduce the crop and fruit quality, in addition to loss of cost and time in getting the trees to fruition. Because of continuous vegetation propagation, grape plantations are constantly targets of different viruses and appear to be a sink for viruses from a broad range of families and genera. Viruses are negatively influence the vineyard: reduction in disease resistance to abiotic and biotic stressors, shortening in productive period, weakening in rooting of propagation

materials, reduces yield and quality, and at least early dieback of grape stocks (4).

There are approximately 60 recognized virus and virus-like diseases of grapevines (*Vitis* and *Muscadinia* spp.). Table 1.2.1. Lists the major viruses that are currently known to infect grapevines. Many of the viruses are associated with significant diseases of grapevines, however a number are not widespread and are considered of minor importance to viticulture worldwide. The viruses which are associated with some of the more serious diseases of grapevines occur in the *Closteroviridae* and include viruses associated with leafroll disease, *Betaflexiviridae* and include viruses associated with the rugose wood complex, *Secoviridae* and many viruses are associated with degeneration and decline of grapevines and the *Tymoviridae* associated with fleck diseases.

Grapevine viruses are of great economic importance because of their world wide occurrence and their severe damages they cause. Symptomatic and symptomless canes and leaves will be randomly collected. During the period of mid-June until mid-August at the time of temperature rise, the viruses begin to be less active and are not as readily found out as during the fall or winter, cooler spring. *Grapevine fanleaf virus* is particularly active in the spring, likewise can be detected in fall and winter testing, mostly if testing cane wood. This virus is heat-sensitive and its concentration during summer in vines becomes very low, making disease testing more unreliable (5).

Table1.2.1: Viruses and virus like infecting *Vitis* and *Muscadinia* spp(70).

Family	Genus	Species
A. Viruses belonging to genera including into families		
Viruses with a single-stranded DNA genome		
GEMINIVIRIDAE	Undetermined	Grapevine cabernet franc-associated virus (GCFaV)
Viruses with a double-stranded DNA genome		
CAULIMOVIRIDAE	Badnavirus	Grapevine vein clearing virus
Viruses with a double-stranded RNA genome		
REOVIRIDAE	Oryzavirus	Unnamed virus
ENDORNAVIRIDAE	Endomavirus	Two unnamed viruses
PARTITIVIRIDAE	Alphacryptovirus	Raphanussativus cryptic virus 3 (RsCV-3) like
		Beet cryptic virus 3 (BCV-3) like
Viruses with a negative-sense single-stranded RNA genome		
BUNYAVIRIDAE	Tospovirus	*Tomato spotted wilt virus* (TSWV)
Viruses with a positive-sense single-stranded RNA genome (filamentous particles)		
CLOSTEROVIRIDAE	Closterovirus	Grapevine leafroll-associated virus 2 (GLRaV-2)
	Ampetovirus	Grapevine leafroll-associated virus 1 (GLRaV-1)
	Velarivirus	Grapevine leafroll-associated virus 3 (GLRaV-3)
		Grapevine leafroll-associated virus 4 (GLRaV-4)
		GLRaV-4 strain 5
		GLRaV-4 strain 6
		GLRaV-4 strain 9
		GLRaV-4 strain Car
		Grapevine leafroll-associated virus 7 (GLRaV-7)
ALPHAFLEXIVIRIDAE	Potexvirus	Potato virus X (PVX)
BETAFLEXIVIRIDAE	Foveavirus	Grapevine rupestris stem pitting-associated virus (GRSPaV)
	Trichovirus	Grapevine berry inner necrosis virus (GINV)
	Vitivirus	Grapevine Pinot gris virus (GPGV)
		Grapevine virus A (GVA)

		Grapevine virus B (GVB) Grapevine virus D (GVD) Grapevine virus E (GVE) Grapevine virus F (GVF)
POTYVIRIDAE	Potyvirus	Unidentified potyvirus-like virus isolated in Japan from a Russian cv Bean common mosaic virus (BCMV). Peanut strain
Viruses with a positive-sense single-stranded RNA genome (rod-shaped particles)		
VIRGAVIRUDAE	Tobamovirus	*Tobacco mosaic virus* (TMV)
		Tomato masaic virus (ToMV)
Viruses with a positive-sense single-stranded RNA genome (isometric particles)		
SECOVIRIDAE	Fabavirus Nepovirus Sadwavirus	Broadbean wilt virus (BBWV) Artichoke Italian latent virus (AILV) Arabis mosaic virus (ArMV) Blueberry leaf mottle virus (BBLMV) Cherry leafroll virus (CLRV) Grapevine Bulgarian latent virus (GBLV) Grapevine deformation virus (GDefV) Grapevine chrome mosaic virus (GCMV) Grapevine fanleaf virus (GFLV) Grapevine Tunisian ringspot virus (GTRV) Peach rosette mosaic virus (PRMV) Raspberry ringspot virus (RpRV) Tobacco ringspot virus (TRSV) Tomato ringspot virus (ToRSV) Tomato blacking virus (TBRV) Strawberry latent ringspot virus (SLRSV)
BROMOVIRIDAE	Alfarnovirus Cucumovirus Ilarvirus	Alfalfa mosaic virus (AMV) Cucumber mosaic virus (CMV) Grapevine line pattern virus (GLPV) Grapevine angular mosaic virus (GAMoV)

TOMBUSVIRIDAE	Crmovirus Necrovirus Tombovirus	Carnation mottle virus (CarMV) Tobacco necrosis virus D (TNV-D) Grapevine Algerian latent virus (GALV) Petunia asteroid mosaic virus (PAMV)
TYMOVIRIDAE	Marafivirus Maculavirus	Grapevine asteroid mosaic-associated virus (GAMaV) Grapevine redglobe virus (GRGV) Grapevine Syrah virus 1 (GSV-1) Grapevine Fleck virus (GFKV) Grapevine rupestris vein feathering virus (GRVFV)
B. Viruses belonging to genera unassigned to families		
	Idaeovirus Sobemovirus	Raspberry bushy dwarf virus (RBDV) Sowbane mosaic virus (SoMV)
C. Taxonomically unassigned viruses		
		Unnamed filamentous virus, Grapevine Ajinashka virus (GAgV), Grapevine stunt virus (GSV) Grapevine labile rod-shaped virus (GLRSV), Southern tomato virus (STV)

1.3. Nepoviruses

Plant viruses are considered as the most important plant pathogens; virus invasion can result in large devastation of the plant, in addition to reduce crop quality and quantity (6). Viruses are obligate intracellular parasites that are simple in their construction, and a virus particle possessing primarily a protein coat which composed a protective layer for viral genome, in addition to nucleic acid either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Extremely small sizes of viruses make the use of

the useful techniques of fungal and bacterial pathogens ineffective for detection of viruses (7).

The genus *Nepovirus* (Nematode transmitted viruses with Polyhedral particle) is classified in the order *Picornavirales*, family *Secoviridae* and one of three member of the subfamily *Comoviridae* along with the genera *Fabavirus* and *Comovirus*. The Nepovirus particle (virion) contained 27-40 % M component (nucleic acid), or 42-46 % B component (nucleic acid) is isometric non-enveloped icosahedral, with a diameter between 28-30 nanometer (8). These viruses comprises of 28-30 nm isometric particles consisted of 60 copies of a single coat protein species encapsidating every one of the each components of the positive-sense, bipartite, single-stranded RNAs species (RNA-1 and RNA-2) (Figure 1.3.1.), each are translated as single polyproteins; RNA-1 encodes the polymerase, proteinase and helicase, and RNA-2 encodes for the movement proteins and capsid. The 3' ends of both genomic RNA segments are polyadenylated , and the 5' ends are covalently attached to the VPg protein . Each one of the RNA segment consists of a single open reading frame encoding a polyprotein (9).

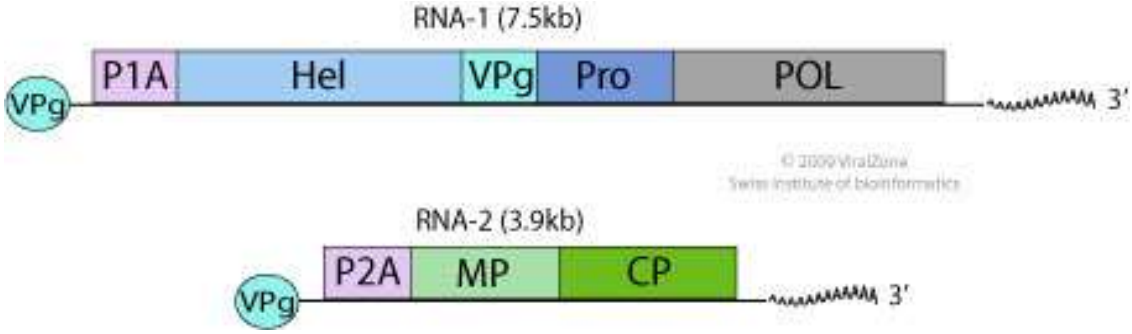

Figure 1.3.1. Nepoviruses genome; genomic organization and expression of RNA-1 and RNA-2 of *grapevine fanleaf virus* (GFLV) (67).

RNA-1 is the larger RNA has about 7.1-8.4 kb depending on the virus, while RNA-2 is more changeable with about 3.4-7.2 nucleotides (10). Depending on the length of RNA-2 and also sequence correspondence and serological relationships nepoviruses categorized in three subgroups: Subgroup A is firstly reported nepoviruses infecting grapevines has RNA2 of 3.7—4 kb and include GFLV, ArMV, GDefV, RpRSV and TRSV; subgroup B has RNA2 of 4.4-4.7 kb and include AILV, GCMV, GARSV and TBRV; subgroup C has RNA2 of 6.4-7.3 kb and include BBLMV, CLRV, GBLV, GTRSV, PRMV and ToRSV (Table 2.2.) (11).

Table 1.3.1. Nepoviruses infecting grapevines (9,11).

Genus *Nepovirus* (Family Secoviridae and subfamily Comovirinae)	Abbreviation	Known prevalence on grapevine	Disease	Vector
Subgroup A				
Arabis mosaic virus	ArMV	Europe	Degeneration, fanleaf, stunt shoot	Nematode
Grapevine deformation virus	GDefV	Turkey	Degeneration, fanleaf, stunt shoot	Unknown
Grapevine fanleaf virus	GFLV	Worldwide	Degeneration, fanleaf	Nematode
Raspberry ringspot virus-grapevine	RpRSV-Gra	Europe	Degeneration, fanleaf, stunt shoot	Nematode
Tobacco ringspot virus	TRSV	USA	Decline	Nematode
Subgroup B				
Artichoke Italian latent virus	AILV	Italy, Balgaria	Fanleaf	Nematode
Grapevine Anatolian ringspot virus	GARSV	Turkey	Degeneration/ yellowing	Unknown
Grapevine chrome mosaic virus	GCMV	Eastern Europe	Mild fanleaf	Nematode
Tomato black ring virus	TBRV	Europe	Degeneration/ decline	Nematode
Subgroup C				
Blueberry leaf mottle virus	BBLMV	USA	Decline on *vitus lambrusca* cv./Yellow mosaic.	Unknown
Cherry leaf roll virus	CLRV	Europe	Latent	Nematode
Grapevine Bulgarian latent virus	GBLV	Eastern Europe, USA	Mild fanleaf	Unknown
Grapevine Tunisian ringspot virus	GTRSV	Tunisia	Decline	Unknown
Peach rosette mosaic virus	PRMV	USA	Decline/ Degradation	Nematode
Tomato ringspot virus	ToRSV	USA	Decline/ yellowing	Nematode

1.3.1 Subgroup A species

All Arabis mosaic virus strains known so far are closely related to one another and distantly related to *Grapevine fanleaf virus* (GFLV). *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV) are two Picornavirus from the genus *Nepovirus* and supposed to have the same origin or even that Grapevine fanleaf virus is the origin of *Arabis mosaic virus*, composed of a bipartite RNA genome encapsidated into a 30 nm icosahedral viral particle formed by of 60 copies of single capsid protein (CP). They are responsible for a severe degeneration of grapevines that takes place in the generality vineyards worldwide (12). The GFLV and ArMV induce significant yield reduction, lowering of the quality of grapevine fruit and reduce the longevity of grapevines. They cause leaves, shoot and fruits malformation, whereas few strains result in yellow discoloration of the leaves (13, 14).

Fanleaf degeneration is one of the most widespread and severe viral disease of grapevine reducing crop product up to 80% lowering the quality of grape and shortening the age of the vine tree in the vineyard (15), causing canes and leaves chlorotic discolorations and malformations of leaves and cause large economic losses, reduce fruit quality and decrease the age of the grapevine (16). It is thought that GFLV has got along with grapes ever after their earliest agriculture and has diffused with vegetatively propagated crop (17). *Xiphinema* index is the ectoparasitic nematode that feed on growing root tips and can also transmit Grapevine fanleaf disease from infected grapevine to healthy one (18).

GFLV results in different of symptoms in grapevines that vary in kind and acuteness so that, moderate to high crop losses caused by GFLV, influence more than 80% of the crop depending on the virus isolate virulence, the susceptibility of the grapevine species, and environmental issues. Distorted and asymmetrical Leaves with clearly closed primary veins and distinguished marginal teeth with open petiolar sinus are the main symptoms (19).

The *Arabis mosaic virus* (ArMV) is considered to be one of the infectious causative virus of the grapevine fanleaf disease, one of the most damaging and widespread viral disease of grapevine. Symptoms are analogous to those caused by *Grapevine fanleaf virus* but are not equal severity; many infections with ArMV are latent and producing symptomless and systemic infection. Transport of propagation material from infected vine primarily lead to long distance spread of viral diseases. *Arabis mosaic virus* is also transmitted by the nematode *Xiphinema* diversicaudatum (20).

Based on molecular data, *Grapevine deformation virus* (GDefV) was allocated in subgroup A of the genus *Nepovirus*, in a position close to that of ArMV and GFLV, with whose RNA-2 it shares sub sequential identity at the nucleotide level. The vine infected by this virus displayed distorted and mottled leaves with irregular branching of the canes and short internodes, asymmetric blades (21).

Tobacco ringspot virus (TRSV) has wide host rang with high efficiency transmission and the symptoms similar to those of grape fan leaf disease as new growth is weak and sparse, internodes are shortened, plants are

stunted, and leaves are distorted and small, in addition of causing ring and line patterns on foliage, dwarfing of plant and small leaves of poor quality (22). Another causative agent of grapevine fanleaf disease is *Raspberry ringspot virus* (RpRSV). Mild Symptoms or often have no symptoms (asymptomatic), on the other hand RpRSV induces symptoms similar to those observed with GFLV, severe yield reduction and the longevity of grapevine is greatly reduced (23).

1.3.2. Subgroup B species

Four nepoviruses of subgroup B are detected in grapevine, *Artichoke Italian latent virus* (AILV) and *Tomato black ring virus* (TBRV) have relatively wide host range, while *Grapevine Anatolian ringspot virus* (GARSV) and *Grapevine chrome mosaic virus* (GCMV) are detected in grapevine only (24). Both of AILV and GARSV show relatively same symptoms with fanleaf-like symptoms, mild yellowing and stunting in infected grapevine. The strain *Artichoke Italian latent virus* (AILV) was isolated first in Italy and reported also from Bulgaria and distinguished serologically in Greek. AILV is transmitted by the nematode *Longidorus apulus*, while a Greek type is transmitted by *Longidorus fasciatus* (25).

Grapevine Anatolian ringspot virus (GARSV) was originally isolated from grapevine with mild fanleaf-like symptoms during a survey performed in the southeastern Anatolia province of Turkey. The virus has no known vector, and not seed-borne infection but may transmitted by infected propagative material that showed chlorotic spots, asymmetric blades and distorted and mottled leaves (26). Another severe infection case of

Grapevine chrome mosaic virus (GCMV), those it can be very damaging in the regions where it is prevalent (27). GCMV was called previously Hungarian chrome mosaic virus due to its first isolation in the area near Lake Balaton in Hungary, and was later detected in Croatia, Czechoslovakia and Austria. This virus can be transmitted through infected seeds and cause noticeable symptoms indistinguishable from yellow mosaic leaves induced by GFLV strain. Affected vine may die and reduce and loss vitality, and in some cases virus induce short internodes or double nodes and leaf deformation, pretty much like *Grapevine fanleaf virus* (28).

Tomato black ring virus (TBRV) is a dangerous pathogen that infects different economically important crops worldwide. TBRV was first detected in 1946 in the United Kingdom, and in grapevines in Germany, then in Greece, Yugoslavia, Turkey, Israel, and Ontario (Canada) as another agent responsible for grapevine fanleaf degeneration. This virus can be transmitted through infected seeds and by nematode vectors of the genus *Longidorus*; *L. attenuatus* and *L. elongates* (29). As in all viruses infect grapevine, TBRV typically causes systemic symptoms on leaves comprising chlorotic or necrosis ringspots, mottling and leaf deformation and distortion, vein yellowing eventually with reduced growth and stunting of shoots in severe systemic infections (30).

To date the complete and available sequence of Nepovirus subgroup B that infected grapevine is only the GCMV isolate from Hungary, so there is no complete basal information to realize the evolution history of these viruses (31).

1.3.3. Subgroup C species

Members of subgroup C are characterized by having a larger RNA2 than the other previous group that includes substantial regions of sequence identity shared with RNA1 (32). Only members of subgroup C of the genus Nepovirus are known to have long 3′-UTRs, which range in size about 1.077 -1.579 kb (33). Cherry leafroll virus is a worldwide virus with fan leaf-like symptoms and yellow mosaic-like symptoms, to date; very little information is available on the molecular or serological variability of CLRV isolates. CLRV is not soil-borne nematodes transmission and considered to be naturally transmitted through pollen grains and seeds unlike the other members of this genus (34).

First detection of *Blueberry leaf mottle virus* (BBLMV) was in 1977 and its presence is limited to Michigan in Canada. Affected leaves with these virus strains are smaller, deformed and mottling symptoms are shown. Infected bushes are stunted and lead to stem dieback, and yield only a portion of their healthy counterparts. In spite of BBLMV member of the genus Nepovirus, no nematodes or aphids transmission vector are detected. The virus is easily transmissible by pollen, mostly via honeybee transmission and seed as in Cherry leaf roll virus. in the C subgroup (35).

Grapevine Bulgarian latent virus (GBLV) was previously detected by in 1977 in Hungary and it describe latent in *Vitis vinifera,* but has been isolated also from vines with fanleaf-like symptoms, yellow mosaic and leafroll. Several cultivars are infected without any symptoms and can

transmit to various herbaceous hosts by mechanical inoculation, for instance *Chenopodium quinoa*. It can cause bud break retardation, irregular protraction of the shoots, poor fruit quality, reduced growth and fanleaf-like symptoms. Particular vector is not known, but with seed and propagative material can be spread (36).

Another C subgroup nepovirus is *Tomato ringspot virus* (ToRSV) the causal agent of grapevine yellow vein infectious; symptoms of ToRSV are very similar to those caused by TRSV of systemic chlorotic or necrosis ringspot, leaf deformation and stunting (37). Several dagger nematode species are ToRSV vectors; *Xiphenema rivesi, X. californicum,* and X. *americanum* and common in vineyard of California in the eastern of United State (38).

Peach rosette mosaic virus (PRMV) was firstly detected in the early 1900s. Symptoms includes smaller leaf than the healthy one, uneven leaf distribution, with apparent deformations, discoloration and deformation occurs on infected fruits and the infected vine generally shows blight and senescence (39). PRMV have a unique property differ from other nematode transmitted viruses that it can be transmitted by several dagger nematode genera; either by *Xiphinema americanum or Longidorus diadecturus* (40).

Grapevine Tunisian ringspot virus (GTRSV) was recorded from Turkey and Tunisia. The strain GTRSV are associated with mild fanleaf symptoms. The amount of available molecular information on these viruses is still limited (41).

1.4. Infectious cycle

1.4.1. Nematode vector

Plant viruses as all viruses are obligatory intracellular parasite, use the plant cells machinery for multiplication and invade their host. Because the plant cells possess in their structure natural physical barriers; cuticle and cell wall, viruses are enter into plant cells through wounds or during the action of vectors (insects, nematodes, fungi) (42). Nematode vectors that belong to the Order *Dorylaimida* and take place within two families *Longidoridae* and Trichodoridae. These are soil-inhabiting ectoparasites which feed on root tips. *Longidorus* and *Xiphinema* are two genera of *Longidoridae* and *Trichodorus* and *Paratrichodorus* are two genera of *Trichodoridae* are known to be vectors (43).

Xiphinema is commonly found in tropics regions whereas *Longidorus* is prevalent in the temperate regions. Adults of *Xiphinema* and *Longidorus* are 3 to 10 mm long. The mouthparts are adapted for probing in all the nematode vectors and composed of a single central stylet, those stylets are hollow and tubular; the proximal or basal part of the stylet is considered to know as odontophore, whereas the distal part is the odontostyle. The length of odontophore and the odontostyle together is about 200 μm in length (44). The stylet of the nematodes can penetrate to the xylem of plants, the virus particles can be attached to the lumen of the pharynx and ondontophore and the cuticular lining for up to 4 years. *Xiphinema* species are important grapevine parasite which worldwide distributed and considered to be the eighth most economically damage ectoparasite

nematode to the agricultural crops (45). The mode of transmission of nepoviruses by nematodes is non-circulative, semi-persistent plant viruses; Nepoviruses which transmitted by nematode are hooked up for several weeks, and in some cases to several years, at particular locations in the feeding apparatus. At the molting stage the nepoviruses are lost completely and not replicate in the nematode vector (46).

1.4.2. Multiplication of positive-sense RNA viruses

Viruses with positive-sense RNA genomes do not have a DNA phase, and act as mRNA encoding for a specific viral proteins; transfer their genomic RNAs directly to cellular ribosomes and start the infectious cycle with translation (47). After the virion enters the infected cell, the genomic RNA release into the host cytoplasm and use as a template to produce replication proteins using the host's machinery, genomic RNA and the host proteins, metabolites, lipids and membranes (organelles and plasma membrane) are considered as viral replication complex (VRC) (48) RNA-dependent RNA polymerase (RdRp) is a viral protein (P1) directly translated from the genome, and encoded at the 5' end. RdRp synthesizes complementary full length negative-sense strand intermediate using the original positive-sense strand as a template, that later use for synthesis of progeny genome (49), see figure 1.4.2.1.

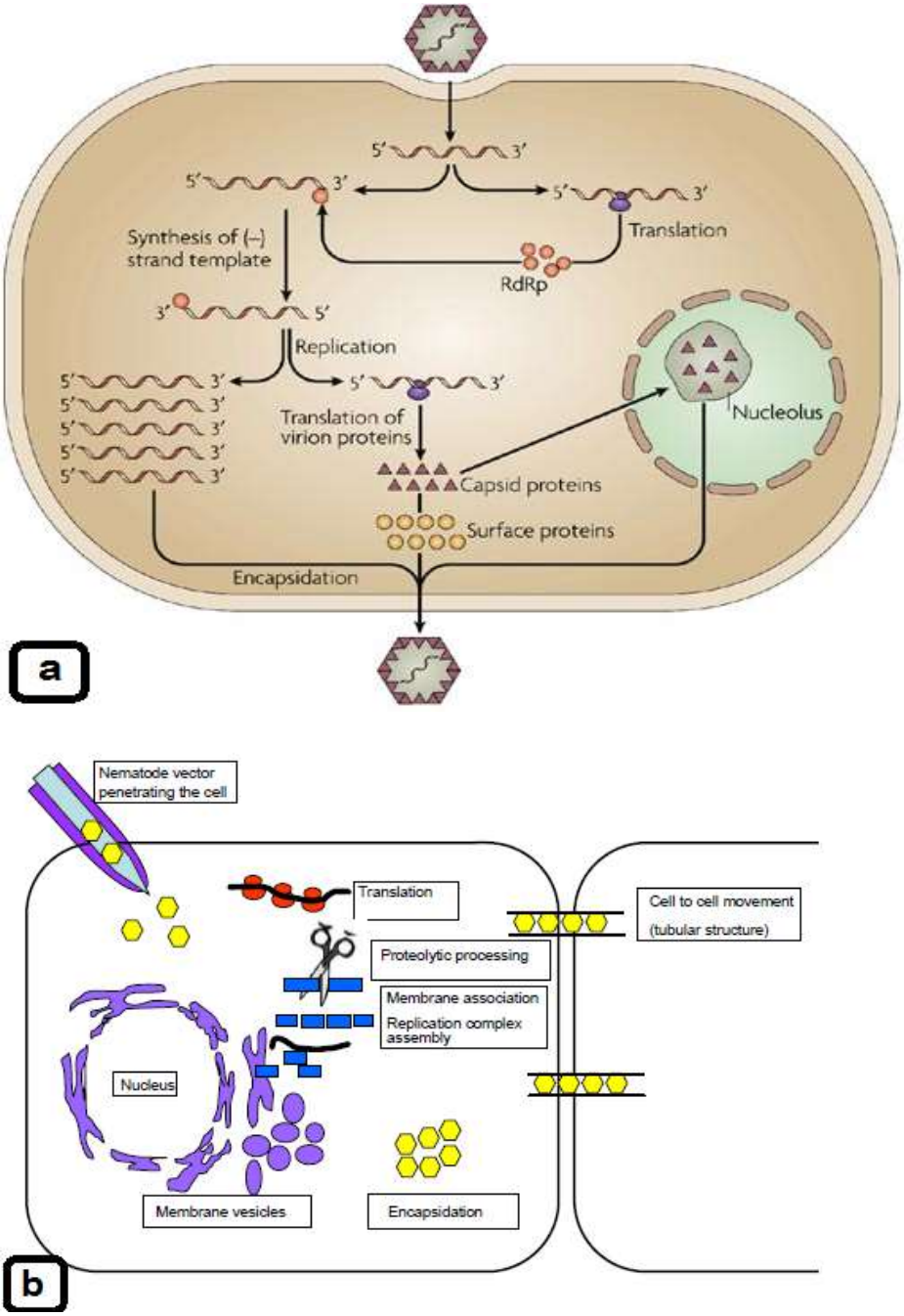

Figure 1.4.2.1. a) Schematic representation of the infection cycle of plant positive-strand RNA virus (66). b) Schematic representation of the infection cycle of nepoviruses starting with nematode vector and replication within the cell and cell to cell transmission (72).

1.5. Methods for the detection of plant virus diseases

1.5.1. Symptomatology

The symptoms represented important signs for plant virus characterization and identification. When symptoms are clear and specific for a certain viral disease, visual screening is relatively easy and commonly used to identify and control a disease. Moreover, the host range, strain of viruses, infection time and the surrounding environment are factors that may influence the symptoms appearance (50).

Symptoms may be inconclusive and very slight, or infected plants probably symptomless. Nevertheless, bioassay with the use of a inoculated indicator test plants still an needful tool for identification and detection of plant viruses and the signs include various symptoms of leaf deformation, mottling, mosaic, petal-color breaking, stunting, ringspots, wilting, chlorotic and necrotic lesions and spots and reduction in yield. Chenopodiaceae families are the most usually used indicator plants to characterize plant viruses (51).

1.5.2. Serology

ELISA is the most significant and popular advance serological tests used for plant virus detection because of its adaptability, simplicity and sensitivity. In this immune-specificity is detected during the activity of the associated enzyme label on an appropriate substrate. The viral identification by serological techniques are based on the properties of virus

coat protein. On the othe hand ELISA does not give a concentration measurement of the infective virus and just detects the viral antigen (52).

The basic principle of the ELISA is in immobilizing the antigen onto a capturing antigen by certain antibodies, or solid surface, and probing with particular immunoglobulin carrying labeled enzyme. The enzyme possessed in the case of positive reaction is detected by supplement the proper substrate. The substrate is converted by the enzyme to product, which can be easily differentiated by its color (53).

The principle of ELISA techniques composed of detecting the antigen-antibody interactions by enzyme produced color reaction. Direct and indirect ELISA are two types of ELISA procedures. The direct ELISA is considered the simplest type of ELISA, the antigen is adsorbed to a microtitre plate, and then an excess of another protein (normally bovine serum albumin) is added to block all the other binding sites (78).

1.5.3. Nucleic acid-based techniques

Nucleic acid-based techniques are widely used for plant viruses' detection and identification, fundamentally after the use of the polymerase chain reaction (PCR) (54). In spite of the fact that culture-based methods and enzyme-linked immunosorbent assay (ELISA) are yet mainly valuable techniques, PCR usually is more sensitive than others and can detect a virus of small amount (55). Serological methods are based on the viral coat proteins and its antigenic differences, which represents only about 10% of the overall virus genome and so they neglect the rest of the virus genome

(56). Furthermore, nucleic acid based methods have important advantage that any part of a viral genome can be targeted to evolve the diagnostic test (57). On the other hand, in some conditions serological techniques cannot be used particularly for the detection of viroids, satellite RNAs, viruses that lack particles (for instance; Groundnut rosette virus (GRV)), viruses that take place as highly diverse serotypes (for instance; Tobacco rattle virus Indian and African Peanut clump virus) and viruses which are not easy purified (50).

Polymerase chain reaction (PCR) is a highly specific, susceptible and versatile in vitro technique that have a great prospect to amplify tiny amounts of targeted nucleic acid utilizing particular primers to the region of amplification, with the use of thermostable DNA polymerase (58, 59). It composed of three step cycles: denaturation in which high temperature is applied on the two original double DNA strands to separate from each other's (raising to temperature between 94-95 °C), annealing of two oligonucleotide primers to their complementary sequences in the opposite strands of the target DNA (annealing temperature be based on the nucleotide construction and extent of the primer (Cooling to temperatures between 35-65 °C), while elongation or extension of any primer through the target region (typically to 72 °C) using a thermo-stable DNA polymerase (usually used *Taq* DNA polymerase). Each one of the result DNA strand made in the first cycle will serve as a template for synthesis of a new DNA strand in the next cycle. The procedure is reduplicate several times (20-40 cycles) (Figure 1.5.3.1). The reaction composed of several

components: dNTP mix, reaction buffer, forward primer, reverse primer, magnesium, template and thermostable DNA polymerase. This procedure is immediatly applied for replication of plant viruses with DNA genomes (for instance Caulimo, Badnaviruses and Gemini) utilizing gene-specific primers to the amplification region. However RT-PCR oftentimes started from a small concentration of target RNA in plant viruses that contained RNA genomes (60), the RNA must be converted to cDNA using reverse transcription (RT) before the starting of PCR procedure. See Figure 3.4.3. In addition to its advantage as a diagnostic technique, PCR is used by several research laboratories worldwide for many goals containing, molecular characterization (61), DNA comparing between linked pathogen species (62) in addition to evolutionary studies (63). In spite of the fact that PCR can realize a very high susceptibility and specificity, distinct comparative trials have been reported a failure of PCR technique to diagnose accurately the infected and non-infected plant. This failure could be a consequence of the "carry-over" impurity of amplicons, this can be responsible for false-positive results and inhibitor components in sample extracts which is the main reason for false negatives (64, 65).

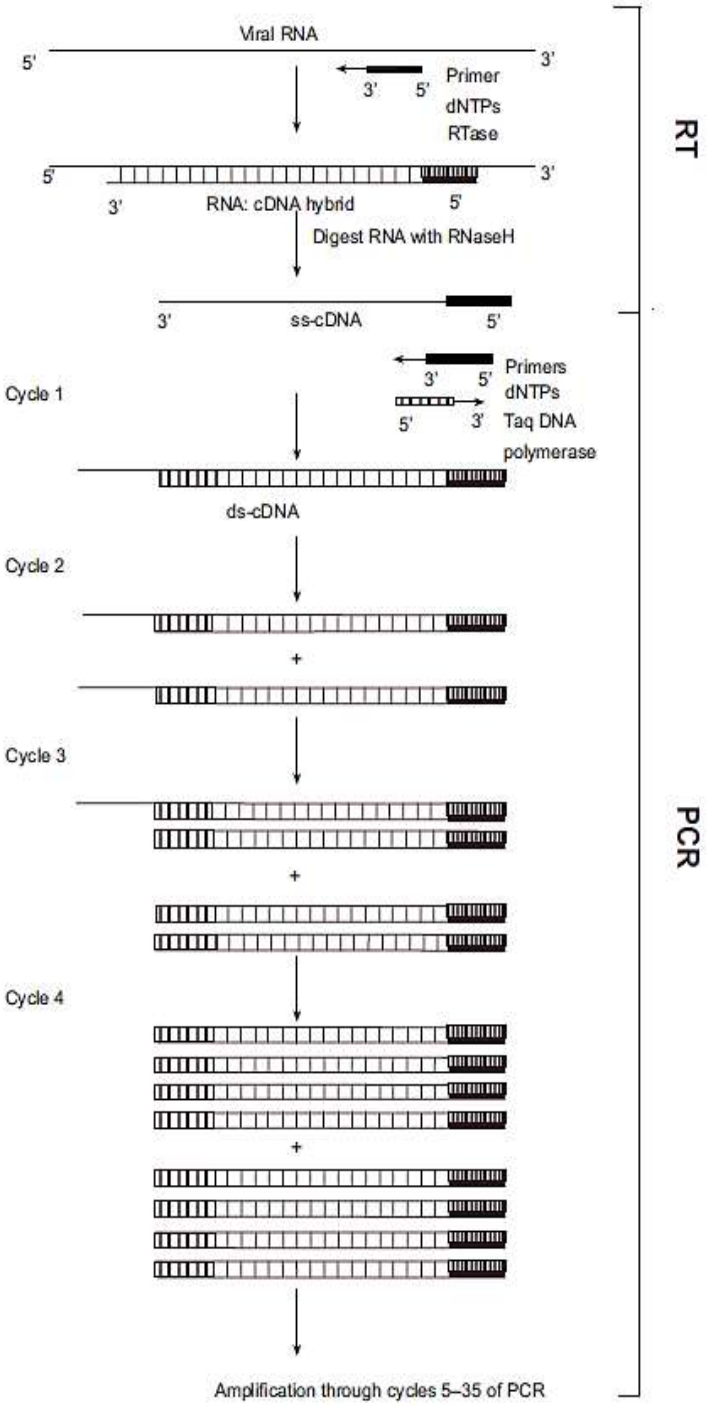

Figure 1.5.3.1. Diagrammatic representation of reverse transcription-polymerase chain **reaction** (RT-PCR). Each one of the PCR cycles consists of denaturation, annealing, and extension. ss–cDNA = single-stranded cDNA , RTse = reverse transcriptase, dNTPs = deoxynucleotide triphosphates, ds-cDNA = double-stranded cDNA (50).

1.6. Objective

Due to scarce in the available information about the prevalence of GFLV in Palestine and within the scope of disseminating of healthy propagating materials to the farmers, this study was aimed to assess the sanitary status of grapevines regarding to the nepoviruses and in particular to the GFLV, using the available biological and molecular techniques. Besides the study was aimed to characterize molecularly the putative detected GFLV isolates.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Field surveys and Plant samples collection

Surveys were carried out in vineyards of the main grapevine-growing areas of West Bank (Hebron, Bethlehem, Jenin, Jericho and Nablus) during the growing seasons of early spring 2015 and late summer 2016 to assess the presence of any viral diseases associated with nepoviruses. A total of 11 different vineyards in 5 districts were surveyed and a total of 80 leaf samples from symptomatic and asymptomatic grapevines were randomly collected (Figure 2.1.1.), in order to test them for the presence of nepovirus diseases in the country.

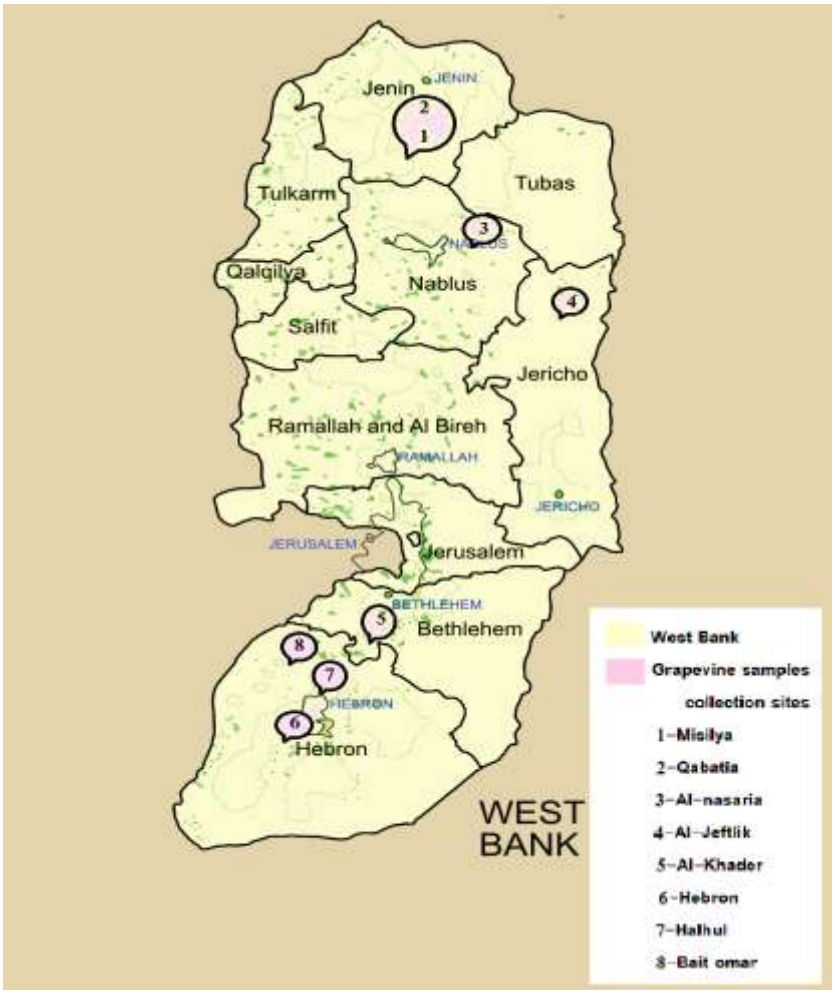

Figure 2.1.1. Locations of the collected Grapevine samples in the West Bank-Palestine.

In Nablus district, sample collection was done from An-Nassaria, Hebron district from Hebron, Halhul and Bait omar, Bethlehem district from Al-Khadr, Jenin from Qabatia and Misilya, other samples were taken from Al Jeftlik in Jericho district.

Each sample was contained several petioles collected from different sites of the tree; grouped and label accordingly. This samples were placed in plastic bags and preserved in ice-bag container and transported at the same day to the laboratory of the Department of Biology and Biotechnology, at An-Najah National University Nablus, Palestine, to be stored at 4^{o}C.

2.2. Mechanical Inoculation (transmission to indicator plants)

Attested seeds of several indicator plants *Nicotiana benthamiana and Nicotiana accidentalis* were cultivated in sterilized soil. Each of 25 symptomatic leaf tissues were ground in a sterile mortar and pestle with the addition of 750 µl of 0.1 M phosphate buffer pH7 and 500 µl of 2.5 % nicotine to make leaf sap (inoculum). Celite powder was already mix with the sap inoculum, before they were applied uniformly over indicator plant leaves with a cotton swab, by starting each stroke from the petiole, and ending at the leaf tip (Figure 2.2.1.). The indicator plants were inoculated with plant mixture extracted from uninfected plants and used as control set. Negative-control plant was also used which was inoculated with phosphate buffer and abrasive (celite) only (75).

Figure 2.2.1. Plants Inoculation; A: *Nicotiana benthamiana,* B: *Nicotiana accidentalis,* C: plants which were already inculated, D: Cotton swap was used to apply the inoculum sap over the indicator plant leaves.

2.3. Molecular detection of Nepovirus

2.3.1. Total Nucleic Extraction

Silica capture protocol was performed (78). For applying this, about 0.5 g of leaves that contained petioles were grinded in sterile sample plastic bags with 3ml grinding buffer (4 sodium acetate, pH 5.2; 25 mM EDTA; 1 M potassium acetate; 2.5 % PVP-40 and 1% sodium metabisulfite). Then the

residue were precipitated by mean of spin centrifuge then left them few minutes on ice. 500 µL of the upper aqueous phase were collected into another tube. After that 150 µL Sarkosyl 10% was added, vortex and the samples were incubated at 70^{o}C for 10 minutes with intermittent shaking. Then, they were incubated for 5 minutes and centrifuged at 1,3000 rpm for 10 minutes. 300 µL of the supernatant were collected and mixture of 500 EtOH and 35 µL of silica were added and left 10 minutes at room temperature. Then the samples were centrifuge 6,000 rpm for 1 minute and the pellets were washed repeatedly by 500 µl of washing buffer. Finally the pellets were resuspended with 150 µl distilled water and directly were exposed to heat for 4minutes at 70^{o}C and then on ice. The samples were centrifuged for three minutes at 13,000 rpm and the supernatant was taken and RNA bands visualized at 1.2% gel electrophoresis in 1x TBE buffer for RNA. Each tube was stored at -20^{o}C until be used.

2.3.2. Reverse transcription of RNA (cDNA synthesis)

cDNA was synthesized by random hexamer primers, 1 µl was added to 10 µL total extracted RNA, then they have been denatured at 95 ^{o}C for 5 minutes and immediately chilled on ice, and then was added to the mixture consists of: 2 µl of M-MLV 10x buffer, 0.5 µl of 10 mM dNTPs, 2 µl of mM DTT, 1 µl of 200 unit/µl reverse transcriptase (M-MLV enzyme) and 1.5 µl of distilled H_2O.The reaction was took place in a final volume of 20 µl. The tubes were shaken, followed by brief centrifugation and incubated

in a thermostat at 37 ^{o}C for 2 hours, after the reverse transcription reaction was completely done the cDNA was store at -20 ^{o}C for later use.

2.3.3. Polymerase chain reaction for gene amplification

For molecular assessment of nepoviruses, RT-PCR detection for the subgroup A in infected grapevines were carried out using the degenerated and species-specific primers as recommended by Digiaro et al. (2007) (Table 2.3.3.1.). Samples were initially tested and screened against GFLV in 55 samples. Then primer set of "Nepo-A-s/a" that able to generate an amplicon of 255 bp from vines that could be infected by any of *Grapevine fanleaf virus* (GFLV), *Tobacco ringspot virus* (TRSV), *Arabis mosaic virus* (ArMV) and *Grapevine deformation virus* (GDefV) were applied.

PCR mix were prepared as 2.5 µl cDNA was added to the amplification mixture: 0.5µl of 20µM sense and antisense of each degenerate primers, 0.5µl of 10mM dNTPs, 2.5 µl of 10x Taq DNA polymerase buffer and 0.2µl Taq polymerase (5 unit/µl) (Promega Corporation, USA) in a final volume of 25 µl.

The recommended PCR protocol included 35 cycles. Firstly an initial denaturation was carried out at 94 ^{o}C for 5 minutes. Each cycle composed of denaturation at 94 ^{o}C for 35 seconds and annealing temperature of 50 ^{o}C for 45 seconds. For subgroub A extension temperature 72^{o}C for 35 seconds and final extension was at 72^{o}C for 7 minutes. The amplified products were subsequently stored at 4 ^{o}C , before amplification products were analyzed by gel electrophoresis.

Table 2.3.3.1. Sequences of degenerate primers for subgroup A detection in RT-PCR, sense and antisense specific primers for GFLV, a member of the subgroup A (11).

Subgroup A viruses	Site of primer Sense (F)	Site of primer Antisense (R)
Nep-A	5'GGHDTBCAKTMYSARRARTGG-3'	5'TGDCCASWVARYTCYCCATA-3'
GFLV	5'-CTAGATTTTAGGCTCAATGG-3'	5'TGCAAACCAGGAAAGAAAAT-3'

s: sense; a: antisense; B (C+G+T); D (A+G+T); H (A+C+T); M (A+C); R (A+G); S (C+G); W(A+T); Y (C+T); K (G+T)

2.3.4. Agarose gel Electrophoresis

Amplified PCR products were visualized at 1.2 % agarose gel depending on the size of the fragments in 1x TAE buffer for DNA, GelRed (Biotium, USA) was added to soluble agarose in buffer which intercalates into DNA and become fluorescent when excited to ultraviolet rays. After separation, the DNA molecules can be visualized under UV-transilluminator. The size of visualized band was determined by comparison with 1 Kb plus DNA marker (0.5 μg/μl a load).

2.4. Colony PCR

2.4.1. Cloning principle

Cloning was performed by mean of InsTAclone PCR Cloning Kit. All PCR product contains 3'-A overhang to both ends, while a cloning vector must have 3'-ddT in its end, as a result this facilitates the binding of insert to the vector, as well as prevent the recircularization of the vector. Recombinant clones were selected with blue white screening.

2.4.2. Preparation of Media and Reagents

SOB Medium

20 gram of tryptone, 5 gram of yeast extract and 0.5 gram NaCl was added to final volume 1 liter deionized water, and then 10 ml of filter-sterilized 1 M MgCl2 and 10 ml of filter-sterilized 1 M MgSO4 was added to mixture.

SOC Medium

It must prepare immediately before use. 2 ml of 20 % filter-sterilized 2 M glucose was added to 100 ml final volume autoclaved SOB medium.

LB-Ampicillin agar

10 gram of NaCl, 10 gram of tryptone, 5 gram of yeast extract and 20 gram of agar was added to 1 liter final volume deionized water. The mixture was adjusted pH to 7.0 with 5 N NaOH, and then autoclaved at 121oC for 30 minutes and cooled to 55oC in order to add 10 ml of 10 mg/ml filter-sterilized ampicillin. Finally, the media was poured in to petri-dishes (25ml/100 mm plate).

LB Broth

10 gram of NaCl, 10 gram of tryptone and 5 gram of yeast extract was added to 1 liter final volume deionized water. The mixture was adjusted pH to 7.0 with 5 N NaOH, and then autoclaved at 121oC for 30 minutes, cooled in order to add 10 ml of 10 mg/ml filter-sterilized ampicillin and put it in 4^{o}C until be used.

TE buffer

5ml of 0.05 M Tris of pH 8 and 2 ml of 0.01 M EDTA were added to 100 ml final volume of deionized water.

Lysis buffer

40 ml of 10% SDS, 5 ml of 0.05 Tris and 2 ml of 1 M EDTA with adjusted pH 12.45 were added to 100 ml final volume of deionized water.

2.4.3. Ligation

1 µl of T4 DNA ligase was added to the following mixture; 3 µl pTZ57R/T cloning vector, 6 µl 5X ligation buffer, 4 µl PCR product and nuclease-free water up to 30 µl total. The mixture was vortexed and centrifuged at high speed for 3-5 seconds. After that the ligation mixture was incubated 1 hour at room temperature and for maximum transformation number it was incubated at 4^{o}C overnight.

2.4.4. Transformation

Two aliquots of 25 µl SoloPack Gold cells were thawed on ice (one tube for each of experimental and control transformations), and then the tubes were swirled gently to mix the cells. 2.5 µl of ligation mixture was added to experimental tube, while 1 µl control DNA of circular supercoiled plasmid vector pTZ57R DNA without insert was added to control tube. The tubes were swirled gently and incubated 30 minutes on ice, after that critical duration heat-pulse carried out; the tubes were incubated 60 seconds in

42°C water bath and directly incubated 2 minutes on ice, also SOC broth was preheated at 42°C and 175 µl was added to each and incubated at 37°C for 1 hour with shaking at 255-250 rpm.

Not more than 200 µl of the transformation mixture was streaked on LB agar plates containing Ampicillin, in addition to IPTG and X-Gal for blue/white screening (). Finally the plates were incubated at 37°C overnight (plates were incubate at least 17 hours to allow the color development or by subsequent 2 hours incubation at 4°C). Next day a sterile toothpick was used to isolate each single culture white colony in 2 ml LB-broth; furthermore the tubes were culture overnight in an incubated orbital shaker at 37ºC at 190-225 rpm.

2.4.5. Plasmid Extraction and Electrophoresis

Plasmid isolation was done (81). 2 ml transformed culture was centrifuged for 5 minutes at high speed and next the pellet was washed in 2 ml of TE buffer (pH 8). 600 µl of freshly prepared lysis buffer was added into eppendorf tubes with gently mixed, additionally the tubes were incubated at 37°C for 30 minutes to be sure that the lysis completely occurred. For neutralization 30 µl of 2 M Tris of pH 7 was added to the mixture with gently mixed and 240 µl 5 M NaCl was added for precipitation of chromosomal DNA and proteins, also it were incubated overnight at 4°C.

Next day the tubes were centrifuged for 10 minutes at 14,000 rpm and the pellet of the bacterial debris was removed from the eppendorf tube with a sterile toothstick. After that the tubes were incubated 45 minutes at 68°C,

and then 6 ml phenol/chloroform (1:1) was added to the hot samples with gently mixed until emulsification occurred. Subsequently, the samples were centrifuge to separate the phases at 10,000 rpm for 20 minutes and the upper aqueous phase was carefully transferred to a new tube contained 1 volume of chloroform. The tubes were mixed and finally were centrifuged and the phases were separated again, aqueous phases were recovered again and used directly for agarose gel electrophoresis. The cloned products were checked using the facilities at Bethlehem university to sequence the cloned PCR.

CHAPTER THREE

RESULTS

3.1. Field surveys and biological identification of virus-associated symptoms in Palestinian Vineyard

Surveys were carried out in vineyards in the main grapevine-growing areas of West Bank (Hebron, Bethlehem, Jenin, Jericho and Nablus) during the growing seasons of 2015 and 2016 to assess the presence and incidence of virus. Symptoms were observed in Hebron, Bethlehem and Jenin in native and imported cultivars showed some symptoms may be caused by Nepoviruses included yellowing, distorted and small leaves and mild flecking of the leaves especially on grafted vines with a high incidence in Hebron, also yellow spots on leaves were observed in vineyard of Misilyah-Jenin (Figure 3.1.1.). However, fanleaf symptoms were scarcely observed especially fan leaf deformation appearance and elongated teeth margin along the leaves, in addition of the symptoms were observed regardless the vine kind.


A


B


C


D


E

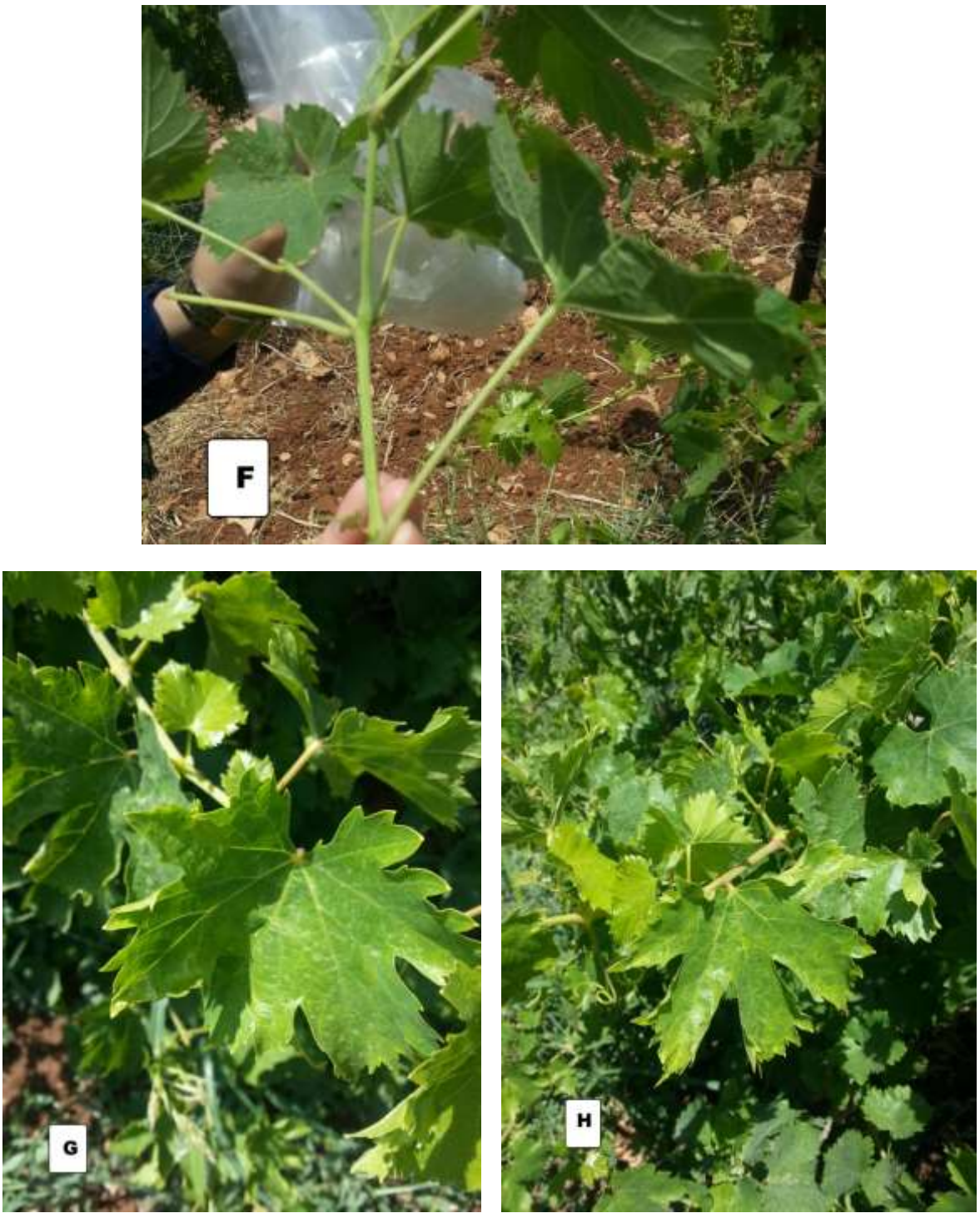

Figure 3.1.1. Viral symptoms were observed in vineyards where it existed in West-Bank of Palestine. Affected vines was showed several symptoms such as: Mild flecking (A), Yellowing and deformation (A,B), leaflets are dwarfed (C), Yellow spots (D,E), Deformed canes, Abnormal branching and different length or short internodes (F) Yellow mosaic symptoms and malformation (G,H).

3.2. Biological assays

Mechanical inoculation was extensively used to isolate any of the infecting viruses. Thus, 25 of collected samples expected to be diseased with virus were inoculated on the herbaceous plants. That mechanical transmission was carried out with these number of symptomatic leaf samples from the growing season 2016 by inoculation onto herbaceous plants *Nicotiana benthamiana* and *Nicotiana accidintalis*. The only symptoms were observed on *Nicotiana benthamiana* with stunting plant growth (Figure 4.5.). This could be referred to nepoviruses infections of any member of subgroup A nepoviruses (such as Tobacco ringspot virus) (22) but not GFLV (79). Stunting symptoms appeared on sample number 16 of Bethlehem after nearly four weeks of inoculation, as it was mentioned in previous study in 2004 (68). *N. benthamiana* plants that inoculated with Nepovirus compared to healthy plant (Figure 3.2.1.). In contrast fanleaf symptoms did not appear on any of the inoculated plant. In contrast, *N. accidintalis* showed no symptoms.

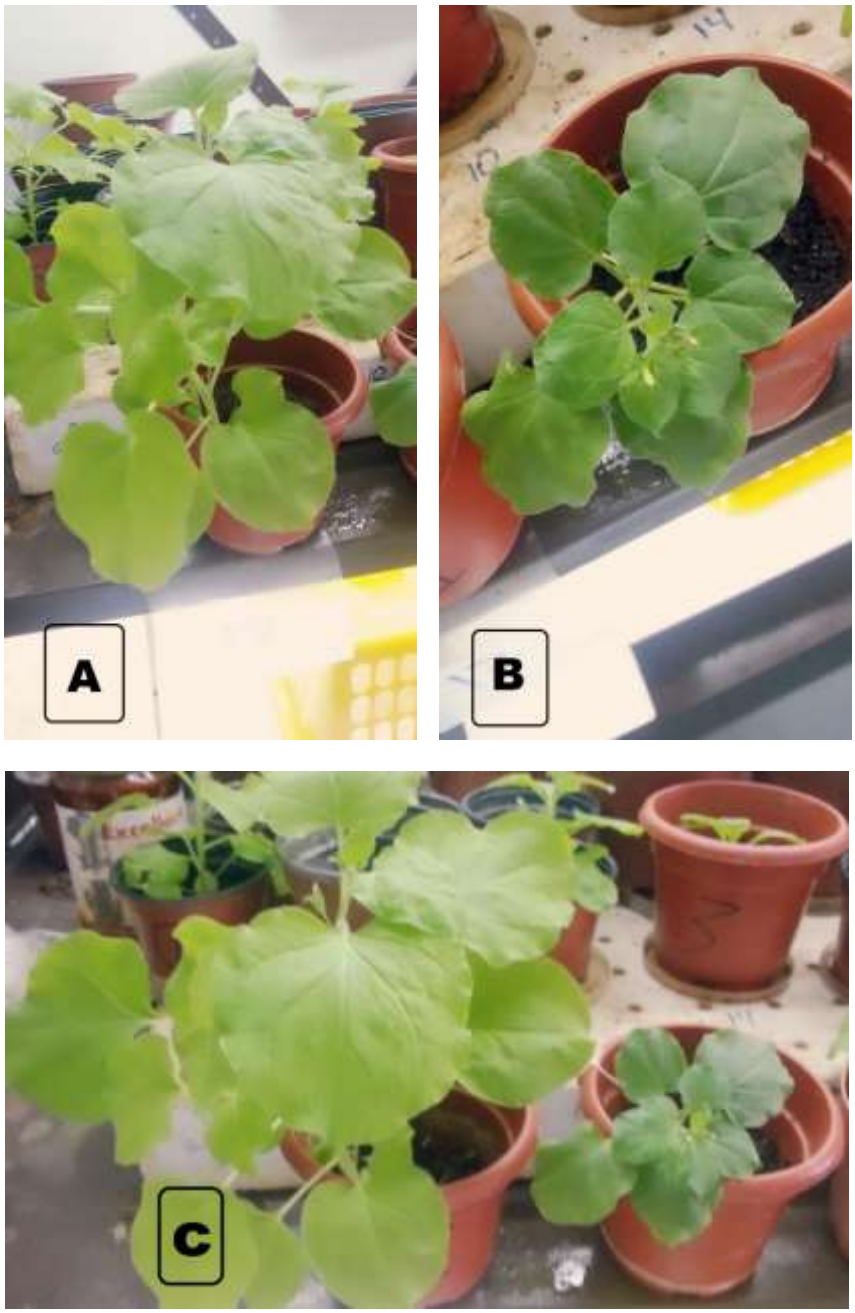

Figure 3.2.1: Mechanical transmission; A: is a healthy control plant, B: Plant of *Nicotiana benthamiana* with stunning symptoms, C: *Nicotiana benthamiana* plant systemically infected with nepoviruses four weeks after mechanical inoculation (on the right); healthy (noninoculated) control plant (on the left).

3.3. Total RNA Extraction

A 1.2% agarose gel was used effectively to evaluate the total extracted nucleic acid, and the quality was analyzed by electrophoresis in agarose gels. Total RNA was successfully extracted from all 55 sample. (figure 3.3.1.).

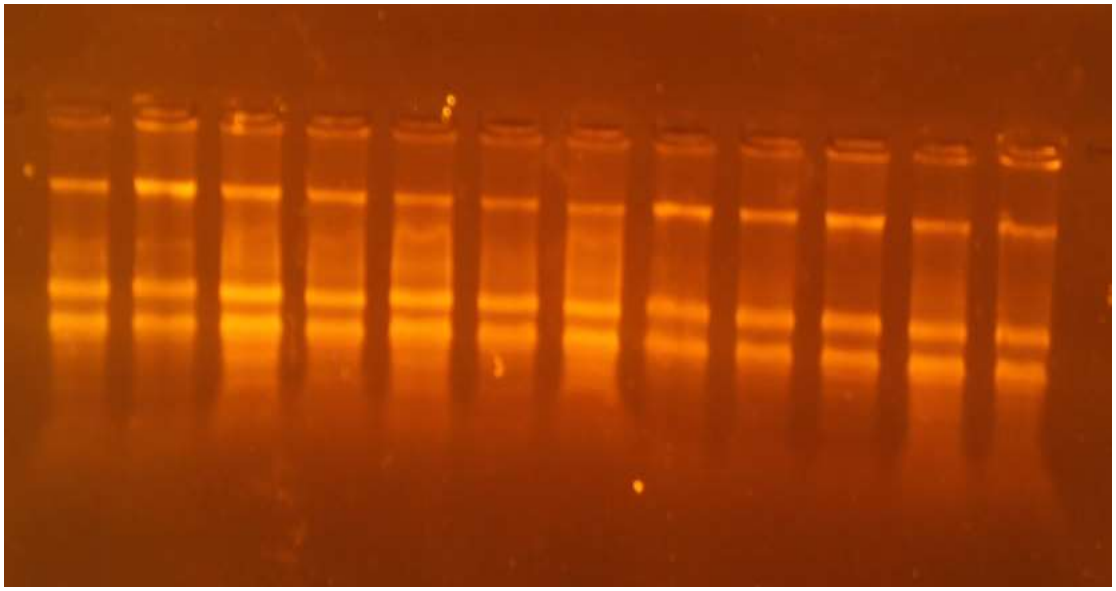

Figure 3.3.1. A 1.2% Agarose Electrophoresis for 12 different RNA extracted samples**.**

3.4. Molecular detection

Degenerate primers designed for the a specific detection of Subgroup A were initially tested and screened versus several nepoviruses in symptomatic and asymptomatic plant tissues. After optimization of the testing situations, the set of primers proved specially efficient for RT-PCR detection. The primer set “Nepo-A-s/a” produced an amplicon of 255 bp from vines infected by either GFLV, ArMV, GDefV or TRSV (figure 3.3.). On the other hand Fanleaf Grapevine Virus specific primers were used, after several trial of the tested samples no specific sequence of fanleaf virus were detected.

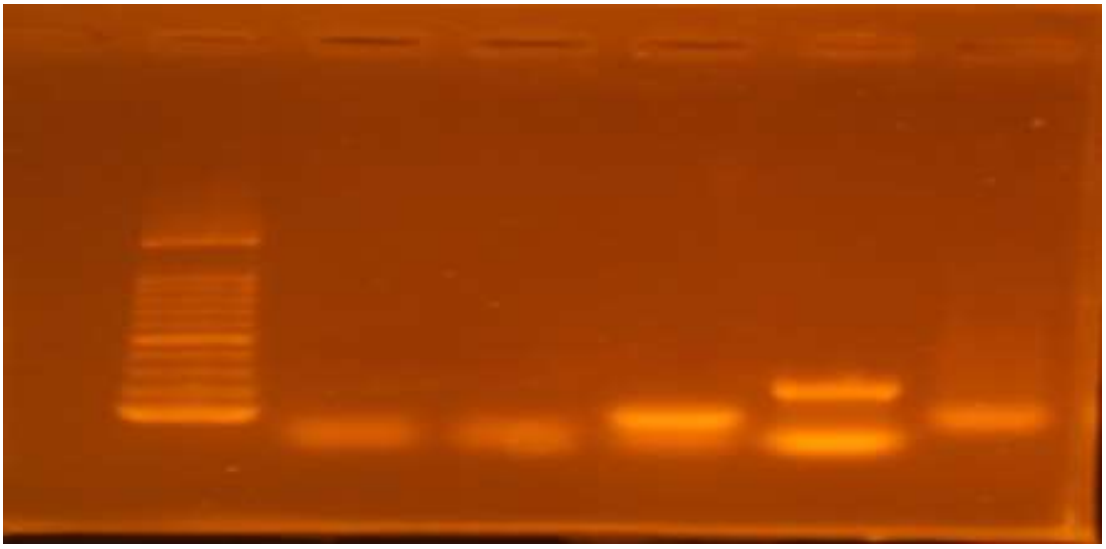

Figure 3.4.1: Molecular Detection Using Degenerate Primer: Gel Electrophoresis showing the 255 bp product bands in each Lane 3,7 and 8 amplified by degenerate primers Nepo-A (s/a) only from infective samples with nepoviruses of subgroup A. Lane M: DNA ladder marker**.**

3.5. Gene Amplification Results

The results of molecular diagnosis of samples of growing season 2015 were limited on nepoviruses of subgroup A, a set of degenerate primers were used “Nepo-A-s/a”. All the 55 samples are reported below in table 3.4.1.; whereas 12 samples show positive results (Table 3.5.1.).

Table 3.5.1. Results of gene amplification (RT-PCR) for the presence of nepoviruses with the use of degenerate primers Nepo-A (s/a)

No.	Place	Grape variety	RT-PCR
1	Halhul-Hebron	Zeany	
17	Halhul-Hebron	Zeany	
32	Hebron	Zeany	
33	Hebron	Zeany	
25	Hebron	Zeany	
9	Hebron	Betuny	
39	Hebron	Zeany	positive
26	Halhul-Hebron	Zeany	
40	Halhul-Hebron	Zeany	positive
18	Halhul-Hebron	Zeany	
19	Halhul-Hebron	Zeany	
10	Halhul-Hebron	Zeany	
11	Halhul-Hebron	Zeany	positive
12	Halhul-Hebron	Zeany	positive
8	Halhul-Hebron	Zeany	
13	Halhul-Hebron	Zeany	positive
20	Halhul-Hebron	Zeany	
34	Halhul-Hebron	Zeany	
35	Halhul-Hebron	Zeany	
2	Jeftlic-Jericho	Sps	
30	Jeftlic-Jericho	Sps	positive
27	Jeftlic-Jericho	Sps	positive
36	Jeftlic-Jericho	Sps	
3	Alnasaria-Nablus	Sps	
37	Alnasaria-Nablus	Sps	
42	Alnasaria-Nablus	Sps	
21	Alnasaria-Nablus	Sps	
22	Alnasaria-Nablus	Sps	
47	Misilyah-Jenin	Biruty	
49	Misilyah-Jenin	Biruty	
28	Qabatia-Jenin	Zeany	
38	Qabatia-Jenin	Zeany	
4	Qabatia-Jenin	Zeany	
51	Misilyah-Jenin	Biruty	
39	Misilyah-Jenin	Biruty	positive
53	Misilyah-Jenin	Biruty	

23	Misilyah-Jenin	Biruty	
54	Misilyah-Jenin	Biruty	
29	Misilyah-Jenin	Biruty	
6	Alnasaria-Nablus	Sps	
24	Alnasaria-Nablus	Sps	
41	Alkhader1-Bethlehem	Biruty	
14	Alkhader1-Bethlehem	Biruty	positive
7	Alkhader1-Bethlehem	Biruty	
43	Alkhader1-Bethlehem	Biruty	
44	Alkhader2-Bethlehem	Zeany	
45	Alkhader2-Bethlehem	Zeany	positive
46	Alkhader2-Bethlehem	Zeany	positive
15	Alkhader2-Bethlehem	Zeany	
55	Alkhader2-Bethlehem	Zeany	
48	Alkhader2-Bethlehem	Zeany	
31	Alkhader2-Bethlehem	Zeany	
16	Alkhader2-Bethlehem	Zeany	Positive
52	Alkhader3-Bethlehem	Zeany	
50	Alkhader3-Bethlehem	Zeany	

3.6. Colony PCR Result

The cloned plasmid and the insert (target DNA) were analyzed at 1.2% agarose gel electrophoresis, bands were noticed (Figure 3.6.1.). Two samples were sent to Bethlehem for sequencing and they were showed negative results.

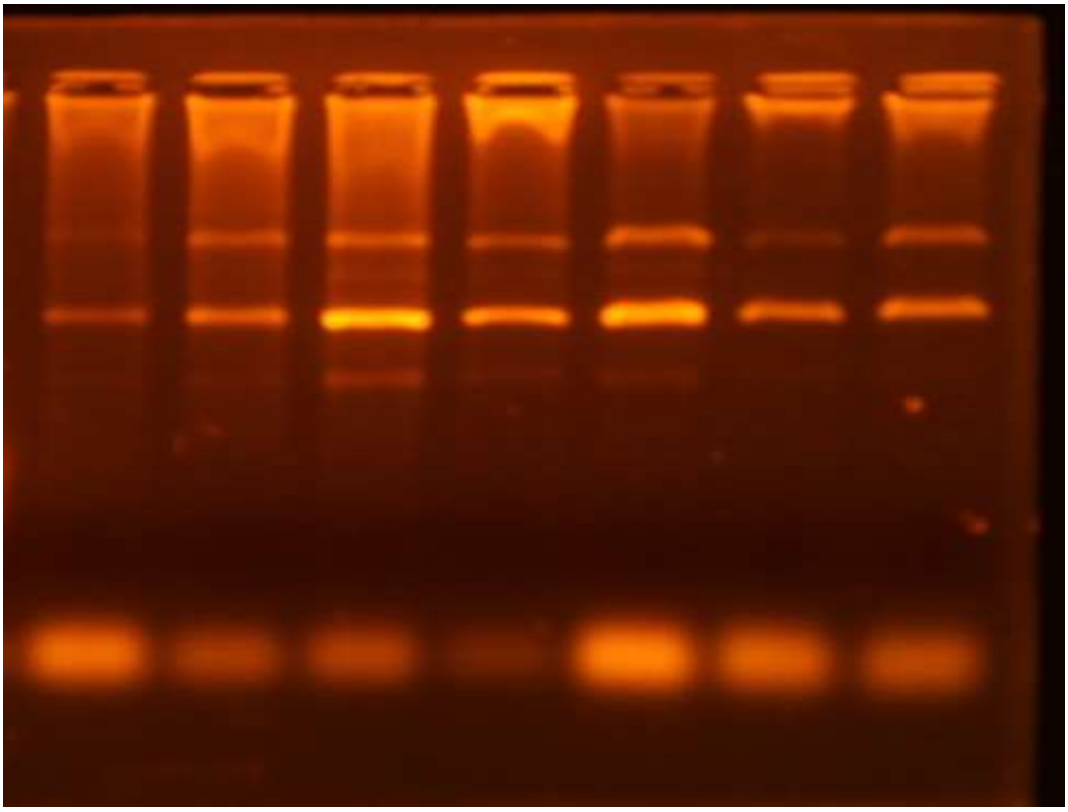

Figure 3.6.1. Gel electrophoretic: Plasmid DNA was extracted using plasmid isolation protocol.

CHAPTER FOUR

DISCUSSION, CONCLUSION, RECOMINDATIONS

4.1. Discussion

Grapevine is the second important fruit crop after olive in Palestine, covering nearly 11 thousand hectares. It is grown everywhere in Palestine due to the suitable climatic region. However, the majority of commercial vineyards are extensively spread in the southern portion of the West Bank (71).

Fanleaf degeneration is a major viticulture problem which caused by Grapevine fanleaf virus (GFLV) , and considered as one of the most severe virus diseases of grapevines worldwide. GFLV causes fundamental crop losses, lowering fruit quality and shortens the aging of grapevines in the vineyard. GFLV is transmitted particularly from grapevine to grapevine by mean of the ectoparasitic nematode Xiphinema index (15).

The incidence of Grapevine Fanleaf virus (a member of subgroup A nepoviruses) was analyzed in 80 grapevine leaf samples. Variety of virus related symptoms (Yellowing and deformation, leaflets are dwarfed, yellow spots , deformed canes, abnormal branching and different length or short internodes, Yellow mosaic symptoms and malformation) were observed in the surveyed vineyards (16,22). Symptoms inspection is not specific with the absence of a particular symptoms of the viral disease, beside that many factors may cause virus-like symptoms such as soil nutrients balance, weather conditions, nonviral pathogens infection, chemical pesticides and malformation caused by pests (nematodes, insects, mites), on the other hand this method is insufficient and not always reliable because the

development of symptoms commonly depends on many factors like growth stage, plant variety, time of infection, virus strain and different environmental factors (50). Furthermore some nepoviruses strains can cause asymptomatic infection in vines (11).

The selected detection technique is influenced by mean of expertise and facilities, information on virus expected to be present, host plant and the duration for completing the experiment. Generally, every detection method must be rapid and extremely specific for the target virus, and must detect virus present in little amounts in the plant tissue in addition to detection during an early stage of disease evolution (73).

Serological techniques have main disadvantage that they are based on the antigenic properties of the virus structural proteins. Consequently immunological approaches without any consideration of the rest of the viral genome. It is possible that viruses which are distantly related or not related, as detected by serological methods, may have highly conserved sequences in the genes other than the coat protein gene on the other hand serologically related viruses may have a few sequence homology. Furthermore, in 2008 ELISA failed to detect four nepoviruses, *Arabis mosaic virus Tobacco ringspot virus*, *peach rosette mosaic virus*, and *Tomato ringspot virus* (ToRSV) in addition to *Grapevine leafroll-associated virus 3* (74).

The majority of the samples (55 samples) were screened using RT-PCR. This technique successfully detected nepoviruses subgroup A in leaf samples even in the leaves that were asymptomatic. Specific Primers

designed for detecting amplified fragment of 255 bp of subgroup A species from samples infected by Arabis mosaic virus (ArMV), Grapevine fanleaf virus (GFLV), Grapevine deformation virus (GDefV) and Tobacco ringspot virus (TRSV), but not from samples infected by another nepovirus species (9, 11).

To investigate the infectivity of virus samples using mechanical inoculation of another 25 symptomatic samples of expected diseased vines was shown infected *Nicotiana benthamiana* with stunting plant growth (Figure 3.5.1.). This could refer in principle to nepoviruses infection specially the symptoms of subgroup A nepoviruses (as Tobacco ringspot virus) (22).

The results of PCR showed successful recovery of nepoviruses subgroup A, and unsuccessful detection of GFLV. Although 12 infected sample were detected, nepovirus-induced diseases specially fanleaf are very little occured in Palestinian vineyards this was observed also in field survey. The scarce findings of GFLV-infected vines in the fields may show that the nematode vector species of Xiphinema index may be evenly rare in Palestine (76).

Viruses and viroids are common pathogens of many important economical plants such as grapevine. These viruses may cause severe losses of yields both in quality and quantity; Grapevine decline, stunted growth are the major symptoms of this disease. There is no recovery for plants already be infected, thus for many farmers, the viral infections are simply tolerated or ignored unless clear symptoms appear, then the affected plants are

discarded. However, these asymptomatic combinations of host and pathogen may not be taken in account, the problem appears when the virus is transmitted to another more sensitive host where acute symptoms or even death may happen. As a result the asymptomatic hosts act as pathogen reservoirs (77).

4.2. Conclusion and Recommendations

Viticulture is rank the second cultivar after olives in Palestine. In addition, vineyards are a relevant feature in southern districts, principally in the Hebron and Bethlehem hills, while in the north districts concentrated in Qabatia town in Jenin.

The incidence of Nepovirus subgroup A infecting grapevine was analyzed in 80 grapevine leaves samples, 55 samples at early spring of 2015 with prevalence of more than 21% of tested samples, while 25 of symptomatic samples were collected at late summer of 2016 and tested by mechanical transmission. Variety of virus related symptoms were observed (mild flecking, dwarfed shoot, yellow spots, deformed canes, abnormal branching and short internodes and Yellow mosaic symptoms and malformation), the majority of symptoms were seen in 2015 season**.** However, in the late summer, the virus become dormant and there is no disease symptoms developed, so it is important for growers to test grape virus diseases in the field before propagation (73).

Although this assessment was limited to 80 trees, the results of biological assay and molecular assay obtained clearly indicator how the sanitary

status of Grapevine in the West-Bank of Palestine. Relatively negative grapevine fanleaf virus result detect a good sanitary status for viticulture, as it was considered the most severe degenerative virus of grapevine worldwide.

Since 1998 Grapevine Fanleaf virus, Palestine in compared with Mediterranean countries had possessed a low infectious level about 1.2% (76). Furthermore, this is the first study of symptomology, molecular analysis of nepoviruses that infect Grapevine in Palestine. Finally, extensive testing must be done throughout all vineyards to ensure suitable detection of infections that may be existed. Additionally, with the use of vegetatively propagated plant materials, it is necessary to start with virus free plant to improve viticulture sanitary status and to avoid the raise of infectious level.

In fact many factors play a crucial role in the sequencing negative results, the most important that always there is a chance for genetic variation of the virus, specially with high replication errors, for example, *Grapevine Fanleaf virus* exists as a quasispecies (82). Even though this study is highly recommended to do more researches on covering the variations among Nepovirus strains that possibly exist on Palestinian soil.

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جامعة النجاح الوطنية

كلية الدراسات العليا

الكشف الجزيئي وتحديد وجود النيبو فيروس (Nepoviruses) في الضفة الغربية– فلسطين

إعداد

ميس مفيد فتحي ساره

إشراف

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

2016

الكشف الجزيئي وتحديد وجود النيبو فيروس (Nepoviruses) في الضفة الغربية–فلسطين

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الملخص

كرمة العنب *(Vitis vinefira)* تنتمي الى عائلة *Vitaceae*، تعد عالميا واحدة من أقدم محاصيل الفاكهة، في حين تحتل في فلسطين المرتبة الثانية بين محاصيل الفاكهة المزروعة هناك بعد الزيتون. وبسبب الاستخدام المتواصل لطريقة التكاثر الخضري، تعد مزارع العنب هدفاً متواصلاً للعديد من الفيروسات، واحدة منهم هي من اكثر الامراض فتكاً بشجرة العنب وتسبب اضرارا كبيرة، مؤدية لمرض الاوراق المروحية (Fanleaf) التي تنتمى لمجموعة من الفيروسات تسمى النيبو فيروس (Nepovirus)، وتنتقل الى الكرمة بشكل رئيسي عن طريق الديدان الخيطية. الهدف من هذه الدراسة هو تقييم الوضع الصحي لشجرة العنب في فلسطين نظرا لعدد وجود بيانات حالية معتمدة لمدى انتشاره.

أجريت عملية مسح ميدانية لكروم العنب الرئيسية في الضفة الغربية– فلسطين، وتم جمع نحو 80 عينة: الخليل (34)، بيت لحم (24)، جنين (11)، نابلس (7) وأريحا (3)، لمراقبة الأعراض خلال في الموسمين 2015 و2016 للكشف عن GFLV، وذلك باستخدام تقنيات حيوية وجزيئية في جامعة النجاح الوطنية – مختبرات التقنيات الحيوية. لمراقبة الأعراض وتحديد الاشجار المصابة. عملية المسح كشفت وجود اعراض مرتبطة بوجود الفيروسات ومن هذه الأعراض (الاصفرار، التشوه، المسافات بين العقد غير متساوية وتفرعات غير طبيعية، التبقيع، الاوراق المتقزمة).

تم اختبار 25 عينة باستخدام التلقيح الميكانيكي لنباتات عشبية كاشفة. كل من النباتين (*Necotiana benthemiana, Necotiana accidentalis*) فشل في عزل فيروس الورقة المروحية GFLV على الرغم من ظهور اعراض لنباتين متقزمين على *N. benthemiana* فقط.

ومع ذلك هي لا تدل على وجود هذا الفيروس. باستخدام تقنية الناسخ العكسي–تفاعل البلمرة المتسلسل RT–PCR ولا عينة من مجمل 55 عينة وجدت فيها أي اصابة بفيروس الورقة المروحية GFLV وذلك باستخدام بادئة (primers) مخصص لمضاعفة هذا الفيروس. بادئة اخرى استخدمت في الكشف عن المجموعة الفرعية A لمجموعة فيروسات النيبو فيروس (Nepovirus)، تم الكشف عن (12) عينة مصابة من مختلف المناطق: الخليل (5)، بيت لحم (3)، أريحا (2)، جنين (1)، بينما لم يتم اكتشاف أي إصابة في عينات نابلس.

تأكد هذه النتائج أنه تم العثور على Nepoviruses، في الكروم لدينا ولكن ليس بالنسبة لفيروس الورقة المروحية GFLV وهذا يؤكد ما تم الوصول اليه في محاولات اجريت في عقود سابقة تتوقع وجود GFLV في الكروم الفلسطينية. نتائج هذه الدراسة ممكن ان تكون بمثابة تقرير موثق للوضع الصحي للعنب بالنسبة لفيروس GFLV، على الرغم من وجود انواع اخرى من Nepoviruses التي تم الكشف عنها في العديد من الكروم من شأنه أن يفتح الباب لمزيد من الدراسات التي ستجري لتحديد هويتهم.

الكلمات الرئيسية: النيبو فيروس، النباتات الكاشفة، الناسخ العكسي_تفاعل البلمرة المتسلسل، كرمة العنب، فيروس الورقة المروحية.