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MOLECULAR DETECTION OF CITRUS VIROIDS IN WEST BANK

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Molecular detection of viroid in citrus of West Bank-Palestine

By

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Dedication

I am dedicating this thesis to beloved people who have meant and continue to mean so much to me. First and for most, to the greatest father in the world who taught me the value of hard work and his endless love, support and encouragement

To greatest woman in my life (my mother) who always believed on me

To my husband

To my lovely son Taleb

To my husband family

To my brothers and sisters who always stay by my side and encourage me to reach my goals.

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

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

MOLECULAR DETECTION OF CITRUS VIROIDS IN WEST BANK

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

Student's Name: _____

Signature: _____

Date: _____

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MOLECULAR DETECTION OF CITRUS VIROIDS IN WEST BANK

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Abstract

Palestine as many of developing countries; its economy depends on many factors including agriculture sector. The citrus cultivated areas for the year 2012/2013, in the West Bank reached approximately 9.831 hectares, which constituted to 9.2% of the total area of fruit trees with annual production of approximately 4% of total production. Citrus are natural hosts to several pathogens like: bacteria, fungi, viruses, and virus-like (viroid). Many of which have an economic ipmpact on the crop A minimum of five viroid species of Pospiviroidae family that are found in citrus trees. The purpose of this study was detection of five citrus viroids (CVdIII , CVdIV , CEVd, HSVd and CBLVd) in West bank by molecular means. Field surveys were conducted during august 2020; where a total of 42 samples were collected from citrus collection plot in NRAC –Jenin. Samples screening for presence of any viroids species were carried out by two-steps RT-PCR. Five viroid were detected by RT-PCR for the first time in West bank. The incidence of Citrus viroid III, Citrus exocortis viroid (CEVd), and Citrus viroid IV were found 35.7%, 23.8%, and 16.6% respectively. Meanwhile Hop stunt viroid and Citrus bent leaf viroid were found in 9.5% and 7%. Portion of CEVd, CVdIII and CVdIV were sequenced to be later deposited in gene bank as Palestinian isolate.

Keywords: Viroid; Citrus; Palestine

Chapter One
Introduction

Diseases caused by viruses and virus-like agents induce annually worldwide losses in fruit trees (Nemeth, 1986; Roistacher, 1992). More than 150 diseases caused by viruses, viroids and unknown graft-transmissible agents have been reported. Citrus, as one of the most important and worldwide distributed fruit trees will be focused in this study. Palestine, as one of the in Mediterranean region countries, constitutes a suitable environment for citrus cultivation.

Despite Palestine's small geographical area that covered, The topography of Palestine varies, and it is possible to split it into four separate sections. Moving eastward from the Mediterranean coast of Palestine, the topography begins with the coastal plain, which stretches from north to south along the coast to the south of Gaza, and the plain becomes more extensive the further south we go. This is followed to the east by the inland hills and mountains, which include the Galilee Mountains, the Nablus Mountains, the Jerusalem and Hebron Mountains, and the fertile Marj b Desert, which lies to the north of the NablusThe Dead Sea is a deep basin connected to a lake with extremely high salinity and no marine life, and to the south lies a desert depression known as Wadi Araba.

the coast plain

The mountains (the mountains of Galilee, Nablus, Jerusalem and Hebron).

Valley of Jordan

The Negev Desert

Heights it is distinguished by a wide difference of terrain and height, as seen in the West Bank, where height ranged from about 1020 meters over sea level in the highlands to 375 meters below sea level in the Jordan valley. Such a difference makes it possible to grow fruit crops (Djelouah et al.,2009).

Agriculture continues to be the most important and controlling sector of the Palestinian economy, accounting for a considerable portion of the GDP and employing a big portion

of the people. Furthermore, the agricultural sector is the primary source of foreign currency and provides for the bulk of the local population's fundamental necessities. During bad times, the agriculture sector has served as a buffer, absorbing significant numbers of jobless individuals who have lost work in Israel or other local industries (Butterfield *et al*., 2000).

Agricultural activity represents the strongest pillar that supplies the Palestinian economy with one of the most important sources of GDP. It contributes to covering part of the final food consumption; In addition to its contribution to the provision of commodities and raw materials that are used as inputs in many manufacturing industries; In addition to being an important component of Palestinian exports. Agricultural activity in Palestine is characterized by its dependence mainly on rain, and its limited dependence on modern agricultural methods. Its added value during the year 2020 decreased compared to the year 2019 by 9.2%; It also recorded a decrease of 8.3% in the West Bank, and a decrease of 11.1% in the Gaza Strip.

According to the Palestinian Central Bureau of Statistics' report "The Performance of the Palestinian Economy, 2020," published in May 2021, the number of agricultural workers in 2020 was around 53,400, with 40,600 from the West Bank and 12,800 from the Gaza Strip; while the number of agricultural workers in 2013 was around 82,700, with 59,900 from the West Bank and 22,800 from the Gaza Strip.

In 2020, the average actual daily pay in the agricultural industry was around 59.2 shekels, with 105 shekels for West Bank workers and 20.5 shekels for Gaza Strip workers.

According to the statistics of the Ministry of Agriculture regarding citrus cultivation for the agricultural year 2012/2013, the cultivated areas in citrus in the West Bank reached approximately 9831 dunums, which constitutes 9.2% of the total area of fruit trees and the annual production is approximately 19,430 tons, or 4% of the total production (ARIJ, 2015)

Tulkarm governorate has the highest citrus production in the West Bank with 49%, followed by Qalqilya governorate with 27% and Nablus with 17% (ARIJ, 2015)

Citrus is belonging to family Rutaceae and subfamily Aurantioideae in the genus of flowering trees and shrubs belongs, which includes significant crops like an orange or lemon-like fruit known as a hesperidium or berry with a unique structure. The luscious pulp of these fruits is made up of vesicles inside segments. In this subfamily, only three genera yield edible juice vesicles (Citrus, Fortunella, and Poncirus) (Baldwin, 1993). The Rutaceae family includes all citrus fruits. This family, which includes blooming plants with a strong aroma, is often known as the rue family. Oranges, grapefruits, limes, and lemons are all members of the Citrus genus. These fruits have been grown since the dawn of time. Although some study suggests they originated in Southeast Asia, they are most likely from Australia, New Caledonia, and New Guinea. Many of the species are hybrids, and it's possible that even true-breeding species in the wild were once hybrids.

Citrus fruits provide humans with a variety of visual and sensory sensations (the beauty of the blooms and the flavor of the fruits), as well as nutritious value. Citrus fruits are known for their fragrant rinds, which contain flavonoids (secondary metabolites) and limonoids (phytochemicals), and most are juice-rich. Citric acid is abundant in the juice, which gives it its characteristic sharp (tart) flavor. They are high in vitamin C, flavonoids, fiber, and folic acid, and hence give significant health advantages.

Citrus trees are known to be attacked by aphids, whiteflies, and scale insects (e.g. California red scale). Some of these ectoparasites act as carriers for viral infections, such as the aphid-transmitted Citrus tristeza virus, which can be fatal to citrine farms if not carefully handled.

Citrus are natural hosts of several pathogens causing number of diseases like: bacteria, fungi viruses, virus-like (viroid). Many of which have an economic impact on the crops.

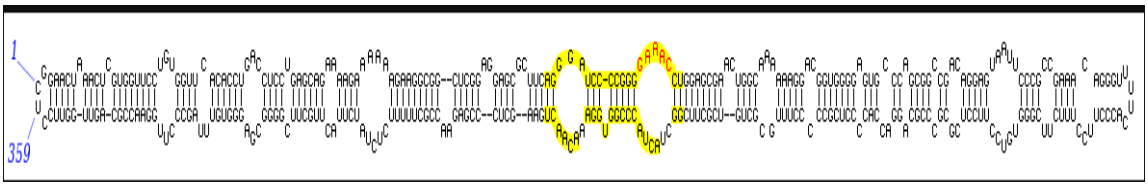
The precise mechanism through which viroids cause illness is uncertain. Because they are substrates for Dicer-like endoribonucleases, which have a high degree of internal base-pairing and stable secondary structures, viruses are both inducers and targets of RNA silencing mechanisms (7). Viroid-derived short RNAs (vd-sRNA) with lengths of 21 to 24 nucleotides (nt) accumulate in infected plants, and the involvement of several silencing components in viroid pathogenesis has been documented in different host–viroid combinations (8–20). Moreover, RNAi-mediated down-regulation of the host's mRNAs by direct interaction with such vd-sRNAs has been explored by several groups.

Stunting, epinasty, staining of vein and leaf clearing , mottling and distortion, chlorotic and necrotic patches, cankers, bark to be scaling or cracking of,also may cause a deformity in tubers also fruits and flowers, and and, in rare cases, plant death are all symptoms of viroids infection. Viroids have structural, functional, and evolutionary properties that are distinct from viral infections in plants (Flores *et al.*, 2005)

This small plant pathogen has a unique structural consistence of single-stranded, circular RNAs, (Fig 1) consisting of a few hundred nucleotides not coded for proteins, classified into two families, Pospiviroidae and Avsunviroidae, that replicate (and accumulate) in the nucleus and chloroplast, respectively, and assimilate the lowest level of complexity for an infectious agent, even less than the smallest known viruses. (Murcia *et al.*, 2015).

Figure 1

PSTVd viroid's putative secondary structure. The highlighted nucleotides are found in the majority of viroids.



Viroids differ from viruses in terms of structure and morphology. They're built up entirely of small circular strands of single-stranded RNA, with no protein coatings.

Crop failures are caused by viroids-infected plants, which cost the agricultural industry millions of dollars each year. These diseases harm potatoes, tomatoes, cucumbers, chrysanthemums, coconut palms, avocados, and other plants.

T.O. Diener was the first to discover viroids in 1971. It was initially discovered in the potato spindle tuber virus, which devastated the potato crop.

Since they are known to be plant parasites, viroids are transcriptional machinery of cell organelles such as the nucleus or chloroplast.

The noncoding nature indicates that viroids must employ their RNA genomes to redirect host machinery for infection. Citrus are natural host of five viroid species from the the Pospiviroidae family Cachexia and exocortis Citrus viroid species are two of the most well-known illnesses in citrus. The causative agents are Citrus exocortis viroid (CEVd)

and Hop stunt viroid (HSVd). Other viroids may have caused particular symptoms ranging from mild to severe stunting (Murica et al., 2015).

Dwarfing may be caused by severe CVd-III, CBLVd, and HSVd variations (Wang et al., 2008) CVd-IV, which is less common than other viroids, can cause severe bark cracking in some species (Wang et al., 2008). The viroid families and species are shown in the table below.

Table 1

List of known Viroids (Di Serio et al., 2017)

Family	Genus	Species
Pospiviroidae	Pospiviroid	*Potato spindle tuber viroid* *Tomato chlorotic dwarf viroid*; *Mexican papita viroid*; (MPVd); *Tomato planta macho viroid*; *Citrus exocortis viroid*; *Chrysanthemum stunt viroid*; *Tomato apical stunt viroid*; *Iresine 1 viroid*; ;*Columnea latent viroid*
	Hostuviroid	Hop stunt viroid
	Apscaviroid	*Apple scar skin viroid*; *Apple dimple fruit viroid*; *Grapevine yellow speckle 1 viroid*; *Grapevine yellow speckle 2 viroid*; *Citrus bent leaf viroid*; *Pear blister canker viroid*; *Australian grapevine viroid*;
	Cocadviroid	*Coconut cadang-cadang viroid*; *Coconut tinangaja viroid*; *Hop latent viroid*; (HLVd); *Citrus IV viroid*; (CVd-IV)
	Coleviroid	*Coleus blumei viroid 1* *Coleus blumei 2 viroid* *Coleus blumei 3 viroid*;
Avsunviroidae	*Avsunviroid*	Avocado sunblotch viroid
	Pelamoviroid	Peach latent mosaic viroid
	Elaviroid;	Eggplant latent viroid

Citrus trees in both commercial and residential plantings can display a variety of symptoms indicating a variety of illnesses that can have variable degrees of influence on their health, vigor, and output. Correctly identifying symptoms is critical in management, since improper therapeutic applications or activities can be expensive and even harmful (Futch, 2021). Identifying symptoms correctly is an important point of management, as awkward remedial applications or actions can be costly and sometimes injurious, so symptoms identification can help prevent viroid spreading through other trees.

The Viroid can spread by Agricultural operations mostly through mechanical contact with farm implements and pruning tools that infected with viroid . Some viroids can be transferred from plant to plant by human hands or foliar contact. Infective vegetative growth, pollen, seed and insects are all ways for viruses to propagate (Gucek *et al*., 2016).

According to citrus orchards, infected budwood propagation and mechanical inoculations with potentially contaminated equipment were described as primary factors for the omnipresence of numerous viroid species, including HSVd. HSVd transmission has been reported mechanically. A transmission study conducted under greenhouse circumstances indicated that all HSVd strains are mechanically transferred from infected to healthy cells by a single knife blade cut. also In the case of CEVd, the probable involvement of gots in HSVd dissemination has been demonstrated under controlled settings. Top working, which is frequent in Mediterranean nations, appears to have aided in the development of HSVd in Mediterranean citrus trees. HSVd does not appear to be seed-borne in citrus and does not have a natural vector.

Vegetative Transmission

Viroids are spread by vegetative mechanisms of replication since they are systemic diseases. All other hosts subject to grafting, budding, cuttings, bulbs, tubers, and other methods of propagation will convey viroid infection to the next planting, with the exception of monocotyledonous palms that host CCCVd and CTiVd and do not have vegetative propagules. CCCVd is planned to be spread by vegetatively propagated ramets using somaclonal propagation, which was developed for oil palm farms.

Viroid Transmission via Mechanical Means

Viroid transmission can occur mechanically through viroid-infected sap or nucleic acids, as well as via viroid-contaminated farming implements and agricultural and horticultural activities that involve viroid-contaminated instruments.-

Seed and Pollen Transmission

A number of viroids have been identified as being spread by seeds and/or pollen. Because epidemic breakouts are dependent on the initial viroid inoculum brought in by viroid-infected seed at the start of the growing season and/or pollen at a later period, these mechanisms of transmission may play a crucial role in the epidemiology of viroid illnesses. The introduction of viroids to new locations, in combination with secondary viroid dissemination by mechanical and/or vector transmission, may result in the emergence of viroid-disease epidemics. Furthermore, international seed and pollen commerce and interchange are thought to be a major contributor to the establishment of viroids and associated illnesses.

The rate of seed and/or pollen transfer is influenced by a number of variables. Plant species and cultivars, viroid variants, ambient circumstances, and infection time are examples of these factors. These viroids require effective phytosanitary treatments to limit pollen and seed mobility.

The difficulty of diagnosing viroid-infected citrus trees during field surveys is due to the lack of disease symptoms, symptomless infected trees and symptoms in viroid-infected plants similar to those in some virus.

In addition, because viroid RNA is non-coding, standard serology-based viral diagnostic techniques are not possible. Therefore, traditional detection of viroids has been achieved by a combination of biological indicators (i.e., bioassays) and molecular biological techniques, as well as plant certification and quarantine programs (Adkar-Purushothama and Perreault, 2020)

Until the physical/chemical nature of viroids was established, the first discovered approach for detecting viroid infections was biological indexing, or bioassay, but it is still a key stage in the detection and identification of viroid infections. However, the number of hosts and host plants necessary for the test, the time it takes to complete

the assay, and the probable lack of symptoms in host plants all pose challenges to using biological indexing as a detection tool.

In viroid research, polyacrylamide gel electrophoresis (PAGE) was used to detect viroids. Because short circular RNAs travel more slowly than linear RNAs in the denaturing phase of electrophoresis, the invention of a simpler purification procedure for low-molecular-weight nucleic acids and two-dimensional, non-denaturing/denaturing PAGE provides a potent tool for viroid identification. After that, the circular RNAs may be seen and collected from the gels for cDNA cloning and characterisation.

The most accurate and valid approach for viroid detection is RT-PCR. Quantitative reverse transcription PCR (RT-qPCR) has revolutionized viroids detection with the development of thermal cyclers with fluorescence detection.With the introduction of thermal cyclers with fluorescence detection, quantitative reverse transcription PCR (RT-qPCR) has revolutionized viroids detection.

The method involves using sequence-specific primers that anneal to the appropriate viroid RNA. Using viroid RNA as a template, the reverse transcriptase enzyme creates a cDNA copy of a portion of the target RNA molecule during reverse transcription. After first-strand cDNA synthesis, the RNA template from the cDNA:RNA hybrid molecule is digested with RNase H to improve the PCR process' sensitivity. Because this cDNA will be used as a template, RT-PCR is a reliable and valid approach for viroid detection.

The most effective methods of controlling viroid illness include preventing the entrance of contaminated plant material into the field or greenhouse, following stringent hygiene measures, and monitoring odd signs on crops. This includes using virus-free seed and germplasm, as well as maintaining hygienic growth conditions (disinfection and cultural controls). Seed certification programs and quarantine enforcement for viroids of quarantine and certification importance by the European and Mediterranean Plant Protection Organization (EPPO) and the North American Plant Protection Organization (NAPPO) have resulted in effective control of several diseases caused by viroids, which rely on sensitive diagnostic and detection methods.

The main goals of this study were to molecularly detect citrus viroid diseases [Citrus exocortis viroid (CEVd); Citrus bent leaf viroid (CBLVd); Hop stunt viroid (HSVd), and

Citrus viroid-III (CVd-III) and Citrus viroid-IV (CVd-IV)] in Palestinian citrus plants for the first time in the West Bank, and to evaluate their incidences.

Chapter Two
Literature Review

2.1 Viroid pathogenicity

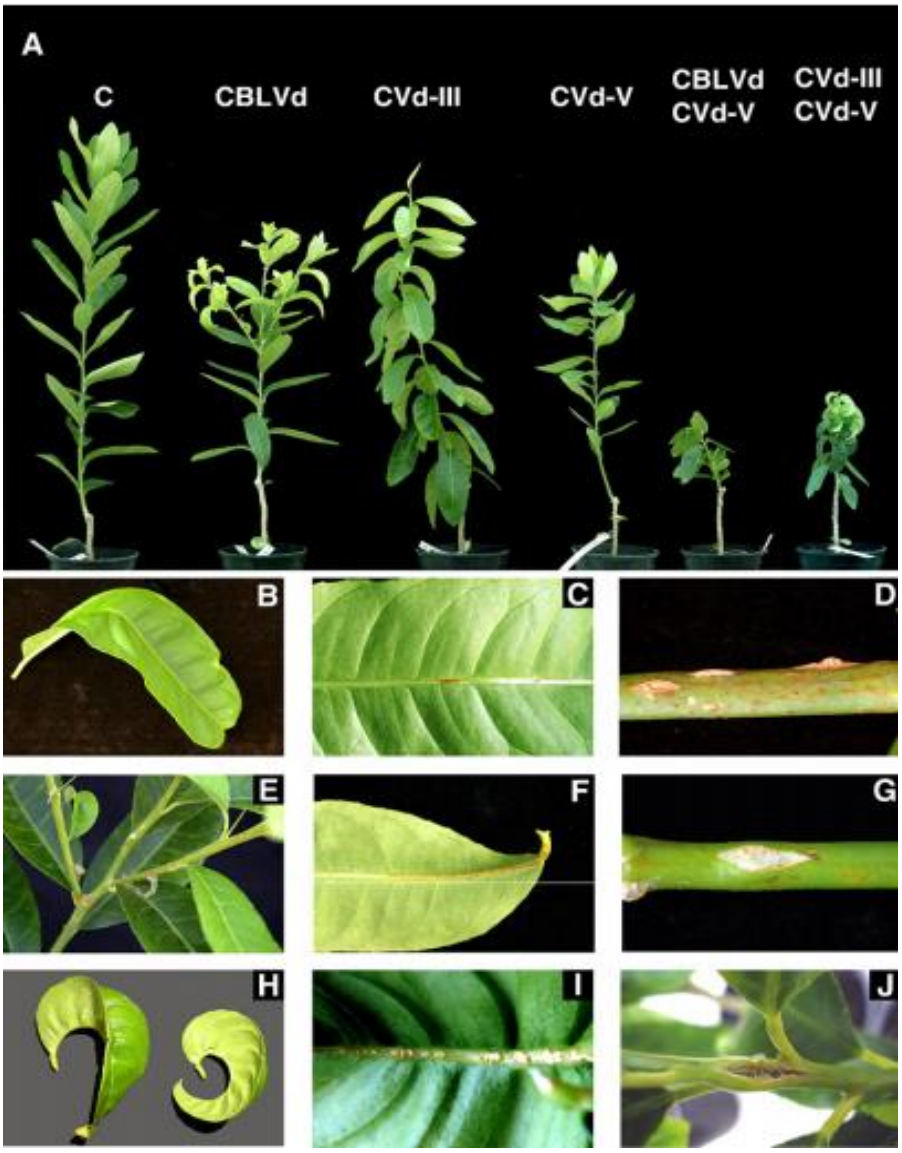
Viroids rely heavily on host factors for mobility and reproduction because to the great simplicity of their circular RNA genome . Furthermore, they can cause widespread transcriptional alterations in host plants by interfering with the cell's silencing machinery [9,10], causing epigenetic modifications , alternative splicing , or, as recently discovered, ribosomal stress . This latter phenomena pertains to the viroid-induced defective processing of the 18S rRNA, which results in ribosome biogenesis abnormalities and significant changes in the host translation machinery [. Furthermore, viroids can activate the plant's defense system by triggering the production of pathogenesis-related (PR) proteins including PR1 , as well as the accumulation of defensive signal molecules like SA and GA .This defensive response activation, however, is ineffective in combating viroid infection, with PR1 induction and SA and GA buildup being linked to viroid symptomatology.

Viroid pathogenicity is a phenomena caused by both the viroid and its host genomes. Stunting, epinasty, vein discoloration, clearing of leaf distortion and mottling, chlorotic or necrotic patches, cankers, bark scaling and bark cracking, tubers, flowers and fruits deformity, and occasionally plant mortality are all macroscopic indications of viroid infection (Fig. 2).

In Gynura aurnatiaca CEVd-infected cells, one of the viroid infections markers (Cytopathic effects) was first observed. There was also a deformation and uneven thickness in cell walls. Both pospi- and avsunviroids have shown abnormal chloroplast formation, however it is unclear if these changes are unique to viral infection or a general response to any biotic stress (Hammann, & Steger, 2012).

Figure 2

Pictures that illustrated viroid infection symptoms. (A) normal plants with no infection (c), infected with one viroid (CBLVd, CVd-III, or CVd-V), or mixed infected with two viroids (CBLVd and CVd-V; CVd-III and CVd-V). (B) leaves Bending. (C) Local necrosis in midvein. (D) bark Cracking and releasing gum. (E) Branching pattern and exudates of gum. (F) necrosis in Petiole and midvein (G) stem Cracking. (H) curling of Leaf (I) midvein Lesions (J) Severe cracks in bark and devoid of gum,



2.2 Viroid replication

Viroids proliferate and spread in their plant hosts in the absence of any other infectious agent. Bioassays, which are also necessary to evaluate if a viroid fits Koch's postulates and is the cause of a plant disease, can be used to test this characteristic experimentally. Despite their time and expense limits, bioassays are essential to identify viroids from other viroid-like RNAs present in plants with a variety of biological properties. Retroviroid-like elements, which are not infectious but have a DNA equivalent, and viroid-like satellite RNAs, which are functionally dependent on a coinfecting helper virus, are examples of this.

RNA-catalyzed The replication of RNA is thought to be a crucial step in the formation of life's first genetic system. The exceptional durability of duplex RNA products, which must be split before the next replication cycle can start, might block RNA replication. In this article, we looked into rolling circle synthesis (RCS) as a viable solution to the strand separation problem.

We detect persistent RCS using a triplet polymerase ribozyme with strand displacement, which produces concatemeric RNA products, in addition to full-length circular synthesis. We also demonstrate that RCS exists in a circular Hammerhead ribozyme capable of self-cleavage and re-circularization. As a result, all phases of a viroid-like RNA replication process can be catalyzed by RNA alone. Finally, we analyze possible RCS mechanisms using molecular dynamics simulations, which demonstrate a slow build-up of conformational alterations.

Viroids multiply in their hosts through two different processes (see Figure 3). The Potato spindle tuber viroid (PSTVd) is a member of the Pospiviroidae family that replicates through an asymmetric rolling circle mechanism that relies on the host DNA-dependent RNA-Polymerase II to identify the nucleus (Jiang et al., 2018). The nuclear-encoded polymerase present in chloroplasts is used to replicate the Avsunviroidae members in a symmetric rolling-circle method (Kovalskayam & Hammond., 2014).

There are two major Viroid families: Avsunviroidae (four species) and Pospiviroidae (27 species). There are significant variations between the two families in terms of structure, replication techniques, and subcellular localization.

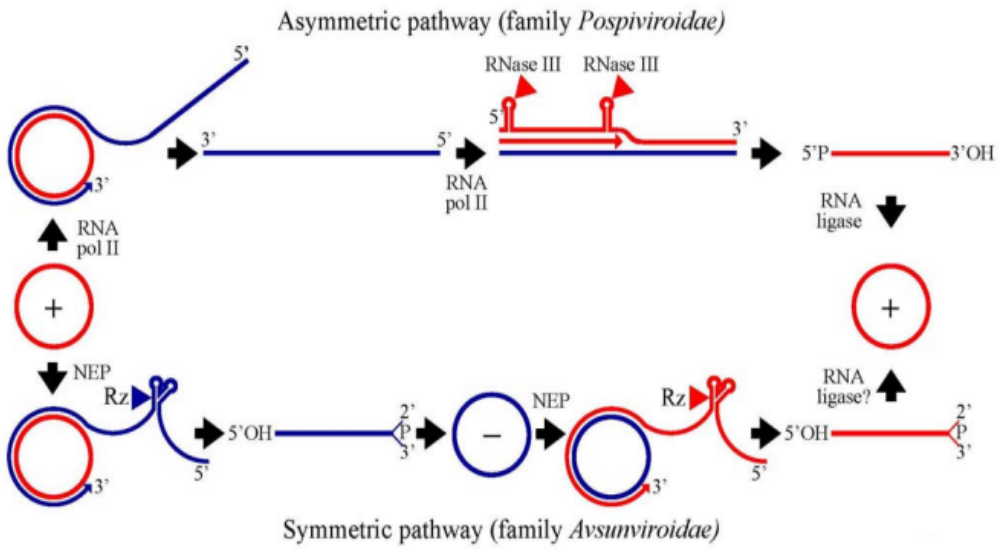
The Pospiviroidae family is named after the potato spindle tuber viroid (PSTVd), but it also contains significant members such as Citrus exocortis viroid (CEVd), Coconut cadang-cadang viroid (CCCVd), and Hop stunt viroid (HSVd).

Pospiviroidae have a rod-like secondary structure and are detected in the nucleolus and maybe in the nucleoplasm of infected plants. The host DNA-dependent RNA-polymerase II, which is responsible for synthesis of a (-) oligomeric linear strand, also acts as a template for the following step of synthesis of (+) oligomeric linear RNA molecules, by employing the template of (+) circular RNA. These (+) linear stranded replication intermediates are subsequently processed and transformed to monomeric circular structure, resulting in asymmetric rolling-circle replication in the Pospiviroidae family.

In contrast to the Pospiviroidae, the Avsunviroidae family is found in the chloroplasts. Avocado sun blotch viroid (ASBVd) was discovered in this viroid family, while Peach latent mosaic viroid is another species in this family (PLMVd). Avsunviroidae is more structurally versatile, with linear structures like (ASBVd) and branching structures like (PLMVd) (Hammann & Steger, 2012)

Figure 3

Symmetric and asymmetric replication of viroid (Flores et al.,2009)



2.3 A Novel citrus viroid found in Australia

In Lisbon lemons, a novel asymptomatic citrus virus has been found. Tree in Victoria, New South Wales.

Biological index, molecular characterisation and detection, tandem sequencing, tandem sequencing combined with in silica-analysis technique for detecting viroid RNA lineament qualities of transmission and replication with specified sequence and structural features

This viroid was termed "citrus viroid VII" (CVd-VII) after one of the recently discovered species in the Apscaviroid genus, family Pospiviroidae (Chambers et al., 2018).

2.4 First Report of Citrus viroid V Naturally that Infecting Calamondin and Grapefruit Trees in California

Citrus samples were obtained from the California Department of Food and Agriculture (CDFA) for the purpose of virus and viroid detection on Citrus Clonal Protection Program–National Clean Plant Network, University of California, Riverside (UCR) citrus. Two samples of red blush grapefruit (RG) and four samples of variegated calamondin were obtained from asymptomatic bud wood sources located in a nursery in Tulare County, CA. For citrus viroids universal detection, TRIzol-extracted RNA was examined with a SYBR green I and quantitative reverse transcription polymerase chain reaction (RT-qPCR).

The CDFA's Plant Pest Diagnostic Center verified the CVd-V identification results, and subsequently the viroid's major source trees were removed (Dang et al., 2018)

2.5 Virus and virus like diseases

A virus is an infectious, non-cellular creature made up of genetic material and protein. It has the ability to infiltrate and proliferate within living microorganisms, plants, and mammals. Because viruses lack the necessary cellular machinery, they cannot proliferate outside of the host cell. As a result, it enters and adheres to a specific host cell before injecting its genetic material. Furthermore, the virus replicates by using the genetic material of the host and finally rips open, releasing new viruses.

It may also crystallize, something no other living cell is capable of. Viruses are small, about 30 to 50 nanometers in length. They don't have a cell wall and are instead wrapped in a protective protein shell called a capsid. It is notable for the concurrent emergence of the virus and the host. It may be viewed as a genetic element.

Viroids are pathogenic viruses that only harm plants. Plant pathogens are another name for them. These critters make new clones of themselves using the biological machinery of plant cells. It affects all higher plant kinds in general.

Viroids differ from viruses in terms of structure and morphology. There are no protein coatings on these small circular and single-stranded RNA strands. Plants infected with viroids are responsible for crop failures and the annual loss of billions of dollars in agricultural revenues. These viruses affect potatoes, tomatoes, cucumbers, floral plants, and coconut palms, among other plants.

Viruses and viroids are infectious particles that can only multiply within a single host cell. Viruses and viroids differ primarily in that viruses are little infectious organisms that can only reproduce within living cells. Viroids, on the other hand, are the smallest infectious agents that assault plants. Viroids are RNA particles, whereas viruses are nucleoprotein particles having either DNA or RNA nucleic acid. Furthermore, viruses have a capsid protein covering, whereas viroids do not.

Due to a shortage of laboratories with the requisite technology, viruses, viroids, and virus-like infections affecting many citrus species could not be correctly diagnosed. Citrus graft-transmissible pathogens (CGTPS) are the unintended agents. Graft-transmissible diseases are another name for these infections (GTDs). This is a brand-new threat to the citrus industry. Some of the most common viruses and virus-like pathogens are citrus tristeza virus (CTV), citrus yellow vein clearing virus (CYVCV), citrus variegation virus (CVV), concave gum, psorosis, cristacortis, ringspot, exocortis, Cachexia-xyloprosis, Candidatus liberibacter asiaticus, and Spiroplasma citri.

Viruses, viroids, and virus-like illnesses that infect numerous citrus species, on the other hand, have received little attention because to a lack of labs equipped to identify them. 'Citrus graft-transmissible pathogens' are the unintentional agents (CGTPS). Graft-

transmissible illnesses are another name for these infections (GTDs). This is a new danger to the citrus sector (Iftikhar et al., 2021).

Table 2

Major citrus virus and virus-like pathogens

Name	Abb.	Symptoms	Reference
Citrus tristeza virus	(CTV)	leaf chlorosis,seedling yellows and Stem-pitting	Bar-Josephand Dawson, 2008
Citrus variegation virus	CCV	chlorotic blotches, curling, and twisting of the leaves.	Grant, and Cobett , 1961
Concave gum	CCGaV	Scaling of the bark trunks lesions and limbs of sweet orange, mandarin and grapefruit, and occasionally ringspot symptoms may appeared on leaves and fruit. Staining of Wood often accompanies bark scaling in infected branches and trunks. Sour orange, lemon, pummelo and rough lemon	Figueroa J, 2010
Exocortis	CEV	stunting and epinasty	Cottili et al., 2019

2.5.1 Citrus tristeza virus (CTV)

Virus of citrus tristeza (CTV), It's a virus that affects plants. It is a member of the Closterovirus genus of the beet yellow virus family. Citrus trees succumb to the virus, which causes fast deterioration and, in some cases, tree mortality.

It is thought to be the most deadly citrus virus disease, causing damage in many citrus-growing countries across the world. Tristeza, which means "sadness or melancholy" in Spanish, refers to a variety of sickness symptoms, many of which are widespread in Florida. Each form of tristeza has its unique set of symptoms.

CTV is the most commercially significant and devastating citrus virus. It may swiftly spread and harm plants not just by destroying sour orange rootstock trees, but also by pitting the stems of regular citrus trees. Since 1910, it has killed about 80 million trees in South Africa, 10 million in Argentina and 6 million in Brazil since 1970, and 3 million in the United States since 1950. The severity and effect of T. citricida have escalated considerably in Central America and the United States as a result of its dissemination. The output of nearly 40 million sweet orange and mandarin trees in Spain has gradually decreased.

CTV infection symptoms vary greatly and are dependent on a variety of factors, including the host, the virus strain's severity, and the environment. The three most common symptom categories are quick and progressive degradation, stem-pitting, and seedling yellows.

When mandarin, sweet orange, or grapefruit trees are grafted onto sick sour orange rootstock, they frequently succumb. The diseased tree's chlorotic leaves and extensive dieback are indicators of this degradation. After the initial indications and symptoms occur, the deterioration may be slow, lasting months to years. In this circumstance, a hump will emerge above the union of buds and honey collection on the inner face of the original sour orange root bark.

It's also feasible that the collapse will be swift, with the host dying within days (Moreno et al., 2008)

CTV is traditionally detected by grafting ill plant tissue onto a Mexican lime (Citrus aurantifolia). The Mexican lime's effects will be quite predictable. On the leaves, the symptoms start with clear veins that become corky, then chlorosis and leaf cupping. More extreme stressors may cause stunting and stem-pitting as adverse consequences. The presence of aggregates of cross-banded inclusion bodies in the phloem of a sick plant can also be used to detect CTV. Other diagnostic procedures include electron microscopy, double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), tissue-print ELISA, and PCR-based diagnostics. These methods check for signs of the virus, such as its microscopic structure (electron microscopy), antigens in its proteins (ELISA), and its RNA (RNA sequencing).

2.5.2 Citrus yellow vein clearing virus (CYVCV)

Citrus yellow vein clearing disease caused by Citrus yellow vein clearing virus. This disease was firstly reported in Pakistan in 1988s in lemons and lime. It was reported in Turkey and India after that. It causes leaves yields shoots on spring and autumn to show veins clear yellow, crinkling, warping, and water-soaked appearances on the underside of them. It decreases tree vitality, causing significant decrease in (Chen et al., 2016)

CYVC is a new disease that is causing severe economic losses in a variety of citrus speci es and types, particularly lemons.With diseased budwood, mechanical inoculation into ot her plant species, andfeeding insect vectors, the disease looks to be spreading quickly ac ross Asia.It can have a significant impact on tree development and fruit supply.Grapevin es are also susceptible to harm.In California, three of the recognized vectors (spirea aphi d, cowpea aphid, and citrus whitefly) are already common.Most sweet orange, pummelo, tangerine, and mandarin types are asymptomatic, and propagation of asymptomatic but contaminated plant material can aggravate the spread of CYVCV.

2.5.3 Psorosis

Citrus psorosis virus was first reported in 1896 in Florida and graft-transmissible disease confirmed in 1934. Psorosis affects trees worldwide, causing yield, growth, and longevity reductions.

2.5.4. concave gum

Fawcett was the first to recognize concave gum as a condition (1936). The illness is found all over the world, but is most prevalent in the Mediterranean region. It is also seen less often in most other citrus-growing regions.

Concave gum disease has been recorded in Japan (Ieki and Ito, 1996).

Blind pocket disease and concave gum disease are very certainly linked, with the blind pocket having sharper and deeper concavities. Concave gum can be recognized from psorosis-A by trunk and leaf symptoms on field trees, leaf symptoms on inoculation indicator seedlings, internal wood darkening or gumming in branches, and cross protection. Furthermore, antiserum will not work on tissue from damaged trees with concave gum.

Citrus psorosis could affect trunk, leaves, branches, and fruits, and causing different types of symptomes as: decreasing in plant growth, thin foliage appearance, low its bearing of fruit-, and tree death.

Symptoms include the production of elongated, white to yellow-green specks, spots, rings, or huge transparent patches in some immature leaves. As the leaves grow, certain symptoms diminish. On the fruit, rings with depressed grooves may develop. The outer bark of trees aged 6 to 12 years or more usually becomes scaly in isolated spots, or tiny irregular pustules and gumlike deposits form, staining the wood beneath. The massive limbs and trunk may develop chambers of various sizes or narrow grooves.

Remove severely damaged trees, plant psorosis-free stock, and use only scions and buds from virus-free trees to manage the disease. Many citrus-growing countries have quarantine and certification processes in place to eliminate tainted stock, while many Citrus species may harbor the viruses asymptomatically for more than ten years.

The disease is found all around the world, but is more frequent in the countries of the Concave gum. Sweet oranges, mandarins, tangors, and tangelos show symptoms, although they can retain the pathogen and remain symptomless hosts.

Although the pathogen has not been identified, it is very definitely a virus, as evidenced by weak strain cross protection . Grafting, top working, and perhaps root grafting can all spread the disease to other trees.

2.5.5 *Ilarvirus*

a genus of positive-strand RNA viruses in the family Bromoviridae.[2] Plants serve as natural hosts. There are 22 species in this genus.

it causes slight yellowing of the leaves or is asymptomatic. PYV has also been demonstrated to be transferred by pollen and botanical seed, unlike AMV. As a result, PYV and AMV were classified as two separate viruses.

For routine diagnosis of particular Ilarvirus species, specific reverse transcription-polymerase chain reaction (RT-PCR) assays have been frequently utilized. The infection of some plant hosts by many Ilarvirus species, on the other hand, makes species identification difficult and needs the employment of several separate RT-PCR techniques for detection. Furthermore, among Ilarvirus species, sequence variety occurs , making the construction of particular primers challenging and affecting their identification by RT-PCR assays.

2.6 A new citrus virus has been discovered in Australia.

In New South Wales, Australia, a new citrus viroid was found in a nonsymptomatic Lisbon lemon tree.

Bioindexing, molecular detection, and characterization of the viroid-RNA hallmark properties of transmissibility and autonomous replication, as well as specific sequence and structural motifs, suggest that this viroid is a member of a new species in the genus Apscaviroid, family Pospiviroidae, which we have tentatively named "citrus viroid VII" (CVd-VII). Chambers and colleagues (2017)

Chapter Three
Materials and Methods

3.1 Field Survey and Collection of Plant Materials.

Surveys for investigating the general pathogenicity status of citrus trees, and symptoms examination, were carried out during summer time (August 2020) in a collection plot at National Agriculture Research Center (NARC), Jenin-Palestine. Forty-two citrus samples (sample represents a set of young leaves were collected from different branches) were collected for testing in laboratory. Samples were labeled and stored in wet condition in plastic bags at 4°C until use for the laboratory assays.

A detailed map of the field was drawn and information about any abnormal symptoms was registered.

3.2 Molecular detection.

3.2.1. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Two-steps RT-PCR protocol was used to detect the five most common citrus viroid belonging to *Pospiviroidae* family: Citrus exocortis viroid(CEV), Citrus viroid IV (CVd IV), Hop stunt viroid(HSVd), Citrus viroid III (CVdIII) and Citrus bent leaf viroid(CBLVd).

Specific sets of primers for each viroid were designed according to the available nucleotide sequences in Genbank (Table 3).

RT-PCR consists in two main steps: Reverse Transcription (RT) and Polymerase Chain Reaction (PCR). In the first step viral RNA is used by reverse transcriptase DNA polymerase (RNA dependent DNA polymerase) as a template for the complementary DNA (cDNA). The second step was performed by employing thermo-resistant DNA polymerase, in presence of specific primers (sense and anti-sense primers) localized at the end of the fragment to be amplified, to permit an exponential amplification of the pre-determined fragment through different cycles of denaturation, annealing and extension.

To perform RT-PCR assay the following two steps are needed:

- Extraction of Total Nucleic Acids (TNA)
- viroid cDNA synthesis and PCR amplification.

3.2.2 RNA extraction (modified plant total nucleic acid extraction with silica)

Citrus leaf tissues (100mg) were ground in a sterile mortar. The powder was homogenized in 1.0 ml of grinding buffe with 0.5% of sodium metabisulfite, and then 500 µl of aqueous phase was mixed with 150 µl of Na-Sarkosyl , incubated at 70°C for10 min, and cooled in ice for 5 minutes. After a centrifugation at 13,000 rpm for 3 min, 500 µl of the supernatant transferred to new eppendorf tube to which 250 µl ethanol,500 µl NaI 6M and 35µl Re-suspended silica were added. The mix was gently agitated at room temperature for 10 min and then centrifuged at 6,000 rpm for 1 min. pellet that formed was washed 2 -3 times by washing buffer by re-suspending in 500 µl of it, and then centrifuged at 6,000 rpm for 1min per time.

After drying the pellet for few minutes by putting the tube upside down on tissue paper pellet was dissolved with 150 µl distilled water, and incubating at 70°C for 4 min and centrifuged at 13,000 rpm for 3 min. Finally, the supernatant was formed transferred to a new eppendorf tube and stored at -20°C (Annex 1). In order to check the quality of the TNA extraction,2µl/ sample were measured by NanoDrop 2000/2000c UV-Vis spectrophotometers (Fig. 4) (Also see Annex2).

Figure 4

NanoDrop 2000/2000c UV-Vis spectrophotometers

3.2.3 Viroid cDNA Synthesis

cDNA Synthesis mix (Figure 5) was prepared by adding of 1µl of (1:1 random hexamers primer (50ng/µl) and oligodT (5OµM)) and 1µL dNTPs, to 500ng of TNA solution, completed to 10µL with DEPC-treated water, then incubated at 65°C for 5 min and kept directly in ice for at least one minute.

Reverse transcription reaction was done for 1h (10 min at 25°C followed by 50 min at 50°C) by adding 2µl 10x RT buffer, 2µl of 0.1M DTT, 4µl of 25mM $MgCl_2$, 1µL RNaseOUT (40U/µL) ,1µL SuperScriptIII RT(200U/µL), to 10µL of cDNA synthesis mix in a final volume of 20µl.

Reaction Terminated at 85º c for 5 min and cooled at 4ºc for 10 min.

Figure 5

SuperScript™ III First-Strand Synthesis kit

3.2.4 PCR reaction

The presences of five viroids were detected using multiplex PCR and single PCR. The specific sets of primers for the five viroids are listed in Table 3.

Table 3

Sets of DNA primers used for the RT-PCR detection of citrus -infection viroids.

Primer	Sequence 5→-3	Size bp	Position	Reference	Conc.
CEV-R	CCGGGGATCCCTGAAGGACTT	371	78–98	Gross *et al*. (1982)	1.25 µl
CEV-F	GGAAACCTGGAGGAAGTCGAG		99–119		1.25 µl
CBLVd-R	TTCGTCGACGACGACCAGTC	234	86–104	Ashulin *et al*. (1991)	0.5 µl
CBLVd-F	CCCTTCACCCGAGCGCTGCTT		188–208		0.5 µl
HSVd-R	CCGGGGCTCCTTTCTCAGGTAAG	302	59–82	Sano *et al*. (1988)	0.25 µl
HSVd-F	GGCAACTCTTCTCAGAATCCAGC		83–105		0.25 µl
CVd-III-R	CGTCACCAACTTAGCTGCCTTCGT	269	91–112	Sieburth *et al*. (2002)	0.5 µl
CVd-III-F	GTCTCCGCTAGTCGGAAAGACTCCG		135–159		0.5 µl
CVd-IV-R	CCGGGGATCCCTCTTCAGGT	138	52–71	Puchta *et al*. (1991)	0.5 µl
CVd-IV-F	GGTGGATACAACTCTTGGGTTGT		217–239		0.5 µl
18s[a]-R	TTCAGCCTTGCGACCATACT	844		Gambino, & Gribaudo (2006)	0.35 µl
18s[a]-F	CGCATCATTCAAATTTCTGC				0.35 µl

R antisense primer,F sense primer ; [a] Internal control.

Multiplex PCR

Except for CEVd (0.5 M) and HSVd (0.1 M), the reaction was completed with a mix of primer pairs from all viroid strains at a final concentration of 0.2 M.

2. l cDNA mixture was processed for PCR amplification in 5 l of 10x Taq polymerase buffer (Promega Corporation, USA), 2 l of 50 M MgCl2, 0.5 l of 10 M dNTPs, primers added as described in table 3, and Taq polymerase (5 unit/l) in a final volume of 25 l. After a 5-minute denaturation at 94°C, 45 cycles of cDNA amplification were performed. Denaturation at 94°C for 50 seconds, annealing at 58°C for 50 seconds, and extension at 72°C for 2 minutes were the phases in each cycle.

Single PCR (for each viroid).

Each viroid was subjected to a single PCR with its own set of primers. Except for CEVd (0.5 M) and HSVd (0.1 M), the reaction began with a mix of each viroid primer pair at a final concentration of 0.2 M.

Each set of primers was added to each reaction as described in table3, and 0.25 l of Taq polymerase (5 unit/l) in a 25 l final volume. After a 5-minute denaturation stage at 94°C, 45 cycles of cDNA amplification were performed. Each cycle consisted of a 50-second denaturation stage at 94°C followed by a 55°C annealing step. around 50 seconds, followed by a 2-minute extension at 72°C, followed by a 10-minute extension at 72°C.

PCR Analysis by gel electrophoresis.

The PCR products were resolved by electrophoresis through 2 % agarose in TBE buffer gel to determine the size of amplified fragment after GelRed (5 µl) staining.

10µl of PCR producte ,5µl DNA ladder were loaded in gel and gel was run For 30-40 min at 100Mv. Then Gel was viewed under UV light detector.

3.3 Sequencing

DNA sequencing is a technique for determining the nucleic acid sequence, or the order of nucleotides in DNA. Included is any technique or technology for establishing the order of the four bases: adenine, guanine, cytosine, and thymine. Rapid DNA sequencing technology has boosted biological and medical research and discoveries substantially.

Despite the lack of time and financial inability, some positive samples were transferred to An-Najah National Hospital-Nablus, for sequencing to confirm the obtained results and to investigate new Isolates that could be registered as Palestinian isolates.

Samples were prepared for sequencing in the laboratories of the National Agricultural Research Center (NARC)- Jenin, with correct labeling and Correct preservation during transfer.

Chapter Four

Results and Discussion

4.1 Field Survey

Field surveys and sampling were carried out in: August 2020. A total of 42 samples (citrus leaf) (Fig. 6) were collected from citrus germplasm at National Agriculture Research Center *NARC*, Jenin, *Palestine*.

Figure 6

Citrus collection plot in NARC –Jenin

Symptoms of possible viroid origin were sometimes observed during the field surveys, i.e. epinasty, stunting, clearing and, discoloration of vein distortion of leaf and mottling, necrotic or chlorotic spots, scaling ,cankers, and bark cracking .

Figure7

Infected trees showed viral symptoms; (A) cankers of bark; (B) Leaf curling; (C) leaf distortion and mottling

(A) (B) (C)

Each picture show an example of citrus viroid expected symptoms as :cankers of bark,leaf curling and leaf distortion and mottling .

4.2 Detection of citrus-infecting viroid.

4.2.1 Multiplex and single PCR Test.

Specific sets of primers to five citrus-infecting viroid species ((CEVd), (CBLVd), (HSVd), (CVd-III) and (CVd-IV)) were used in two-step PCR analysis for testing all the 42 samples collected from the collection plot, in order to evaluate the relative incidence of each single viroid in the tested samples. The expected size of PCR amplifications of the tested viroid was visualized by agaros gel electrophoresis (Fig. 8 and 9).

Figure 8

Multiplex PCR product for five viroid (M: 50bp Marker ,-ve: negative control)

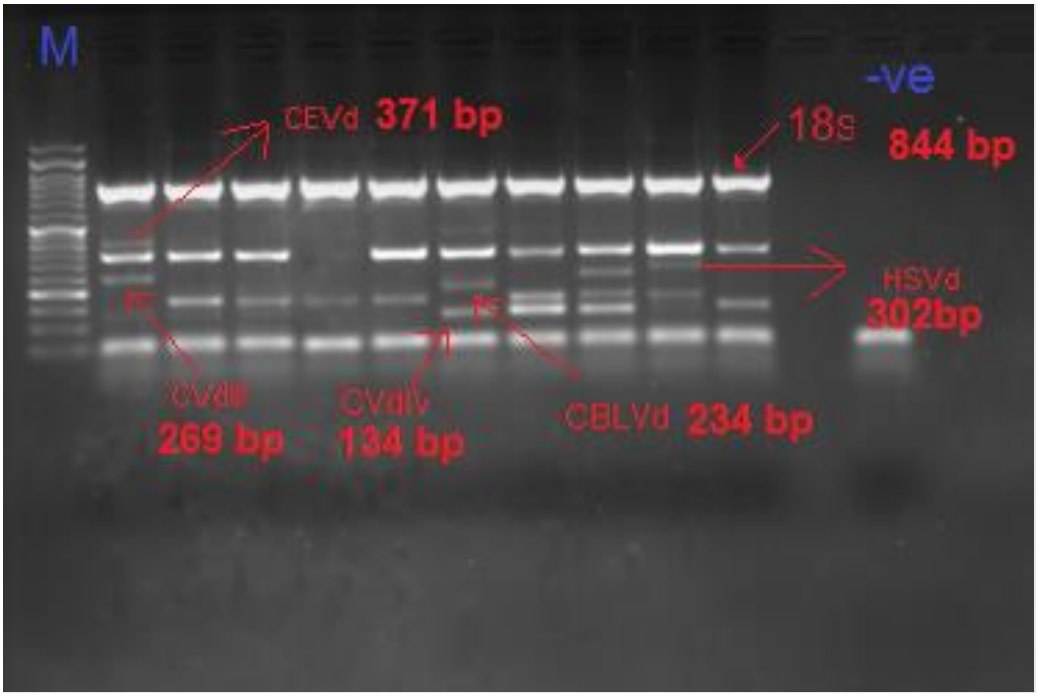
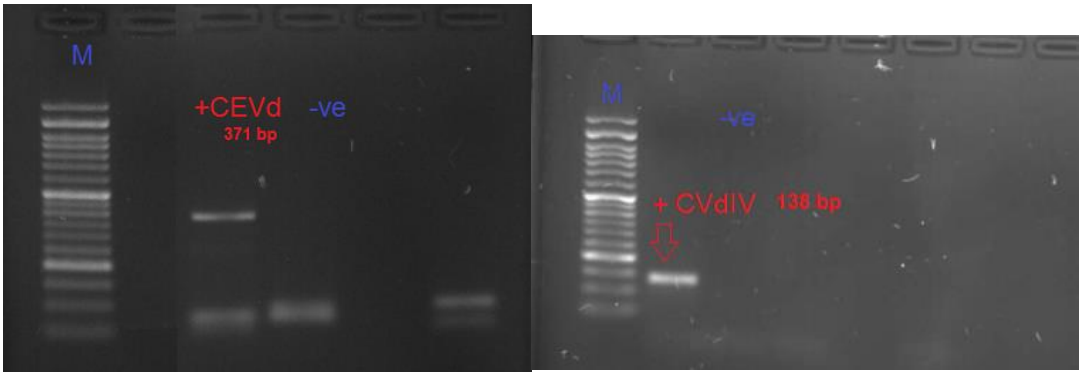
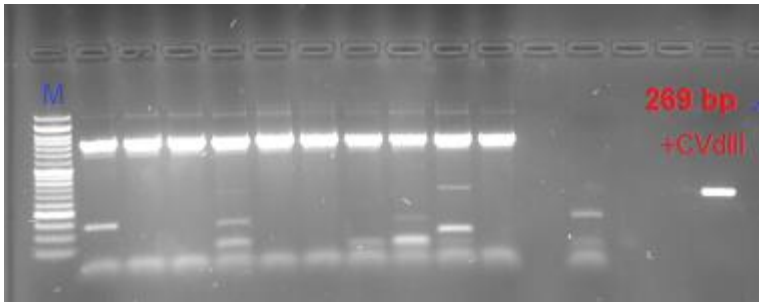


Figure 9

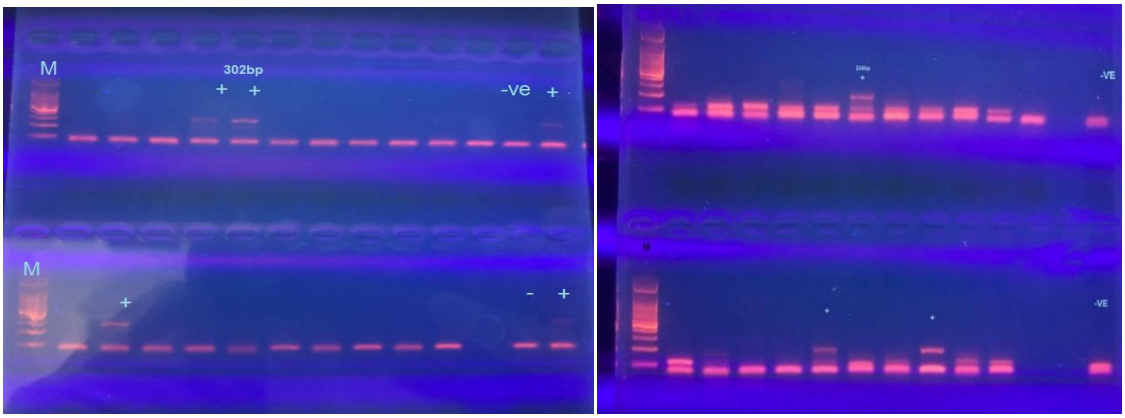
Single viroid PCR detection gel. (M) represent 50 bp marker.; (-ve): negative control. , (+): positive. (a) PCR products of CEVd; (b) for CVdIV; (c) CVdIII viroid; (d) HSVd; and (e) for CBLVd.



a b



c



d e

The most common viroids were CVd IV and CEVd, which were found in 35.7 percent and 23.8 percent of samples, respectively (Fig.10). Contrary to earlier studies conducted in Campania, Italy, CVdIII, HSVd, and CBLVd were ranked third in terms of incidence (16.6 percent, 9.5 percent, and 7%, respectively). Citrus exocortis viroid (CEVd), also known as Hop stunt viroid (HSVd), and Citrus viroid III (CVd-III), were found to be the most common, accounting for 67.9%, 86.6, and 84.8 percent of all infected samples, respectively. Citrus viroid IV (CVd-IV) and Citrus bent leaf viroid (CBLVd) were discovered in only 24.1 and 13.4% of the sources, respectively (Malfitano et al, 2007).

The presence of these viroids in citrus fruits and in these percentages is a matter that is necessary to follow because of its impact on citrus trees.

An unexpected high level of infection was detected in the collection plot under study 59.5% Moreover, mixed infection (mixture of two-three or four viroid) was also frequent, interesting about 30% of samples (Fig. 10). Contrarily to what observed in previous survey done in Campania (southern Italy), as 82.1% of citrus trees were infected with two or more viroid species (Malfitano *et al*, 2007)

4.2.2 Sequencing and genome analysis.

(BLAST) The Basic Local Alignment Search Tool can find local similarity between sequences the regions . The program calculating the statistical significance of matches by firstly comparing the nucleotide sequences needs to sequence databases.

BLAST can be used to infer functional and evolutionary relationships between sequences and also help members of gene families identification .

These are the blast results for each sequenced sample, which interpreted as the list of hits starts with the best match (most similar),smaller the E-value and have a high percentage of identity,. E-value: it’s the expected number of chance alignments . First in the list is the query sequence itself, which has the best score (Fig 12, 13, 14, 15).

Chapter Five

Conclusions and Recommendations

Citrus is a worldwide important fruit crop with substantial domestic, export, and industrial potential. Citrus orchards are experiencing lower yields as a result of citrus decline. Graft-transmissible virus and viral-like infections are all significant factors, as are filthy nursery operations and poor orchard management. However, nurseries are to blame for the majority of the problems. Nurseries must now operate on highly technical and scientific lines, focused on delivering disease-free and certified plants to citrus growers. In order to construct disease-free nurseries, indexing viral and virus-like infections is a critical issue that must be addressed.

The present study brings an important contribution in molecular detection of five citrus viroid in west bank -Palestine which is reported for the first time in west bank and three of them will registered in gene bank as a Palestinian isolate.

Two-step RT-PCR testing revealed the presence of five citrus viroids. These findings show that the two-step multiplex molecular detection methods proposed in this work are a viable tool for studying the genetic diversity of viroid isolates and quantifying viroids in their citrus hosts. The percentage of infected trees was around 59.5 percent, which is a relatively high number that necessitates the development of effective preventative techniques to avoid infection in citrus plants.

Citrus viroids, are distributed in general by introduction and propagation infected budwoods by mechanical transmission and by top working. viroids are known for their ability to infect large number of host plants .as in CEVd and HSVd they are destructive to certain citrus varieties and can lead to yield losses.

The mixed infection is likely to have an effect on the severity of symptoms which is affect the growth and productivity of citrus tree which have a mix infection affected. Therefore, it is very important to focus on this point in subsequent studies**.**

Due to poor financial capabilities and lack of time, the sequencing process was not completed for all viroid detected, so the sequencing process was performed for three viroids only.

This opens prospects to another study in the same field to complete the sequencing process for the rest of the viroid (CBLVd, HSVd) and it may also be as Palestinian isolates to be registries in gene bank.

List of abbreviations

Abbreviation	Meaning
cDNA	Complementary deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
dH_2O	Distilled Water
EDTA	Ethylene Diamino Tetra Acetic Acid (disodium salt)
EtOH	Ethanol
HCl	Hydrochloridric Acid
KOAc	Potassium acetate
NaCl	Sodium chloride
Na_2CO_3	Anhydrous sodium carbonate
NaI	Sodium iodide
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaOAc	Sodium acetate
Nt	Nucleotide
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Spp.	Species
TBE	Tris- Borate-EDTA
TE	Tris- EDTA
TNA	Total Nucleic Acid
Tris	Tris (Hydroxymethyl) aminomethane
U	Enzymatic Unit
UV	Ultraviole

Measurement unit

%	Percentage
µ	Micro
µg	Microgram
µl	Microliter
µM	Micromolar
°C	Degree Celsius
bp	Base pair
g	Gram
H	Hour
L	Litre
M	Molar
Min	Minute
ml	Milliliter
mM	Millimolar
pg	picogram
sec	Second

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Appendices

Appendix A: Figures of Study

Figure 10

Incidence of viroids infection in the citrus trees in NARC –Jenin. as detected by PCR.

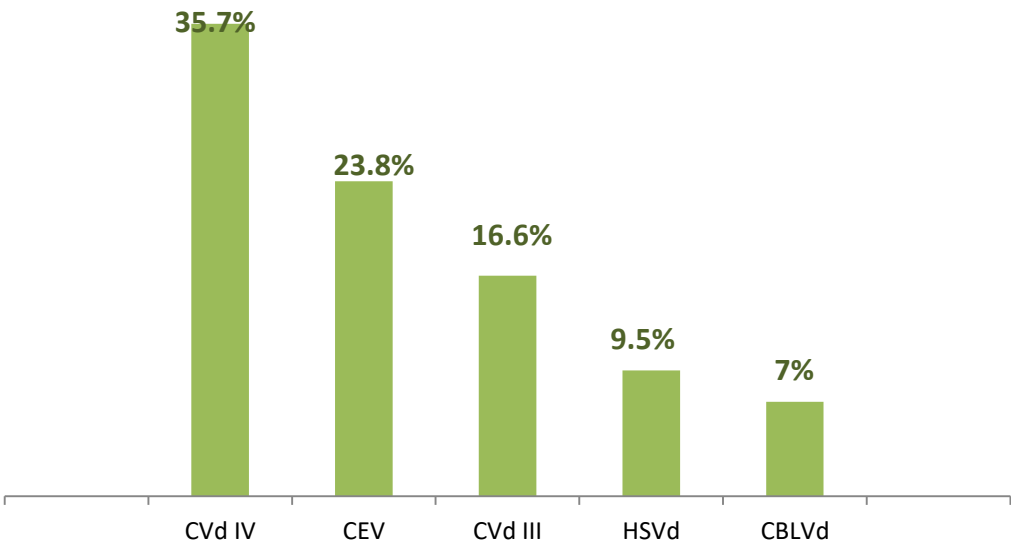

Figure 11

*single /mix infection percentage***.**

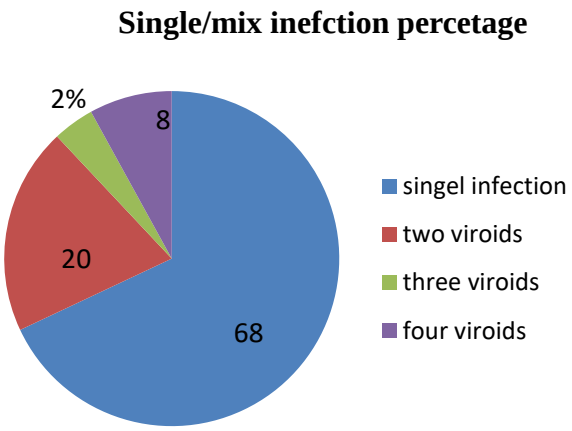

Figure 12

BLAST search results for the amplified portion of CVd IV

Sample 1 CVd IV

CGACATCCAGAGTTGTTACCCGGAATGGCCCGCGTTTGAGACCCCTCTGGGG
AATTTCTCTGCGGGACCAAATAAAAACAGCTTGTGGAGGGAACATACCTGA
AGAGGGATCCCCGGAATCTCTCAGAAGCTTGCGATATAAATATGTTTTACAA
GCTCATCTAAAGGTTTCTCCATCTTTAGTCAAAACATTGATTTTCTTTAAATA
AGAATCTGATTCAAAATCACTACTGCTAGATGCAGAGGTATCTGAGTCAATA
AGCTCATCGACCTGAAGAGGGATCCCCGG

The BLAST search

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Citrus exocortis viroid isolate 159-1, complete genome	Citrus exocortis viroid	544	544	87%	3e-150	99.01%	371	KF973205.1
☑	Citrus exocortis viroid isolate CEVd-XNM-XY Guangdong-YC, complete genome	Citrus exocortis viroid	538	538	87%	1e-148	98.68%	371	DQ431993.1
☑	Citrus exocortis viroid isolate CEVd-CY218, complete genome	Citrus exocortis viroid	364	511	84%	2e-96	98.55%	380	MH771135.1
☑	Citrus exocortis viroid strain CBO-D, complete genome	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	371	KC290927.1
☑	Citrus exocortis viroid isolate T1-1, complete genome	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	370	FJ904296.1
☑	Citrus exocortis viroid isolate soV1, complete genome	Citrus exocortis viroid	364	524	87%	2e-96	98.55%	371	EU564183.1
☑	Citrus exocortis viroid isolate CEVd-f-1, complete genome	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	372	EF494677.1
☑	Citrus exocortis viroid isolate CEVd-i-3, complete genome	Citrus exocortis viroid	364	530	87%	2e-96	98.55%	373	EF488066.1
☑	Citrus exocortis viroid isolate CEVd-i-4, complete genome	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	372	EF488065.1
☑	Citrus exocortis viroid isolate CEVd-i-7, complete genome	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	371	EF488062.1
☑	Citrus exocortis viroid isolate CEVd-i-12, complete genome	Citrus exocortis viroid	364	534	87%	2e-96	98.55%	371	EF488057.1
☑	Citrus exocortis viroid isolate 9669-FI-I, complete genome	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	372	AY517496.1
☑	Citrus exocortis viroid complete genome, isolate E117, haplotype V8	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	371	AJ564802.1
☑	Citrus exocortis viroid complete genome, isolate E117, haplotype V5	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	372	AJ564799.1
☑	Citrus exocortis viroid complete genome, isolate E117, haplotype V4	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	371	AJ564798.1
☑	Citrus exocortis viroid complete genome, isolate E117, haplotype V2	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	372	AJ564796.1
☑	Citrus exocortis viroid isolate 454-FI-I, complete genome	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	371	AY229990.1
☑	Citrus exocortis viroid variant E55K genomic RNA, complete sequence	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	371	AB054596.1
☑	Citrus exocortis viroid variant BF genomic RNA, complete sequence	Citrus exocortis viroid	364	535	87%	2e-96	98.55%	373	AB054594.1
☑	Citrus exocortis viroid isolate CEVd-XNM-HJ Guangdong-YC, complete genome	Citrus exocortis viroid	532	532	87%	6e-147	98.36%	372	DQ431992.1

Figure13

BLAST search results for the amplified portion of CVdIII

Sample 4: CVdIII

TCCTCCGGCGCCCCGCTAGCTCGCCGCTAGTCGAGCGGACCACGGGAAGTAGCCCT
ACTCCTAATCTGTTTTTATTTAGGCTAGAAGGGGATTGGGCCTCCAGGGTAAAACAC
GATTGGTGTTTTCCCCGGAAAACTCCGTGTGGTTCCTGTGGGGCACACCCCCTTGCC
GAAAATAAAACGCAGAGAGGGAAAGGGAACTTACCTGTCGTCGTCGACGAAGGCA
GCTAAGTTGGTGACGCCGCTAAGTTCCCTTTCCTCTCTGCGTTTTATTTTCTGCAAGG
GGGTGTGCCCCACAGGAACCACACGGAGTTTTCCGGGGAAAACACCAATCGTGTTT
TACCCTGGAGGCCCAATCCCTTCTTGCCTAAATAAAAACAGATTAGGAGTAGGGCT
ACTTCCGTGGTCGCTGACTAGCGGCGAGCTAGCGGGTCTGCGGAGGATGCGGAGTC
TTTCCTACTAGCGGAGACAGCA

The BLAST search

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Citrus dwarfing viroid isolate lot1-1/2-19, complete genome	Citrus dwarfing vir...	206	669	98%	2e-48	100.00%	293	KM214215.1
☑	Citrus viroid III isolate 16 complete genome	Citrus dwarfing vir...	206	687	97%	2e-48	100.00%	293	AF123868.1
☑	Citrus dwarfing viroid clone CV20, complete genome	Citrus dwarfing vir...	206	695	98%	2e-48	99.13%	291	MF421248.1
☑	Citrus dwarfing viroid isolate VR-15, complete genome	Citrus dwarfing vir...	206	678	98%	2e-48	99.13%	293	JF812070.1
☑	Citrus dwarfing viroid isolate LS-4, complete genome	Citrus dwarfing vir...	206	700	98%	2e-48	99.13%	293	JF812069.1
☑	Citrus dwarfing viroid isolate IIIbl11, complete genome	Citrus dwarfing vir...	206	656	98%	2e-48	99.13%	292	GQ260214.1
☑	Citrus dwarfing viroid isolate GBS28, complete genome	Citrus dwarfing vir...	206	689	98%	2e-48	99.13%	293	GQ246203.1
☑	Citrus dwarfing viroid isolate c, complete genome	Citrus dwarfing vir...	206	689	98%	2e-48	99.13%	293	GQ166530.1
☑	Citrus dwarfing viroid isolate b, complete genome	Citrus dwarfing vir...	206	680	98%	2e-48	99.13%	293	GQ166529.1
☑	Citrus dwarfing viroid clone A35-3-4, complete genome	Citrus dwarfing vir...	206	706	98%	2e-48	99.13%	292	FJ773280.1
☑	Citrus dwarfing viroid isolate 584-4, complete genome	Citrus dwarfing vir...	206	700	98%	2e-48	99.13%	293	EU934020.1
☑	Citrus viroid III isolate Tahiti lime 7, complete genome	Citrus dwarfing vir...	206	667	98%	2e-48	99.13%	293	EU549775.1
☑	Citrus viroid III isolate Tahiti lime 6, complete genome	Citrus dwarfing vir...	206	663	98%	2e-48	99.13%	293	EU549774.1
☑	Citrus viroid III isolate CQ clone 2, complete genome	Citrus dwarfing vir...	206	665	98%	2e-48	99.13%	294	EU382206.1
☑	Citrus viroid III isolate E21a, complete genome	Citrus dwarfing vir...	206	706	98%	2e-48	99.13%	292	AF447788.1
☑	Citrus viroid III isolate 23 complete genome	Citrus dwarfing vir...	206	678	98%	2e-48	99.13%	293	AF123874.1
☑	Citrus viroid III isolate 22 complete genome	Citrus dwarfing vir...	206	689	98%	2e-48	99.13%	293	AF123873.1
☑	Citrus viroid III isolate 19 complete genome	Citrus dwarfing vir...	206	667	98%	2e-48	99.13%	293	AF123870.1
☑	Citrus viroid IIIb complete genome	Citrus viroid IIIb	206	667	98%	2e-48	99.13%	296	AF184148.1
☑	Citrus dwarfing viroid clone CV22, complete genome	Citrus dwarfing vir...	202	704	98%	2e-47	99.12%	288	MF421249.1
☑	Citrus viroid III isolate 20 complete genome	Citrus dwarfing vir...	202	685	98%	2e-47	99.12%	293	AF123871.1
☑	Citrus dwarfing viroid isolate lot1-1/2-2, complete genome	Citrus dwarfing vir...	200	660	98%	9e-47	99.11%	294	KM214214.1
☑	Citrus dwarfing viroid isolate 19-1.17-CDVd, complete sequence	Citrus dwarfing vir...	200	639	97%	9e-47	99.11%	294	JX259432.1

Sample 5 CVdIII

TCCTCCGCGCCCCTCCTTGCTCGCCGCTAGTCGAGCGGACTTCAGGAGAGTAGCCCC
AATCCTAACCTGTTTTTATCTAGGCTAGAAGGGGATTGGGCCTCCAGGGTAAAACAC
GATTGGTGTTTTCCCCGGGAAACTCCGTGTGGTTCCTGTGGGGCACACCCCCTTGCC
GAAAATAAAACGCAGAGAGGGAAAAGGGAACTTACCTGTCGTCGTCGACGAAGGC
AGCTAAGTTGGTGACGACAGGTAAGTTCCCTTTTCCCTCTCTGCGTTTTATTTTCGGC

AAGGGGGTGTGCCCCACAGGAACCACACGGAGTTTCCCGGGGAAAACACCAATCGT
GTTTTACCCTGGAGGCCAATCCCTTCTGCCTAGATAAAAACAGGGTTAGGATTGGGG
GCTACTCTCTGAAGTCCCTTGACTAGCGCCAAGAAGGAAGGGTCTGCCGGAAGGAT
GCGGAATCTTTCCAACTAGGGGAAACAA

The BLAST search

	Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☐	Citrus dwarfing viroid isolate GB5-7, complete genome	Citrus d...	NA	551523	206	704	93%	2e-48	100.00%	293	EU934021.1
☐	Citrus viroid III isolate 18 complete genome	Citrus d...	NA	551523	206	660	99%	2e-48	100.00%	294	AF123869.1
☐	Citrus dwarfing viroid clone CV22, complete genome	Citrus d...	NA	551523	202	687	96%	3e-47	99.11%	288	MF421249.1
☐	Citrus dwarfing viroid clone CV20, complete genome	Citrus d...	NA	551523	202	672	95%	3e-47	99.11%	291	MF421248.1
☐	Citrus dwarfing viroid isolate IIIbl11, complete genome	Citrus d...	NA	551523	202	652	99%	3e-47	99.11%	292	GQ260214.1
☐	Citrus viroid III isolate E21a, complete genome	Citrus d...	NA	551523	202	684	95%	3e-47	99.11%	292	AF447788.1
☐	Citrus viroid III isolate 23 complete genome	Citrus d...	NA	551523	202	509	62%	3e-47	99.11%	293	AF123874.1
☐	Citrus viroid III isolate 22 complete genome	Citrus d...	NA	551523	202	647	95%	3e-47	99.11%	293	AF123873.1
☐	Citrus viroid III isolate 20 complete genome	Citrus d...	NA	551523	202	647	95%	3e-47	99.11%	293	AF123871.1
☐	Citrus viroid III isolate 19 complete genome	Citrus d...	NA	551523	202	667	99%	3e-47	99.11%	293	AF123870.1
☐	Citrus dwarfing viroid isolate lot1-1/2-2, complete genome	Citrus d...	NA	551523	200	641	99%	9e-47	99.10%	294	KM214214.1
☐	Citrus dwarfing viroid isolate lot1-1/2-19, complete genome	Citrus d...	NA	551523	198	637	99%	3e-46	99.10%	293	KM214215.1
☐	Citrus dwarfing viroid isolate CI-0, complete sequence	Citrus d...	NA	551523	198	630	98%	3e-46	99.10%	291	MN136646.1
☐	Citrus dwarfing viroid, complete genome	Citrus d...	NA	551523	198	630	99%	3e-46	99.10%	291	HQ219183.2
☐	Citrus viroid III variant 10SA1-IW genomic RNA, complete sequence	Citrus d...	NA	551523	198	624	98%	3e-46	99.10%	291	AB054631.1
☐	Citrus viroid III variant OS2 genomic RNA, complete sequence	Citrus d...	NA	551523	198	630	99%	3e-46	99.10%	291	AB054626.1
☐	Citrus viroid III variant OS1 genomic RNA, complete sequence	Citrus d...	NA	551523	198	641	98%	3e-46	99.10%	291	AB054625.1
☐	Citrus viroid III variant AD2 genomic RNA, complete sequence	Citrus d...	NA	551523	198	630	99%	3e-46	99.10%	291	AB054620.1
☐	Citrus viroid IIIc complete genome	Citrus vi...	NA	110139	198	641	99%	3e-46	99.10%	291	AF184149.1
☐	Citrus viroid III isolate TL1, complete genome	Citrus d...	NA	551523	198	689	99%	3e-46	99.09%	296	EU564176.1
☐	Citrus dwarfing viroid isolate NRCV04, complete sequence	Citrus d...	NA	551523	196	654	99%	1e-45	98.21%	296	KT725632.1
☐	Citrus viroid III isolate CVd-III-tun/cl2, complete genome	Citrus d...	NA	551523	195	624	98%	4e-45	98.21%	293	AF540965.1
☐	Citrus dwarfing viroid clone CV16, complete genome	Citrus d...	NA	551523	195	660	99%	4e-45	98.20%	294	MF421246.1
☐	Citrus dwarfing viroid isolate 19-1.17-CDVd, complete sequence	Citrus d...	NA	551523	195	632	99%	4e-45	98.20%	294	JX259432.1

Figure 14

BLAST search results for the amplified portion of CEVd

Sample 6 CEVd

TCGCAGTCTAGTGTGTAACGGGTAAAGTCCTTCGGGATCCCCGGAGGGGAA
AACAGGAGTCGTCTCCTTCCTTTCGCTGCTGGCTCCACATCCGATCGTCGCTG
AAGCGCCACGCCCCCTCGCCCGGAGCTTCTCTCTGGCTACTACCCGGTGGAT
ACAACTGAAGCTTCAACCCCAAACCGCTTTTCTTATATCTTCACTGCTCTCCG
GGCGAGGGTGAAAGCCCTCGGAACCCTAGATTGGGTCCCTCGGGATCTTTCT
TGAGGTTCCTGTGGTGCTCACCTGACCCTGCAGGCAGGAAAAGAAAAAAGA
GGCGGCGGGGAAGAAGTCCTTCAGGGATCCCCGG

The BLAST search

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Citrus exocortis viroid isolate 159-1, complete genome	Citrus exocortis viroid	544	544	87%	3e-150	99.01%	371	KF973205.1
☑	Citrus exocortis viroid isolate CEVd-XNM-XY Guangdong-YC, complete genome	Citrus exocortis viroid	538	538	87%	1e-148	98.68%	371	DQ431993.1
☑	Citrus exocortis viroid isolate CEVd-CY218, complete genome	Citrus exocortis viroid	364	511	84%	2e-96	98.55%	380	MH771135.1
☑	Citrus exocortis viroid strain CBO-D, complete genome	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	371	KC290927.1
☑	Citrus exocortis viroid isolate T1-1, complete genome	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	370	FJ904296.1
☑	Citrus exocortis viroid isolate soV1, complete genome	Citrus exocortis viroid	364	524	87%	2e-96	98.55%	371	EU564183.1
☑	Citrus exocortis viroid isolate CEVd-f-1, complete genome	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	372	EF494677.1
☑	Citrus exocortis viroid isolate CEVd-i-3, complete genome	Citrus exocortis viroid	364	530	87%	2e-96	98.55%	373	EF488066.1
☑	Citrus exocortis viroid isolate CEVd-i-4, complete genome	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	372	EF488065.1
☑	Citrus exocortis viroid isolate CEVd-i-7, complete genome	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	371	EF488062.1
☑	Citrus exocortis viroid isolate CEVd-i-12, complete genome	Citrus exocortis viroid	364	534	87%	2e-96	98.55%	371	EF488057.1
☑	Citrus exocortis viroid isolate 9669-FI-I, complete genome	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	372	AY517496.1
☑	Citrus exocortis viroid complete genome, isolate E117, haplotype V8	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	371	AJ564802.1
☑	Citrus exocortis viroid complete genome, isolate E117, haplotype V5	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	372	AJ564799.1
☑	Citrus exocortis viroid complete genome, isolate E117, haplotype V4	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	371	AJ564798.1
☑	Citrus exocortis viroid complete genome, isolate E117, haplotype V2	Citrus exocortis viroid	364	539	87%	2e-96	98.55%	372	AJ564796.1
☑	Citrus exocortis viroid isolate 454-FI-I, complete genome	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	371	AY229990.1
☑	Citrus exocortis viroid variant E55K genomic RNA, complete sequence	Citrus exocortis viroid	364	545	87%	2e-96	98.55%	371	AB054596.1
☑	Citrus exocortis viroid variant BF genomic RNA, complete sequence	Citrus exocortis viroid	364	535	87%	2e-96	98.55%	373	AB054594.1
☑	Citrus exocortis viroid isolate CEVd-XNM-HJ Guangdong-YC, complete genome	Citrus exocortis viroid	532	532	87%	6e-147	98.36%	372	DQ431992.1

Appendix B: Buffers used for TNA extraction with Silica

Buffer	Material	Quantity	Note
Grinding buffer pH 5.6-5.8	Guanidine thiosianate. NaOAc, pH 5.2 EDTA. KOAc PVP-40 Sodium bisulphate	4M 0.2 M 25 mM 1.0 M 2.5% 2%	Adjust pH using CH_3COOH Sterilize by autoclaving Keep it at 4°C. Add Sodium bisulphate before using.
NaI	Na_2SO_3 NaI (Sigma S8379)	0.75 g 36 g	Dissolve in 40 ml distilled water. Sterilize by autoclaving Keep it in dark at4°C.
Silica particles solution pH 2.0	silica particles (Sigma S5631)	12%	Add 60 g silica to 500 ml H_2O Mix and let settle for 24 hours. Discard the upper 470 ml supernatant (90% of the supernatant). Add H_2O up to 500 ml and mix well Let settle 5 h. Discard 440 ml (85% of the supernatant). Adjust the remaining 60 ml slurry to a pH 2.0 with HCl. Autoclave and store in dark at room temperature
Washing buffer 1x	Tris-HCl, pH7.5 (1 M) EDTA (5 M) NaCl (0.5 M) Ehanol	10.0 mM 0.5 mM 50.0 mM 50%	Sterilize by autoclaving before adding ethanol. Keep it at 4°C .

Appendix C: Amount of RNA extracted from each sample (ng/µl)

Sample No#	RNA amount (ng/ µl)
1	72.4 ng/ µl
2	58.2 ng/ µl
3	90 ng/ µl
4	97 ng/ µl
5	60 ng/ µl
6	83.6 ng/ µl
7	100 ng/ µl
8	82 ng/ µl
9	83 ng/ µl
10	87 ng/ µl
11	65 ng/ µl
12	75 ng/ µl
13	63 ng/ µl
14	41 ng/ µl
15	58 ng/ µl
16	79 ng/ µl
17	63 ng/ µl
18	37 ng/ µl
19	42 ng/ µl
20	53 ng/ µl
21	65 ng/ µl
22	56 ng/ µl
23	112 ng/ µl
24	78 ng/ µl
25	110 ng/ µl
26	122 ng/ µl
27	dry tree
28	92 ng/ µl
29	82.5 ng/ µl
30	99 ng/ µl
31	90 ng/ µl
32	142 ng/ µl
33	89 ng/ µl
34	93 ng/ µl
35	88 ng/ µl
36	146 ng/ µl
37	112 ng/ µl
38	161 ng/ µl
39	128 ng/ µl
40	94 ng/ µl
41	72.8 ng/ µl
42	93 ng/ µl
43	90 ng/ µl

جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

الكشف الجزيئي عن الفايرودات الموجودة في الحمضيات في الضفة الغربية

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قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجة الماجستير في العلوم الحياتية من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.

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الملخص

تعتبر الزراعة في فلسطين من اهم القطاعات الانتاجية التي تساهم بشكل كبير في الحفاظ على عجلة الاقتصاد الفلسطيني،حيث تساهم الزراعة في جزء كبير من الناتج القومي الاجمالي، وبالنسبة للحمضيات ف انها تعبتر من الرزاعات المهمة من ناحية استهلاك السكان او من ناحية البيع والتجارة ،لذلك الحفاظ على صحة الاشجار والحفاظ على انتاج جيد يعتبر امر ضروري . قد تصاب اشجار الحمضيات بالعديد من الآفات منها ما هو ناتج عن عدوى بالبكتيريا، فايروسات'فطريات او فايرودات. تعتبر الفايرودات كئن غير حي شبه بالفايروس لكنه يحتوي فقط على حمض اميني بدون غلاف بروتيني ولا اعضاء داخلية ولا يمكن للحمض الاميني الخاص به ترجمة اي بروتين لذلك يقوم بتسخير الخلية واجهزتها الداخلية للعمل لصالحه ولصالح تكاثره. وتسبب الاصابة بالفايروويد بظهور العديد من الاعراض التي تذهر على الشجرة المصابة ومن اهم هذه الاعراض: تجعد الورق، ظهور بقع على اوراق الاشجار، تصمغ وتقشر اللحاء.

من خلال هذه الدراسة تم التركيز على الفايرودات حيث تم الكشف جزئيا عن خمس اصناف منها حيث تم اخذ 42 عينة من اشجار حمضيات مزروعة داخل بيت بلاستيكي مخصصة لامور البحث في مركز البحوث الوطني الفلسطيني–جنين، حيث تم جمع العينات واجراء بعض الفحوصات اللازمة للكشف عن هذة الفايرودات .ومن النتائج التي تم الحصول عليها هي اكتشاف خمس اصناف من الفايرودات اللتي تصيب الحمضيات لاول مرة في الضفة الغربية وتم اجراء تسلسل جيني لثلاثة منها لتسجل اول ثلاث عزلات فلسطينية في بنك الجينات العالمي. من الفايرودات الخمسة التي تم الكشف عنها ما يلي :CVdIII ,CEVd,CVdIV,HSVdand CBLVd حيث ظهر

كل واحد منها بنسبة:35,7% ،23,8%،16.6% ، 9.5و7% لكل فايرويد بالترتيب. وظهور هذه النسب امر يدعو لاتخاذ التدابير الصحيحة والتعامل الصحيح مع الاشجار المصابة خصوصا ان مثل هذه الفايرودات تنتقل بواسطة الادوات الزراعية وادوات التقليم، لذلك وجب الكشف عن الاشجار المصابة والتعامل معها لمنع انتشار العدوى بين باقي الاشجار والحفاظ على انتاج جيد ووفير من الحمضيات .