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Etiology and Epidemiology of Phytoplasma-Associated Diseases of Stone Fruits and Grapevine in Jordan

By

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Matriculation No. R12361

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Matriculation No. Matr. R12361

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in *Agriculture, Environment and Bio-energy*

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Supervisor: Prof. Fabio Quaglino

Co-Supervisor: Prof. Rosemarie Tedeschi

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Date: January 10, 2022

Dedication

This work is dedicated to soul of my father, sister and Rayan, may ALMIGHT ALLAH have mercy on them.

and to my great and tenderness mother, my wife, and my sweet BAN, without whose constant support this thesis work was not possible. They always inspire me.

At the same time, my thanks also go to my whole family siblings, nieces and nephew

Declaration

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions.

This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Signature *Date. January 10, 2021*

Preface

This dissertation is submitted for the degree of Doctor of Philosophy at the University of Milano (Università degli Studi di Milano). The present research is original work and it was conducted under the supervision and guidance of ***Prof. Fabio Quaglino*** at the Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy (**DiSAA**) *and* ***Prof. Rosemarie Tedeschi*** *at the Department of Agricultural, Forestry and Food Sciences (DISAFA) University of Turin (Dipartimento di Scienze Agrarie, Forestali e Alimentari (****DISAFA****) Università degli Studi di Torino), between October 2018 and November 2021.*

Parts of this work have been communicated into scientific congresses and papers under submission in peer-reviewed journals and proceedings, as follows:

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Chapter 3
Association of seven distinct 'Candidatus Phytoplasma' species with almond diseases in Jordan, and preliminary information on their putative insect vectors. *Asem Habes Abu Alloush, Piero Attilio Bianco, Enrico Busato, Amre AlMahasneh, Alberto Alma, Rosemarie Tedeschi, Fabio Quaglino. Under submission to* ***Crop Protection****.*

Chapter 4
Association of four distinct 'Candidatus Phytoplasma' species with pomegranate witches'-broom and leaf alteration in Jordan, and preliminary insights on their putative vectors and reservoir plants. *Asem Habes Abu Alloush, Piero Attilio Bianco, Enrico Busato, Amre Mahasneh, Mahmoud AlShoubaki, Alberto Alma, Rosemarie Tedeschi, Fabio Quaglino. Under submission to* ***Annals of Applied Biology****.*

Chapter 5
Grapevine yellows in Jordan: associated phytoplasmas, putative insect vectors, and reservoir plants. *Asem Habes Abu Alloush, Piero Attilio Bianco, Enrico Busato, Yousef AlKhawaldeh, Alberto Alma, Rosemarie Tedeschi, Fabio Quaglino. Under submission to* ***Annals of Applied Biology.***

Abstract

In this study, a national survey on phytoplasma-associated diseases was conducted in Jordan from 2019 to 2021 targeting almond, pomegranate, and grapevine, three of the main fruit crops cultivated in all country as commercials and family farming. The activities included: (i) monitoring and sampling symptomatic and symptomless plants from early summer to autumn; (ii) total nucleic acids extraction and phytoplama detection by 16S rDNA amplification in nested PCRs using the primer pairs P1/P7 followed by F1/R0; (iii) sequencing and bioinformatic analyses (BlastN, iPhyClassifier) of F1/R0 amplicons.

During field surveys, almond yellows and witches'-broom (incidence ranging from 20-85%), pomegranate exhibiting leaf chromatic alteration and rolling, little leaf and witches'-broom (incidence ranging from 30- 65%), and grapevine yellows (incidence ranging from 10-55%) were observed. Nested PCR-based amplification of 16S rRNA gene detected phytoplasmas in 23, 17, 22 and 15.7% of collected symptomatic almond, pomegranate trees and symptomatic wine and table grape cultivar plants, respectively. Molecular detection and 16S rDNA nucleotide sequence analyses revealed the presence of different '*Candidatus* Phytoplasma' species within samples from symptomatic plants, while no amplification was obtained from symptomless plant samples.

Five categories of phytoplasma-like symptoms, including early flowering along with evergreen pattern; witches'-broom, yellowing, and dieback; slim leaf and leaf rolling; stem fasciation, were observed in almond trees. Disease incidence in the investigated orchards ranged from 20 to 85%. Nested PCR-based amplification of *16S rRNA* gene detected phytoplasmas in 23% of collected symptomatic almond trees. Amplicon nucleotide sequence analyses allowed attributing the detected phytoplasmas to '*Candidatus* Phytoplasma asteris' (taxonomic subgroups 16SrI-B and -R), '*Ca.* P. aurantifolia' (16SrII-B and -C), '*Ca.* P. omanense' (16SrXXIX-A and -B) (16SrXXIX-B described for the first time), '*Ca.* P. phoenicium'(16SrIX-B), '*Ca.* P. pyri' (16SrX-C), '*Ca.* P. solani' (16SrXII-A), and '*Ca.* P. ulmi'(16SrV-A). Moreover, further investigation identified '*Ca*. P. asteris' (subgroup 16SrI-R) in putative insect vectors *Agalmatium* sp., *Empoasca* sp., *Reptalus quinquecostatus*, and *Hyalesthes obsoletus*, '*Ca*. P. pyri' in *Cacopsylla bidens, Cicadulina bipunctata*, *Laodelphax striatellus*, and *Tettigometra* sp., and '*Ca*. P. omanense' (subgroup 16SrXXIX-B) in the non-crop plant *Amaranthus* sp.

In pomegranate symptomatic plants, four genetically distinct phytoplasmas were identified and attributed to '*Candidatus* Phytoplasma solani' (16SrXII-A), '*Ca.* Phytoplasma aurantifolia'

(16SrII-B), '*Ca.* Phytoplasma asteris' (16SrI-B, -R), and '*Ca.* Phytoplasma ulmi' (16SrV-A). Additionally, the presence of three cicadellids (*Macrosteles sexnotatus, Cicadulina bipunctata, Psammotettix striatus*) and two non-crop plants (*Plantago major, Capsicum annuum*) hosting the same pomegranate-infecting '*Ca.* Phytoplasma asteris' strains, and one cicadellid (*Balclutha incisa*) carrying the same pomegranate-infecting '*Ca*. Phytoplasma solani' strain was found. In conclusion, this study described a new pomegranate disease, called pomegranate witches'-broom and leaf alteration, associated with multiple phytoplasmas. Interestingly, '*Ca*. P. ulmi', *Ca*. P. pyri', and '*Ca*. P. omanense' in association with almond, and '*Ca*. P. ulmi' in association with pomegranate are reported for the first time in this study. The other phytoplasma species identified in almond and pomegranate were previously reported in the Middle East.

In grapevine yellows (GY) affected plants, amplicon nucleotide sequence analyses allowed attributing the detected phytoplasmas to '*Candidatus* Phytoplasma solani' (taxonomic subgroup 16SrXII-A), '*Ca*. P. omanense' (16SrXXIX-A and -B), '*Ca*. P. aurantifolia' (16SrII-C), and '*Ca*. P. asteris' (16SrI-R) in 72.4%, 17.2%, 6.9%, 3.4% of infected plants, respectively. Further investigation allowed identifying '*Ca*. P. solani' in the putative insect vectors *Orosius cellulosus* (firstly reported in Jordan), *Euscelidius mundus, Laodelphax striatellus*, and *Circulifer* sp., and in *Convolvulus arvensis*; '*Ca*. P. aurantifolia' in the insect *O. cellulosus* and in bindweed; '*Ca*. P. omanense' in the insect *Psammotettix striatus*; '*Ca*. P. asteris' in the insects *Arboridia adanae, Cicaduliana bipunctata, Circulifer* sp., *L. striatellus*, *Hyalesthes obsoletus*, and *P. striatus*. Based on this preliminary data, ecological cycles of such phytoplasmas are discussed. Obtained results suggest that GY phytoplasma diversity and ecology in Jordan are more complex than previously known, leading to a potential risk of disease outbreaks.

Data obtained in this study revealed a great genetic diversity of phytoplasmas infecting important crops in Jordan. Further studies concerning the epidemiology of these phytoplasma-associated diseases, including the identification of putative insect vectors and reservoir plants, are in progress. Overall results will allow developing integrated strategies for the management of such diseases.

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Your spirituality made my journey

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Chapter 1. INTRODUCTION

The evidence that numerous yellows-type diseases of plants, believed to be caused by viruses, were associated with phloem colonization by prokaryotes morphologically resembling mycoplasmas (mycoplasma-like organisms: MLOs) was first shown in 1967 (Doi *et al.,* 1967; Bertaccini *et al.,* 2014). These findings were a new era in plant diseases and open the gate for new pipeline of research and further studies in plant diseases and molecular biology. Several hundreds of plant diseases have been associated with phytoplasma-associated complex, and many of them were destructive and listed as quarantine pathogens (Parrella et al., 2008; Pierro et al., 2019. Phytoplasma classification adopted the highly conserved rRNA gene sequences for phytoplasma classification in ribosomal groups. The molecular based- technique depicted that phytoplasmas constitute a large monophyletic group within the class Mollicutes (Lee et al., 1992; Lee et al., 1998; Quaglino et al., 2009; pierro et al., 2019) and allowed the retrived of a new taxon named "*Candidatus* Phytoplasma" (IRPCM, 2004). Currently, 33 groups were identified, and each of them has been proposed to represent in at least one species, and a threshold of 97.5% similarity with any previously described species is used to propose any new species. Several groups and subgroups were officially designed as "species" under the provisional status "Candidatus," while other provisional "species" have not been formally described yet (i.e., "Candidatus Phytoplasma vitis") (IRPCM, 2004; Perez-Lopez *et al*., 2016). During the last decade, the number of phytoplasma strains reported worldwide has increased exponentially, and based on the PCR\RFLP and nested PCR\ 16rDNA genes, 47 '*Candidatus* Phytoplasma' species have being placed under the identified 33 groups with more than 200 subgroups (Bertaccini and Lee, 2018, Wei *et al.,* 2021**).**

Plants infected by phytoplasmas exhibit a broad range of symptoms causing developmental and morphological changes, modulate and regulate plant host genes, hormones, and secondary metabolite biosynthesis (Sugio *et al.,* 2011; Sugio *et al*., 2014; Orlovskis, 2017; Nampa, 2019; Hemmati *et al,* 2021). They exhibited mild to severe of virescence/phyllody (development of green leaf like structures instead of flowers), sterility of flowers, proliferation of axillary (side) buds resulting in a witches' broom behaviour, abnormal and short internode elongation, generalized stunting and little leaf, early flowering and emerging new grow during dormancy season (Lee *et al.,* 2000, Bertaccini, 2007; Muirhead *et al*., 2019). They are insect-vectored microorganisms and transmitted in a persistent manner (Figure 1). The major vectors

are leafhoppers and planthoppers that belong to different families of Auchenorrhyncha such as Cicadellidae, Cixiidae, Issidae, Delphacidae and Derbidae, and the family of Psyllidae (Hemiptera: Sternorryncha) (Weintraub and Beanland, 2006).

From plant pathogens and economic point of views, phytoplasmas is a large plant pathogenic bacterial group, and phytoplasmas diseases are associated with thousand plants worldwide including fruit and woody trees, vegetables and ornamental plants, and weeds. They are significantly impact the agriculture sector and crop productivities (Oshima *et al.,* 2013). They are impacting crop quality and quantity and in many outbreak cases they are leading to plant death. Phytoplasma epidemics have destroyed the livelihoods of many people in different parts of the world, who depend on the trees (coconuts) for nourishment, building materials, and income improvement (Oshima *et al.,* 2013).

Grape yellows (GY), apple proliferation (AP), citrus witches'-broom, almond witches'-broom (AlmWB), palm lethal yellowing, peach X-disease were among the most destructive and epidemic phytoplasma diseases, and excellent indicators about the economic importance of phytoplasma diseases (Davis *et al*., 2013; Bertaccini *et al*., 2014). Furthermore, phytoplasma infection led to significant yield losses in vegetables crops (tomato, eggplant, cucumber, and others) up to 30-100% over the globe (Bogoutdinov *et al.,* 2008; Navratil *et al.,* 2009; Kube *et al*., 2012, Rao and Kumar, 2017, Kumari *et al*, 2019).

Phytoplasma-related diseases are expected to increase because global warming/climate change is advantageous to the cold-sensitive phytoplasma vectors (Hogenhout *et al.,* 2008). They are transregional and their effects are subjected to double with expected outbreaks due to the climate change and unmanaged epidemiology. Further studies covering all their aspects, collaborative efforts among and within the regions and various agencies and authorized bodies must be designed, coordinated, activated, and updated.

In MENA Region, phytoplasmas have been paid more attention in the last decades especially in Iran and Lebanon. Many of economic crops were infected, and several epidemic diseases associated with significant losses were reported (Abou-Jawdah *et al*., 2003; Salehi *et al*., 2018). Stone fruits, grape vines and poem fruits were among such crops, and the following but not limited important diseases were reported associated with these crops; the two major GY; "flavescence dorée" (FD) and "bois noir" (BN); AP including pear decline (PD), European stone fruits yellows (ESFY); AlmWB in (Iran and Lebanon), citrus wichs'- broom (Abou-Jawdah *et al*., 2014, Lova *et al.,* 2014; Al-Salehi *et al.,* 2014). In General, the epidemiology and potential vectors still poorly studied in this region. In addition, more attempts should be

done to identify the role of alternative host species in phytoplasma diseases epidemiology (Hemmati et al., 2021). Few studies were conducted in Jordan concerning phytoplasma associated diseases, and many phytoplasma- like symptoms were observed on various economic crops in different parts of the Country including grapevine, stone fruits, and pome fruits. Consequently, the potential spread of their phytoplasmas associated diseases, the current study was implemented targeting detecting the presence and identifying the etiology and epidemiology of such phytoplasmas associated diseases. Moreover, the study was highlighted and review the phytoplasma in MENA region with more focus on that one infecting grapevines and stone fruits.

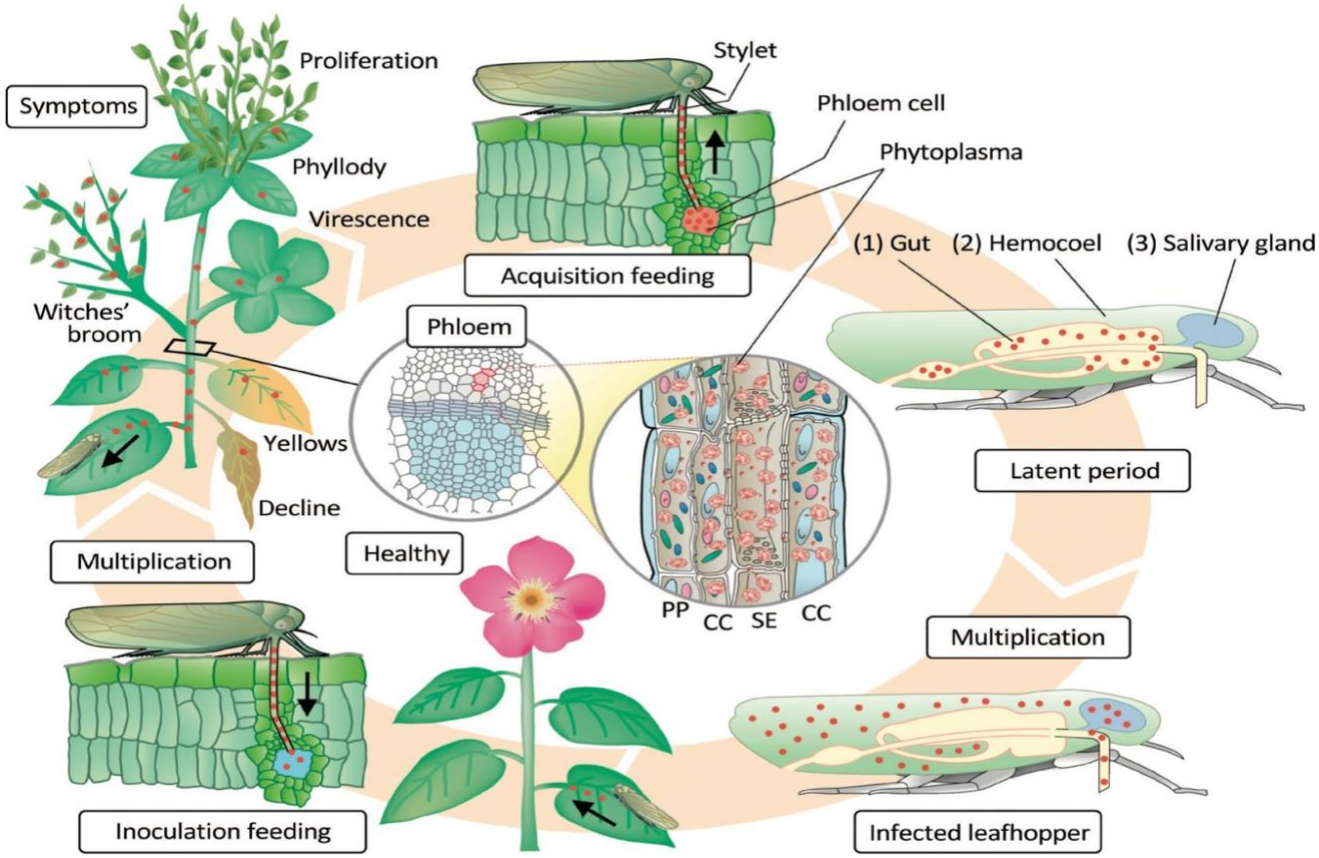

Figure 1. The life cycle of phytoplasmas; these are acquired by insects from plant phloem (via the stylets) and then enter the insect gut (acquisition feeding). Phytoplasmas must overcome three insect barriers to phytoplasma transmission (gut, hemocoel, and salivary gland barriers) if they are to be transmitted to plants. Phytoplasmas are transmitted from the insect to the phloem (of another plant) via inoculation feeding, and then multiply and establish a systemic infection, causing many unique symptoms. PP: phloem parenchymal cell, CC: companion cell, SE: sieve element (Namba, 2019).

1.1 Research statement and objectives

In Jordan, agriculture is a very important sector, considered the backbones of the socioeconomic, improving the livelihoods and food security situation for various stakeholders, among the family and commercial farming. Although its importance, and the reported important diseases infecting economic crops such as '*Candidatus* Phytoplasma solani' in the country and bordered areas (South of Syria) or '*Ca*. P. mali', phytoplasmas and their epidemiology in Jordan are still poorly understood and there are lack of information and implemented studies. Therefore, the present search was designed to study the phytoplasmas of grapevines, pomegranate, and almond in Jordan. The target crops were selected based on their economic importance, observed symptoms, epidemiological elements in terms of associated weeds, putative Hemiptera vectors and suitable climate. The current study is designed to investigate the etiology and epidemiology of phytoplasma diseases infecting grapevines, almond, and pomegranate and for that the following specific objectives were addressed:

Objectives

(i) Highlighted the phytoplasma in MENA region, and review the phytoplasma associated diseases of grapevine, stone fruits, and pome fruits (pomegranate in particular).

(ii) Survey phytoplasma-associated diseases of grapevine, almond, and pomegranate in Jordan.

(iii) Identify and type the phytoplasmas associated with such diseases and their potential insect vectors and non-crop host plants.

Chapter 2. Phytoplasma background

2.1 Phytoplasma biology and properties

Phytoplasmas are a large group of phloem-restricted, cell wall-less bacteria (non-helical prokaryotes) that infect nearly a thousand plant species and cause serious economic loss worldwide. In nature, phytoplasmas are transmitted by phloem sap feeding insect vectors, mainly leafhoppers and planthoppers, in a persistent-propagative manner (Weintraub and Beanland, 2006). In insect hosts, phytoplasma can induce host range expansion and host shift (Wie *et al.*, 2021). Due to possible vector-mediated host range expansion and host shift, phytoplasma may "infect" nonspecific plants without showing symptoms (Purcell, 1988, Wei et al, 2021).

They are polymorphic with small size ranging from 200 to 800 nm diameter and possess a very small genome ranging from 530 to 1350 kb. The development of molecular-based technique and phylogenetic- based DNA sequencing, approved that MLOs are a large monophyletic group within the class Mollicutes. With a small, A-T rich, and distinctively organized genome, phytoplasmas are a well-defined clade inside the class Mollicutes, derived from an Acholeplasma-like ancestor (Lee *et al.*, 2010; Zhao *et al.*, 2014, 2015).

They have one chromosome and several small plasmids. Their plastic genomes allow them to reproduce successfully in two types of biologically distant hosts, such plant and insect vectors. The smallest chromosome, about 530 kb, is known to occur in the Bermuda grass white leaf agent '*Ca.* P. cynodontis'. This value represents the smallest mollicute chromosome reported to date. Phytoplasmas inhabit the phloem sieve elements of plants and the gut, hemolymph, salivary gland, and other organs of sap-sucking insects. They are sensitive to tetracycline antibiotics, but not to penicillin. They are characterized by low content of guanine and cytosine (23 – 29 mol%), UGA is used as a stop codon instead as a tryptophan codon as in several other mycoplasmas and membranes are resistant to digitonin and sensitive to hypotonic salt solutions. Specific nucleotide signatures that are characteristic of '*Ca*. Phytoplasma' are: adenine at position 242, thymine at position 286 and at position 1247 (in the sequence of *Oenothera* phytoplasma 86-7 with GenBank accession number M30790) (IRPCM, 2004).

Phytoplasmas can adopt two diverse environments as intra-cellular parasites of both plants and insects. Microarray analysis of '*Ca*. P. asteris' OY-M strain revealed that expression of approximately 33% of the genes changes during host switching between plant and insect,

suggesting that phytoplasmas dramatically alter gene expression in response to their host (Oshima *et al.*, 2011) and may use transporters, secreted proteins, and metabolic enzymes in a host-specific manner (Oshima *et al.*, 2013).

In summary phytoplasmas are a large group of plant-pathogenic unculturable gram-positive wall-less microorganisms, non-helical, small size chromosome bacteria parasitizing the plants and causing different diseases and transmitted by insects. They are collectively referred to as yellows diseases, in more than a thousand plant species worldwide, and their classification is based on RFLP of 16SrDNA (Marcone, 2014; Lee et al., 2013; Bertaccini and Lee, 2018, Namba, 2019).

2.2 Phytoplasma taxonomy and genetic diversity

"*As phytoplasmas are discovered at an ever-increasing pace in emerging and re-emerging plant diseases worldwide, the scheme for classification of phytoplasmas into 16S rRNA gene RFLP (16Sr) groups and subgroups is experiencing an ongoing rapid expansion. Improper delineation or designation of new groups and subgroups can open potential conflicts in classifying newly identified phytoplasma strains. To maintain the integrity of the classification scheme, criteria for the delineation of new groups and subgroups must be followed, and proper registration should be required to track established groups and subgroups*" (Zhao and Davis, 2019).

Phytoplasmas are difficult to culture and that created real difficulties in their classification. The development and use of molecular-based techniques to detect, identify, and classify the phytoplasmas was resulted into new insight and knowledge of broad aspects of phytoplasmas including their plant hosts, insect vectors, geographical distributions and dispersal, phylogenetic relationships and genetic diversity, and the classification systems has emerged (Lee *et al*., 2000). The molecular approach showed that phytoplasmas constitute a large monophyletic group within the class Mollicutes (Lee *et al.*, 1998; Quaglino *et al.*, 2009) and allowed the designation of a new taxon named '*Candidatus* Phytoplasma' (IRPCM, 2004).

Based on the similarity of 16S rRNA gene sequences supported by phylogenetic analysis, 47 '*Candidatus* Phytoplasma' species have been described, (Davis *et al.*, 2013; Harrison *et al.*, 2014; IRPCM, 2004; Nejat *et al.*, 2013, Quaglino *et al.*, 2013, preez-lopes *et al*.,2016, bertatccini *et al.* 2018). Classification of phytoplasmas is further supported by the 16S rRNA gene using restriction fragment length polymorphism (RFLP) of the 16S rRNA F2nR2 fragment with a set of seventeen endonucleases. This approach identifies at least 33 groups of phytoplasmas, and each group including subgroups designated by letters and the

majority of such groups have various number of subgroups (Harrison *et al.*, 2014; Pérez-López *et al.*, 2016a; Zhao *et al.*, 2009).

The validation of a computer simulated (*in silico*) RFLP as an alternative to the actual (*in vitro*) RFLP, along with the development of the interactive online phytoplasma classification tool *i*PhyClassifier, increased the accuracy of phytoplasma classification based on 16S rRNA gene sequences (Wei *et al*., 2007,; 2008; Zhao *et al*., 2009; Quaglino *et al*., 2009). A novel '*Ca.* Phytoplasma' species can be named only if its 16S rRNA gene sequence has <97.5% similarity to that of any of the previously described species or if there are sufficient biological and genetic characteristics to warrant the designation of the new taxon (*IRPCM, 2004*). However, many other genes have been used to identify, characterize, and differentiate between the various phytoplasmas species and their groups. The threshold to delineate new *cpn60* UT subgroups (0.97), corresponds with the threshold to delineate new 16S rRNA gene subgroups (Wei *et al*., 2007; Perez-Lopez *et al*., 2016).

2.3 Phytoplasma whole genomes

Presently, six complete phytoplasma genomes have been sequenced (there are other 15 in progress) (Monje and Lara, 2019), and that led to identify a significant number of genes considered playing a major role in phytoplasma-host (plant and insect) interactions (Marcone, 2014). The full genome sequence has been completed for two strains of aster yellows ('*Candidatus* Phytoplasma asteris'), two strains of '*Ca*. P. australiense', and a strain of '*Ca*. P. mali' (Oshima *et al.*, 2004; Bai *et al*. 2006; Kube *et al.* 2008; Tran-Nguyen et al. 2008; Andersen *et al*. 2013). In addition, draft genome sequences were determined for several phytoplasmas including four strains of the X-disease phytoplasma group, two strains of the stolbur phytoplasma, the peanut witches'-brooms, wheat blue dwarf agents and one strain of Almond witches'-broom (Saccardo *et al*., 2012; Chung *et al.*, 2013; Chen *et al.*, 2014; Quaglino *et al*., 2015; Zamorano and Fiore 2016).

Some phytoplasma strains which may warrant designation of a new taxon but fail to meet the requirement of sharing <97.5% sequence similarity with existing '*Ca*. Phytoplasma', can be differentiated and classified using additional unique biological properties such as antibody specificity, host range, and vector transmission specificity (Seemüller and Schneider, 2004; Bertaccini and Lee, 2018).

2.4 Symptomatology of phytoplasma-associated diseases

Phytoplasma refers collectively to yellows diseases. Plants infected by phytoplasmas express a wide range of symptoms. Certainly, there is no specific symptoms indicating certain phytoplasma species or group and subgroups. Moreover, mixed infections by one or more species/ groups or/ and with viral infections have been quietly reported. However, symptoms of affected plants may vary with the phytoplasma strain, host plant, stage of the disease, age of the plant at the time of infection, phytoplasma concentration in infected tissues, strain interactions and environmental conditions. The most common reported symptoms were virescence (the development of green flowers and the loss of normal flower pigments), phyllody (the development of floral parts into leafy structures), sterility of flowers, early flowering, big bud, proliferation of axillary shoots resulting in a witches'-broom appearance, abnormal elongation of internodes resulting in slender shoots, generalized stunting (small flowers and leaves and shortened internodes), discolorations of leaves or shoots, leaf curling or cupping, bunchy appearance of growth at the ends of the stems, and generalized decline (stunting, die back of twigs, and unseasonal yellowing or reddening of the leaves) (Figure 2) (Lee *et al*., 2000; Kumari *et al*, 2019; Omar *et* al, 2018; Namba, 2019).

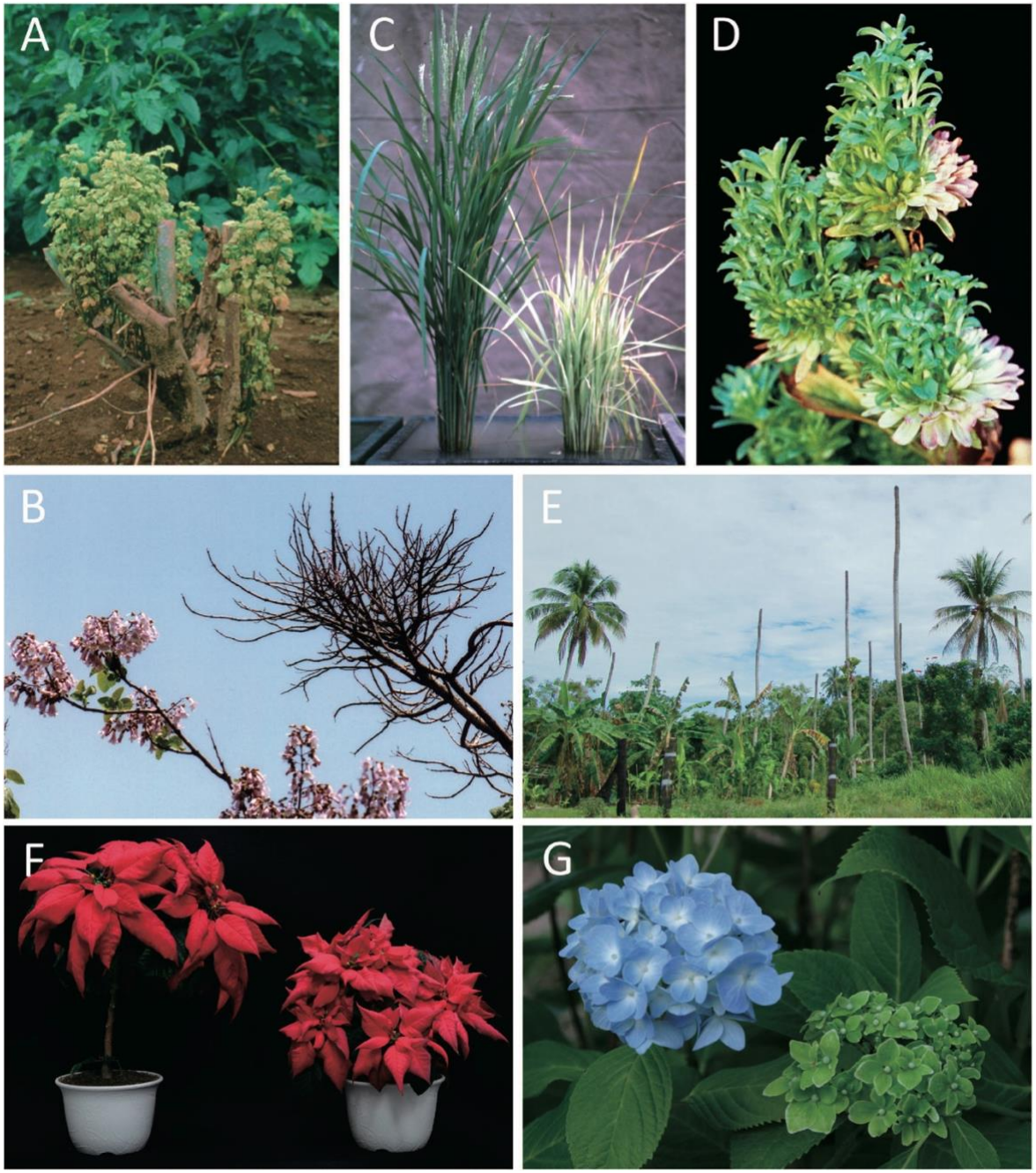

Figure 2. Various symptoms caused by yellows diseases. (A) Mulberry dwarf. (B) Paulownia witches' broom. (C) Rice yellow dwarf. (D) Aster yellows. (E) Coconut lethal yellowing. (F) Poinsettia witches' broom. (G) Hydrangea phyllody. (B, C, F, G) right side: infected plants; left side: healthy plants (Namba, 2019).

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Chapter 3. PHYTOPLASMAS IN MENA REGION

The MENA region is a transcontinental region that includes Western Asia, Iran, Turkey, and North Africa including Sudan, in which agriculture plays a vital economical role, improving livelihoods and food security situation. Many informative phytoplasmas reviews have been published and discussed various phytoplasmas aspects and concerning their vectors (Weintraub and Beanland, 2006), global status of phytoplasmas diseases in vegetables crops (Kumari *et al*., 2019), grape yellows in Southern and East Asia (Pierro *et al*., 2019), and phytoplasma in Iran (Siampour, *et al.,* 2019). Recently, phytoplasmas in Middle East have been described (Hemmati *et al*., 2021), summarizing the phytoplasma groups per crop. In the current review, general background about phytoplasmas in wider area (MENA) and focusing on the phytoplasma groups and subgroups, the most common phytoplasmas species and associated diseases, potential and insect vectors, symptoms, and mixed infections were highlighted and provided. Then, the review was more focused on the phytoplasmas infecting grapevines and stone fruits in this region, and the available information from Jordan was covered and discussed.

3.1 Literature survey

The following web sites were accessed to collect the target literature and obtain the essential and important information and Knowledge; FAO (***https://www.fao.org/faostat/***), Scopus (***www.scopus.com***), web of science (www.webofknoledge.com), google scholar (www.scholar.google.com) and research gate (www.researchgate.net). The references provided by each article were checked carefully. The access dates in these websites were lasting from October 2020 until the End of August 2021. The survey focused on all articles reporting phytoplasma detecting, occurrence, identification, characterization, and associated diseases in the MENA countries. The search terms included: phytoplasmas, MENA countries, grape yellows, phytoplasma solani, phytoplasmas and crops, vectors, witches'-broom and other phytoplasma related symptoms and aspects.

3.2 Background

Fruit trees including stone fruit, grapevine, and pome fruit, are largely cultivated, highly commercial revenues and exporting value, with special important for global agricultural industry as well as consider a major food nutrition and security crops and play a significant role in the economy over the world and for MENA region, with significantly expanding areas.

MENA Countries are among top ten largest s producers of Grapes, stone fruit, and pome fruits. For example, six out of the top ten almond producers were MENA's countries in 2019, including Iran, Turkey, Morocco, Syria, Tunisia, and Algeria. Turkey and Iran were the largest two producers of apricot in the world, sharing into 27.82 and 16.08%, respectively, and all of Algeria, Morocco and Egypt were among the top 10 largest ones. Turkey was the 5th highest exported dollar-value worth of fresh apricot, and its dried apricot exported to 112 countries. Three out of ten larger producers of peach and nectarine including Turkey, Iran, and Egypt. Turkey was the fourth largest producers of almond, and fifth one of peaches and nectarines. Iran also, is the third largest producer of almond with harvested area 80, 000 ha producing 177,000 t in 2019, and ranking among top ten largest producers of apple, peach, nectarine, and apricot in 2019. In terms of the importance of stone fruit, almond in particular, Syria is ranked the 7th larger producers over the world with harvested area 71,000 ha and total production around 80,000 t (FAOSTAT,2019).Tunisia is the eight largest producers of Almond with harvested area estimated in 2019 by 225,500 ha produced around 80,000 tones. The total areas of plum, apricot, grapes and pear were 2,270; 7,000; 26,200 and 2,200 ha, respectively in 2019. (FAO STAT, 2019; Asgharipour, Mousavinik, & Enayat, 2016). Based on FAO STAT (2019), 5, 000 ha of almond produced around 30, 000 t. peaches and nectarines distributing on 3,400 ha and produced 47,000 t in Lebanon.

In Turkey, grapevine cultivation is present since more than 6000 years (Ertunc *et al,* 2015), and the total harvested area in 2019 was 405, 000 ha produced around 4,100,000 t. Grape is widely growing in Iran with harvest area around 155,000 ha producing 2,000,000 tones in 2019 (FAO STAT, 2019). Lebanon produced around 62, 000 t of Grapes from 7,000 cultivated ha. in 2019, Syria harvested 45,000 ha of grapes, that producing more than 252,000 tons, and 772,000 tons (FAOSTAT,2019).

Pomegranate is growing economically in all MENA countries, Iran and Turkey are contributing to considerable portion of global production and exporting's. MENA is the leading of global dates production and exporting's. (FAO STAT, 2019; ***https://www.intracen.org/news/The-global-trade-in-dates***). Iran is one of the most important pomegranate producers and exporters in the world with an annual production of about 900,000 tonnes, and annual export of more than 150,000 tonnes (Tehranifar *et al.*, 2010; Salehi *et al.*, 2016). Total pomegranate production reached 315.000 tons in 2012 that corresponds to place Turkey as one of the largest pomegranate economies in the world (Gazel *et al*., 2014).

In Jordan, Grapevines, stone fruit, and pome fruits are major and very important exporting crops. They consider as food nutrition and security crops and various stakeholders are depending on their revenues. Jordan Valley, Al-Mafraq, rift valley and southern desert are the most cultivated areas in the country. The total amount of grapes harvested area in Jordan is around 3,000 ha produced about 54,000 ton in 2019, and the total exporting quantities was around 494 ton in 2017 (MOA,2021). The harvested area of stone fruit including peach, nectarine, plum, apricot, and sweet cherry was 6100 ha produced 117,000 ton in 2019 (FAO STAT, 2019). Almond cultivation is expanding in several rural areas and family farming instead of olive. Almond green fruits are gaining huge popularity and recorded unprecedented prices (€14/kg). The Jordanian exporting of stone fruit was estimated by 50,000 ton in 2017. Pomegranate is one of the eldest fruit trees in the country, and grow in the whole country as family farming, home gardens and expanding as a commercial farm in irrigated areas. Pomegranate is gaining huge popularity and marketing for good prices (MOA, 2021).

Many pests and diseases are threat the global food production. Phytoplasma and their associated diseases are emerging as a new major challenge facing the agricultural crops worldwide with significant losses of quantity and quality productivity and threat their surveillance over the globe (Bertaccini *et al.,* 2014; Rizza *et al.*, 2016; Kumari *et al.,* 2019).

Phytoplasma in MENA region back to four decades ago, where the first destructive phytoplasma diseases Witches' broom of lime (WBDL) '*Candidtaus* Phytoplasma aurntifolia' appeared in Oman, and rapidly spread to UAE and Iran (Al-Yahya, 2015; Al-Abadi et al, 2016). In Iran too, sesame phyolldy was reported three decades ago and the new phytoplasma era was started in this country with large body of research. Twenty years ago, an epidemic destructive almond witch's broom disease '*Ca*. P. phoenicium' (16SrIX-B) was reported in Middle East, in Lebanon and Iran, with huge losses (Abou-Jawdah et al., 2002; Verdin et al., 2003; Salehi et al., 2006; Abou-Jawdah et al., 2011). Phytoplasma disease have paying attention in many MENA countries due to their socio-economic impact and rapid spread, huge of susceptible plant hosts with many phytoplasma disease. Many phytoplasma groups and diver's species which associated with big number of diseases that infecting economic vegetables, fruit trees, forage, and animal feeding crops as well as alternative hosts, weeds, ornamental, and landscaping plant host were reported in this region.

3.3. Symptoms of phytoplasma-associated diseases in MENA Region

The typical and most common symptoms of phytoplasmas disease are varied and could be exhibited and observed everywhere over the world. In MENA region most of such

symptoms were associated with infected fruit trees, vegetables, and open field crops as well as herbaceous weeds and forestry wooden plants (trees). Almond early flowering, off-season growth, number of axillary branches (Lebanon and Iran), almond new growth during dormancy and leafed out before flowers opened in addition to early defoliation in summer in Tunisia, leaf scorch (grapevine in Turkey and Lebanon, respectively), malformation of fruits (where two or three fruits were attached together) in Egypt, (Abou-Jawdah *et al*, 2003; El-Saghir, 2017; Mahrous *et al*, 2018; El-Attar and Aljamali, 2013; Salem *et al*, 2013; Cronje *et al*, 2000; Hemmati *et al*, 2020; Omar *et al*., 2014; Omar *et al*, 2020, Hosseini et al, 2015; Çağlar *et al*, 2018; Ben Khalifa and Hatem Fakhfakh, 2011; Kumari *et al*, 2019; Ermacora and Osler 2019; Wei *et al*, 2019). Plum decline disease 1(6SrX-A) where the infected trees exhibited leaf and fruit wilting followed by tree death in a few weeks (Ben Khalifa and Fakhfakh, 2011).

Severe redness, shriveling of berries, and inward curling of leaves were associated with BN diseases in Turkey (Ertunc et al., 2015). Leaf reddening, leaf rolling, and yellowing were exhibited by BN infected grapes in Iran (Mirchenari et al., 2015). However, typical symptoms of grape yellows were reported in Syria, Jordan, and Lebanon (Contaldo et al., 2011; Salem et al., 2013, Foissac et al., 2018).

Globally, it seems to be that phytoplasma diseases and its groups have various patterns of symptoms and infections (plants and groups- based symptoms patterns). It is very common (confirmed) that the different phytoplasmas depict different symptoms in varied plant hosts. Good example is with ribosomal group of 16SrII, where the little leaf, yellowing, leaf rolling and red coloration, virescence and phyllody stunting, in different crops including pomegranate and grapevines in Iran (Omar *et al*., 2012). Furthermore, different phytoplasma groups may be induced different symptoms in one plant. For instance, almond witches-broom, almond little leaf, and almond yellows diseases in the center of Iran were associated with the new phytoplasmas that have never been described to be associated with almond diseases anywhere. They were phytoplasmas related to members of peanut WB (16SrII), clover proliferation (16SrVI) and stolbur (16SrXII) groups in almond (Zirak and Ahoonmanesh, 2009). Moreover, some studies indicated two or more different phytoplasma groups in single plant. The 16SrX-B (‘*Ca.* P. prunorum’), 16SrX-C (‘*Candidatus* Phytoplasma pyri’) and mixed infection of 16SrX-A/16SrX-C and 16SrX-C/16SrI (aster yellows) were detected in the apricot in Turkey (Orel *et al*, 2019). Diverse ‘*Ca*. phytoplasma’ species were associated with grapevine decline in Iran and the following four ‘*Ca.* P. species were reported; ‘*Candidatus* Phytoplasma fraxini,

‘*Ca*. P. aurantifolia’, ‘*Ca*. P. solani and ‘*Ca.* P. phoenicium’-related strains (Zamharir *et al,* 2017).

Additional example was the grapevine yellow (GY) in grapevines in Syria with stolbur (16SrXII) and the other tentatively related to clover proliferation (16SrVI) (Contaldo *et al,* 2011), and manifested in Iran were three phytoplasmas diseases from two different groups (16SrI and 16SrX) associated with pear decline including ‘*Ca.* P. asteris, ‘*Ca*. P. pyri’ and ‘*Ca.* Phytoplasma prunorum. ‘*Ca.* P. asteris associated with PD was the first report in the world (Hashemi- Tameh *et al,* 2014).

Moreover, the phytoplasma infection could be mixed with virus infections, and such kind of infections were manifested (reported) in peach trees in Egypt with Prunus necrotic ringspot virus, and the mixed infection by Phytoplasma (16SrII) and Sugarcane Yellow Leaf Virus Associated with Leaf Yellowing of Sugarcane (16SI) (ElSayed *et al,* 2016).

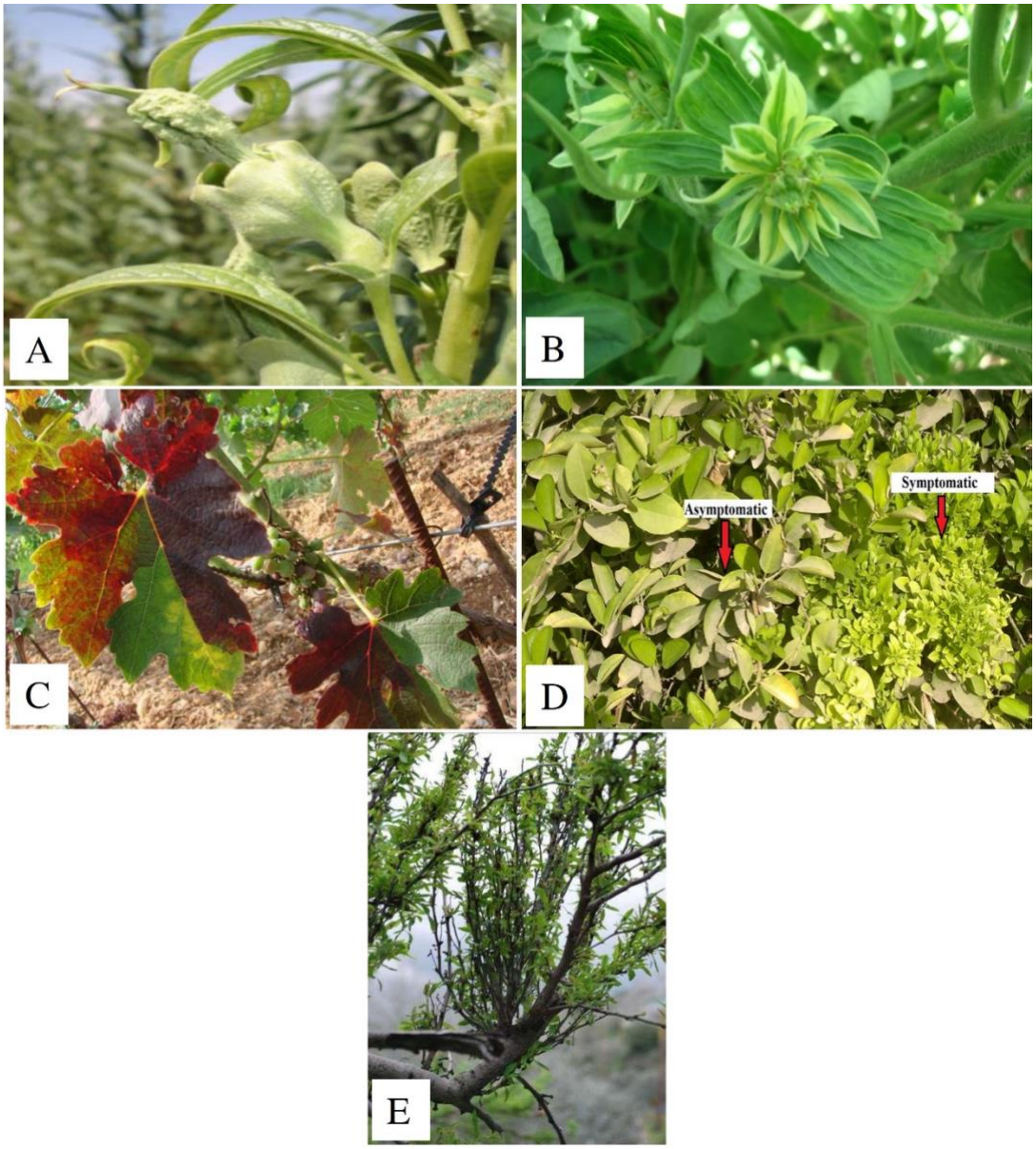

Figure 3. Symptoms on selected infected crops in MENA region, A. Tomato big bud (TBB) floral phyllody and virescence in Iran (Jamshidi *et al*, 2014), B. internode shortening symptoms of sesame phyllody disease in Iran (Salehi *et al*, 2016). C. Typical symptoms of grapevine yellows: reddening of leaves and shriveling of berries in cv. Merlot in Turkey (Ertunc et al, 2015), D. Symptomatic and asymptomatic branches on an acid lime infected by '*Ca.* P. aurantifolia (Al-Abadi et al, 2016), E. Almond witches'-broom in Lebanon (Abu Jawdah).

3.4 Phytoplasma Taxonomy, Genetic Diversity, and Geographical Distribution In MENA

More than 12 '*Candidatus* Phytoplasma' species, that belong to 14 out of 33 taxonomic phytoplasma groups, were reported in MENA region, with diversity of subgroups. The major MENA phytoplasma groups are shown in Figure 4. Draft genome to genetic diversity among strain populations of '*Candidatus* Phytoplasma phoenicium' associated with almond witches'-broom disease was completed and published (Qualingo *et al*, 2015). Several '*Candidatus* Phytoplasma' species and novel subgroups, diseases and infected plant were reported in MENA for the first time in the world (Hashemi-Tameh, 2014; AlSubhi,2017; Oksal 2020; Omar *et al*, 2020), and other genetic diversity of phytoplsama subgroups and disease were also, reported. Mixed infections by different phytoplasma groups and/ or subgroups as well as with viral diseases were identified. Various alternative and reservoir weeds plants that belong to many plant families were associated with many phytoplasma diseases. Less studies investigated the Auchenorrhyncha community is majority of insect putative/ potential and / or vectors are not identified and poorly understood, but some of the important potential and vectors were identified, and successful transmission trial were implemented. Iran and Turkey reported the highest phytoplasma groups followed by Oman, Saudi Arabia, and Lebanon, respectively.

The 14 reported MENA's phytoplasma groups were 16SrI, 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 6SrXIV, 16SrXV, 16SrXXIX. The major and most common group was Peanut witches' broom group (16SrII), and associated with broad range of various crops, and five out of 24 global subgroups belong to it were reported in MENA, as a second diverse group after 16SrIX Pigeon pea witches'-broom group which presented nine subgroups. Both groups of 16SrIII and 16SrV were very restricted into geographical area and host. Phytoplasma 16SrIII, '*Ca*. P. pruni' (X- diseases) was limited to Iran, associated with one plant and the disease of pomegranate decline. 16SrV was detected in Tunisia with potential vector and in Turkey infected Grapevine. Six phytoplasma groups were associated with dates. Five out of seven global groups were reported on Solanaceae crops. Seven out of seven global groups were infected grapevines, BN disease was common in many countries, while the FD diseases was reported in Turley. Six and four groups were infected stone fruit and apple, respectively. Three '*Candidatus* Phytoplasma' species of AP group were presented. '*Ca*. P. pronourum' ended the infected apricot trees within few weeks in Tunisia and three years in Turkey and was able to reduce the apricot yield up 77% and infect all local and

imported stone fruits. Two groups were infected cucurbits, and cucumber phyllody was very destructive with 100% losses in some seasons. Three phytoplasma groups were infected legumes and four were infected pomegranate. Four and five out of 12 groups were infected oilseeds and alfalfa crops, respectively. Eight and nine out of 14 reported groups were detected in weeds and insect fauna (potential and vectors), respectively.

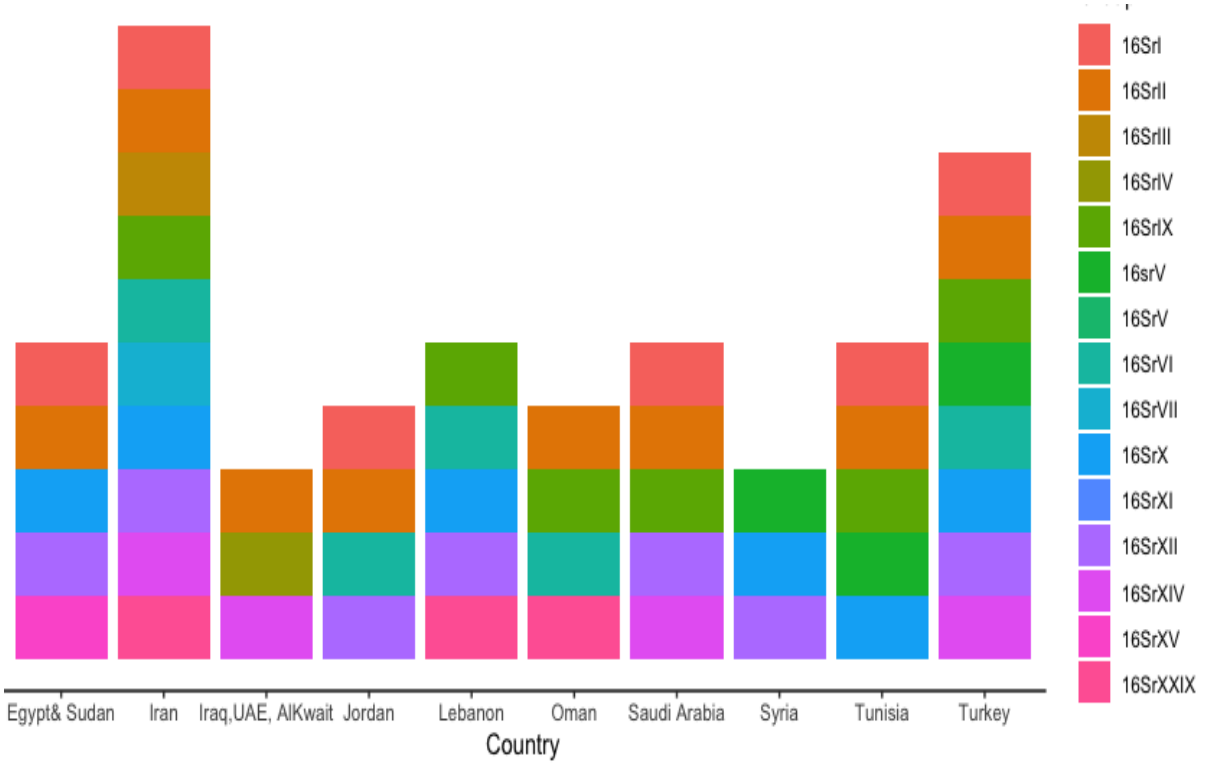

Figure 4. Phytoplasma distribution in MENA region.

Among seven phytoplasma groups infecting grapevine in the world (Angelini 2010; Constable 2010; Duduk *et al*. 2010), four of them were reported in Turkey. They are 16SrXII-A, 16SrV, 16SrI-B, and 16SrIX. Both Stolbur BN and FD are very important and widespread in main viticulture areas in Turkey. It was reported that the wine varieties infections were more comparing with the table varieties. Delphacid *Laodelphax striatellus* and *Cicadelids Empoasca sp*., *Euscelis incisus* and *Psammotettix sp* were potential vectors of BN disease. (Canik *et al*, 2011; Ertunc *et al*, 2015; Arabiçak *et al*, 2020).

Four phytoplasma groups including 6SrXII, 16SrVII, 16Sr I- B-C were reported infection grapes in Iran. Bois noir" (BN) is the most important and widespread phytoplasma disease on grapevine in Iran. The incidence of BN throughout the world has been over 80% in a year in some vineyards. The first BN outbreak was reportd in five Persian provinces in 2015. *Hyalesthes obsoletus* is the main vector of BN present in Iranian vineyards (Constable, 2010;

Karimi *et al.,* 2009; Salehi *et al*., 2014; Ghayeb Zamharir *et al*., 2017; Mirchenari *et al,* 2015; Ghayeb Zamharir *et al*., 2016; Babaei *et al*, 2019).

BN disease of Stolbur 16SrXII-A subgroup was reported in Lebanon in 2007 (Choueiri *et al*, 2007). Recently, in addition to Candidatus Phytoplasma solani, grapvines in Lebanon were associated with '*Ca. P. omanense'* which belong to 16SrXXIX, *Cassia* witches' broom group (Foissac et al., 2018). Interstingly both diseases were detected in Cixiid planthoppers *Hyalesthes obsoletus* and *Reptalus sp.,* (Choueiri, et al, 2019). Both grapevines phytoplasma diseases were detected in bindweed *Convolvulus arvensis* (Choueiri, *et al*, 2019). Interestingly, two phytoplasmas species belonging to two different groups (Stolbur - 16SrXII and clover proliferation- 16SrV) were infected the grapevines in Syria. Grape yellows in Tunisia were associated with aster yellows (16SrI-B), exhibited plant weakness, incomplete lignification, flexible shoots, and drooping. Affected leaves were thicker than normal, brittle, rolled downward and showed veinal yellowing and necrosis. In addition, grape bunches became dry and shrivelled before fruit could fully develop and ripen (Nahdi *et al.,* 2020). Among 10 Cicadomorpha insect species collected from grapevine in Tunisia, the following phytoplasma were detected: Clover phyllody and strawberry green petal phytoplasmas in *Euscelis lineolatus* Blöte; 16SrV, 16SrIX and Bermuda grass white leaf phytoplasmas in *Exitianus capicola* (Stål); "stolbur" phytoplasmas in *Psammotettix alienus* (Dahlbom); European stone fruit yellows (16SrX-B) phytoplasmas in *Empoasca sp*.; and "Stolbur", aster yellows and 16SrII phytoplasmas in *Austroagallia sinuate* (Mulsant & Rey) (Nahdi et al 2020).

The most destructive phytoplasma diseases was AlmWB which is dwelling by the etological agent of '*Ca*. P. phoenicium') of 16SrIX group, in Iran and Lebanon. it was the most common and diverse phytoplasma in Lebanon with five subgroups; B-D-F-G and C. AlmWB '*Candidatus* Phytoplasma phoenicium' sp. nov., a novel phytoplasma associated with an emerging lethal disease of almond trees is proposed for the phytoplasma associated with almond witches'-broom in Lebanon and Iran (Choueiri et al., 2001; Abou-Jawdah et al., 2002, 2009; Verdin et al., 2003). The infection of AlmWB was associated with no flowers and no fruit and losing the trees within few years. Two phytoplasma subgroups 16SrIX-B and -D were identified at the beginning. In 2009, '*Ca*. P. phoenicium' was identified also in association with a severe disease of peach and nectarine in southern Lebanon (Abou-Jawdah et al., 2009) and more than 40,000 newly diseased trees were observed in 2010 throughout the country. The disease spread rapidly in Lebanon from coastal areas to elevations exceeding 1200 m, killing over 150,000 trees in a span of two decades (Abou-Jawdah *et al,* 2014). Further studies discovered two new subgroups F and -G, in group 16SrIX of this disease and to the five global

previous subgroups. Therefore, Lebanon is hosting five out of seven subgroups of Pigeon Pea Witches-Broom (PPWB- 16SrIX). 16SrIX B-D-F- and G Subgroups were presented in Nectarine, 16SrIX -D- G were detected in almond and peach (Lova *et al,* 2011). The genetic diversity within '*Ca.* P. phoenicium' strain populations in Lebanon suggested that AlmWB disease could be associated with phytoplasma strains derived from the adaptation of an original strain to diverse hosts. Moreover, the identification of a putative inhibitor of apoptosis-promoting Bax factor (BI-1) in '*Ca*. P. phoenicium' draft genome and within genomes of other '*Ca.* Phytoplasma' species suggested its potential role as a phytoplasma fitness-increasing factor by modification of the host-defense response (Quaglino *et al,* 2015).

Grafting experiments and molecular analyses have revealed that '*Ca*. P. phoenicium' does not affect plum, apricot, and cherry trees (Abou-Jawdah *et al*., 2003). Although, most of the vectors fauna of this disease is not understood yet, several Surveying missions and transmitting trials were led to identifying several potential and vectors of AlmWB in Lebanon (discussed in the epidemiology section). However, *Asymmetrasca decedens* is a vector of AlmWB in Lebanon (Abou-Jawdah et al, 2014). Additionally, AlmWB can be graft transmitted to almond, nectarine, and peach (Abou-Jawdah, et al, 2002). Abou-Jawdah et al, 2011) detected the Aster yellows (AY) group 16SrI-F subgroup in *Psammotettix provincialis.* Infections of 12 weed plants were reported in Lebanon harbored 16SrIX-C subgroup (Table 1) (Casati *et al* ,2016).

Phytoplasma of 16SrIX group reported six subgroups, namely, 16SrIX-B, 16SrIX-C, 16SrIX-E, 16SrIX-M, 16SrIX-J and 16SrIX-I. Wild almond was reported as a potential sources of AlmWB (16SrIX-B) in Iran (Salehi *et al.,* 2015). Also, it was associated with apricot yellows (Salehi *et al*., 2018), peach witches'-broom disease (PWIB) (subgroup 16SrIX-B) and '*Ca.* P. aurantifolia' (subgroup 16SrII-C) (Salehi *et al,* 2019). Almond witches-broom, almond little leaf and almond yellows diseases were associated with peanut WB (16SrII), clover proliferation (16SrVI) and stolbur (16SrXII) groups in almond (Zirak; M. Bahar; A. Ahoonmanesh 2009). '*Ca.* P. phoenicium' (16SrIX) was reported with sweet orange with declining symptoms (Abbasi et al, 2019). Also, it was associated with apricot yellows (Salehi *et al*., 2018), peach witches'-broom disease (PWIB) (subgroup 16SrIX-B) and '*Ca.* P. aurantifolia' (subgroup 16SrII-C) (Salehi *et al,* 2019). Almond witches-broom, almond little leaf and almond yellows diseases were associated with peanut WB (16SrII), clover proliferation (16SrVI) and stolbur (16SrXII) groups in almond (Zirak; M. Bahar; A. Ahoonmanesh 2009). '*Ca.* P. phoenicium' (16SrIX) was reported with sweet orange with

declining symptoms (Abbasi et al, 2019. '*Ca*. P. prunorum' has been associated with plum yellow leaf stunt disease. '*Ca*. P. asteris' and peanut WB group (16SrII) infect sweet cherry trees (Zirak et al, 2010; Allahverdi *et al*, 2014). '*Ca*. P. pruni (16SrIII, X- disease), and 16SrII-D were infected pomegranate (refs). More information about pomegranate phytoplasma is available in chapter five. Plum decline disease (16SrX- where the infected trees exhibited leaf and fruit wilting followed by tree death in a few weeks (Ben Khalifa and Fakhfakh, 2011). ESFY phytoplasma '*Ca*. P. prunorum' was associated with stone fruit in Egypt (Al khazindar and Abdel-Salam, 2010).

ESFY (16SrX-B) was associated with two major stone fruit in Tunisia. '*Ca.* P. prunorum' was detected in almond trees. The infected trees produced new growth during dormancy and leafed out before flowering in addition to early defoliation in summer (Ben Khalifa and Fakhfakh, 2011). Additionally, ESFY was reported on apricot (Ben Khalifa et al, 2011).

AP is listed in quarantine in Turkey and economically limits apple production in the world. Three main phytoplasma diseases of AP group were reported in Turkey infected apple, pear and stone fruit, and three subgroups were manifested (Orel *et al,* 2019), '*Ca.* P. *mali* of subgroup 16SrX-A, occurred in infected apple Golden Delicious orchards. The infected trees were cut down due to great yield losses that reached about 65%. (Canik and Ertunc, 2007; Yavuz *et al,* 2019). Pear decline (PD), associated with '*Ca*. P. pyri phytoplasma was reported in the northeastern part of Turkey (Çağlayan *et al.,* 2006) with serious outbreak leading to high 55% infection rate in various pear cultivars. It is widely distributed in pear trees with a high incidence, and it is a big threat for pear production in Turkey. (Gazel *et al*., 2007). Recently, a mixed infection by two subgroups, 16SrX-A and 16SrX-C was reported on pear trees (Orel *et al*., 2019). The 16SrX-B ('*Candidatus* Phytoplasma prunorum'), 16SrX-C ('*Ca.* P. pyri') and mixed infection of 16SrX-A/16SrX-C and 16SrX-C/16SrI (aster yellows) were detected in the apricot samples (Çağlayan *et al*., 2006, Serce *et al.,* 2006; Orel *et al*, 2019; OKSAL 2020). However, '*Ca.* P. prunorum', the causal agent of European stone fruit yellows (ESFY), is one of the most important pathogens causing considerable economic losses in stone fruit orchards (Nečas, 2018). It was infecting both local and imported Japanese stone fruit such as apricot, japanese plum, and almond (Serce *et al*; 2006), but the apricot was more devastating with apricot *P. armeniaca* (Çaglayan, *et al* 2011.), reduced its yield up to 80 %, and the trees death were reported within few years (Gazel *et al.,* 2009). Two phytoplasma subgroups were infected pomegranate trees in Turkey, 16SrI-B and 16SrXII-. (Gaze *et al,* 2014). *Cacopsylla pyri* and *C. pruni* were reported as insect vectors of PD and ESFY phytoplasmas respectively in Turkey

(Ulubaş Serçe *et al*. 2006, 2011). *C. pyricola* is known to transmit both PD and peach yellow leaf roll (PYLR) phytoplasmas. Stolbur", aster yellows and 16SrII phytoplasmas was detected in *Austroagallia sinuate* in Tunisia (Nahdi *et al* 2020).

3.5 Phytoplasmas in Jordan

The phytoplasmas diseases were associated with four phytoplasmas groups in Jordan. They were found associated with peach, tomato, grapevines, potato and plum. Tomato Big bud disease (clover proliferation - 16SrVI) was the first phytoplasma disease reported in Jordan in 2003 in Al-Mafraqa area (Anfoka *et al*, 2003). Followed by aster yellows phytoplasma (16SrI) on peach in 2004 in two regions (Anfoka and Fattash, 2004). '*Ca.* P. solani' (16SrXII) has been associated with grapevine ("bois noir" 16SrXII-A) (Salem *et al*, 2013) and plum (*Prunus domestica*) in Al-Mafraq area (desert) (Salem *et al*, 2019). '*Candidatus* Phytoplasma aurantifolia' was reported on potato in 2019 (Salem *et al*, 2019). Notably, that Al- Mafraq is growing multiple economic crops (grape, pome fruit, stone fruits, vegetable, forage, and feeding crops). It is sharing long borders with Syria and the bordered Syrian regions are growing the same crops too. Therefore, it is expected the natural dispersal of phytoplasmas from Jordan and vice versa. For example, '*Candidatus* phytoplasma mali' (Apple proliferation group- 16SrX) was reported on apple in Al-swidaa region. This region is very close to Al-Mafraq region and both areas have the same climate and growing apple (could be the same varieties). Further monitoring, detecting and surveys and further epidemiology research must be implemented in both countries and consider that the area is reported number of severe and epidemic diseases such as AlmWB, TBB and apple proliferation with abundant and huge diversity of Auchenorrhyncha species and psyllids. Approximately 114 Auchenorrhyncha species have been reported in Jordan in addition to 33 Psylloidea species (Nabas 2020; AI-Khawaldeh, *et al*, 1997).

3.6 Epidemiology of phytoplasma diseases in MENA Region

Phytoplasmas are quite often associated with severe and rapidly spreading plant diseases, modify insect fitness, and leading to various symptoms and plant morphological changes; in other case they are associated with severe decline and death of the infected plants (Bertaccini *et al.*, 2014; Hemmati et al., 2017; Rao, 2019). They are transmitted plant-to-plant by phloem feeders of the order Hemiptera, mostly leafhoppers (Cicadellidae), planthoppers (Fulgoroidea) and psyllids (Psylloidea), which feed on the phloem sap of infected plants, therefore, their host range is dependent upon feeding habits of their insect vectors (Weintraub and Beanland, 2006;

Bertaccini, 2007; Quaglino *et al.*, 2015). Phytoplasmas are also efficiently spread via vegetative propagation such as cuttings, grafting, seeds and micropropagation practices (Bertaccini 2007; Çağlar *et al* 2018).

In MENA region several destructive and epidemic phytoplasma diseases were reported. Witches'-broom disease in lime (WBDL) which is associated with '*Ca.* P. aurantifolia' in Oman, United Arab Emirates (UAE) and Iran killed more than a million lime trees. It was reported in certain regions of Oman and rapidly spread in many other parts. WBDL was reported in UAE in 1989 in Iran 1990s' (Al-Abadi *et al*, 2016; Al-Yahya, 2015). Almond witches'- broom (AlmWB) is very epidemic and destructive and devastated the almond orchards and killed over 100,000 almond trees in Lebanon and in Iran (Abou-Jawdah *et al.*, 2002; Verdin *et al.*, 2003; Abou-Jawdah *et al*., 2011, Salehi *et al*., 2006). In 2009, the natural and epidemic spread of '*Ca.* P. phoenicium' in peach and nectarine was reported, and the growers were advised to eradicate the infected trees immediately (Abou-Jawdah *et al*., 2009). The rapid geographical spread of this epidemic disease suggests an efficient vector(s). This etiological agent was associated with Peach in many regions and infected the stone fruits like peach, nectarine, and apricot (Salehi *et al*, 2020; Salehi *et al.*, 2018). The rapid spread of this devastating disease is alarming not only Iran and Lebanon but also other stone fruit growing countries worldwide (Siampour *et al.*, 2019 in). Zamharir (2011) expected that the disease could be challenged for almond producing countries. in conclusion '*Ca*. P. phoenicium' has all the characteristics of a severe quarantine pathogen (Abou-Jawdah *et al.*, 2014).

Another clear example about the spread of epidemic phytoplasma diseases in MENA is the AP diseases in Turkey with number of vectors and potential vectors. '*Ca.* P. prunorum' causing ESFY is transmitted by *Cacopsylla pruni* Scopoli in Turkey (Cieślińska, 2011) and its capability to infect broad range of local and imported stone fruits. The apricot losses were up to 75% and 65% of apple trees. Both pear decline and '*Ca*. P. mali were very severe and led to significant impact. Pear decline is associated with three phytoplasmas in Iran; '*Ca*. P. pyri', '*Ca*. P. asteris' and '*Ca.* P. prunorum' (Hashemi-Tameh *et al*, 2014). It seems that the cultivars and geo-climate conditions are playing a vital role in the type of disease. Plum decline in Tunisia kills the infected plum trees within few weeks. Grapevine yellows (GV), associated with '*Ca.* P. solani', the "bois noir" (BN) etiological agent is widespread in MENA (Lebanon, Syria, Jordan, Iran, Turkey). FD is a major grapevine disease in Turkey (Salem *et al*, 2013; Contaldo *et al.*, 2011; Choueiri *et al.*, 2007; Canik *et al.*, 2011. Ertunc *et al.*, 2015, Arabiçak *et al*, 2020). However, several phytoplasma groups were reported on grapes in the region (Table 1) with rapid dispersal. Additionally, in Lebanon, '*Ca*. P. omanense'-related strain was detected

in yellowing grapevine, bindweed (*Convolvulus arvensis*) and in collected Cixiidae planthoppers *Hyalesthes obsoletus* and *Reptalus* sp., (Foissac et al., 2019). The importance of such findings is that these phytoplasma species are detected in Oman in wild perennial legumes and Iran in bindweed (Al- Saady et al. 2008), and therefore, it is broader hosts. Considering that the two planthoppers *H. obsoletus* and *Reptalu*s sp. are widespread in the Mediterranean area and already known to vector '*Ca*. P. solani' strains associated with BN disease in grapevine, *'Ca*. P. omanense' could constitute a new threat.

Although phytoplasma diseases on date palm are not lethal so far, considerable losses were observed in Arab Gulf region. For example, fruit failure at harvest has been reported due to Al-Wijam in Kuwait and Saudi Arabia (Alhudaib *et al*. 2007; Hemmati *et al*, 2017), and some reports indicated that the losses associated with Wijam disease were more than 30–40% of date production and many palm trees were died (Alhudaib *et al.*, 2008). Significant vegetables phytoplasma diseases were reported in MENA, and some of them were resulted into huge losses. TBB have been reported in many MENA countries like Jordan, Syria, Iran, Lebanon, Turkey, and Egypt. It was associated with several phytoplasm groups. Potential vectors and wide range of such groups could be associated with unexpected epidemic crisis.
Vectors play a vital role phytoplasma distribution and spread (Rashidi *et al*. 2014; Linck and Reineke, 2019). Three major taxonomic insect groups were confirmed as vectors of phytoplasma diseases including Cicadellidae (belong to Cicadopmorpha group), Fulgoromorpha and the smallest one is the s Sternorrhyncha, in which only two genera in the Psyllidae are confirmed as vectors (Weintraub and Beanland, 2006). The epidemiology of phytoplasma-associated diseases is a consequence of the vectors' ability in acquisition, inoculation, dispersal, survival, host range, and habitat colonization. Within the same vector species, acquisition efficiency may depend on the phytoplasma load in source plants and on the vectors' life instar (nymphs versus adults) (Alma *et al*., 2019).

There is an information gap about the phytoplasma epidemiology in MENA region because the vectors are unknown. However, some studies have been investigated the phytoplasma putative vectors in MENA region, but their role is still very poor understood, and the list of vectors and potential vectors is not completed yet. In Saudi Arabia, *Cicadulina bipunctata* (Melichar) is a potential vector of Al-Wijam date palm disease (Alhudaib *et al*, 2007). One of the most important vectors of '*Ca.* P. phoenicium' (16SrIX-B) is the cicaddelid *Asymmetrasca decedens* (Paoli) (Hemiptera, Cicadellidae) (Abou-Jawdah *et al,* 2014).'*Ca*. Phytoplasma phoenicium' strains in Lebanon, were detected in four genera including *Cixius, Tachycixius, Eumecurus* and *Hyalesthes*. *Tachycixius* sp. specimens were able to transmit the

detected strains to healthy peach potted seedlings (Tedeschi *et al*, 2015). Nine species of leafhoppers belonging three subfamilies Phrodinae, Deltocephalinae, and Megophthalminae were reported as potential vectors of AlmWB in Lebanon. (Dakhila *et al* 2010). In Iran *Frutioidea bisignata* (Mulsant & Rey) was reported as potential vector of AlmWB (Siampour *et al.*, 2004, Zamharir, 2011). Vectors of '*Ca*. P. solani' (CaPsol) in most MENA's countries are unidentified, but in Turkey like other parts in Euro-Mediterranean regions the main insect vector is the cixiid *H. obsoletus* (Hemiptera, Cixiidae). This species was one of 114 Auchenorrhyncha species listed in Jordan (Nabas, 2020). It is well known that the potential vector doesn't confirm its ability to transmit the disease. It is expected that the insect is the dead-end host. So, to confirm the vector ability, transmission trials must be implemented and the phytoplasma to be detected using 16SrDNA molecular technique.

Both wild perennial and annual plants (herbaceous weeds and other wild plants) are important as they play a key role in the hosting and reservoir of the pathogens. As many parts of the world, MENA region reported many infected species that can play a key role in the diffusion the phytoplasmas diseases. The bindweed *C. arvensis* is reported in all surveyed MENA's countries. For example, "stolbur" phytoplasma (16SrXII-A) and for Pigeon pea witch broom in Labanon, Iran and Jordan. In Iran, Bindweed *C. arvensis* has double role. firstly, as a reservoir for some phytoplasmas also detected in alfalfa, and the second as infection source for '*Ca.* P. omanense' (Hosseini *et al*, 2016).

In addition to *C. arvensis*, 12 wild species were reported as reservoir for the AlmWB phytoplasma in Lebanon (Casati *et al.*, 2016). The potential adaptation of 16SrIX-C phytoplasma to numerous wild plants highlights the elevated risk of its spread throughout the Middle East and neighbouring geographic regions. It is well known that periwinkle is a natural host for most of the known phytoplasmas in various regions of the world, where two main group of symptoms have been reported: virescence and proliferation (Omar *et al*. 2010; Chen *et al*. 2011; Nejat *et al*. 2013; Omar and Alsohim 2016).

In conclusion phytoplasmas are associated with a broad range (hundreds) of plant diseases and many of them have significant economic losses and have quarantine status worldwide. MENA region reported considerable portion of the classified phytoplasmas groups and suffering of very destructive diseases (AlmWB, WBL, TBB). Moreover, the reservoir and alternative hosts are available and some of them with double role. Auchenorryncha community is present in the region. Based on many bio-ecological indicators and real climate change the phytoplasmas disease are subject to outbreak and diffusion in new parts of MENA countries. Therefore, more economic losses, food shortage and vulnerable to food insecurity are expected.

Little is known about the epidemiology of phytoplasma diseases due to lack of information about their vectors in the whole area, and the information about the status of phytoplasma in many countries still poor understood. More attention should be paid, and further studies and research must be implemented about the phytoplasmas and their epidemiology and be aware that phytoplasmas were detected in asymptomatic plants in the region.

Studies on insect vectors and their interaction with plants have yet to be made, which may lead to an understanding of the increasing incidence of phytoplasma diseases (Mitrovic *et al*. 2012). This information will provide a basis for future investigations concerned with epidemiology of the disease, host range studies and identification of possible insect vectors by molecular techniques (Win Nang and Jung, 2012). In the absence of proper control methods, this information is useful for the early detection of infected material to prevent further spread of the phytoplasma diseases (ElSayed *et al*, 2014).

3.7 Conclusion

The geographical distribution and impact of phytoplasma diseases primarily depends on the host range as well as the feeding preference of insect vectors (Kumari et al, 2019). Out of 33 phytoplasma groups, 14 were reported in MENA detected in symptomatic and asymptomatic plants. All epidemic and outbreaks elements in terms of broad range of host plants and diseases diversity, alternatives and reservoirs plant, diversity and abundant of insect vectors were reported Several destructive and epidemic diseases leading to significant losses have been reported. Agri-trading rules, and certificate measures must be activated with special attention for the seedling movement. Further studies about the epidemiology of phytoplasmas, surveying missions as well as must be implemented along with increase the awareness about the importance of phytoplasmas aspects. Listing and identifying the vectors, and beneficial fauna that could be associated with phytoplasma vectors must be considered, and for more precise identification, adopting molecular techniques will be useful. The surveying and detecting missions must include both symptomatic and symptomless plants. Sustainable management approach is powerful solution. Phytoplasmas are transregional and transcontinental, therefore exchange and sharing the information and collaboration in the region are useful and inevitable.

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Chapter 4. Association of seven distinct '*Candidatus* Phytoplasma' species with almond diseases in Jordan, and preliminary information on their putative insect vectors

4.1 Abstract

During field surveys carried out from June to October 2020 and in January 2021 in northern Jordan, five categories of phytoplasma-like symptoms, including early flowering along with evergreen pattern; witches'-broom, yellowing, and dieback; slim leaf and leaf rolling; stem fasciation, were observed in almond (*Prunus dulcis* (Mill.) D.A. Webb, 1967) trees. Disease incidence in the investigated orchards ranged from 20 to 85%. Nested PCR-based amplification of *16S rRNA* gene detected phytoplasmas in 23% of collected symptomatic almond trees. Amplicon nucleotide sequence analyses allowed attributing the detected phytoplasmas to '*Candidatus* Phytoplasma asteris' (taxonomic subgroups 16SrI-B and -R), '*Ca.* P. aurantifolia' (16SrII-B and -C), '*Ca.* P. omanense' (16SrXXIX-A and -B), '*Ca.* P. phoenicium'(16SrIX-B), '*Ca.* P. pyri' (16SrX-C), '*Ca.* P. solani' (16SrXII-A), a cnd '*Ca.* P. ulmi'(16SrV-A). Such phytoplasmas were found associated with specific symptoms and differentially distributed in the considered locations. Moreover, further investigation identified '*Ca*. P. asteris' (subgroup 16SrI-R) in putative insect vectors *Agalmatium* sp., *Empoasca* sp., *Reptalus quinquecostatus*, and *Hyalesthes obsoletus*, '*Ca*. P. pyri' in *Cacopsylla bidens*, *Cicadulina bipunctata*, *Laodelphax striatellus*, and *Tettigometra* sp., and '*Ca*. P. omanense' (subgroup 16SrXXIX-B) in the non-crop plant *Amaranthus* sp. In conclusion, this study described an almond disease complex associated with multiple phytoplasmas, including '*Ca*. P. pyri', '*Ca*. P. omanense', and '*Ca*. P. ulmi' never reported before in this crop. Further studies are needed to survey the diffusion of this almond disease complex in the region, demonstrate the transmission capability of the identified putative vectors, and in-depth investigate the ecologies of all phytoplasmas associated with the disease.

4.2 Introduction

Almond (*Prunus dulcis* (Mill.) D.A. Webb, 1967) is one of the most important stone fruit crops in Middle East and North Africa (MENA) region, in which also wild almond is very common. In 2019, six out of the top ten almond producers over the world were from MENA including Iran, Turkey, Morocco, Syria, Tunisia, and Algeria (https://www.fao.org/faostat/en/#rankings/countries_by_commodity). In Jordan, stone fruits are the most important exported fruit crops (59,425 ton in 2020) (MOA, 2021). Within stone fruits, almond cultivation is expanding in several rural rainfed areas as family farming instead of olive, the most common inherited economic tree. Recently, almond commercial orchards have been established in the irrigated areas of the Country, and the green fruits of almond are gaining huge popularity and recorded unprecedented prices. Many phytoplasmas were reported in association with almond diseases in MENA countries including Iran, Lebanon, Turkey, and Tunisia (Hemmati et al., 2021). Phytoplasmas are a large group of phloem-restricted, cell wall-less bacteria that infect nearly a thousand of plant species worldwide (Gasparich, 2010). They are transmitted plant-to-plant by phloem-feeding insects, mainly leafhoppers (Cicadellidae) (Weintraub and Beanland, 2006; Alma et al., 2015)**,** and their sever epidemic outbreaks can induce losses up to 70-100% (Bertaccini et al., 2014; Kumari et al., 2019). Almond witches'-broom (AlmWB) is the most destructive phytoplasma disease of almond, associated with '*Candidatus* Phytoplasma phoenicium' (taxonomic subgroup 16SrIX-B and its variants) in Middle East (Abou-Jawdah et al., 2002; Verdin et al., 2003; Salehi et al., 2006; Molino Lova et al., 2011; Mosayyebi et al., 2021). The most characteristic symptoms of AlmWB are shoot proliferation on the main trunk with the appearance of a witches'-broom, perpendicular development of many axillary buds with small and yellowish leaves, and general tree decline with final dieback. More than 100,000 of almond trees were eradicated in Lebanon due to AlmWB outbreak in 2002 (Abou-Jawdah et al., 2003). In Lebanon, AlmWB epidemiological cycle involves *Asymmetrasca decedens* (Paoli) (prevalent in almond), possibly responsible for the transmission of '*Ca*. P. phoenicium' from almond to almond, and cixiids of the genus *Tachycixius* (prevalent in *Smilax aspera* L. and *Anthemis* sp.), possibly responsible for the transmission from weeds to almond (Abou-Jawdah et al., 2014; Tedeschi et al., 2015). In Iran, *Prunus scoparia*, a wild almond species harboring '*Ca*. P. phoenicium', could play a role in the phytoplasma transmission pathways to fruit trees (Salehi et al., 2015). Based on detection of '*Ca*. P. phoenicium' in insect body and saliva and the presence of consistent populations, the leafhopper *Frutioidea bisignata* Mulsant and Rey can be considered as potential vector of this

phytoplasma in Iran (Taghizadeh and Salehi, 2002; Siampour et al., 2004). In the last years, '*Ca*. Phytoplasma phoenicium' was associated with diseases of stone fruits including peach, nectarine, apricot, and cherry (Abou-Jawdah et al., 2009; Salehi et al., 2018, 2020). '*Ca.* Phytoplasma phoenicium' is a quarantine pathogen in the European Union, being included in the List A1 of the European Plant Protection Organization (EPPO) by September 2018. In 2019, AlmWB was firstly reported in south Italy (Nigro et al., 2019). In Iran, AlmWB was found associated also with phytoplasmas belonging to subgroup 16SrIX-C (Salehi et al., 2006) that, in Lebanon, were reported only in wild plant species (Casati et al., 2016). Symptoms including decline, early fall, rosette, witches'-broom, yellowing, little leaf, leaf rolling, leaf scorch, and leaf reddening were observed in almond trees in Iran in association with *'Ca.* Phytoplasma aurantifolia, *'Ca.* P. solani, *'Ca.* P. trifolii', and '*Ca*. P. asteris' (Zirak et al., 2009, 2021). Moreover, '*Ca*. P. prunorum', the causal agent of European stone fruit yellows (ESFY), was identified in almond showing early leaf reddening in autumn, off-season growth in winter followed by dieback, and bore small and tasteless fruits in Tunisia (Ben Khalifa et al., 2011). In Jordan, few studies focused on phytoplasma-associated diseases as well as their epidemiology. Therefore, based on the increased attention for both almond cultivation and phytoplasma-associated diseases in Jordan, the current study aimed to (i) survey the presence of phytoplasma-like diseases of almond in orchards localized in northern Jordan, (ii) detect and identify by molecular analyses the phytoplasmas associated with such diseases, (iii) preliminarily investigating the presence of putative vectors and reservoir plants of the identified phytoplasmas.

4.3 Materials and Methods

4.3.1 Phytoplasma-like symptom observation, plant sampling, and insect collection

Phytoplasma-like symptoms were surveyed, from June to October 2020 and in January 2021, in almond orchards localized in seven rainfed locations in two governorates in northern Jordan. In detail, the surveys were conducted in Kharja, Ezrit, Hofa and Sydoor in Irbid governorate and in Ain Jana, Zatarah, and Sikhrah in Ajloun governorate (Figure 5). Around 23 rainfed almond orchards (19 in Irbid and 4 in Ajloun). The dimension of surveyed orchards was ranging from 0.3 up to 1.2 ha (0.4 ha in average), and more than 3,600 almond trees were observed. In each location, incidence of phytoplasma-like diseases was estimated as the

percentage of symptomatic trees out of the observed ones. Most of the surveyed orchards were surrounded by olive trees, stone fruits, grapevine, and scattered pears. Many of these orchards were very close to home gardens with mixed fruit trees. A lack of associated green cover weeds was observed in the orchards during summer due to the dry season and the agronomic soil management.

Leaves were collected from 140 almond trees showing phytoplasma-like symptoms and 16 symptomless almond trees (Table 1). Moreover, leaves were sampled from 20 symptomatic plants of four weed species (*Convolvulus arvensis* L., *Chenopodium* sp., *Amaranthus* sp., *Capparis* sp.) observed within and around investigated almond orchards (Table 1). Collected samples were labeled, signed by the areas coordinates, transported to the laboratories of National Agricultural Research Center (NARC), Baqaà, Jordan, and maintained at 4°C until total nucleic acids extraction. Additionally, during the field survey carried out in both governorates, insects within almond orchards were collected in summer 2020, by entomological sweeping net and transferred to the NARC laboratories. Stereomicroscope observation was conducted for preliminary selection of phloem feeding Hemiptera taxa. The selected insects were kept in 99% ethanol until their identification carried out at the Department of Agriculture, Forest, and Food Science (DISAFA), University of Turin, Italy. The insect identification was based on stereomicroscope observation of phenotypic characters and male genitalia after their dissection and clarification in a 10% potassium hydroxide solution. Insects recognized at genus/species level were maintained in 99% ethanol at -20°C until total nucleic acids extraction.

4.3.2 Total nucleic acids extraction

Total nucleic acids (TNAs) were extracted from 0.5 g of petiole and midrib tissues of all the collected plant samples using a CTAB-based extraction protocol previously describes by Angelini *et al.*, (2001). TNAs were extracted from single insect specimens or from insect pools (2-5 specimens) based on their size and number of collected specimens using a CTAB-based extraction method previously described (Marzachì *et al.*, 1998). The obtained TNAs were solved in 50 (insects) to 100 µl (plants) distilled sterile water and stored at −20 °C until further use. DNA quality and concentration were measured by Nanodrop system.

4.3.3 Phytoplasma detection and identification

TNAs extracted from plants and insects were used as templates in nested PCR reactions conducted to detect the presence of phytoplasmas. Nested PCRs were carried out to amplify

the phytoplasma 16S rRNA gene using the primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by the primer pair R16F1/R16R0 (Lee et al., 1995). Reaction mixtures and reaction conditions were as previously described (Lee *et al*., 1998; Quaglino *et al*., 2009). TNAs extracted from periwinkle [*Catharanthus roseus* L. (G. Don)] plants, infected by phytoplasma strains STOL ('*Ca*. Phytoplasma solani', subgroup 16SrXII-A) and AY1 ('*Ca*. P. asteris', subgroup 16SrI-B) and maintained in greenhouse at Department of Agricultural and Environmental Sciences, University of Milan (Italy), were employed as positive controls. TNAs extracted from healthy periwinkle and reaction mixtures devoid of TNAs were used in as negative controls. PCR products (6 µl) were analyzed by electrophoresis on 1% (w/v) agarose gels in 1X TBE buffer, stained with Midori Green, and visualized on UV transilluminator.

Nested PCR products (F1/R0 fragment), amplified from plants and insects, were sequenced in both strands (3X coverage per base position) by a commercial service (Eurofins Genomics, Germany). 16S rDNA nucleotide sequence assembling and trimming to the annealing sites of primers F1/R0 were carried out using the software BioEdit, version 7.1.3.0 (Hall, 1999). Edited sequences, obtained in this study, were compared using the ClustalW Multiple Alignment program and analyzed by Sequence Identity Matrix in the sofware BioEdit to estimate their genetic diversity. 16S rDNA nucleotide sequences, representative of the phytoplasma populations detected in this study, were deposited on NCBI GenBank (Accession Number OL873123-OL873133) and aligned with those of representative strains of the 47 '*Ca*. Phytoplasma' species described in literature and checked for their sequence identity in the software Bioedit. Species attribution was confirmed searching the species-specific signature sequences, and by analysis on iPhyClassifier online tool (Wei *et al*., 2007). For group/subgroup attribution, 16S rDNA sequences were analyzed by virtual RFLP using the online tool iPhyClassifier (Zhao *et al*., 2009). Actual RFLP analysis was carried out to confirm the virtual restriction patterns.

Phylogenetic analyses were carried out on the alignment of 16S rRNA gene nucleotide sequences of phytoplasmas identified in the present study and reference strains of '*Ca*. Phytoplasma' species retrieved from NCBI GenBank. The Minimum-Evolution method was employed using the Neighbor-Joining algorithm and bootstrap replicated 1,000 times with the software MEGAX to obtain a phylogenetic tree (Kumar et al., 2018).

4.4 Results

4.4.1 Description of phytoplasma-like symptoms in almond and weeds

During the survey conducted in almond orchards in seven locations in North Jordan, five main categories of phytoplasma-like symptoms were observed in almond trees. (i) Witches'-broom, yellowing, and dieback (Figure 6A, B) were found in orchards localized in Ezrit, Hofa, and Sydoor (Irbid governorate), and Ain Jana (Ajloun governorate) with an incidence (percentage of symptomatic trees) of 40%, 25%, 30%, and 80%, respectively. (ii) Early flowering (started 30 days before the expected blossom period) along with evergreen pattern (leaf canopy was maintained during the winter season) (Figure 6C) was observed in orchards localized in Sikhrah (Ajloun), with an incidence around 85%. (iii) Witches'-broom, yellowing and leaf rolling (Figure 6D) were found in orchards localized in Kharja with an incidence around 55%. (iv) Slim leaves (Figure 6E) were observed in orchards localized in Zatarah (Ajloun) with an incidence around 20%. (V) Flat stem (Figure 6F) was exhibited by almond trees in Kharja (Irbid), with an incidence around 40%. Additionally, symptoms of yellowing on *Capparis* sp., reddening on *Chenopodium* sp., and little leaves and colour alteration on *Amaranthus* sp. (Figure 6G) were observed within and around almond orchards in Kharja and Ezrit.

4.4.2 Molecular detection and identification of phytoplasmas in plants

Nested PCR reactions allowed amplifying phytoplasma 16S rRNA gene in leaf samples of 31 out of 176 plants. In detail, F1/R0 amplicons of the expected size (around 1370 bp) were obtained in 30 out of 140 symptomatic almond trees (21.4%), and in one (*Amaranthus* sp.) out of 20 non-crop weeds (5%) (Table 1). No amplificons were obtained in leaf samples collected from symptomless almond trees and from *C. arvensis*, *Chenopodium* sp., and *Capparis* sp. Goodness of PCR reactions was supported by the amplification of F1/R0 fragment from periwinkles infected by phytoplasma strains STOL and AY1 (positive controls), while no amplification was obtained from healthy periwinkle and reaction mixture devoid of TNA (negative controls). Infection rates (percentage of phytoplasma-infected trees) varied among the surveyed localities as it follows: 67% in Sikhrah, 36.7% in Kharja, 32% in Hofa and Ain Jana, 13% in Zatarah 13%, and 12% in Sydoor and Ezrit.

Based on 16S rDNA sequence identity versus the reference strains of '*Ca*. Phytoplasma' species and on the presence of species-specific signature sequences, the phytoplasma strains detected in the present study in 31 symptomatic almond trees were attributed to the species '*Ca.* P. solani' (40%; 12 strains out of 31), '*Ca.* P. ulmi' (16.7%; 5 out of 31), '*Ca.* P. omanense'

(16.7%; 5 out of 31), '*Ca*. P. asteris' (9.7 %; 3 out of 31), '*Ca.* P. pyri'(10%; 3 out of 31), '*Ca.* P. aurantifolia' (3.3%; 1 out of 31), and '*Ca.* P. phoenicium' (6.6 %; 2 out of 31) (Table 3). In detail, '*Ca*. P. solani' strains have identical 16S rDNA nucleotide sequence (GenBank Acc. No. OL873130), distinct from the reference strain STOL by four single nucleotide polymorphisms (SNPs) at positions 504 (T/A), 595 (A/G), 888 (C/T), and 1084 (T/C) from the annealing site of the primer R16F1. Within '*Ca*. P. ulmi', '*Ca.* P. omanense', and '*Ca*. P. asteris' the identified strains of each species have diverse 16S rDNA nucleotide sequences. In '*Ca*. P. ulmi', sequences of strains AL2, AL3, AL5, and AL10, identical between them (GenBank Acc. No. OL873125), and AL1 (GenBank Acc. No. OL873124) are distinct from the reference strain EY1 by three [positions 95 (C/T), 117 /A/C), 346 (A/C)] and two [positions 95 (C/T), 346 (A/C)] SNPs, respectively. In '*Ca.* P. omanense', sequences of strains AL163, AL1052, AL1056, and AL1058, identical between them (GenBank Acc. No. OL873126) are distinct from the reference strain IM-1 by SNPs at positions 152 (G/A), 274 (T/C), 331 (C/T), 344 (G/A), and 712 (G/A), while the sequence of strain AL408 (GenBank Acc. No. OL873127) is identical to the reference strain IM-1. In '*Ca*. P. asteris', sequences of strains AL7 and AL2C, identical between them (GenBank Acc. No. OL873123), and AL831 (GenBank Acc. No. OL873129) are distinct from the reference strain OAY by three [323 (G/-), 346 (G/-), 539 (C/T)] and seven [323 (G/-), 346 (G/-), 488 (C/T), 539 (C/T), 698 (C/T), 984 (A/G), 1122 (G/A)] SNPs, respectively. In '*Ca*. P. pyri', the sequences of the strains AL198, AL222, and AL225 (GenBank Acc. No. OL873132) were identical to the reference strain PD1. In '*Ca*. P. aurantifolia', the sequence of the strain AL214 (GenBank Acc. No. OL873131) is distinct from the reference strain WBDL by six SNPs at positions 62 (T/A), 83 (G/A), 285 (C/T), 559 (-/T), 793 (-/C), and 1032 (T/C). In '*Ca*. P. phoenicium', the sequence of the strain AL1067 (GenBank Acc. No. OL873128) is identical to the reference strain A4. Based on similarity coefficient obtained by comparison of virtual RFLP patterns, confirmed by actual enzymatic digestion profiles (data not shown), '*Ca*. P. solani' strains were attributed to taxonomic subgroup 16SrXII-A, '*Ca*. P. ulmi' strains to subgroup 16SrV-A (strain AL1) and its variant (strains AL2, AL3, AL5, and AL10), '*Ca*. P. omanense' strain AL408 to subgroup 16SrXXIX-A, '*Ca*. P. asteris' strains to subgroups 16SrI-B (strains AL7, AL2C) and a variant of the subgroup 16SrI-R (strain AL831), '*Ca*. P. pyri' strains AL198, AL222, and AL225 to subgroup 16SrX-C, '*Ca*. P. aurantifolia' strain AL214 to a variant of the subgroup 16SrII-C, and '*Ca*. P. phoenicium' strain AL1067 to subgroup 16SrIX-B (Figure 7). Moreover, '*Ca*. P. omanense' strains AL163, AL1052, AL1056, and AL1058 were characterized by a common collective restriction profile sharing the higher similarity coefficient (0.97) with the profile of the

reference strain of subgroup 16SrXXIX-A; such digestion patterns are distinguished by the enzyme *Alu*I. Due to the similarity coefficient value, these four '*Ca*. P. omanense' strains were inserted in the new taxonomic subgroup 16SrXXIX-B (Figure 7). Phytoplasmas identified in symptomatic almond trees were found differentially distributed in the examined locations and associated with different symptoms. '*Ca*. P. solani' (16SrXII-A) was found in Hofa (7 strains out of 8 detected phytoplasmas), Sydoor (2 out of 3), Kharja (2 out of 5), and Ezrit (1 out of 3) in association with witches'-broom, yellowing, leaf rolling, and dieback (Figure 6A, B, D). '*Ca*. P. ulmi' was found only in Sikhrah (5 strains out of 7) in association with early flowering and evergreen pattern (Figure 6C). '*Ca*. P. omanense' was found in Ain Jana (2 strains out of 3), Ezrit (1 out of 3), and Sydoor (1 out of 3) in association with witches'-broom, yellowing, and dieback (Figure 6A, B), and was the sole phytoplasma identified in Zatarah (1 out of 1) in association with slim leaves (Figure 6E). '*Ca*. P. asteris' was identified in Sikhrah (2 strains out of 7) in association with early flowering and evergreen pattern (Figure 6C), and in Ezrit (1 out of 3) in association with witches'-broom, yellowing, and dieback (Figure 6A, B). '*Ca*. P. pyri' (3 strains out of 5) was identified in Kharja in association with flat stem. Finally, '*Ca*. P. aurantifolia' and '*Ca*. P. phoenicium' were identified in Hofa (1 strain out of 8) and Ain Jana (1 out of 3), respectively, in association with witches'-broom, yellowing, and dieback (Figure 6A, B) (Table 3). Concerning non-crop weeds, a '*Ca*. P. omanense strain, sharing identical 16S rDNA sequence with almond-infecting strains AL163, AL1052, AL1056, and AL1058 (newly reported subgroup 16SrXXIX-B), was identified in *Amaranthus* sp. exhibiting little leaf and yellowing in Ezrit (Figure 6G). Phytoplasma clustering in phylogenetic tree confirmed the attribution to phytoplasma species and taxonomic subgroups (Figure 8).

4.4.3 Molecular detection and identification of phytoplasmas in insects

During the field survey carried out in Ezrit, Kharja, and Sydoor localities in July and August 2020, 122 Auchenorrhyncha and 4 Sternorrhyncha adults were collected and classified, based on stereomicroscope analyses, in 12 taxonomic groups defined at genus (4) and species (8) level. Most of Auchenorrhyncha insects belong to the family Cicadellidae (106 specimens), while the remnant 16 collected specimens belong to the families Cixiidae, Issidae, Delphacidae, and Tettigometridae. Within Cicadellidae, the more abundant insect taxa were *Zygina flammigera* (Fourcrov) (25 specimens) (firstly reported in Jordan), *Anaceratagallia frisia* (Wagner) (20 specimens) (firstly reported in Jordan), *Cicadulina bipunctata* (Melichar), (18 specimens), *Balclutha incisa* (Matsumura) (15 specimens), and *Empoasca* sp. (15 specimens). All Sternorrhyncha insects (4) belong to the family Psyllidae. In Kharja, 10 out of 12 insect

taxa were captured; in Ezrit and Sydoor only two and one insect taxa were captured, respectively. In these last two locations, the following three insect taxa were captured, namely *Z. flammigera*, *Hyalesthes obsoletus* (Signoret), and *B. incisa* (Table 3). Molecular analyses for phytoplasma detection and identification were conducted on 48 insect pools (35 from Kharja, 7 from Ezrit, and 6 from Sydoor) representative of the observed diversity. Nested PCR allowed detecting phytoplasmas in 13 insect pools (infection rate 27.1%), belonging to 8 different insect taxa, collected in Kharja (infection rate 34.3%) and Ezrit (infection rate 14.3%). No positive insect pools were found in Sydoor. Among Cicadellidae, two out of 34 insect pools (5.9%), belonging to the taxa *C. bipunctata* and *Empoasca* sp., were found phytoplasma-infected. Among the other families, 11 out of 14 insect pools (78.6%), belonging to six taxa, were found phytoplasma-infected. In detail, infection rate among such taxa was 66.7% in *Reptalus quinquecostatus* (Dufour) (2 pools out of 3) (firstly reported in Jordan), and 50% in *H. obsoletus* (1 pool out of 2) (Cixiidae); 100% in *Agalmatium* sp. (Issidae) (3 pools out of 3), *Cacopsylla bidens* (Psyllidae) (2 pools out of 2), and *Laodelphax striatellus* (Fallén) (Delphacidae) (2 pools out of 2); 50% in *Tettigometra* sp. (Tettigometridae) (1 pool out of 2) (Table 3; Figure 5). Analyses of 16S rDNA nucleotide sequences allowed attributing the phytoplasma strains detected in insects to the species '*Ca*. P. asteris' (7 pools out of 13) and '*Ca*. P. pyri' (6 pools out of 13) (Table 4). In detail, '*Ca*. P. asteris' strains found in *Empoasca* sp., *R. quinquecostatus*, *H. obsoletus*, and *Agalmatium* sp. share identical 16S rDNA nucleotide sequence with the almond-infecting strain AL831, attributed to a variant of taxonomic subgroup 16SrI-R. '*Ca*. P. pyri' strains found in insects *L. striatellus*, *Tettigometra* sp., *C. bidens*, and *C. bipunctata* shares identical 16S rDNA nucleotide sequence between them (GenBank Acc. No. OL873133), distinct from almond-infecting strains by SNPs at positions 251 (A/C), 470 (C/A), and 723 (G/A). Such strains were attributed to taxonomic subgroup 16SrX-C (Table 3). Phytoplasma clustering in phylogenetic tree confirmed the attribution to phytoplasma species and taxonomic subgroups (Figure 8).

4.5 Discussion

Recently, almond became a very important stone fruit crop in Jordan, gaining huge popularity among family farming, investors, and consumers. In this work, field surveys allowed observing almond trees exhibiting five different symptom categories in North Jordan, and molecular analyses identified in symptomatic almond trees the presence of seven genetically distinct '*Ca*. Phytoplasma' species, including '*Ca*. P. solani', '*Ca*. P. aurantifolia', '*Ca*. P. asteris', '*Ca*. P.

ulmi', '*Ca* P. pyri', '*Ca*. P. phoenicium', and '*Ca*. P. omanense', belonging to nine taxonomic subgroups (16SrI-B, I-R, II-C, V-A, IX-B, X-C, XII-A, XXIX-A and -B). Remarkably, '*Ca*. P. solani', '*Ca*. P. aurantifolia', '*Ca*. P. asteris', and '*Ca*. P. ulmi', identified in almond in the present study, were previously reported in Jordan in association with diseases affecting grapevine, plum, peach, tomato, date palm, and pomegranate (Anfoka et al., 2003; Anfoka & Fattash, 2004; Alhudaib et al., 2019; Salem et al., 2013, 2019, 2020; Abu Alloush et al., unpublished). On the other hand, '*Ca.* P. pyri', '*Ca*. P. phoenicium', and '*Ca*. P. omanense' are firstly reported in Jordan. Furthermore, '*Ca*. P. phoenicium', '*Ca.* P. solani', '*Ca*. P. aurantifolia', and '*Ca*. P. asteris' were already found in association with almond diseases in MENA region (Abou-Jawdah et al., 2003; Salehi et al., 2006; Zirak et al., 2009; Ghayeb Zamharir et al., 2014). Based on the findings of this study, '*Ca*. P. pyri', '*Ca*. P. omanense', and '*Ca*. P. ulmi' are reported for the first time in almond around the world.

Most of the symptoms observed in almond trees in Jordan (early flowering along with evergreen pattern, leaf rolling, witches'-broom, yellowing, and dieback) were already reported in MENA countries (Abou-Jawdah et al., 2003; Salehi et al., 2006), except for flat stem (stem fasciation) and slim leaves, firstly reported here in association with infection by '*Ca*. P. pyri' and '*Ca*. P. omanense' (newly described subgroup 16SrXXIX-B), respectively. Such results suggested that the symptoms observed in almond could be related to differences in phytoplasma-plant interactions and/or in specific environmental features of the examined locations, as reported for other crops (Bisognin et al., 2008; Hren et al., 2009; Murolo and Romanazzi, 2015; Quaglino et al., 2016). Even if the incidence of phytoplasma-like symptoms was high in examined orchards, only 21% of collected symptomatic almond trees were found phytoplasma infected. This can be due to: (i) the uneven distribution of phytoplasmas in phloem tissues of infected plants (Constable et al., 2003); (ii) the possible low concentration of phytoplasma cells in plant tissues in the different sampling periods (from July to November) (Martini et al., 2011); (iii) the possibility that observed symptoms are caused by other etiological agents or to abiotic stresses.

Among the seven phytoplasma species identified in almond in this study, '*Ca*. P. solani was the most prevalent and detected in all Irbid locations. Such finding confirms previous reports about the large dispersal of this phytoplasma species throughout the Country in association with grapevine 'bois noir' disease, plum yellowing and witches'-broom (Salem et al., 2013, 2020), and pomegranate yellowing and little leaf (Abu Alloush et al., unpublished). In Europe and in MENA countries, diffusion of '*Ca*. P. solani' is strictly related to the vectoring activity of the polyphagous planthopper *H. obsoletus* and the presence of its preferred host plants

(mainly nettle and bindweed) in agroecosystems (Maixner, 1994; Choueiri et al., 2019; Kosovac et al., 2019; Jamshidi et al., 2019; Quaglino et al., 2021). In this study, '*Ca*. P. solani' was not detected in both *H. obsoletus* (very low number of captured specimens) and bindweed (low number of collected plants in restricted areas), suggesting that its spread to almond could be related to a different ecology. However, '*Ca*. P. solani' was not detected in other captured insects, including its known vectors *L. striatellus* and *R. quinquecostatus* (Chuche et al 2016; Quaglino et al., 2019). Considering that insect survey was limited throughout the season and that study of putative reservoir plants should be extended, further studies are necessary to investigate accurately the ecological cycle of '*Ca*. P. solani' in Jordan, with a particular focus on its transmission pathways to almond.

Four phytoplasma-infected insect taxa, including *Agalmatium* sp., *Empoasca* sp., *H. obsoletus* and *R. quinquecostatus* (firstly reported in Jordan), were found in Kharja carrying '*Ca*. P. asteris' strains belonging to a variant of taxonomic subgroup 16SrI-R, undistinguishable from a strain identified only in one almond tree exhibiting witches'-broom, yellowing, and dieback in Ezrit. Phytoplasma strains of subgroup 16SrI-R were previously identified in association with diseases of cherry and pomegranate in Lituania and Jordan, respectively (Jomantiene et al., 2011; Abu Alloush et al., unpublished). *H. obsoletus* and *R. quinquecostatus* are known as '*Ca*. P. solani' vectors and putative vectors of '*Ca*. P. asteris' (Maixner et al., 1994; Chuche et al., 2016; Zambon et al., 2018; Pierro et al., 2020); *Empoasca* sp. was described as putative vector in field and experimental vector in controlled condition of '*Ca*. P. asteris' (Galetto et al., 2011; Kumar et al., 2015; Perilla-Henao et al., 2016); while *Agalmatium* sp. was never found infected by phytoplasmas. On the other hand, '*Ca*. P. asteris' strains belonging to subgroup 16SrI-B were identified in almond trees showing early flowering along with evergreen pattern in Sikhrah, but no weeds and insects were found infected by this phytoplasma. Based on these findings, it is reasonable to suggest that diffusion of at least 'Ca. P. asteris' subgroup 16SrI-R to almond in Jordan can involve the insects *H. obsoletus*, *R. quinquecostatus*, and *Empoasca* sp. Upscaling the surveyed orchards and surroundings could provide better insights on the '*Ca*. P asteris' diffusion in almond and its epidemiology.

Interestingly, '*Ca*. P. pyri' (16SrX-C), associated with pear decline (PD) and other diseases of stone fruits (Paltrinieri et al., 2001; Bohunická *et al.,* 2018) and included in the EPPO A2 List (OEPP/EPPO, 2007), was identified in almond trees showing stem fasciation in Kharja location. Molecular analyses allowed identifying '*Ca*. P. pyri′ in insects taxa *C. bidens*, *L. striatellus*, *Tettigometra* sp., and *C. bipunctata* in the same location. It is well known that

Cacopsylla pyri is one of the main vectors of '*Ca*. P. pyri' in the Mediterranean area (Garcia-Chapa et al., 2005), where it transmits this phytoplasma to stone fruits (Sabaté et al., 2018). Consequently, based on evidence from this and previous study (Etropolska et al., 2015), it is reasonable to hypothesize that *C. bidens* can be a vector of '*Ca*. P. pyri' to almond in Jordan. Previous studies reported that *L. striatellus* is a vector of '*Ca*. P. solani' to grapevine (Quaglino et al., 2019), *C. bipunctata* is a potential vector of '*Ca*. P. asteris'-related strain to date palm (Alhudaib et al., 2007), and *Tettigometra* sp. was never found infected by phytoplasmas. Further studies should be planned to investigate the vector activity of these insects by transmission trials, and to describe its ecology, including cultivated and wild host plants.

'*Ca*. P. omanense', previously reported in MENA Countries in association with grapevine 'bois noir' and yellowing, reddening, dwarfing, die-back and decline diseases in *Prunus persica*, *Prunus domestica*, *Diospyros kaki*, and *Sophora alopecuroides* (Foissac et al., 2019; Esmaeilzadeh-Hosseini et al., 2019, 2020), was identified in this study for the first time in almond trees exhibiting three symptom categories (witches'-broom, yellowing, dieback; little leaf, yellowing; slim leaves) in three Jordanian localities. Interestingly, the majority of '*Ca*. P. omanense' strains have been classified in a new taxonomic subgroup, named 16SrXXIX-B, and are undistinguishable from strains recently identified in table grapes in Jordan (Abu Alloush et al., unpublished). Molecular analyses of weeds revealed the presence of '*Ca*. P. omanense', subgroup 16SrXXIX-B, in *Amaranthus* sp., suggesting a potential role of this plant as reservoir for phytoplasma diffusion. Recent studies reported *H. obsoletus* and *Reptalus* sp. as putative vectors of '*Ca.* P. omanense' in Lebanon (Foissac et al., 2019), but in the present work these and the other examined insects were not found infected by this phytoplasma. Due to the association of the new subgroup 16SrXXIX-B to almond and grapevine diseases in Jordan, it will be useful to focus further studies on improving the knowledge on its epidemiology throughout the Country, in different agroecosystems.

Early flowering is one of the most common symptoms of almond witches'-broom disease, associated with *'Ca*. P. phoenicium' in Lebanon and Iran (Salehi et al., 2006; Molino Lova et al., 2011). In this study, early flowering along with evergreen pattern was associated with '*Ca.* P. asteris' (16SrI-B) and '*Ca.* P. ulmi' (16SrV-A), not detected in examined weeds and insects. To the best of our knowledge, '*Ca.* P. ulmi', previously reported in Jordan in association with date palm stunting and yellowing (Alhudaib et al., 2019), is firstly associated in this study with an almond disease worldwide.

Interestingly, '*Ca*. P. phoenicium' (16SrIX-B), the etiological agent of AlmWB in Middle East (Fiore et al., 2018), was reported in one almond tree in Ain Jana. Studies investigating '*Ca*. P.

phoenicium' epidemiology reported that insect vectors *A. decedens* and *Tachycixius* sp. and reservoir plants *Slimax aspera* and *Anthemis* sp. are involved in its diffusion to almond (Abou-Jawdah et al., 2014; Tedeschi et al., 2015; Casati et al., 2016). Moreover, several insect species were reported as putative vectors in Lebanon and Iran (Dakhil et al., 2011; Zirak et al., 2021). Except for *H. obsoletus*, none of these putative and confirmed vectors were collected during field surveys in Jordan, and 'Ca. P. phoenicium' was not detected in collected insects and putative host plants. However, considering the capability of this pathogen to adapt to diverse environmental conditions and novel agroecosystems (Quaglino et al., 2015; Salehi et al., 2018, 2020), and to quickly spread in large areas (Abou-Jawdah *et al*., 2009), its report in Jordan sounds like an alarm and needs to be accurately monitored in the next years. In particular, the movement of propagation materials between MENA Countries and within the local areas suggests giving special attention to stone fruits nurseries with regularly sampling to investigate the phytoplasma presence.

In conclusion, the present study evidenced that seven '*Candidatus* Phytoplasma' species are associated with an almond disease complex characterized by five symptom categories, including witches'-broom, yellowing, leaf rolling, and stem fasciation. Most phytoplasma species, identified in almond in this study, are associated with destructive plant diseases in different parts of the world. Moreover, obtained results provided new insights on almond phytoplasmas diffusion, identifying putative vectors and non-crop plants at least for '*Ca*. P. asteris', '*Ca*. P. pyri', and '*Ca*. P. omanense'. Further studies have to focus on (i) verify the capability of putative insect vectors, identified in the present work, to transmit '*Ca*. P. asteris' and '*Ca*. P. pyri' to almond by transmission trials; (ii) survey the insect population diversity and dynamics throughout the whole season in the almond cultivation areas; (iii) upscale the survey of almond disease complex in the whole Country, focusing on differences among the available cultivars; (iv) controlling the sanitary status of the propagation materials by molecular analyses of regularly collected samples, with special attention for the presence of '*Ca*. P. phoenicium' and '*Ca*. P. pyri'.

Table 1. Collected and phytoplasma- infected almond and non-crop plant samples from surveyed locations in north Jordan.

Governorate	Location	Plant	No. of collected samples	No. of phytoplasma-infected samples
Irbid	Kharja	Symptomatic almond	30	5
		Asymptomatic almond	3	0
		Convlovulus arvensis L.	6	0
		Chenopodium sp.	2	0
		Amaranthus sp.	4	0
	Ezrit	Symptomatic almond	25	3
		Asymptomatic almond	2	0
		Amaranthus sp.	4	1
		Capparis sp.	2	0
		Chenopodim sp.	2	0
	Hofa	Symptomatic almond	25	8
		Asymptomatic almond	3	0
	Sydoor	Symptomatic almond	25	3
		Asymptomatic almond	2	0
Ajloun	Sikhrah	Symptomatic almond	12	7
		Asymptomatic almond	2	0
	Ain Jana	Symptomatic almond	8	3
		Asymptomatic almond	2	0
	Zatarah	Symptomatic almond	15	1
		Asymptomatic almond	2	0
		Overall total	176	31

Table 2. Attribution to species and taxonomic subgroups of phytoplasmas detected in plants.

Strain	Plant host	Location	Symptoms	Phtoplasma species	Identity % versus reference strain	16Sr subgroup	Acc. No.
AL7	Almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. asteris'	99.9	I-B (1.00)	OL873123 (a)
AL2C	Almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. asteris'	99.9	I-B (1.00)	A
AL1	Almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.9	V-A (1.00)	OL873124
AL2	Almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.8	V-A* (0.98)	OL873125 (b)
AL3	Almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.8	V-A* (0.98)	B
AL5	Almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.8	V-A* (0.98)	B
AL10	Almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.8	V-A* (0.98)	B
AL163	Almond	Zatarah	slim leaves	'*Ca*. P. omanense'	99.6	XXIX-B (new)	OL873126 (c)
AL1056	Almond	Ain Jana	witches'-broom, yellowing, dieback	'*Ca*. P. omanense'	99.6	XXIX-B (new)	C
AL1058	Almond	Ain Jana	witches'-broom, yellowing, dieback	'*Ca*. P. omanense'	99.6	XXIX-B (new)	C
AL1067	Almond	Ain Jana	witches'-broom, yellowing, dieback	'*Ca*. P. phoenicium'	100	IX-B (1.00)	OL873128
AL831	Almond	Ezrit	witches'-broom, yellowing, dieback	'*Ca*. P. asteris'	99.6	I-R* (0.98)	OL873129 (d)
AL1042	Almond	Ezrit	witches'-broom, yellowing, dieback	'*Ca*. P. solani'	99.7	XII-A (1.00)	OL873130 (e)
AL1052	Almond	Ezrit	witches'-broom, yellowing, dieback	'*Ca*. P. omanense'	99.6	XXIX-B (new)	C
AL1054	*Amaranthus* sp.	Ezrit	little leaf, yellowing	'*Ca*. P. omanense'	99.6	XXIX-B (new)	C
AL169	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	E
AL170	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	E
AL171	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	E
AL172	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	E
AL173	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	E
AL174	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	E
AL175	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL214	almond	Hofa	witches'-broom, yellowing, dieback	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	OL873131
AL408	almond	Sydoor	witches'-broom, yellowing, dieback	'*Ca*. P. omanense'	100	XXIX-A (1.00)	OL873127
AL167	almond	Sydoor	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL168	almond	Sydoor	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL11	almond	Kharja	witches'-broom, yellowing, leaf rolling	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL1299	almond	Kharja	witches'-broom, yellowing, leaf rolling	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL198	almond	Kharja	flat stem	'*Ca*. P. pyri'	100	X-C (1.00)	OL873132 (f)
AL222	almond	Kharja	flat stem	'*Ca*. P. pyri'	100	X-C (1.00)	f
AL225	almond	Kharja	flat stem	'*Ca*. P. pyri'	100	X-C (1.00)	f

Table 3. Collected and phytoplasma- infected insects from surveyed locations in northern Jordan.

Governorate	Location	Insect code	Family	Species	Collection date	No. of collected insects	No. of pools	No. of positive pools	Infection rate (%)
Irbid	Sydoor	SH2	Cicadellidae	*Zygina flammigera*[a]	Jul	25	6	0	0
Irbid	Kharja	K6	Cicadellidae	*Anaceratagallia frisia*[a]	Aug	20	6	0	0
Irbid	Kharja	K17	Cicadellidae	*Cicadulina bipunctata*	Aug	18	6	1	16.7
Irbid	Kharja	KPS	Psyllidae	*Cacopsylla bidens*	Aug	4	2	2	100
Irbid	Kharja	K24	Issidae	*Agalmatium* sp.	Aug	3	3	3	100
Irbid	Kharja	K25	Cixiidae	*Reptalus quinquecostatus*[a]	Aug	3	3	2	66.7
Irbid	Ezrit	EA	Cixiidae	*Hyalesthes obsoletus*	Jul	2	2	1	50
Irbid	Ezrit	ED	Cicadellidae	*Balclutha incisa*[a]	Jul	15	5	0	0
Irbid	Kharja	K3	Cicadellidae	*Zygina flammigera*	Aug	12	5	0	0
Irbid	Kharja	K7	Delphacidae	*Laodelphax* sp.	Aug	4	2	2	100
Irbid	Kharja	K11	Tettigometridae	*Tettigometra* sp.	Aug	4	2	1	50
Irbid	Kharja	K28	Cicadellidae	*Empoasca* sp.	Aug	15	5	1	20
Irbid	Kharja	K4	Cicadellidae	*Eupelix* sp.	Aug	1	1	0	0
					Overall	126	48	13	27.1

[a] insect firstly reported in Jordan

Table 4. Attribution to species and taxonomic subgroups of phytoplasmas detected in insects.

Strain	Species	Location	Phytoplasma species	Identity % versus reference strain	16Sr subgroup	Acc. No.
K7-15	*Laodelphax* sp.	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	G
K7-16	*Laodelphax* sp.	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	G
K11-17	*Tettigometra* sp.	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	G
K17-18	*Cicadulina bipunctata*	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	G
K28-19	*Empoasca* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	D
KPS-20	*Cacopsylla bidens*	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	OL873133 (g)
KPS-21	*Cacopsylla bidens*	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	G
K24-7	*Agalmatium* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	D
K24-8	*Agalmatium* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	D
K24-9	*Agalmatium* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	D
K25-10	*Reptalus quinquecostatus*	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	D
K25-11	*Reptalus quinquecostatus*	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	D
EA-12	*Hyalesthes obsoletus*	Ezrit	'*Ca*. P. asteris'	99.6	I-R* (0.98)	D

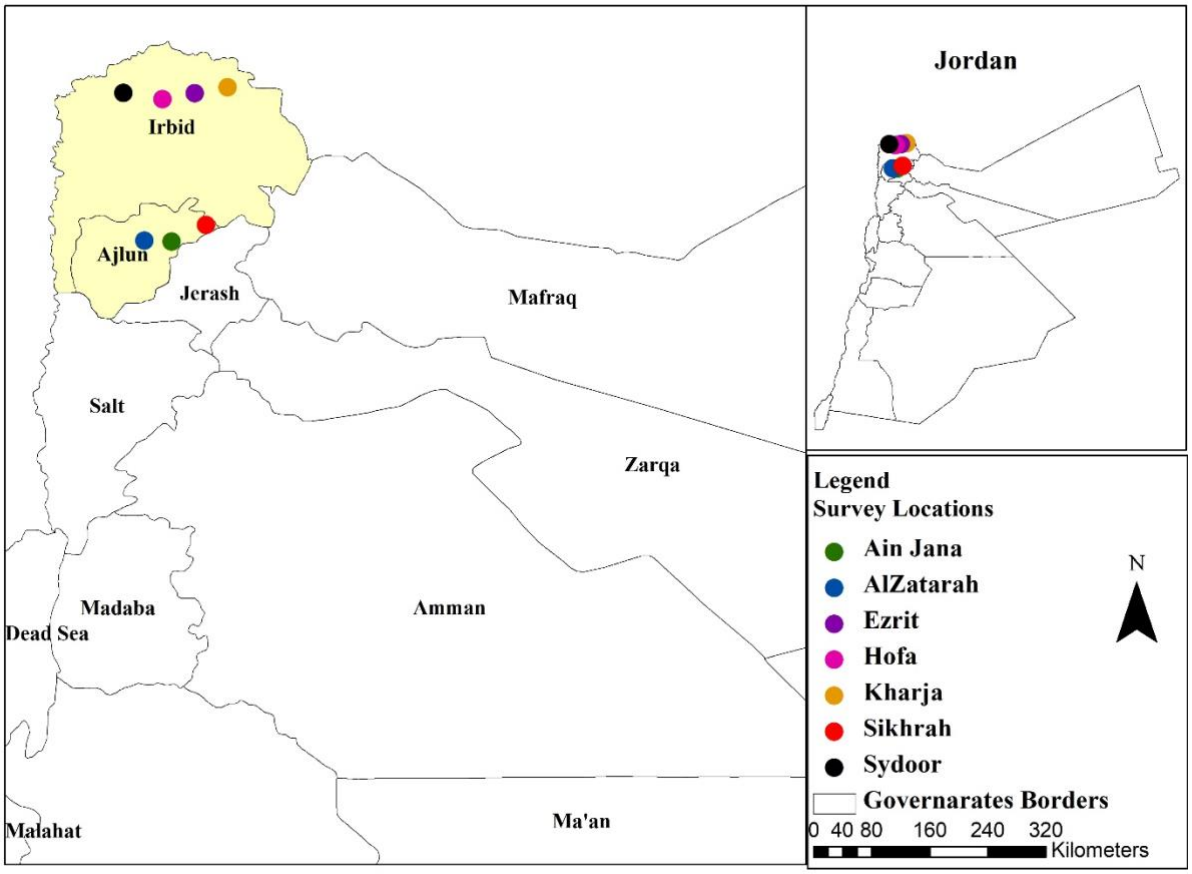

Figure 5. Maps of governorates and locations in North Jordan where the surveys on phytoplasma-like diseases in almond orchards were conducted.

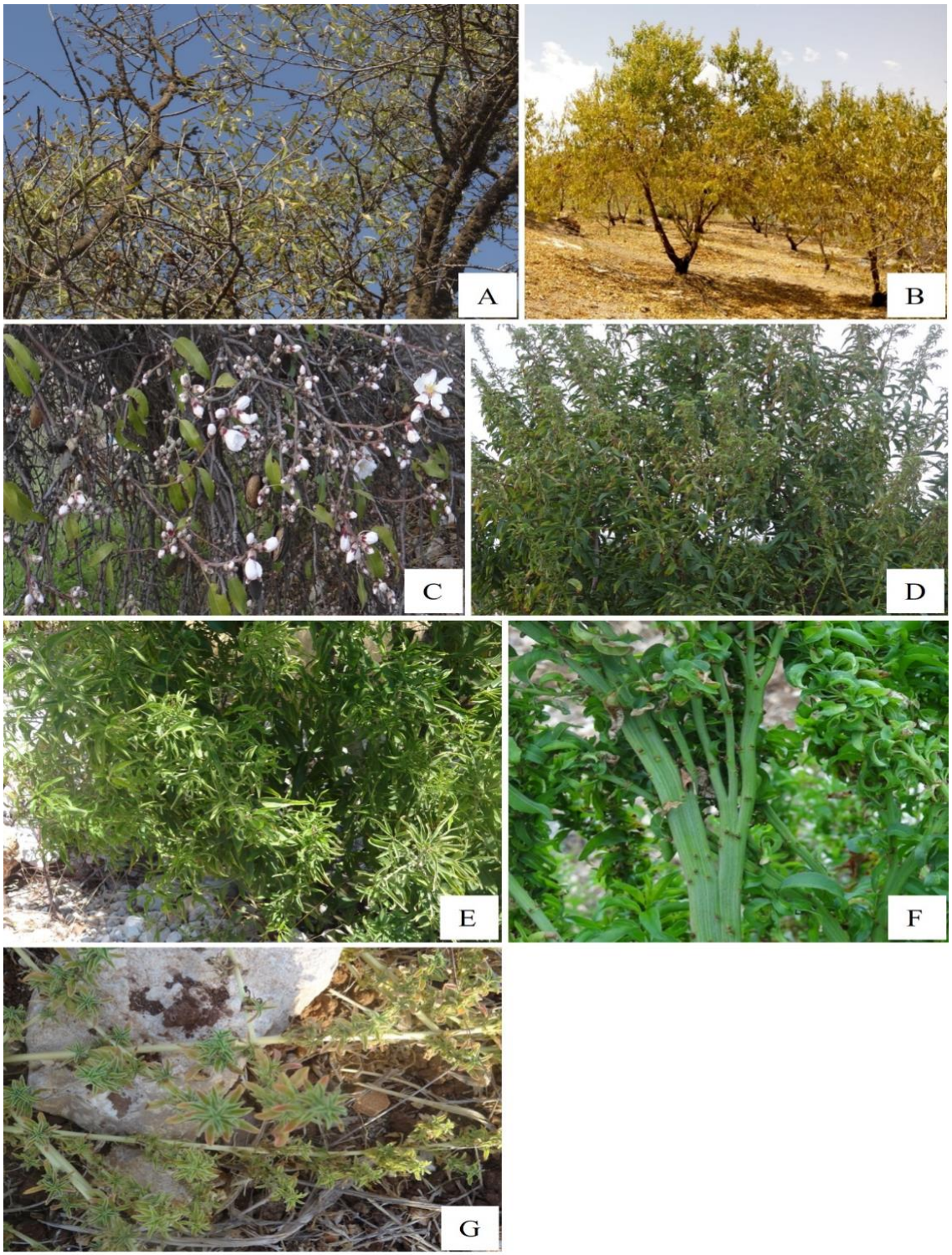

Figure 6. Phytoplasma-like symptoms observed in almond trees and weeds in northern Jordan. Witches'-broom, yellowing, and dieback in Ezrit, Hofa, Sydoor, and Ain Jana (A, B); (ii) early flowering along with evergreen pattern in Sikhrah (C); witches'-broom, yellowing and leaf rolling in Kharja (D); (iv) slim leaves in Zatarah (E); (v) flat stem in Kharja (F); little leaves and colour alteration on *Amaranthus* sp. in Kharja and Ezrit (G).

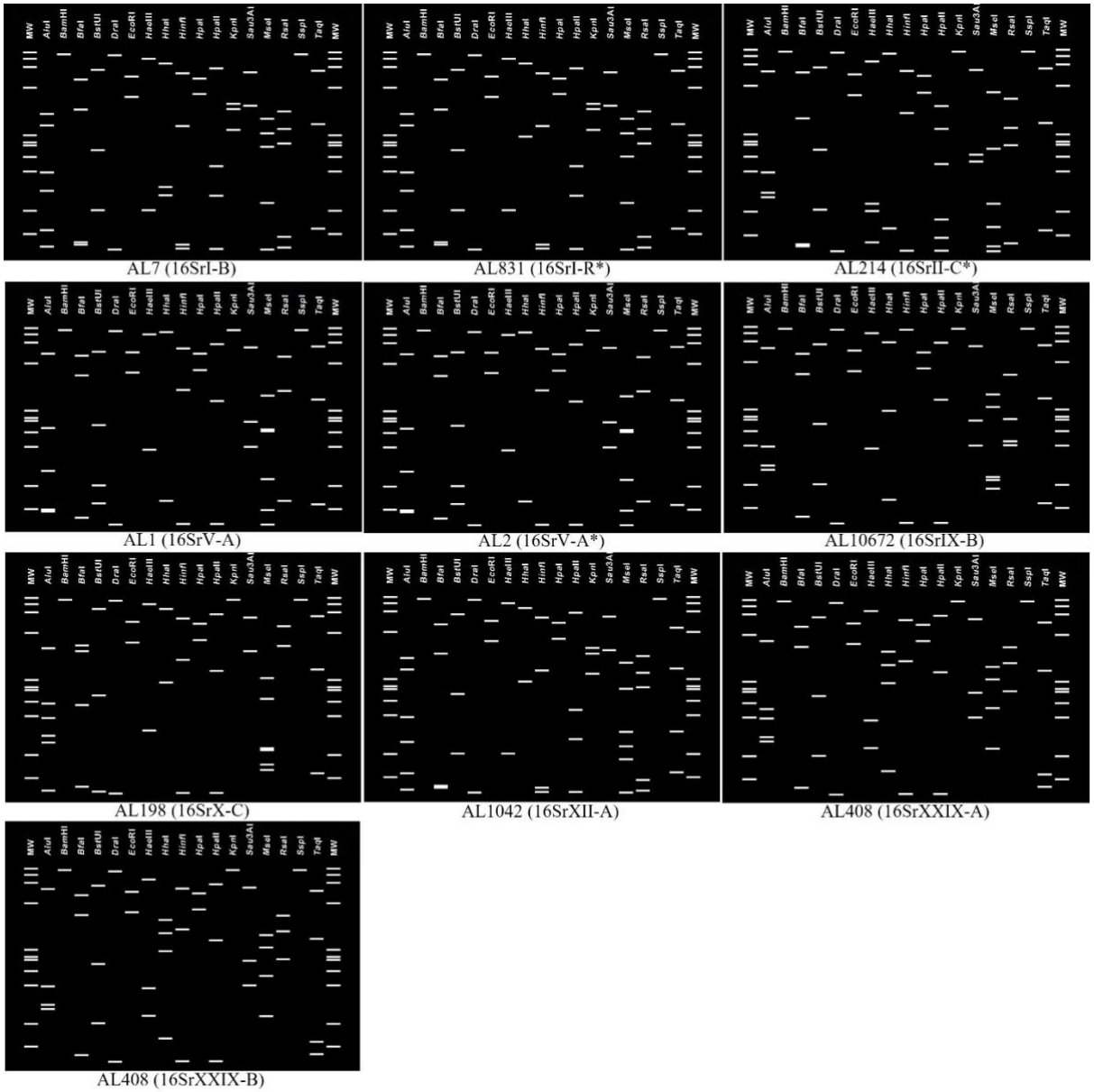

Figure 7. Virtual RFLP profiles of 16S rDNA nucleotide sequences of phytoplasma strains identified in almond, weeds, and insects in northern Jordan. One strain among those sharing identical 16S rDNA sequence (Tables 2 and 4) was selected as representative strain for iPhyClassifier analyses.

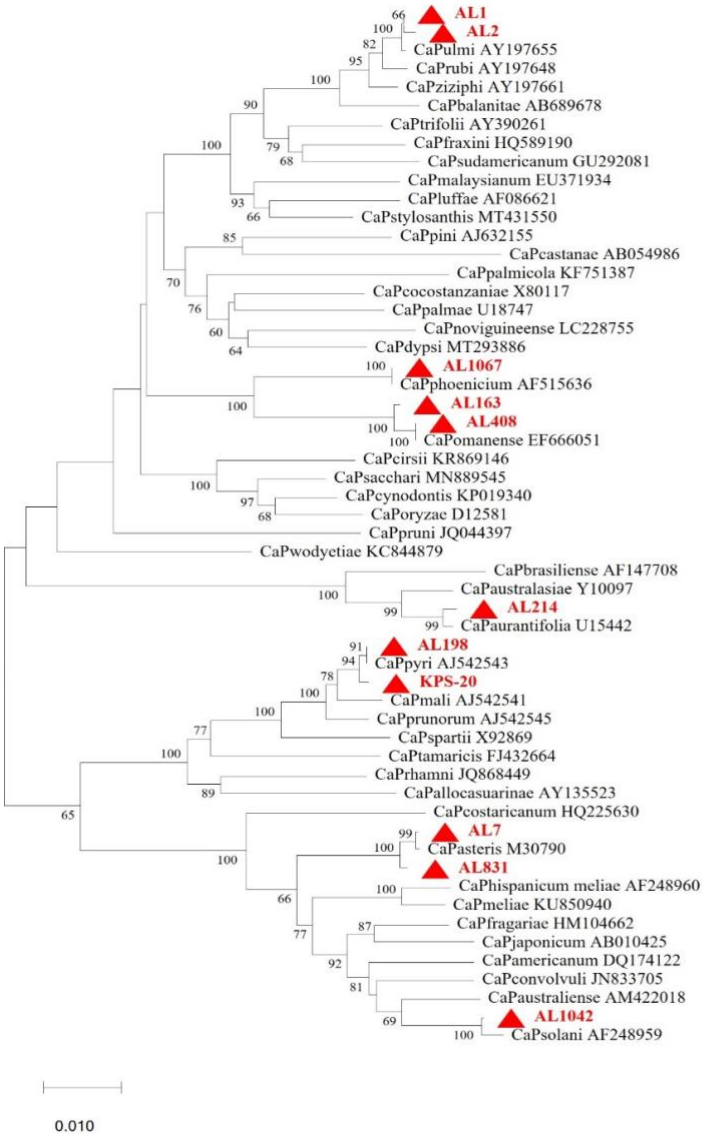

Figure 8. Phylogenetic tree based on the alignment of 16S rDNA nucleotide sequences of representative phytoplasmastrains identified in almond, putative vectors, and reservoir plants in Jordan (red bold characters), and reference strains of previously described '*Candidatus* Phytoplasma' species. Regarding phytoplasmas identified in this study, one strain among those sharing identical 16S rDNA sequence (Tables 2 and 4) was selected as representative strain for phylogenetic analysis. The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 0.91431819 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. This analysis involved 56 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1424 positions in the final dataset.

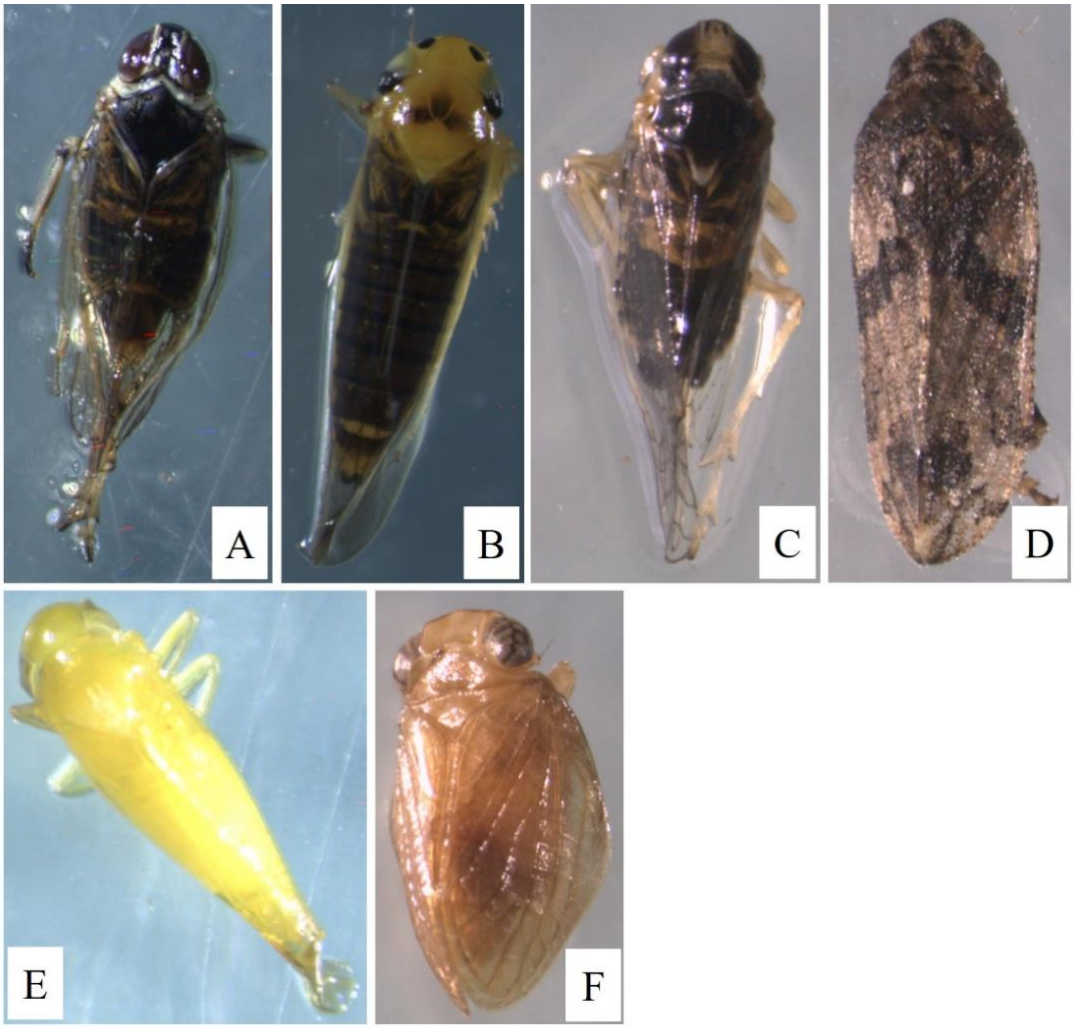

Figure 9. Putative insect vectors of phytoplasmas infecting almond in northern Jordan. (A) *Hyalesthes obsoletus*, (B) *Cicadulina bipunctata*, (C) *Laodelphax* sp., (D) *Tettigometra* sp., (E) *Empoasca* sp., (F) *Agalmatium* sp.

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Chapter 5. Association of four distinct '*Candidatus* Phytoplasma' species with pomegranate witches'-broom and leaf alteration in Jordan, and preliminary insights on their putative vectors and reservoir plants

5.1 Abstract

During field surveys conducted in northern Jordan from June to November 2020, phytoplasma-like symptoms, including leaf color alteration and rolling, little leaf, and witches'-broom were observed in pomegranate (*Punica granatum* L.). Disease incidence ranged from 30 to 65% in the surveyed areas. Nested PCR-based amplification of *16S rRNA* gene detected phytoplasmas in 17% of collected symptomatic pomegranate trees. Amplicon nucleotide sequence analyses allowed attributing the detected phytoplasmas to '*Candidatus* Phytoplasma solani' (16SrXII-A), '*Ca.* Phytoplasma aurantifolia' (16SrII-B), '*Ca.* Phytoplasma asteris' (16SrI-B, -R), and '*Ca.* Phytoplasma ulmi' (16SrV-A). Such phytoplasmas were found associated with specific symptoms and differentially distributed in the considered locations. Additional preliminary investigation highlighted the presence of three cicadellids (*Macrosteles sexnotatus*, *Cicadulina bipunctata*, *Psammotettix striatus*) and two non-crop plants (*Plantago major*, *Capsicum annuum*) hosting the same pomegranate-infecting '*Ca.* Phytoplasma asteris' strains, and one cicadellid (*Balclutha incisa*) carrying the same pomegranate-infecting '*Ca*. Phytoplasma solani' strain. In conclusion, this study described a new pomegranate disease, called pomegranate witches'-broom and leaf alteration, associated with multiple phytoplasmas, including '*Ca*. Phytoplasma auranfitolia' and '*Ca*. Phytoplasma ulmi', never reported before in this host plant. Moreover, it furnished preliminary indications on the epidemiology of this disease in Jordan, involving two putative vectors (*M. sexnotatus*, *B. incisa*) firstly reported in the Country. Further studies are needed to survey the diffusion of this pomegranate disease in the region, demonstrate the transmission capability of the identified putative vectors, and in-depth investigate the ecologies of all phytoplasmas associated with the disease.

5.2 Introduction

Pomegranate (*Punica granatum* L.) is a high value horticultural crop of tropical and subtropical regions of the world and is a native to Central Asia and Northern India (Still, 2006). It is a woody deciduous shrub or a small tree and sustains wider range of environments from mild temperate to sub-tropical, and relatively drought tolerant (Kahramanoglu & Usanmaz, 2016). Pomegranate fruits have nutritional benefits and are used to obtain many pharmacological products (Calani et al., 2013; Fahmi et al., 2020; Sun et al., 2021). Thus, in the last years, the worldwide demand for pomegranate has been increasing (Kandylis & Kokkinomagoulos, 2020). MENA (Middle East and North Africa) is one of the leading producers and exporters of pomegranate over the world. Almost, all MENA's countries are growing pomegranate with significant expanding areas. Turkey and Iran are ranked second and third producers and exporters over the world. In Jordan, pomegranate is one of the oldest fruit trees and considered as a major source of income for the farmers and various stakeholders throughout the last decades. Its plantation extends from North (200 m below the sea level) to the rift valley as well as inland and the wider parts of Padia. In the last decade, the investment on pomegranate cultivation and industry in Jordan has been increased with significant expanding areas because of a national project promoting the pomegranate sector in the Country.

Recently, pomegranate diseases associated with infection by phytoplasmas were reported in Turkey and Iran (Gazel et al., 2015; Salehi et al., 2016), two MENA Countries.

Considering the increasing importance of both pomegranate and phytoplasma diseases in Jordan, the present study aimed to (i) survey the presence of phytoplasma-like diseases of pomegranate in familiar and commercial orchards in Jordan, (ii) detect and identify by molecular analyses the phytoplasmas associated with such diseases, (iii) preliminarily explore the presence of putative vectors and reservoir plants of the identified phytoplasmas in pomegranate.

5.3 Materials and Methods

5.3.1 Field surveys, plant sampling, and insect collection

From June to November 2020, field surveys for phytoplasma-like symptoms were conducted in 15 family farming and two commercial pomegranate orchards, including local (Khdary, Mawardy, Erqaby) and imported (Wonderful) cultivars, in five locations in the governorates of Irbid (Kufr Soum and Jdita), Ajloun (Arjan), and Al-Mafraq (Sabha and Umjmal) in northern

Jordan (Figure 10). The size of surveyed orchards ranging from 0.2 up to 0.7 ha, with plnat density ranging from 45- 60 pomegranate trees per 0.1 ha. In each location, incidence of phytoplasma-like diseases was estimated as the percentage of symptomatic trees out of the observed ones. Around 3, 400 pomegranate trees were observed in different locations. Leaf samples were collected from 112 pomegranate trees exhibiting different phytoplasma-like symptoms and from 13 symptomless trees. Moreover, leaf samples were collected from 30 plants of 11 non-crop weed species, showing phytoplasma-like symptoms, observed within and around the surveyed orchards (Table 5). Collected samples were transferred to the laboratories of National Agricultural Research Center, Baqaà, Jordan, and maintained at 4°C until total nucleic acids extraction. In parallel, during the field survey carried out in Irbid and Ajloun governorates, insects within pomegranate orchards were collected by entomological sweeping net and transferred to the NARC laboratories. Stereomicroscope observation was conducted for preliminary selection of phloem-feeding insects (putative phytoplasma vectors). The selected insects were kept in 99% ethanol until their identification carried out at the Department of Agriculture, Forest, and Food Science (DISAFA), University of Turin, Italy. The insect identification was based on stereomicroscope observation of phenotypic characters and male genitalia after their dissection and clarification in a 10% potassium hydroxide solution. Insects recognized at genus/species level were maintained in 99% ethanol at -20°C until total nucleic acids extraction.

5.3.2 Phytoplasma detection

Total nucleic acids (TNA) were extracted from the collected plants and insects as previously described respectively by Angelini et al. (2001) and Marzachì et al. (2008), with some modifications. Concerning plant samples, leaf midribs and petioles (0.5 g) were grounded in 3 ml of prewarmed 2% CTAB-based buffer in sterile mortars. Regarding insects, for each species, TNA extraction was done from single, or pooled (2 to 5) individuals based on their size and/or number of captured specimens. Individual or pooled insects were crashed by sterile pestles in a 1.5 ml tubes containing sand and 0.5 ml of prewarmed 2% CTAB-based buffer. Extracted TNA was washed by 0.3 ml of 70% ethanol, dissolved in 50 (insects) or 100 (plants) µl of TE-based buffer, measured for quantity and quality by Nanodrop system, and stored at -20°C until molecular analyses.

TNAs extracted from plants and insects were used as templates in nested PCR reactions conducted to detect the presence of phytoplasmas. Nested PCRs were carried out to amplify the phytoplasma 16S rRNA gene using the primer pair P1/P7 (Deng & Hiruki, 1991; Schneider

et al., 1995) followed by the primer pair R16F1/R16R0 (Lee et al., 1995). Reaction mixtures and reaction conditions were as previously described (Quaglino et al., 2009). TNAs extracted from periwinkle [*Catharanthus roseus* L. (G. Don)] plants, infected by phytoplasma strains STOL ('*Ca*. Phytoplasma solani', subgroup 16SrXII-A) and AY1 ('*Ca*. P. asteris', subgroup 16SrI-B) and maintained in greenhouse at Department of Agricultural and Environmental Sciences, University of Milan (Italy), were employed as positive controls. TNAs extracted from healthy periwinkle and reaction mixtures devoid of TNAs were used in as negative controls. PCR products (6 µl) were analyzed by electrophoresis on 1% (w/v) agarose gels in 1X TBE buffer, stained with Midori Green, and visualized on UV transilluminator. In each location, phytoplasma infection rate was estimated as the percentage of infected plants out of the examined ones.

5.3.3 Phytoplasma identification

Nested PCR products (F1/R0 fragment), amplified from plants and insects, were sequenced in both strands (3X coverage per base position) by a commercial service (Eurofins Genomics, Germany). Nucleotide sequences were assembled by the Contig Assembling Program and trimmed to the annealing sites of the nested PCR primer pairs in the sofware BioEdit, version 7.1.3.0 (Hall, 1999). Trimmed nucleotide sequences were aligned using the ClustalW Multiple Alignment program and analyzed by Sequence Identity Matrix in the sofware BioEdit to estimate their genetic diversity. For attribution to '*Ca*. Phytoplasma' species, 16S rDNA nucleotide sequences, representative of the phytoplasma populations detected in this study, were aligned with those of representative strains of the 47 '*Ca*. Phytoplasma' species described in literature and checked for their sequence identity in the software Bioedit. Species attribution was confirmed searching the species-specific signature sequences within the analyzed F1/R0 nucleotide sequences, and by analysis on iPhyClassifier online tool (Wei et al., 2008). For group/subgroup attribution, 16S rDNA sequences were analyzed by virtual RFLP using the online tool iPhyClassifier (Zhao et al., 2009). Actual RFLP analysis was carried out to confirm the virtual restriction patterns.

Nucleotide sequences of 16S rRNA gene of phytoplasmas, identified in the present study, and reference strains of '*Ca*. Phytoplasma' species were employed for phylogenetic analyses. The Minimum-Evolution method was employed using the Neighbor-Joining algorithm and bootstrap replicated 1,000 times with the software MEGAX to obtain a phylogenetic tree (Kumar et al., 2018).

5.4 Results

5.4.1 Phytoplasma-like symptoms observed in pomegranate trees and weeds

During field surveys conducted in pomegranate orchards of five locations in northern Jordan, different phytoplasma-like symptoms were observed. In Irbid governorate, Khdary pomegranate cultivar trees in Kufr Soum orchards were found exhibiting leaf reddening and rolling, leaf yellowing and little leaf, and leaf discoloration (Figure 11 A, B, C) with an incidence (percentage of symptomatic trees) of around 40%. In the same governorate in Jdita orchards, Mawardy pomegranate cultivar trees showed witches'-broom, little leaf, and yellowing (Figure 11 D, E) with an incidence of around 50%. In both locations, symptoms ranged from mild to severe. In Ajloun governorate, pomegranate trees showing mild leaf reddening (Figure 10F) were observed with an incidence of around 65%. In Al-Mafraq governorate, mild leaf reddening and/or yellowing symptoms (Figure 11 G) were observed in Wonderful (SabhaWsubhieh) and Erqaby (Umjmal) pomegranate cultivars with an incidence of around 30%. Within and around pomegranate orchards, leaf wrinkle, yellowing and/or reddening, and little leaf were observed on *Convolvulus arvensis* L., *Capsicum annuum* L., *Rubus* sp., *Malva sylvestris* L., *Chenopodium album* L., *Plantago major* L., *Origanum vulgare* L., *Bidens* sp., *Inula* sp., *Amaranthus* sp., and *Lactuca* sp. (Figure 11H).

5.4.2 Molecular detection and identification of phytoplasmas in plants

Nested PCRs allowed detecting the presence of phytoplasmas in 26 out of 155 analyzed plant samples. In detail, F1/R0 amplicons of the expected size (around 1310 bp) were obtained in 19 out of 112 symptomatic pomegranate trees (17%), and in 7 out of 30 non-crop weeds (23%). No amplification was obtained in symptomless pomegranate plants (Table 5). Robustness of PCR reactions was proved by the amplification of the expected PCR product from periwinkles infected by phytoplasma strains STOL and AY1 (positive controls), and by the absence of amplification in healthy periwinkle and reaction mixture devoid of TNA (negative control). Concerning pomegranate, infection rate (percentage of phytoplasma-infected trees) changed in relation to the orchard locations. Higher infection rate was found in Jdita (35%), followed by Kufr Soum (22%), and SabhaWsubhieh (10%). No phytoplasma-infected trees were identified in Arjan and Umjmal locations. Regarding weeds, three out of 11 analyzed weed species were

found infected by phytoplasmas: *C. arvensis* (5 out of 8), *C. annuum* (1 out of 2), and *P. major* (1 out of 1) (Table 5).

Based on 16S rDNA sequence identity versus the reference strains of '*Ca*. Phytoplasma' species and on the presence of species-specific signature sequences, the phytoplasma strains detected in the present study in 19 symptomatic pomegranate trees were attributed to the species '*Ca*. P. solani' (52%; 10 strains out of 19), '*Ca*. P. aurantifolia' (21%; 4 out of 19), '*Ca*. P. ulmi' (16%; 3 out of 19), and '*Ca*. P. asteris' (10%; 2 out of 19) (Table 6). In detail, '*Ca*. P. solani' strains have identical 16S rDNA nucleotide sequence (GenBank Acc. No. OL873110), distinct from the reference strain STOL by four single nucleotide polymorphisms (SNPs) at positions 504 (T/A), 595 (A/G), 888 (C/T), and 1084 (T/C) from the annealing site of the primer R16F1. '*Ca*. P. aurantifolia' strains have identical 16S rDNA nucleotide sequence (GenBank Acc. No. OL873109), distinct from the reference strain WBDL by four SNPs at positions 285 (C/T), 559 (-/G), 793 (-/C), and 1032 (T/C) from the annealing site of the primer R16F1. Within '*Ca*. P. asteris' and '*Ca*. P. ulmi', the identified strains of each species have diverse 16S rDNA nucleotide sequences. In '*Ca*. P. ulmi', sequences of strains PG1 and PG17, identical between them (GenBank Acc. No. OL873111), and PG7 (GenBank Acc. No. OL873112) are distinct from the reference strain EY1 by two [positions 95 (C/T), 346 (A/C)] and three [positions 95 (C/T), 117 /A/C), 346 (A/C)] SNPs, respectively. In '*Ca*. P. asteris', sequences of strains PG18 (GenBank Acc. No. OL873113) and PG784 (GenBank Acc. No. OL873108) are distinct from the reference strain OAY by three [323 (G/-), 346 (G/-), 539 (C/T)] and seven [323 (G/-), 346 (G/-), 488 (C/T), 539 (C/T), 698 (C/T), 984 (A/G), 1122 (G/A)] SNPs, respectively. Based on similarity coefficient obtained by comparison of virtual RFLP patterns, confirmed by actual enzymatic digestion profiles (data not shown), '*Ca*. P. solani' strains were attributed to taxonomic subgroup 16SrXII-A, '*Ca*. P. aurantifolia' strains to subgroup 16SrII-B, '*Ca*. P. ulmi' strains to subgroup 16SrV-A (strains PG1 and PG17) and its variant (strain PG7), and '*Ca*. P. asteris' strains to subgroups 16SrI-B (strain PG18) and a variant of subgroup 16SrI-R (strain PG784) (Figure 12). Phytoplasma clustering in phylogenetic tree confirmed the attribution to phytoplasma species and taxonomic subgroups (Figure 13). Phytoplasmas identified in symptomatic pomegranate trees were found differentially distributed in the examined locations and associated with different symptoms. '*Ca*. P. solani' (16SrXII-A) was found in Kufr Soum (4 strains out of 11 detected phytoplasmas) in association with yellowing and little leaf, in Jdita (5 out of 7) in association with witches'-broom and little leaf, and in SabhaWsubhieh (1 out of 1) in association with yellowing. '*Ca*. P. aurantifolia' (16SrII-B) was found in pomegranate exclusively in Kufr Soum orchards (4 strains out of 11) in association

with leaf reddening and rolling. '*Ca*. P. ulmi' was found in Kufr Soum (2 strains out of 11) in association with leaf reddening and rolling, and in Jdita (1 strain out of 7) in association with witches'-broom and little leaf. '*Ca*. P. asteris' was found in Kufr Soum (1 strain out of 11) in association with leaf discoloration, and in Jdita (1 strain out of 7) in association with witches'-broom and little leaf (Table 5; Figure 12).

Concerning non-crop weeds, '*Ca*. P. asteris' (16SrI-B) strains sharing identical 16S rDNA sequence between them and with pomegranate-infecting strain PG18 were identified in *P. major* and *C. annum* in Kufr Soum. Moreover, '*Ca*. P. aurantifolia' strains sharing identical 16S rDNA sequence (GenBank Acc. No. OL873114) were identified in *C. arvensis* from Kufr Soum (one plant) and Umjmal (four plants). Such strains, attributed to a variant of taxonomic subgroup 16SrII-C by virtual iPhyClassifier analysis, are distinct from '*Ca*. P. aurantifolia' pomegranate-infecting strains identified in Kufr Soum (subgroup 16SrII-B) (Table 5; Figure 13). Phytoplasma clustering in phylogenetic tree confirmed the attribution to phytoplasma species and taxonomic subgroups (Figure 13).

5.4.3 Molecular detection and identification of phytoplasmas in insects

During the field survey carried out in Irbid and Ajloun governorates from August to November 2020, 1918 Cicadomorpha and Fulgoromorpha adult insects were collected and distinguished, based on stereomicroscope analyses, in 10 taxonomic groups defined at species (5) and genus (5) level. Most of such insects belong to the family Cicadellidae (1830 specimens), while only 90 specimens belong to the family Delphacidae. Within Cicadellidae, the more abundant species Macrosteles *sexnotatus* (Fallén) (700 specimens), *Cicadulina bipunctata* (Melichar) (505 specimens), *Balclutha incisa* (Matsumura) (395 specimens), and *Zyginidia sohrab* Zachvatkin (135 specimens). In Kufr Soum, 9 out of 10 insect taxa were captured; in Jdita and Arjan only four and three insect taxa were captured, respectively. In these last two locations, *Cicadulina*, *Balclutha*, and *Laodelphax* (Table 8) were captured. Molecular analyses for phytoplasma detection and identification were conducted on 187 insect pools (146 from Kufr Soum, 27 from Jdita, and 14 from Arjan) representative of the observed diversity. Nested PCR allowed detecting phytoplasmas in eight insect pools (infection rate 4.3%), belonging to five different insect taxa, collected in Kufr Soum (infection rate 4.8%) and Jdita (infection rate 3.7%). No positive insect pools were found in Ajloun governorate. Infection rate of the phytoplasma-infected insect taxa was 100% in *Psammotettix striatus* (Linnaeus) (2 pools ou of 2), 14.3% in *Z. sohrab* (2 pools out of 14) and *B. incisa* (1 pool out of 7), 6.7% in *M. sexnotatus* (2 pools out of 30), and 2.5% in *C. bipunctata* (1 pool out of 40) (Table 7; Figure 5). Analyses

of 16S rDNA nucleotide sequences allowed attributing the phytoplasma strains detected in insects to the species '*Ca*. P. asteris' (7 pools out of 8) and '*Ca*. P. solani' (1 pool out of 8) (Table 7). In detail, '*Ca*. P. asteris' strains KF1-27 and -28 (found in *M. sexnotatus*), and KF2-33 (found in *C. bipunctata*) share 16S rDNA nucleotide sequences undistinguishable from those of pomegranate-infecting strain PG18, attributed to taxonomic subgroup 16SrI-B. '*Ca*. P. asteris' strains RK3A-1 and -2 (found in *P. striatus*) share 16S rDNA nucleotide sequences undistinguishable from those of pomegranate-infecting strain PG784, attributed to a variant of taxonomic subgroup 16SrI-R. '*Ca*. P. asteris' strains KF4-40 and -41 (found in *Z. sohrab*) have identical 16S rDNA nucleotide sequences (GenBank Acc. No. OL873115) distinct from the reference strain OAY by three SNPs at positions 488 (C/T), 539 (C/T), 698 (C/T). Based on similarity coefficient obtained by comparison of virtual RFLP patterns, confirmed by actual enzymatic digestion profiles (data not shown), '*Ca*. P. asteris' strains KF4-40 and -41 were attributed to a variant of taxonomic subgroup 16SrI-B (Table 7; Figure 13). '*Ca*. P. solani' strain RJ2-44 (found in *B. incisa*) shares identical 16S rDNA nucleotide sequence with the pomegranate-infecting strain PG797, attributed to taxonomic subgroup 16SrXII-A (Table 7). Phytoplasma clustering in phylogenetic tree confirmed the attribution to phytoplasma species and taxonomic subgroups (Figure 13).

5.5 Discussion

Results obtained from field surveys and molecular analyses conducted during this study revealed the association of four distinct '*Ca*. Phytoplasma' species ('*Ca*. P. solani', '*Ca*. P. aurantifolia', '*Ca*. P. asteris', '*Ca*. P. ulmi'), including five 16Sr taxonomic subgroups (16SrXII-A, II-B, I-B, I-R, V-A), to pomegranate witches'-broom and leaf alteration in orchards located in northern Jordan. Interestingly, the majority of these phytoplasmas identified in symptomatic pomegranate trees were already reported in Jordan in association with diseases of other important crops. Four phytoplasmas groups were found in Jordan. They were found associated with peach, tomato, grapevines, potato and plum. Tomato Big bud disease (clover proliferation - 16SrVI) was the first phytoplasma disease reported in Jordan in 2003 in Al-Mafraqa area (Anfoka *et al*, 2003). Followed by aster yellows phytoplasma (16SrI) on peach in 2004 in two regions (Anfoka and Fattash, 2004). '*Ca.* P. solani' (16SrXII) has been associated with grapevine ("bois noir" 16SrXII-A) (Salem *et al*, 2013) and plum (*Prunus domestica*) in Al-Mafraq area (desert) (Salem *et al*, 2019). '*Candidatus* Phytoplasma aurantifolia' was reported on potato in 2019 (Salem *et al*, 2019).

tomato, grapevine, plum, potato, date palm) (Anfoka et al., 2003; Anfoka & Fattash, 2004; Alhudaib et al., 2019; Salem et al., 2013, 2019, 2020). Up to now, phytoplasma diseases of pomegranate were found associated with five '*Ca*. Phytoplasma' species inducing different symptoms around the world. Pomegranate yellows were found associated with '*Ca.* P. asteris' (16SrI-B) and '*Ca.* P. solani' (16SrXII-A) in Turkey (Gazel et al., 2016). Pomegranate decline and little leaf were found associated respectively with '*Ca.* P. pruni' (16SrIII) and '*Ca*. P. australasia' (16SrII-D) in Iran (Karimishahri et al., 2015; Salehi et al., 2016). Pomegranate fasciation in China was found associated with '***Ca.*** P. asteris' (16SrI-B) (Gao et al., 2018). In India, pomegranate little leaf, yellows and malformation were associated with infections by '*Ca.* P. australasia' (16SrII-D), while pomegranate leaf yellowing and reddening were associated with '*Ca*. P. oryzae' (16SrXI-B) (Rao et al., 2020). In Guadalupe (central America), pomegranate little leaf, yellows and dried branch were associated with '*Ca*. P. asteris', subgroups 16SrI-B and -F (Castañeda-Alvarez et al., 2018). Thus, to the best of our knowledge, the association of pomegranate diseases with '*Ca*. P. aurantifolia' (16SrII-B), '*Ca*. P. ulmi' (16SrV-A), and '*Ca*. P. asteris' (variant of subgroup 16SrI-R) was reported for the first time in this study. Also in Jordan, different symptoms exhibited by pomegranate were found associated with infection by distinct phytoplasmas. However, such symptom range can be related also to differences in phytoplasma-plant interactions in pomegranate cultivars and/or in specific environmental features of the examined locations, as reported for other crops (Bisognin et al., 2008; Hren et al., 2009; Murolo and Romanazzi, 2015; Quaglino et al., 2016).

Even if the incidence of phytoplasma-like symptoms was high in examined orchards, only 17% of collected symptomatic pomegranate trees were found phytoplasma infected. This can be due to: (i) the sporadic distribution of phytoplasmas in phloem tissues of infected plants (Constable et al., 2003); (ii) the possible low concentration of phytoplasma cells in plant tissues in the different sampling periods (from July to November) (Martini et al., 2011); (iii) the possibility that observed symptoms are caused by other etiological agents or to abiotic stresses. Concerning the distribution of '*Ca*. Phytoplasma' species identified in pomegranate orchards, obtained data indicated that '*Ca*. P. solani' is the prevalent phytoplasma infecting pomegranate throughout the investigated areas. This evidence confirmed previous studies conducted in the Country revealing the large prevalence of such phytoplasma associated with 'bois noir' of grapevine and plum yellowing and witches'-broom (Salem et al., 2013, 2020). Interestingly, '*Ca*. P. solani' strains, undistinguishable based on 16S rDNA nucleotide sequences, were found in three different pomegranate cultivars (Khdary, Mawardy, Wonderful), in three distinct

locations, showing distinct symptoms. Based on this fact, it is reasonable to hypothesize that symptom diversity can be due to different, specific interaction between the same phytoplasma strain and pomegranate cultivars. On the other hand, further analyses should be conducted to type more accurately the '*Ca*. P. solani' strains using molecular markers on hyper-variable genes, such as *secY*, *stamp* and *vmp1*, recently correlated to differences in strain virulence (Pierro et al., 2018; 2020). Molecular analyses on weeds and putative insect vectors figured out that '*Ca*. P. solani' was identified in the insect *B. incisa* (with 14.3% infection rate) in Jdita location, while no weeds were found infected. *B. incisa* (Cicadellidae), firstly reported in this study in Jordan, is known as vector of '*Ca*. P. australasiae' (16SrII-D) associated with Fenugreek phyllody in Pakistan (Malik et al., 2020), and '*Ca*. P. aurantifolia' (16SrII) associated with cactus witches'-broom in Indonesia (Wulandari et al., 2021). This insect is present worldwide and prefers feeding on grasses (Narhardiyati & Bailey, 2005). These overall evidence supports the fact that *B. incisa* can be a potential vector of '*Ca*. P. solani' in Jordan, with preferential activity limited to weeds present in the orchards. Thus, it could be suggested that *B. incisa* can increase the diffusion of '*Ca*. P. solani' in reservoir wild herbaceous plants and that other insects, not found in the present study, could acquire the phytoplasma from weeds and transmit it to pomegranate or other crops and/or forest trees present in the area. Remarkably, even if '*Ca*. P. asteris' (subgroups 16SrI-B and 16SrI-R variant) was found only in two pomegranate trees in Irbid governorate, most phytoplasma-infected insects (7 specimens belonging to four distinct taxa) collected in the same locations were found infected by this phytoplasma species. Molecular analyses evidenced that *M. sexnotatus* (firstly reported in Jordan in this study) and *C. bipunctata* hosted phytoplasma strains identical to pomegranate-infecting strain PG18 ('*Ca*. P. asteris', 16SrI-B), while *P. striatus* hosted phytoplasma strains undistinguishable from pomegranate-infecting strain PG784 ('*Ca*. P. asteris', 16SrI-R variant). Previous studies reported that *M. sexnotatus* and *P. striatus* are vector of 16SrI group phytoplasmas ('*Ca*. P. asteris' and '*Ca*. P. tritici', respectively), while *C. bipunctata* was found as potential phytoplasma vector (Alma et al., 2015; Alhudaib et al., 2007; Weintraub & Beanland, 2006; Wu et al., 2010). Moreover, '*Ca*. P asteris' (16SrI-B) strains, identical to those identified in pomegranate and *M. sexnotatus*, were found also in non-crop plants *Plantago major* L. and *Capsicum annuum* L. in Kufr Soum. Based on this evidence, it could be proposed that at least two ecological cycles can be involved in diffusion of '*Ca*. P. asteris' strains found in pomegranate in Jordan: the first one, related to '*Ca*. P asteris' (16SrI-B), could include pomegranate (crop), *M. sexnotatus* (vector), *P. major* and *C. annuum* (reservoir plants); the second one, related to '*Ca*. P asteris' (16SrI-R variant), could include pomegranate (crop) and

P. striatus (vector). Moreover, *Z. sohrab*, one of the four insect taxa infected by '*Ca*. P. asteris' in Kufr Soum, carried phytoplasma strains attributed to a subgroup 16SrI-B variant, not identified in pomegranate and weeds. Upscaling sample collection from pomegranate, non-crop plants, and other crops should be investigated to clarify if this phytoplasma strain could be present, giving importance to *Z. sohrab* as putative vector. Noteworthy, '*Ca*. P. aurantifolia' (16SrII-B) and '*Ca*. P. ulmi' (16SrV-A and its variant) strains, identified in pomegranate, were not detected in both analyzed insects and non-crop plants. In fact, all phytoplasma-infected bindweeds were found hosting '*Ca*. P. aurantifolia' strains attributed to a variant of subgroup 16SrII-C. Considering that insect survey was preliminary and conducted in short period and restricted arena in each location, limited description of the entomofauna diversity present in the pomegranate orchards was acquired. Consequently, the sole identification of '*Ca*. P. asteris' and '*Ca*. P. solani' in the collected insects is understandable.

In conclusion, this study described a new pomegranate disease complex, including witches'-broom and leaf alteration, associated with four distinct '*Ca*. Phytopalsma' species in northern Jordan, and provided preliminary insights on its epidemiology, indicating putative insect vectors and reservoir plants potentially involved in spreading of these phytoplasmas. Further studies focusing on the following topics are necessary: (i) transmission trials to verify the capability of putative insect vectors, identified in the present work, to transmit '*Ca*. P. asteris' and '*Ca*. P. solani' to pomegranate; (ii) insect population diversity and dynamics conducted throughout the whole season in the affected orchards; (iii) upscaling survey of pomegranate disease complex in the whole Country.

Table 5. Collected and phytoplasma-infected pomegranate and non-crop plant samples from surveyed locations in northern Jordan.

Governorate	Location	Plant	No. of collected samples	No. of phytoplasma-infected samples
Irbid	Kufr Soum	Symptomatic *Punica granatum* L. (Khdary)	50	11
		Asymptomatic *Punica granatum* L. (Khdary)	5	0
		Convolvulus arvensis L.	4	1
		Capsicum annuum L.	2	1
		Rubus sp.	2	0
		Malva sylvestris L.	1	0
		Chenopodium album L.	4	0
		Plantago major L.	1	1
	Jdita	Symptomatic *Punica granatum* L. (Mawardy)	20	7
		Asymptomatic *Punica granatum* L. (Mawardy)	2	0
		Chenopodium album L.	2	0
		Malva sylvestris L.	1	0
		Origanum vulgare L.	2	0
		Bidens sp.	2	0
Ajloun	Arjan	Symptomatic *Punica granatum* L. (unknown)	12	0
		Asymptomatic *Punica granatum* L. (unknown)	2	0
		Inula sp.	1	0
		Malva sylvestris L.	1	0
		Amaranthus sp.	2	0
		Lactuca sp.	1	0
Al-Mafraq	SabhaWsubhieh	Symptomatic *Punica granatum* L. (Wonderful)	10	1
		Asymptomatic *Punica granatum* L. (Wonderful)	2	0
	Umjmal	Symptomatic *Punica granatum* L. (Erqaby)	20	0
		Asymptomatic *Punica granatum* L. (Erqaby)	2	0
		Convolvulus arvensis	4	4
		Overall total	155	26

Table 6. Attribution to species and taxonomic subgroups of phytoplasmas detected in plants.

Strain	Plant host	Pomegranate cultivar	Location	Symptoms	Phytoplasma species	Identity % versus reference strain	16Sr subgroup	Acc. No.
PG784	*Punica granatum* L.	Khdary	Kufr Soum	leaf discoloration	'*Ca*. P. asteris'	99.6	I-R* (0.98)	OL873108 (a)
PG795	*Punica granatum* L.	Khdary	Kufr Soum	leaf reddening and rolling	'*Ca*. P. aurantifolia'	99.9	II-B (1.00)	OL873109 (b)
PG789	*Punica granatum* L.	Khdary	Kufr Soum	leaf reddening and rolling	'*Ca*. P. aurantifolia'	99.9	II-B (1.00)	b
PG791	*Punica granatum* L.	Khdary	Kufr Soum	leaf reddening and rolling	'*Ca*. P. aurantifolia'	99.9	II-B (1.00)	b
PG793	*Punica granatum* L.	Khdary	Kufr Soum	leaf reddening and rolling	'*Ca*. P. aurantifolia'	99.9	II-B (1.00)	b
PG797	*Punica granatum* L.	Khdary	Kufr Soum	yellowing, little leaf	'*Ca*. P. solani'	99.7	XII-A (1.00)	OL873110 (c)
PG799	*Punica granatum* L.	Khdary	Kufr Soum	yellowing, little leaf	'*Ca*. P. solani'	99.7	XII-A (1.00)	c
PG806	*Punica granatum* L.	Khdary	Kufr Soum	yellowing, little leaf	'*Ca*. P. solani'	99.7	XII-A (1.00)	c
PG798	*Punica granatum* L.	Khdary	Kufr Soum	yellowing, little leaf	'*Ca*. P. solani'	99.7	XII-A (1.00)	c
PG1	*Punica granatum* L.	Khdary	Kufr Soum	leaf reddening and rolling	'*Ca*. P. ulmi'	99.9	V-A (1.00)	OL873111 (d)
PG7	*Punica granatum* L.	Khdary	Kufr Soum	leaf reddening and rolling	'*Ca*. P. ulmi'	99.8	V-A* (0.98)	OL873112 (e)
PM14	*Plantago major* L.		Kufr Soum	Symptomless	'*Ca*. P. asteris'	99.9	I-B (1.00)	OL873113 (f)
CAn22	*Capsicum annuum* L.		Kufr Soum	Yellowing	'*Ca*. P. asteris'	99.9	I-B (1.00)	f
CAr403	*Convolvulus arvensis* L.		Kufr Soum	yellowing, little leaf	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	OL873114 (g)
PG624	*Punica granatum* L.	Mawardy	Jdita	witches'-broom, little leaf	'*Ca*. P. solani'	99.7	XII-A (1.00)	c
PG621	*Punica granatum* L.	Mawardy	Jdita	witches'-broom, little leaf	'*Ca*. P. solani'	99.7	XII-A (1.00)	c
PG623	*Punica granatum* L.	Mawardy	Jdita	witches'-broom, little leaf	'*Ca*. P. solani'	99.7	XII-A (1.00)	c
PG619	*Punica granatum* L.	Mawardy	Jdita	witches'-broom, little leaf	'*Ca*. P. solani'	99.7	XII-A (1.00)	c
PG620	*Punica granatum* L.	Mawardy	Jdita	witches'-broom, little leaf	'*Ca*. P. solani'	99.7	XII-A (1.00)	c
PG17	*Punica granatum* L.	Mawardy	Jdita	witches'-broom, little leaf	'*Ca*. P. ulmi'	99.9	V-A (1.00)	d
PG18	*Punica granatum* L.	Mawardy	Jdita	witches'-broom, little leaf	'*Ca*. P. asteris'	99.9	I-B (1.00)	f
PG404	*Punica granatum* L.	Wonderful	SabhaWsubhieh	Yellowing	'*Ca*. P. solani'	99.7	XII-A (1.00)	c
CAr399	*Convolvulus arvensis* L.		Umjmal	yellowing, little leaf	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	g
CAr400	*Convolvulus arvensis* L.		Umjmal	yellowing, little leaf	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	g
CAr401	*Convolvulus arvensis* L.		Umjmal	yellowing, little leaf	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	g
CAr402	*Convolvulus arvensis* L.		Umjmal	yellowing, little leaf	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	g

[a] Representative sequence utilized for phylogenetic analysis

Table 7. Collected and phytoplasma-infected insects from surveyed locations in northern Jordan.

Governorate	Location	Insect code	Family	Species	Collection date	No. of collected insects	No. of pools	No. of positive pools	Infection rate (%)
Irbid	Kufr Soum	KF1	Cicadellidae	**Macrosteles sexnotatus*	November	700	30	2	6.7
Irbid	Kufr Soum	KF2	Cicadellidae	*Cicadulina bipunctata*	August	440	40	1	2.5
Irbid	Kufr Soum	KF3	Cicadellidae	*Anaceratagallia* sp.	November	40	20	0	
Irbid	Kufr Soum	KF4	Cicadellidae	*Zyginidia sohrab*	August	135	14	2	14.3
Irbid	Kufr Soum	KF7	Cicadellidae	**Balclutha incisa*	November	175	24	0	
Irbid	Kufr Soum	KF5	Cicadellidae	**Eupteryx stachydearum*	November	49	10	0	
Irbid	Kufr Soum	RK2	Delphacidae	**Laodelphax striatellus*	August	35	6	0	
Irbid	Kufr Soum	RK3A	Cicadellidae	*Psammotettix striatus*	August	4	2	2	100
Irbid	Jdita	RJ1	Cicadellidae	*Balclutha incisa*	October	120	7	1	14.3
Irbid	Jdita	RJ1A	Delphacidae	*Laodelphax striatellus*	October	25	9	0	
Irbid	Jdita	RJ1C	Delphacidae	*Laodelphax* sp.	October	10	4	0	
Irbid	Jdita	RJ3	Cicadellidae	*Cicadulina bipunctata*	October	55	7	0	
Ajloun	Arjan	AR1	Cicadellidae	*Balclutha incisa*	October	100	6	0	
Ajloun	Arjan	AR2	Delphacidae	*Laodelphax striatellus*	October	20	5	0	
Ajloun	Arjan	AR3	Cicadellidae	*Cicadulina bipunctata*	October	10	3	0	
				Total		1918	187	8	4.3

- Insect species are firstly reported in Jordan.

Table 8. Attribution to species and taxonomic subgroups of phytoplasmas detected in insects.

Insect	Species	Region	Phytoplasma species	Identity % versus reference strain	16Sr subgroup	Acc. No.
KF1-27	*Macrosteles sexnotatus*	Kufr soum	'*Ca*. P. asteris'	99.9	I-B (1.00)	F
KF1-28	*Macrosteles sexnotatus*	Kufr soum	'*Ca*. P. asteris'	99.9	I-B (1.00)	F
KF2-33	*Cicadulina bipunctata*	Kufr soum	'*Ca*. P. asteris'	99.9	I-B (1.00)	F
KF4-40	*Zygidinia sohrab*	Kufr soum	'*Ca*. P. asteris'	99.8	I-B* (0.98)	OL873115 (h)
KF4-41	*Zygidinia sohrab*	Kufr soum	'*Ca*. P. asteris'	99.8	I-B* (0.98)	H
RK3A-1	*Psammotettix striatus*	Kufr soum	'*Ca*. P. asteris'	99.6	I-R* (0.98)	A
RK3A-2	*Psammotettix striatus*	Kufr soum	'*Ca*. P. asteris'	99.6	I-R* (0.98)	A
RJ2-44	*Balclutha incisa*	Jdita	'*Ca*. P. solani'	99.7	XII-A (1.00)	C

[a] Representative sequence utilized for phylogenetic analysis

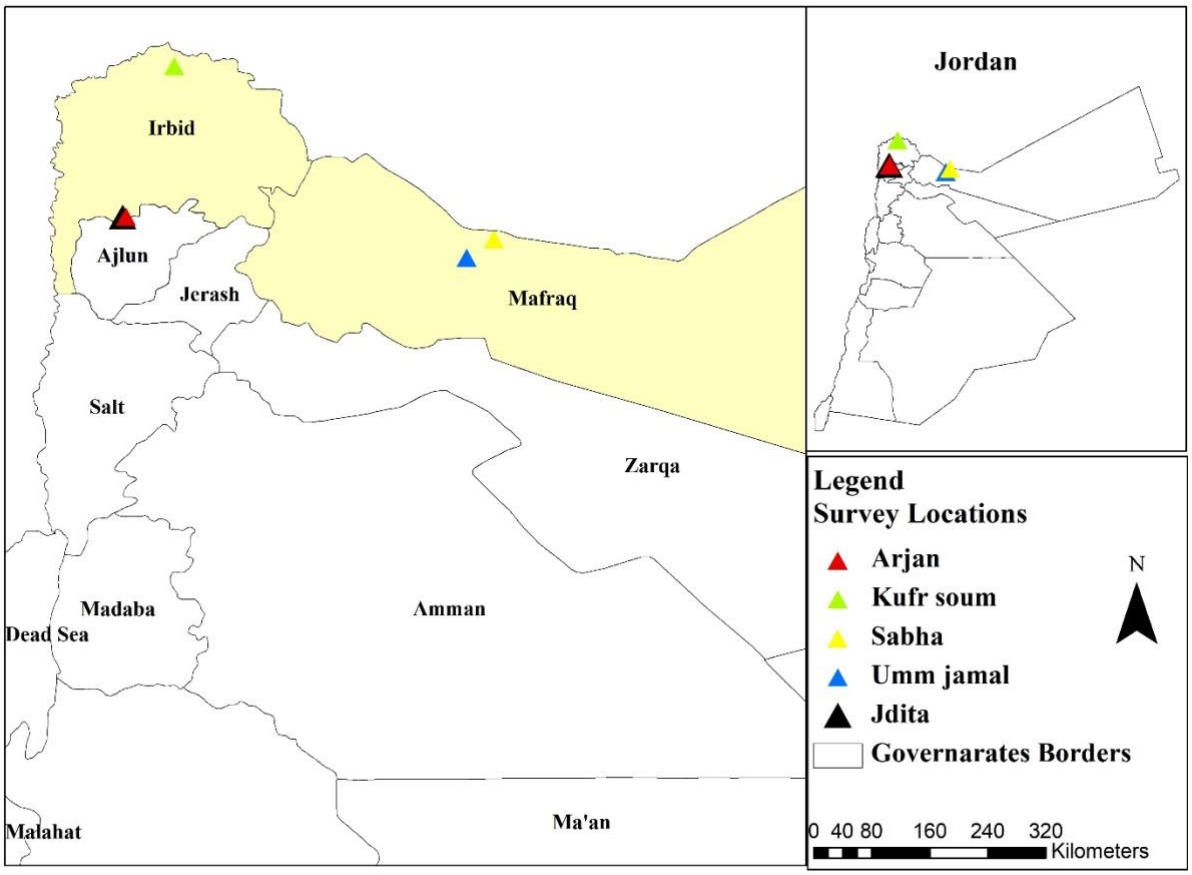

Figure 10. Maps of governorates and locations in North Jordan where the surveys on phytoplasma-like diseases in pomegranate orchards. were conducted.

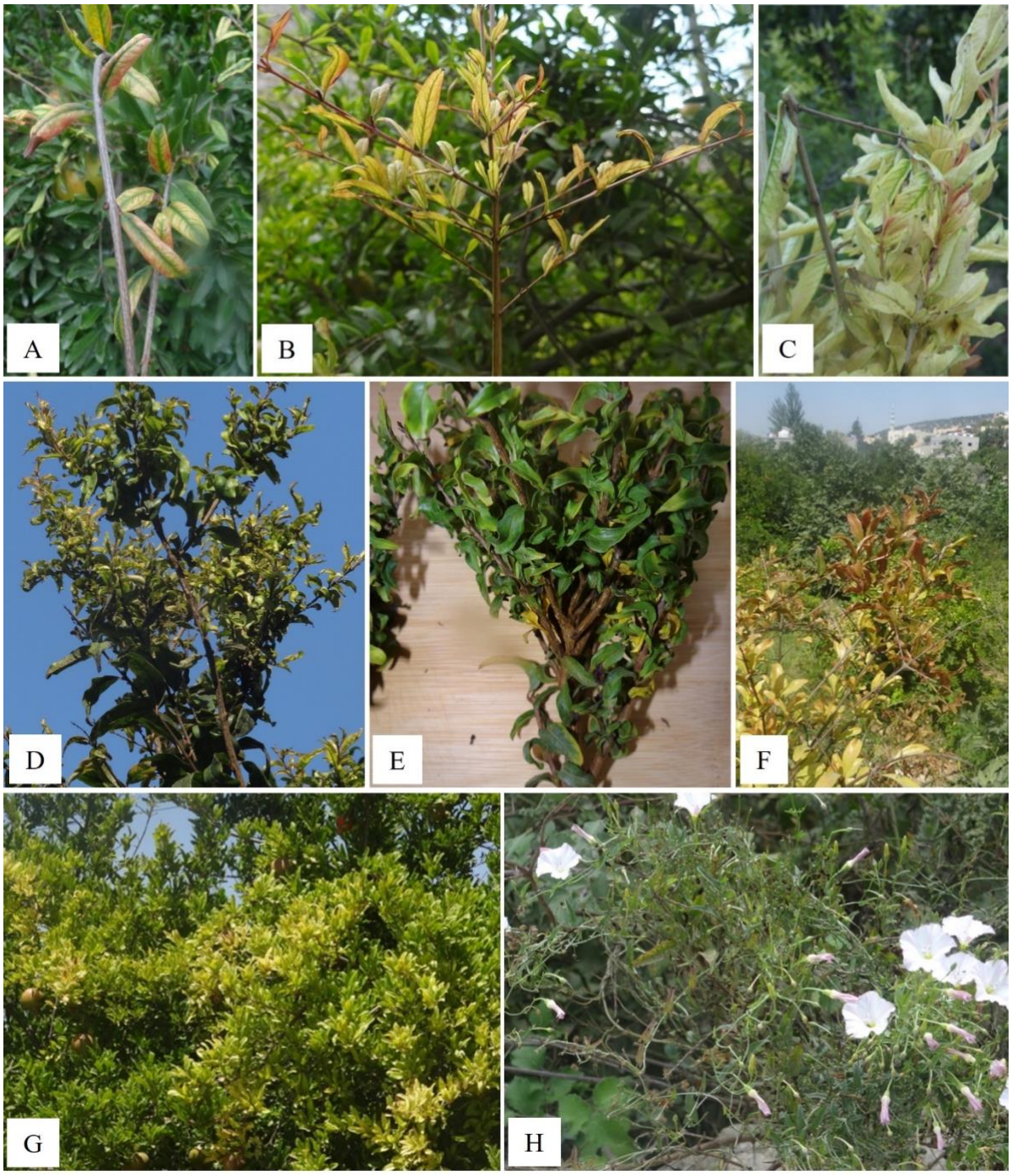

Figure 11. Phytoplasma-like symptoms observed in pomegranate trees and weeds in northern Jordan. Leaf reddening and rolling (A), leaf yellowing and little leaf (B), and leaf discoloration (C) exhibited by Khdary pomegranate cultivar in Kufr Soum, Irbid governorate; witches'-broom, little leaf, and yellowing (D, E) exhibited by Mawardy pomegranate cultivar in Jdita, Irbid governorate; leaf reddening (F) observed in pomegranate in Arjan, Ajloun governorate; leaf reddening and/or yellowing (G) observed in Wonderful and Erqaby pomegranate cultivars respectively in SabhaWsubhieh and Umjmal, Al-Mafraq governorate; leaf yellowing and little leaf (H) observed on *Convolvulus arvensis* in Kufr Soum, Irbid governorate.

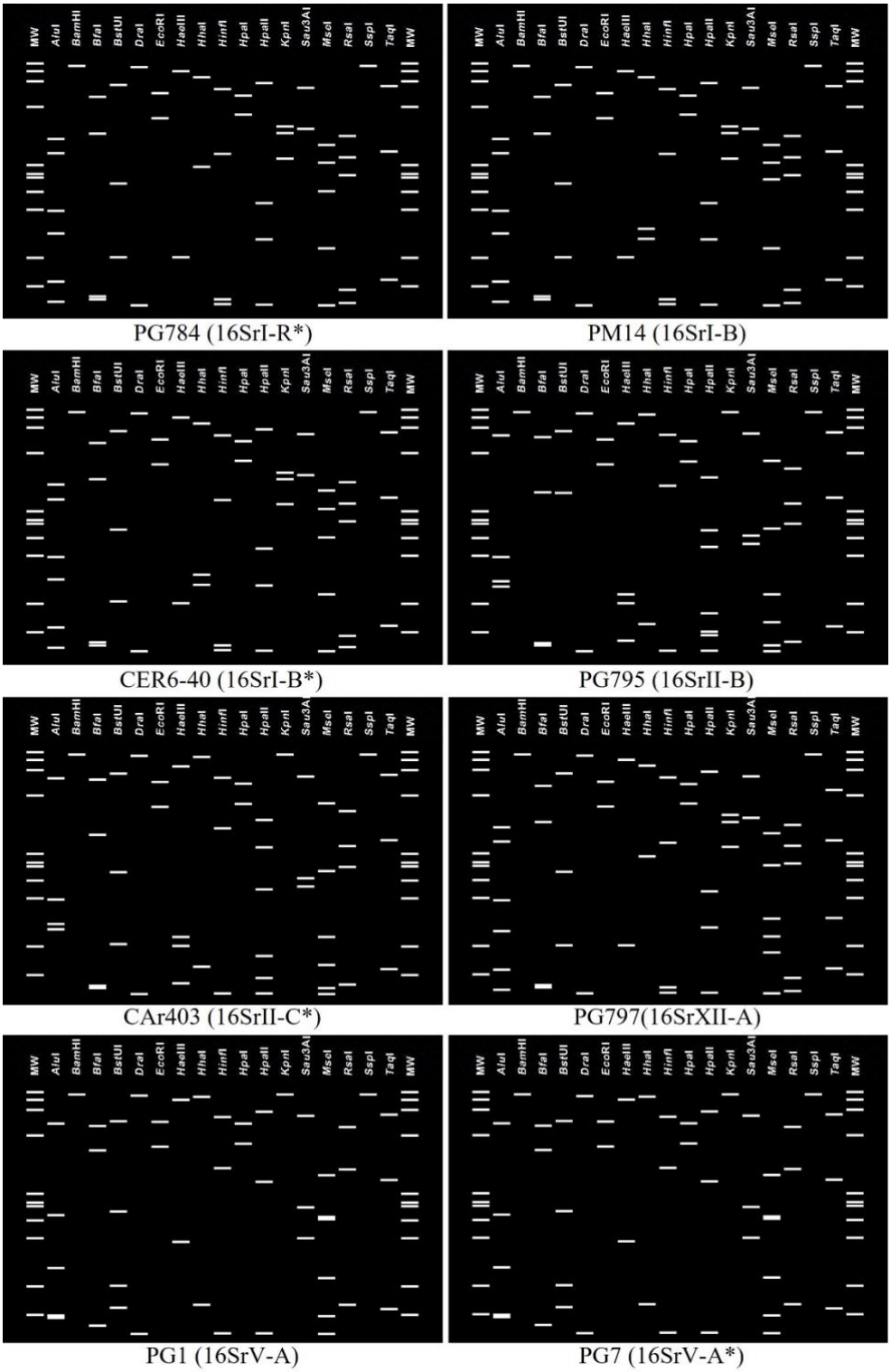

Figure 12. Virtual RFLP profiles of 16S rDNA nucleotide sequences of phytoplasma strains identified in pomegranate, weeds, and insects in northern Jordan. One strain among those sharing identical 16S rDNA sequence (Tables 2 and 4) was selected as representative strain for iPhyClassifier analyses.

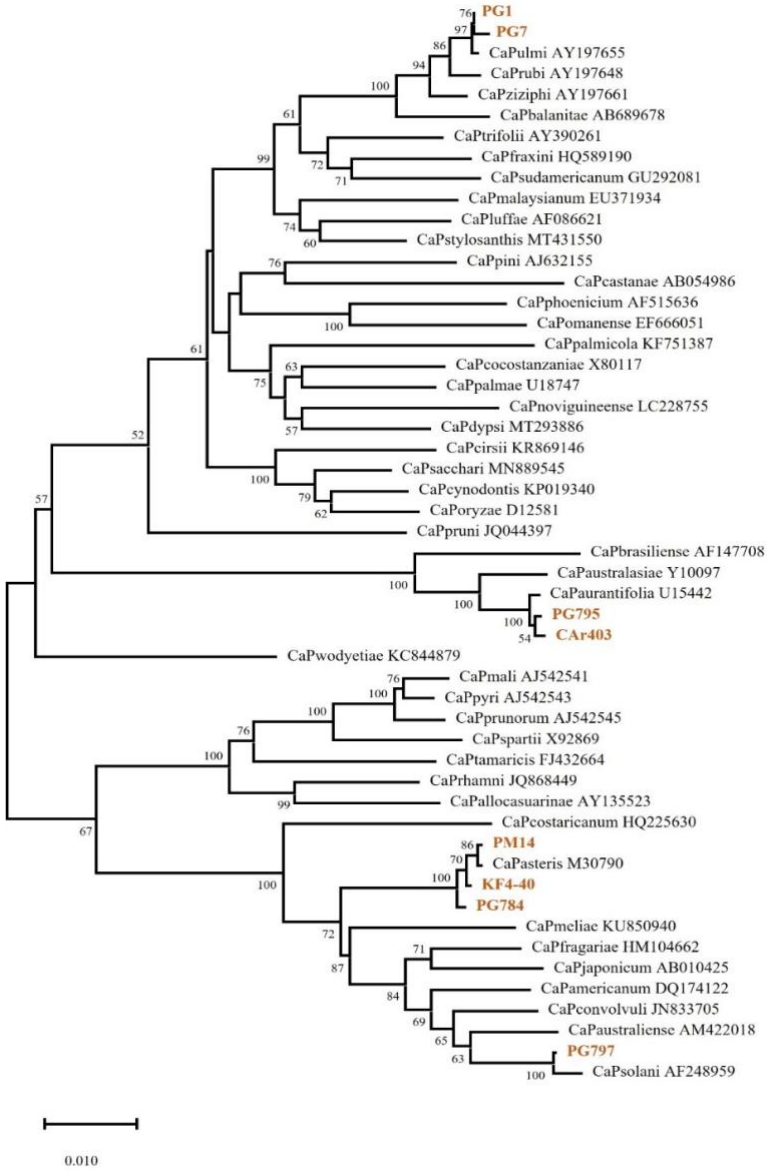

Figure 13. Phylogenetic tree based on the alignment of 16S rDNA nucleotide sequences of representative phytoplasma strains identified in pomegranate, putative vectors, and reservoir plants in Jordan (orange bold characters), and reference strains of previously described '*Candidatus* Phytoplasma' species. Regarding phytoplasmas identified in this study, one strain among those sharing identical 16S rDNA sequence (Tables 2 and 4) was selected as representative strain for phylogenetic analysis. The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 0.84447270 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. This analysis involved 52 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option**).**

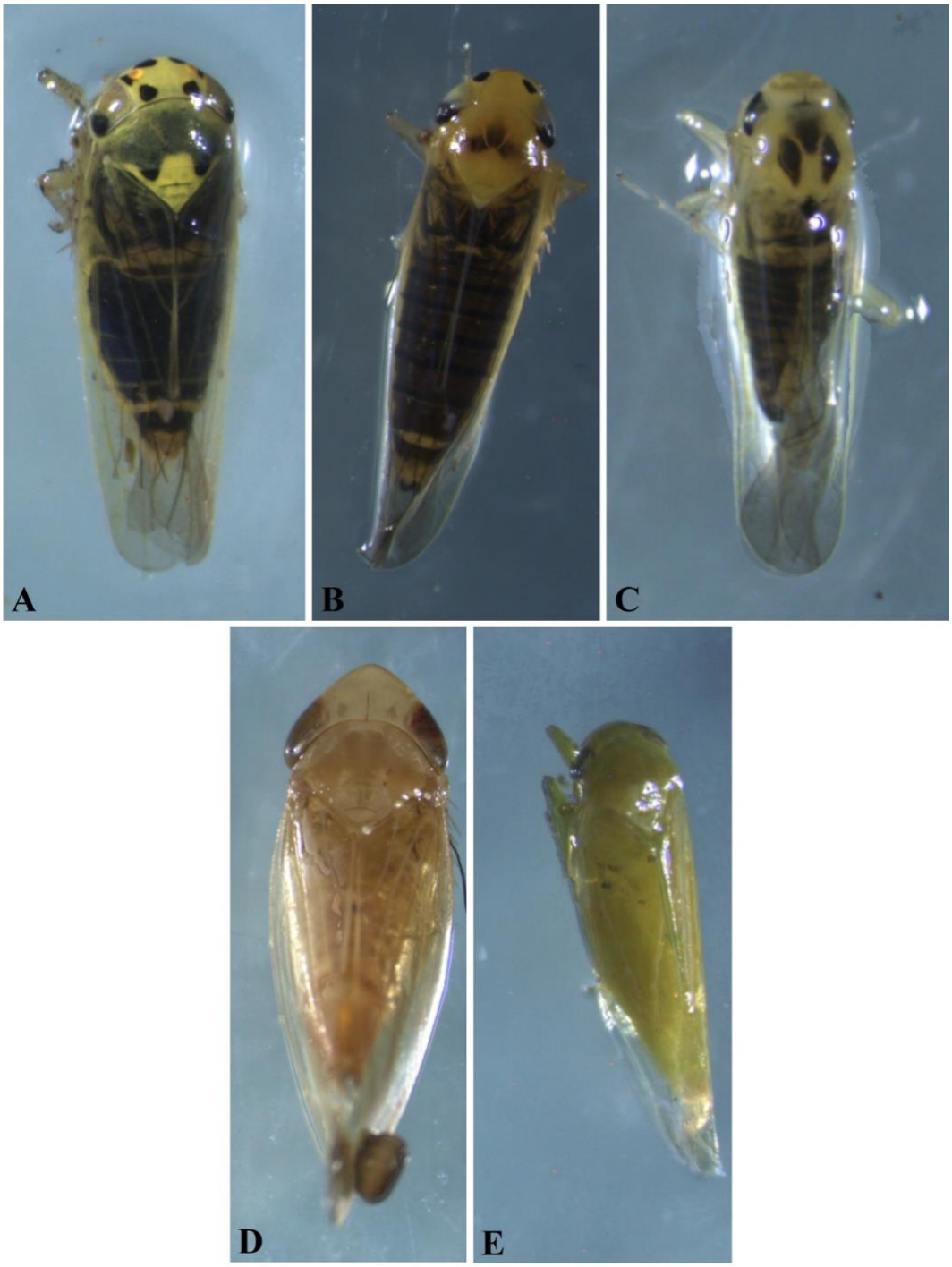

Figure 14. Putative insect vectors of phytoplasmas infecting pomegranate in northern Jordan. (A) *Macrosteles sexnotatus*, (B) *Cicadulina bipunctata*, (C) *Zygidinia sohrab*, (D) *Psammotettix striatus* (F) *Balclutha incisa*.

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Chapter 6. Grapevine yellows in Jordan: associated phytoplasmas, putative insect vectors, and reservoir plants

6.1 Abstract

During field surveys conducted from June to October 2020 in 13 locations belonging to five governorates in North and South Jordan, typical grapevine yellows (GY) symptoms, including leaf reddening/yellowing and rolling, were observed in wine and table grape cultivar vineyards. Disease incidence in the investigated vineyards ranged from 10 to 55%. Nested PCR-based amplification of *16S rRNA* gene detected phytoplasmas in 22% and 15.7% of symptomatic wine and table grape cultivar plants, respectively. Amplicon nucleotide sequence analyses allowed attributing the detected phytoplasmas to '*Candidatus* Phytoplasma solani' (taxonomic subgroup 16SrXII-A), '*Ca*. P. omanense' (16SrXXIX-A and -B), '*Ca*. P. aurantifolia' (16SrII-C), and '*Ca*. P. asteris' (16SrI-R) in 72.4%, 17.2%, 6.9%, 3.4% of infected plants, respectively. Such phytoplasmas were found differentially distributed in wine and table grape cultivar vineyards in the considered locations. Further investigation allowed identifying '*Ca*. P. solani' in the putative insect vectors *Orosius cellulosus* (firstly reported in Jordan), *Euscelidius mundus*, *Laodelphax striatellus*, and *Circulifer* sp., and in *Convolvulus arvensis*; '*Ca*. P. aurantifolia' in the insect *O. cellulosus* and in bindweed; '*Ca*. P. omanense' in the insect *Psammotettix striatus*; '*Ca*. P. asteris' in the insects *Arboridia adanae*, *Cicaduliana bipunctata*, *Circulifer* sp., *L. striatellus*, *Hyalesthes obsoletus*, and *P. striatus*. Based on this preliminary data, ecological cycles of such phytoplasmas are discussed. Obtained results suggest that GY phytoplasma diversity and ecology in Jordan are more complex than previously known, leading to a potential risk of disease outbreaks. Further studies are needed to survey the GY diffusion covering more areas throughout the full vegetative season of grapevine and non-crop reservoir plants, demonstrate the transmission capability of the identified putative vectors, and investigate the insect population dynamics.

6.2 Introduction

\Grapevine yellows (GY) are a complex of diseases associated with genetically distinct phytoplasmas causing undistinguishable symptoms on *Vitis vinifera* L., including desiccation of inflorescences, berry shrivel, leaf discolorations, reduction of growth and irregular ripening of wood (Belli et al., 2010; Angelini et al., 2018). The main GY diseases are: (i) Flavescence dorée (FD), present mainly in Europe, associated with 16SrV-C and -D phytoplasmas transmitted mainly by the vector *Scaphoideus titanus* Ball (Arnaud et al., 2007; Malembic-Maher et al., 2020); (ii) Bois noir (BN), widespread throughout Europe, Asia, South America, and South Africa, associated with '*Candidatus* Phytoplasma solani' (16SrXII-A) transmitted by *Hyalesthes obsoletus* Signoret and *Reptalus panzeri* Loew (Quaglino et al., 2013; Cvrković et al., 2014; Pierro et al., 2019); (iii) Palatinate grapevine yellows (PGY), present in Germany and in other European countries, associated with 16SrV-C phytoplasmas transmitted by *Oncopsis alni* Schrank (Angelini et al., 2001); (iv) Australian grapevine yellows (AGY), present in Australia and New Zealand, associated with '*Ca*. P. australiense' (16SrXII-B), transmitted by *Oliarus atkinsoni* Meyers (Padovan et al., 1995; Liefting et al., 1997); (v) North America grapevine yellows (NAGY), associated with '*Ca.* P. pruni' (16SrIII-A), transmitted by *Jikradia olitoria* Say, and '*Ca*. P. asteris' (16SrI-A) (Davis et al., 1998, 2015; Lenzi et al., 2019); (vi) South Africa grapevine yellows, associated with '*Ca*. P. asteris' (16SrI-B) transmitted by *Mgenia fuscovaria* Stål (Engelbrecht et al., 2010; Pietersen et al., 2018).

In Jordan, grapevine is a key commercial fruit crop with two farming styles: family and commercial farms. More than 8,960 ha are cultivated with total production estimated by 56,000 ton in 2019 (MAO STAT, 2021). Table grape cultivars, very popular and cultivated in the whole Country, are characterized by a long harvesting season extending from May (for the early seedless cultivar growing in Jordan valley) to October. Concerning GY in Jordan, BN associated with '*Ca.* P. solani' (16SrXII-A) was reported in wine grape cultivars and bindweed in one vineyard in North Padia (Salem *et al.,* 2013). No further studies were conducted in the Country.

Following the first report of BN and based on the recent observation of phytoplasma-like symptoms in several viticultural areas in Jordan, the present study aimed at (i) surveying the diffusion of GY symptoms throughout the Country focusing on both table grape and wine grape cultivars, (ii) collecting putative insect vectors and reservoir plants (non-crop plants), (iii) detect and identify the phytoplasmas in grapevines, potential insect vectors, and reservoir plants.

6.3 Materials and Methods

6.3.1 Field surveys, plant sampling, and insect collection

Field surveys on grapevine yellows-like symptoms were conducted from June to October 2020 in 13 locations belonging to five governorates in North (12) and South (1) Jordan, including vineyards with table grape cultivars (11 locations) and wine grape cultivars (2 locations- two huge orchards with several modern blocks) (Figure 15). Around 15 table grape farms All surveyed orchards (farm) in Al-Mafraq (including five table grape farms and two wine) and Al-disi (one farm) areas were characterized by high density plantation, modern turning system, and growing under irrigated conditions. While all surveyed orchards (farms) in Ajloun and Irbid were under rainfed conditions, cultivated in ground and netting systems, and ranging from 0.3 up to 2 ha. More than 20, 000 grapes in different locations. were observed. In each location, incidence of phytoplasma-like diseases was estimated as the percentage of symptomatic trees out of the observed ones. Leaf samples were collected from 50 plants of wine grape cultivars, and 102 plants of table grape cultivars exhibiting grapevine yellows-like symptoms, and from 25 symptomless plants (five from wine grape cultivars; 20 from table grape cultivars). Moreover, leaf samples were collected from 22 symptomatic and 8 symptomless bindweed (*Convolvulus arvensis* L.) plants observed within and around the surveyed vineyards (Table 9). Collected samples were transferred to the laboratories of National Agricultural Research Center (NARC), Baqaà, Jordan, and maintained at 4°C until total nucleic acids extraction. Additionally, insects within the surveyed vineyards were collected by entomological sweeping net and transferred to the NARC laboratories. Stereomicroscope observation was conducted for preliminary selection of phloem-feeding insects (putative phytoplasma vectors). The selected insects were kept in 99% ethanol until their identification carried out at the Department of Agriculture, Forest, and Food Science (DISAFA), University of Turin, Italy. The insect identification was based on stereomicroscope observation of phenotypic characters and male genitalia after their dissection and clarification in a 10% potassium hydroxide solution. Insects recognized at genus/species level were maintained in 99% ethanol at -20°C until total nucleic acids extraction.

6.3.2 Phytoplasma detection

Total nucleic acids (TNA) were extracted from the collected plant samples as previously described by Angelini et al. (2001) with some modifications, while the insect TNA were extracted based on the protocol designed by Marzachì et al. (1998). Concerning plant samples, leaf midribs and petioles (0.5 g) were grounded in 3 ml of prewarmed 2% CTAB-based buffer in sterile mortars. Regarding insects, for each species, TNA extraction was done from single or pooled (2 to 5) individuals based on their size and/or number of captured specimens. Individual or pooled insects were crashed by sterile pestles in 1.5 ml tubes containing sand and 0.5 ml of prewarmed 2.5% CTAB-based buffer. Extracted TNA was washed by 0.5 ml of 70% ethanol, dissolved in 50 (insects) or 100 (plants) µl of TE-based buffer, measured for quantity and quality by Nanodrop system, and stored at -20°C until molecular analyses.

TNAs extracted from plants and insects were used as templates in nested PCR reactions conducted to detect the presence of phytoplasmas. Nested PCRs were carried out to amplify the phytoplasma 16S rRNA gene using the primer pair P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995) followed by the primer pair R16F1/R16R0 (Lee et al., 1995). Reaction mixtures and conditions were as previously described (Quaglino et al., 2009). TNAs extracted from periwinkle [*Catharanthus roseus* L. (G. Don)] plants, infected by phytoplasma strains STOL ('*Ca*. Phytoplasma solani', subgroup 16SrXII-A) and AY1 ('*Ca*. P. asteris', subgroup 16SrI-B) and maintained in greenhouse at Department of Agricultural and Environmental Sciences, University of Milan (Italy), were employed as positive controls. TNAs extracted from healthy periwinkle and reaction mixtures devoid of TNAs were used as negative controls. PCR products (6 µl) were analyzed by electrophoresis on 1% (w/v) agarose gels in 1X TBE buffer, stained with Midori Green, and visualized on UV transilluminator. In each location, phytoplasma infection rate was estimated as the percentage of infected plants out of the examined ones.

6.3.3 Phytoplasma identification

Nested PCR products (F1/R0 fragment), amplified from plants and insects, were sequenced in both strands (3X coverage per base position) by a commercial service (Eurofins Genomics, Germany). Nucleotide sequences were assembled by the Contig Assembling Program and trimmed to the annealing sites of the nested PCR primer pairs in the sofware BioEdit, version 7.1.3.0 (Hall, 1999). Trimmed nucleotide sequences were aligned using the ClustalW Multiple Alignment program and analyzed by Sequence Identity Matrix in the sofware BioEdit to estimate their genetic diversity. For attribution to '*Ca*. Phytoplasma' species, 16S rDNA nucleotide sequences, representative of the phytoplasma populations detected in this study,

were aligned with those of representative strains of the 47 '*Ca*. Phytoplasma' species described in literature and checked for their sequence identity in the software Bioedit. Species attribution was confirmed searching the species-specific signature sequences within the analyzed F1/R0 nucleotide sequences, and by analysis on iPhyClassifier online tool (Wei et al., 2007). For group/subgroup attribution, 16S rDNA sequences were analyzed by virtual RFLP using the online tool iPhyClassifier (Zhao et al., 2009). Actual RFLP analysis was carried out to confirm the virtual restriction patterns.

Nucleotide sequences of 16S rRNA gene of phytoplasmas, identified in the present study, and reference strains of '*Ca*. Phytoplasma' species were employed for phylogenetic analyses. The Minimum-Evolution method was employed using the Neighbor-Joining algorithm and bootstrap replicated 1,000 times with the software MEGAX to obtain a phylogenetic tree (Kumar et al., 2018).

6.4 Results

6.4.1. Grapevine yellows symptoms observed in vineyards

During field surveys, undistinguishable leaf reddening/yellowing and rolling symptoms (Figure 16 A, B, C), typical of grapevine yellows (GY) disease complex, were observed in wine and table grape cultivars in vineyards localized in all the 13 considered locations. The disease incidence (percentage of symptomatic trees out of the observed ones) was ranging from 10 to 55%. The highest incidence (55%) was observed in wine grape cultivar vineyards in Alsalhieh, followed by table grape cultivar vineyards in Jaber Alsarhan (25%), both in Almafraq governorate. The lowest incidence (10%) was reported in table grape cultivar vineyards situated in Aldisi (Aqaba governorate) and in the three locations of Ajloun governorate. The remnant seven locations had a disease incidence ranging from 12 to 15%. Within and around surveyed vineyards, 22 bindweed plants exhibited little leaf and reddening (Figure 16 D).

6.4.2 Molecular detection and identification of phytoplasmas in plants

According to results of universal nested PCR-based amplification, phytoplasmas were detected in 29 out of 152 symptomatic grapevines (infection rate 19.1%), and 12 out of 22 symptomatic bindweeds (infection rate 54.5%). In detail, F1/R0 amplicons of the expected size (around 1370 bp) were obtained in 16 out of 102 symptomatic table grape cultivars (15.7%), in 11 out of 50 symptomatic wine grape cultivars (22%). No amplification was obtained in symptomless wine

and table grape cultivars, and in symptomless bindweed (Table 9). Robustness of PCR reactions was proved by the amplification of the expected PCR product from periwinkles infected by phytoplasma strains STOL and AY1 (positive controls), and by the absence of amplification in healthy periwinkle and reaction mixture devoid of TNA (negative controls). Concerning wine grape cultivars, infection rate ranged from 20 to 27.5% in SamaAlSarhan and Alsalhieh locations, respectively. Concerning table grape cultivars, the highest infection rates were reported in plant samples collected in vineyards located in Jaber AlSarhan (AlMafraq governorate) (33%) and UmAlyanabee (Ajloun) (30%). Samples from Alfuhaisnad (AlBalqa) and Alboedah (Irbid) shared the same infection rate (20%). The lowest infection rate (8.3%) was found in Kufranjeh (Ajloun). No phytoplasma-infected table grape samples were identified in Ain Jana, Alkom Alahmar, and Thagrat AlJob. Regarding *C. arvensis*, the infection rates were 45% (9 plants out of 20) in AlSalhieh, 40% (2 plants of 5) in Sama AlSarhan and AlDisi (Table 9).

Based on 16S rDNA sequence identity versus the reference strains of '*Ca*. Phytoplasma' species and on the presence of species-specific signature sequences, the phytoplasma strains detected in the present study in 29 symptomatic grapevine plants were attributed to the species '*Ca*. P. solani' (72.4%; 21 strains out of 29), '*Ca*. P. omanense' (17.2%; 5 out of 29), '*Ca*. P. aurantifolia' (6.9%; 2 out of 29), and '*Ca*. P. asteris' (3.4%; 1 out of 29) (Table 10). '*Ca*. P. solani' and '*Ca*. P. aurantifolia' strains were found in both table and wine grape cultivars, while '*Ca*. P. omanense' and '*Ca*. P. asteris' were detected exclusively in table and wine grape cultivars, respectively. In detail, '*Ca*. P. solani' strains have identical 16S rDNA nucleotide sequence (GenBank Acc. No. OL873119), distinct from the reference strain STOL by four single nucleotide polymorphisms (SNPs) at positions 504 (T/A), 595 (A/G), 888 (C/T), and 1084 (T/C) from the annealing site of the primer R16F1. '*Ca*. P. aurantifolia' strains have identical 16S rDNA nucleotide sequence (GenBank Acc. No. OL873116), distinct from the reference strain WBDL by six SNPs at positions 62 (T/A), 83 (G/A), 285 (C/T), 559 (-/G), 793 (-/C), and 1032 (T/C) from the annealing site of the primer R16F1. '*Ca*. P. asteris' strain VV112 (GenBank Acc. No. OL873120) is distinct from the reference strain OAY by seven [323 (G/-), 346 (G/-), 488 (C/T), 539 (C/T), 698 (C/T), 984 (A/G), 1122 (G/A)] SNPs. Within '*Ca*. P. omanense', the identified strains have diverse 16S rDNA nucleotide sequences. Sequences of strains VV95, VV103, VV1007, and VV1034 identical between them (GenBank Acc. No. OL873118) are distinct from the reference strain IM-1 by SNPs at positions 152 (G/A), 274 (T/C), 331 (C/T), 344 (G/A), and 712 (G/A), while the sequence of strain VV1259 (GenBank Acc. No. OL873117) is identical to the reference strain IM-1. Based on similarity coefficient

obtained by comparison of virtual RFLP patterns, confirmed by actual enzymatic digestion profiles (data not shown), '*Ca*. P. solani' strains were attributed to taxonomic subgroup 16SrXII-A, '*Ca*. P. aurantifolia' strains VV162 and VV632 to a variant of subgroup 16SrII-C, '*Ca*. P. omanense' strain VV1259 to subgroup 16SrXXIX-A, 'and '*Ca*. P. asteris' strain VV112 to a variant of subgroup 16SrI-R. Moreover, '*Ca*. P. omanense' strains VV95, VV103, VV1007, and VV1034 were characterized by a common collective restriction profile sharing the higher similarity coefficient (0.97) with the profile of the reference strain of subgroup 16SrXXIX-A; such digestion patterns are distinguished by the enzyme *Alu*I. Due to the similarity coefficient value, these four '*Ca*. P. omanense' strains were inserted in the new taxonomic subgroup 16SrXXIX-B (Figure 17). Phytoplasma clustering in phylogenetic tree confirmed the attribution to phytoplasma species and taxonomic subgroups (Figure 18).

Phytoplasmas identified in symptomatic almond trees were found differentially distributed in the examined locations.

Concerning the phytoplasma distribution, '*Ca*. P. solani' was found in table grape cultivars (10 out of 16 plants: 62.5%) from 5 out 11 considered locations, and in wine grape cultivars (11 out of 13 plants: 84.6%) from both AlSalhieh and SamaAlSarahn. '*Ca*. P. omanense' was found exclusively in table grape cultivars (5 out of 16 plants: 31.3%) from Kufranjeh (subgroup 16SrXXIX-A), UmAlYanabee and Aldisi (subgroup 16SrXXIX-B). '*Ca*. P. aurantifolia' was found in both table grape (1 out of 16 plants: 6.3%) and wine grape (1 out of 13 plants: 7.7%) cultivars from Javer AlSarahan and AlSalhieh, respectively. '*Ca*. P. asteris' was found only in one wine grape cultivar plant (1 out of 13 plants: 7.7%) from AlSalhieh.

Regarding the non-crop weeds, the phytoplasma strains detected in the present study in 12 symptomatic bindweed plants were attributed to the species '*Ca*. P. solani' (83.3%; 10 strains out of 12 identified in SamaAlSarahn and AlSalhieh), and '*Ca*. P. aurantifolia' (16.7%; 2 out of 12 identified in Aldisi) (Table 10). '*Ca*. P. solani (16SrXII-A) and '*Ca*. P. aurantifolia' (variant of 16SrII-C) strains, found in bindweed, shared identical 16S rDNA sequence with strains of the same species found in *V. vinifera* plants.

6.4.3 Molecular detection and identification of phytoplasmas in insects

During the field survey carried out in the localities AlSalhieh, Alkom AlAhmar, Sabha (AlMafraq governorate), Kufranjeh and Aain Jana (Ajloun governorate) in August, September and November 2020, 1173 Auchenorryncha adults (557 and 616 from wine and table grape cultivar vineyards, respectively) were collected and distinguished, based on stereomicroscope analyses, in 11 taxonomic groups defined at species (8) and genus (3) level. Most of such

insects belong to the family Cicadellidae (1133 specimens), while the remnant 40 collected specimens belong to the families Delphacidae (33) and Cixiidae (7). Within Cicadellidae, the more abundant insect taxa were *Cicadulina bipunctata* (Melichar), (393 specimens), *Empoasca* sp. (350 specimens), *Arboridia adanae* (Dlabola) (271 specimens), *Circulifer* sp. (49 specimens), and *Orosius cellulosus* (Lindberg) (28 specimens) (firstly reported in Jordan). *C. bipunctata*, *Laodelphax striatellus* (Fallén), and *Psammotettix striatus* (Linnaeus) were the only species captured in both wine and table grape vineyards. *Empoasca* sp., *Balclutha* sp., *O. cellulosus*, *Toya propinqua* Fieber, *Circulifer* sp., and *Euscelidius mundus* (Haupt) were collected exclusively in wine grape vineyards in AlSalhieh, while *Hyalesthes obsoletus* Signoret and *A. adanae* exclusively in table grape vineyards in Sabha and Ain Jana (Table 12). Molecular analyses for phytoplasma detection and identification were conducted on 112 insect pools (54 from wine grape vineyards located in AlSalhieh; 58 from table grape vineyards located in Alkom AlAhmar, Sabha, Kufranjeh, and Ain Jana) representative of the observed diversity. Nested PCR allowed detecting phytoplasmas in 34 insect pools (infection rate 34.8%), belonging to 10 different insect taxa. Infection rate in insect pools from wine and table grape vineyards was 38.9% (21 out of 54 pools) and 22.4% (13 out of 58 pools), respectively. No positive insect pools were found in *Balclutha* sp. and *T. propinqua*. Among insects captured in wine grape vineyards, infected rates were 100% in *E. mundus*, 83.3% in *P. striatus*, 50% in *Circulifer* sp., 40% in *O. cellulosus*, 33.3% in *C. bipunctata*, and 11.1% in *Empoasca* sp. Concerning the insects captured in table grape vineyards, infected rates were 100% in *P. striatus*, 60% in *L. striatellus*, 50% in *H. obsoletus*, 19% in *C. bipunctata*, and 10.7% in *A. adanae* (Table 11; Figure 19).

Analyses of 16S rDNA nucleotide sequences allowed attributing the phytoplasma strains detected in insects to the species '*Ca*. P. asteris' (23 pools out of 34), '*Ca*. P. solani' (8 pools), '*Ca*. P. aurantifolia', '*Ca*. P. omanense', and '*Ca*. P. pyri' each in one pool (Table 12).

In detail, 22 out of 23 '*Ca*. P. asteris' strains were found in *P. striatus* and *C. bipunctata* in both wine and table grape vineyards, in *Circulifer* sp. in wine grape vineyards, in *L. striatellus*, *H. obsoletus*, and *A. adanae* in table grape vineyards. Such strains share identical 16S rDNA nucleotide sequence with the wine grape-infecting strain VV112, attributed to a variant of taxonomic subgroup 16SrI-R. The '*Ca*. P. asteris' strain SUF5-4, identified in one pool of *P. striatus* from table grape vineyard in Alkom AlAhmar, is distinct from the reference strain OAY by three [323 (G/-), 346 (G/-), 539 (C/T)] SNPs. This strain was attributed to taxonomic subgroup 16SrI-B. Concerning '*Ca*. P. solani', its strains were identified in *O. cellulosus* (three strains), *E. mundus* (three strains), and *Circulifer* sp. (one strain) from wine grape vineyards,

and in *L. striatellus* (one strain) from table grape vineyard in Alkom AlAhmar. Such strains share identical 16S rDNA nucleotide sequence with the ones infecting wine and table grape cultivars and bindweed, attributed to taxonomic subgroup 16SrXII-A. '*Ca*. P. aurantifolia' strain MH8-16 and '*Ca*. P. omanense' strain MH5-11, found respectively in *O. cellulosus* and *P. striatus* in wine grape vineyards, share identical 16S rDNA sequences respectively with '*Ca*. P. aurantifolia' (variant of 16SrII-C) strains identified in grapevine and bindweed and '*Ca*. P. omanense' (16SrXXIX-A) strain identified in grapevine. Moreover, a '*Ca*. P. pyri' strain, sharing identical 16S rDNA sequence with the reference strain PD1 (subgroup 16SrX-C), was found in one *Empoasca* sp. pool in AlSalhieh. Phytoplasma clustering in phylogenetic tree confirmed the attribution to phytoplasma species and taxonomic subgroups (Figure 18).

6.5 Discussion

This study provided new insights on GY diffusion, associated phytoplasmas and their putative vectors and reservoir plants in Jordan. Obtained results confirmed the large diffusion of Bois noir (BN), associated with '*Ca*. P. solani' (16SrXII-A), in wine grape cultivars, as previously reported by Salem and colleagues (2013). Moreover, '*Ca.* P. asteris' (variant of 16SrI-R) and '*Ca*. P. aurantifolia' (variant of 16SrII-C) were firstly reported in the Country in association with GY-affected wine grape cultivars. In previous studies, '*Ca.* P. asteris'- and '*Ca*. P. aurantifolia'-related strains were found in association with peach and potato diseases, respectively (Anfoka & Fatash, 2004; Salem et al., 2019). Moreover, '*Ca*. P. solani' (16SrXII-A) was found as prevalent also in table grape cultivars in different locations, confirming its identification in table grapes in Chile and Syria (Gajardo et al., 2009; Contaldo et al., 2011). Also '*Ca*. P. aurantifolia' (variant of 16SrII-C) was found in table grape, as previous reported in Iran (subgroup 16SrII-B) (Zamharir et al., 2017). Furthermore, '*Ca*. P. omanense' subgroup 16SrXXIX-A, previously reported in association with wine grape cultivars in Lebanon (Foissac et al., 2019), and subgroup 16SrXXIX-B, recently reported in almond in Jordan (Abu Alloush et al., unpublished), were found for the first time in association with GY-affected table grape cultivars in Jordan.

Even if the incidence of phytoplasma-like symptoms was high in examined orchards, only 19.1% of collected symptomatic wine and table grape cultivars were found phytoplasma infected. This can be due to: (i) the uneven distribution of phytoplasmas in phloem tissues of infected plants (Constable et al., 2003); (ii) the possible low concentration of phytoplasma cells in plant tissues in the different sampling periods (from July to November) (Martini et al., 2011); (iii) the possibility that observed symptoms are caused by other etiological agents or associated

with nutritional disorders. Moreover, it should be considered that surveys and sample collection were carried out during a limited period throughout the season.

Interestingly, obtained results evidenced the presence of '*Ca*. P. solani' and '*Ca*. P. aurantifolia' strains, undistinguishable from those found in wine and table grape cultivars, in the putative insect vector *Orosius cellulosus* (firstly reported in Jordan) and in symptomatic bindweed, a non-crop plant known for its epidemiological role in BN diffusion (Quaglino et al., 2021). No studies are available about *O. cellulolus* and its vectoring activity of phytoplasmas, but other *Orosius* species, such as *O. albicinctus* and *O. orientalis*, living on different plant hosts (Rao et al., 2018), were reported as vectors of phytoplasmas belonging to groups 16SrIX and 16SrII in Turkey and Iran (Esmailzadeh-Hosseini et al., 2011; Ikten et al., 2014; Salehi et al., 2017). Based on this evidence, it is reasonable to hypothesize that diffusion of '*Ca*. P. solani' and '*Ca*. P. aurantifolia' in Jordan can involve *O. cellulolus* and bindweed.

Moreover, '*Ca*. P. solani' strains, undistinguishable from those found in wine and table grape cultivars, were found also in *E. mundus*, *L. striatellus*, and *Circulifer* sp. *Euscelidius mundus* was reported as putative vector of '*Ca*. P. phoenicium' in Lebanon (Dakhil et al., 2011), but other species of the genus *Euscelidius*, such as *E. variegatus*, are known as vector of '*Ca*. P. solani' to grapevine (Quaglino et al., 2019). Previous studies reported that *L. striatellus* is a vector of '*Ca*. P. solani' to grapevine (Quaglino et al., 2019). Concerning *Circulifer* sp., different species, such as *C. tenellus* and *C. aematoceps*, were described as vectors of phytoplasmas belonging to taxonomic groups 16SrI, 16SrII, 16SrVI, and 16SrIX (Salehi et al., 2011, 2017; Aslam et al., 2021). Thus, '*Ca*. P. solani' transmission to grapevine in Jordan could involve also *Circulifer* sp., *E. mundus*, and *L. striatellus.*

Concerning '*Ca*. P. omanense', found exclusively in table grape cultivars, it was identified (subgroup 16SrXXIX-A) in *P. striatus*, known as vector of '*Ca*. P. tritici' (16SrI) (Wu et al., 2010). No insects were found infected by 16SrXXIX-B phytoplasma strains. Recent studies reported *H. obsoletus* as putative vector of '*Ca.* P. omanense' in Lebanon (Foissac et al., 2019), but in the present work it was found not infected by this phytoplasma. Due to the association of the new subgroup 16SrXXIX-B to almond and grapevine diseases in Jordan, it will be useful to focus further studies on improving the knowledge on its epidemiology throughout the Country, in different agroecosystems.

'*Ca*. P. asteris' (variant of subgroup 16SrI-R), identified only in one plant of wine grape cultivar, was prevalent in examined insect vectors. It was found in *Circulifer* sp., *P. striatus*, *C. bipunctata*, *L. striatellus*, *H. obsoletus*, and *A. adanae*. As reported above, *Circulifer* sp. and *P. striatus* are known as vectors of 16SrI phytoplasmas, and *L. striatellus* as vector of 16SrXII

phytoplasmas. *C. bipunctata* is a potential vector of '*Ca*. P. asteris'-related strain to date palm (Alhudaib et al., 2007); *H. obsoletus* is known as '*Ca*. P. solani' vector and putative vector of '*Ca*. P. asteris' (Maixner et al., 1994; Zambon et al., 2018). Although *A. adanae* is considered a serious pest of grapewine in Eastern Mediterranean Regions and Europe (Yigit and Erckle 1992; Olivier et al., 2012), no studies are available about its role as vector or putative vector of phytoplasmas. Based on these findings, it is reasonable to suggest that diffusion of '*Ca*. P. asteris' subgroup 16SrI-R to grapevine in Jordan can involve multiple insect species. Upscaling the surveyed vineyards and surroundings could provide better insights on the '*Ca*. P asteris' diffusion in grapevine.

The epidemiology of phytoplasma-associated diseases is determined by the interactions between host plants, pathogen, and environmental conditions (Rotter et al., 2018). Further studies in terms of transmission trials and upscaling the surveying orchards and non-crop plant hosts will be crucial to profound the knowledge about GY etiology and epidemiology in Jordan. Outbreak of GY epidemics could be a concrete risk in the vineyard agroecosystems in all viticultural areas. Monitoring and control strategies against GY are essential to prevent epidemic phytoplasma spread (Pierro et al., 2019).

Most phytoplasmas identified in *Vitis vinifera* in this study were detected also in almond and pomegranate in different areas of the Country, suggesting that phytoplasma diversity and distribution in Jordan are more complex than previously known, leading to a potential risk of disease outbreaks. Studies and knowledge about the insect vectors including their identification, distribution, population dynamics are essential for proper management measures and mitigation of the risk of disease outbreaks. Considering the preliminary results, obtained in the present study, about the GY epidemiology in Jordan, further studies covering more areas throughout the full vegetative season of grapevine and non-crop reservoir plants are essential and will provide comprehensive insights about the GY phytoplasma diversity, ecological complexity, and epidemiology.

Table 9. Collected and phytoplasma-infected plant samples from surveyed locations in Jordan.

Governorate	Location	Plant	No. of collected samples	No. of phytoplasma-infected samples
Almafraq	AlSalhieh	Symptomatic wine grape	40	11
		Asymptomatic wine grape	3	0
		Convolvulus arvensis L.	20	9
	Sama-AlSarhan	Symptomatic wine grape	10	2
		Asymptomatic wine grape	2	0
		Convolvulus arvensis L.	5	1
	Sabha	Symptomatic table grape	10	1
		Asymptomatic table grape	2	0
	Thagrat Aljob	Symptomatic table grape	7	0
		Asymptomatic table grape	2	0
	Alkom AlAhmar	Symptomatic table grape	5	0
		Asymptomatic table grape	2	0
	Jaber Alsarhan	Symptomatic table grape	15	5
		Asymptomatic table grape	2	0
Irbid	Hofa	Symptomatic table grape	4	0
		Asymptomatic table grape	1	0
	Bouida	Symptomatic table grape	5	1
		Asymptomatic table grape	2	0
Ajloun	Kufranjeh	Symptomatic table grape	12	1
		Asymptomatic table grape	2	0
	Ain Jana	Symptomatic table grape	5	0
		Asymptomatic table grape	2	0
	UmALyanabee	Symptomatic table grape	10	3
		Asymptomatic table grape	1	0
Aqaba	Aldisi	Symptomatic table grape	14	2
		Asymptomatic table grape	2	0
		Convulvulius arvensis L.	5	2
AlBalga	Alfuhais	Symptomatic table grape	15	3
		Asymptomatic table grape	2	0
		Overall	207	41

Table 10. Attribution to species and taxonomic subgroups of phytoplasmas detected in plants (part I).

Strain	Plant host	Grapevine cultivar	Location	Phytoplasma species	% id vs ref strain	16Sr subgroup	Acc. No.
VV1259	*Vitis vinifera*	table grape	Kufranjeh	'*Ca*. P. omanense'	100	XXIX-A (1.00)	OL873117 (b)
VV1003	*Vitis vinifera*	table grape	UmAlYanabee	'*Ca*. P. omanense'	99.6	XXIX-B (new)	C
VV1007	*Vitis vinifera*	table grape	UmAlYanabee	'*Ca*. P. omanense'	99.6	XXIX-B (new)	C
VV1034	*Vitis vinifera*	table grape	UmAlYanabee	'*Ca*. P. omanense'	99.6	XXIX-B (new)	C
VV95	*Vitis vinifera*	table grape	Aldisi	'*Ca*. P. omanense'	99.6	XXIX-B (new)	OL873118 (c)
VV37	*Vitis vinifera*	table grape	Aldisi	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV1395	*Vitis vinifera*	table grape	AlFuhais	'*Ca*. P. solani'	99.7	XII-A (1.00)	OL873119 (d)
VV1398	*Vitis vinifera*	table grape	AlFuhais	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV1399	*Vitis vinifera*	table grape	AlFuhais	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV636	*Vitis vinifera*	table grape	Bouida	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV1005	*Vitis vinifera*	table grape	UmAlYanabee	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV635	*Vitis vinifera*	table grape	Jaber AlSarahan	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV629	*Vitis vinifera*	table grape	Jaber AlSarahan	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV633	*Vitis vinifera*	table grape	Jaber AlSarahan	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV634	*Vitis vinifera*	table grape	Jaber AlSarahan	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV632	*Vitis vinifera*	table grape	Jaber AlSarahan	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	A
VV162	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	OL873116 (a)
VV112	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	OL873120 (e)
VV134	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV131	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV110	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV157	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV158	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV159	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV163	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV1	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV2	*Vitis vinifera*	wine grape	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV3	*Vitis vinifera*	wine grape	SamaAlSarahn	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
VV4	*Vitis vinifera*	wine grape	SamaAlSarahn	'*Ca*. P. solani'	99.7	XII-A (1.00)	D

Table 11. Attribution to species and taxonomic subgroups of phytoplasmas detected in plants (part II).

Strain	Plant host	Location	Phytoplasma species	% id vs ref strain	16Sr subgroup	Acc. No.
CAr25	*Convolvulus arvensis*	Aldisi	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	A
CAr26	*Convolvulus arvensis*	Aldisi	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	A
CAr6	*Convolvulus arvensis*	SamaAlSarahn	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
CAr108	*Convolvulus arvensis*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
CAr5	*Convolvulus arvensis*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
CAr65	*Convolvulus arvensis*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
CAr153	*Convolvulus arvensis*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
CAr165	*Convolvulus arvensis*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
CAr168	*Convolvulus arvensis*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
CAr360	*Convolvulus arvensis*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
CAr109	*Convolvulus arvensis*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
CAr112	*Convolvulus arvensis*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D

Table 12. Collected and phytoplasma-infected insects from surveyed locations in northern Jordan.

Governorate	Location	Grapevine cultivar	Insect code	Family	Species	Collection date	No. of collected insects	No. of pools	No. of positive pools	Infection rate (%)
AlMafraq	AlSalhieh	wine grape	MH2	Cicadellidae	*Empoasca* sp.	Aug	350	9	1	11.1
AlMafraq	AlSalhieh	wine grape	MH3b	Cicadellidae	*Balclutha* sp.	Aug	12	1	0	0
AlMafraq	AlSalhieh	wine grape	MH5	Cicadellidae	*Psammotettix striatus*	Aug	15	6	5	83.3
AlMafraq	AlSalhieh	wine grape	MH7	Cicadellidae	*Cicadulina bipunctata*	Aug	65	9	3	33.3
AlMafraq	AlSalhieh	wine grape	MH8	Cicadellidae	*Orosius cellulosus*	Aug	28	10	4	40
AlMafraq	AlSalhieh	wine grape	MH11	Delphacidae	*Toya propinqua*	Aug	23	4	0	0
AlMafraq	AlSalhieh	wine grape	MH12	Delphacidae	*Laodelphax striatellus*	Aug	5	2	0	0
AlMafraq	AlSalhieh	wine grape	MH18	Cicadellidae	*Circulifer* sp.	Nov	49	10	5	50
AlMafraq	AlSalhieh	wine grape	MH19	Cicadellidae	*Euscelidius mundus*	Nov	10	3	3	100
AlMafraq	Alkom AlAhmar	table grape	SUF1	Cicadellidae	*Cicadulina bipunctata*	Aug	88	7	2	28.6
AlMafraq	Alkom AlAhmar	table grape	SUF3	Delphacidae	*Laodelphax striatellus*	Aug	5	5	3	60
AlMafraq	Alkom AlAhmar	table grape	SUF5	Cicadellidae	*Psammotettix striatus*	Aug	5	2	2	100
AlMafraq	Sabha	table grape	YM2	Cixiidae	*Hyalesthes obsoletus*	Aug	7	2	1	50
AlMafraq	Sabha	table grape	YM3	Cicadellidae	*Cicadulina bipunctata*	Aug	135	6	1	16.7
Ajloun	Kufranjeh	table grape	Z1	Cicadellidae	*Cicadulina bipunctata*	Sep	105	8	1	12.5
Ajloun	Ain Jana	table grape	G61	Cicadellidae	*Arboridia adanae*	Sep	271	28	3	10.7
						Overall	1173	112	34	34.8

Table 13. Attribution to species and taxonomic subgroups of phytoplasmas detected in insects.

Strain	Species	Region	Phytoplasma species	% id vs ref strain	16Sr subgroup	Acc. No.
MH2-1	*Empoasca* sp.	AlSalhieh	'*Ca*. P. pyri'	100	X-C (1.00)	OL873122
MH18-3	*Circulifer* sp.	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH18-4	*Circulifer* sp.	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH18-5	*Circulifer* sp.	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH18-6	*Circulifer* sp.	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH18-23	*Circulifer* sp.	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
MH4b-7	*Psammotettix striatus*	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH4b-8	*Psammotettix striatus*	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH5-9	*Psammotettix striatus*	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH5-10	*Psammotettix striatus*	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH5-11	*Psammotettix striatus*	AlSalhieh	'*Ca*. P. omanense'	100	XXIX-A (1.00)	B
MH7-12	*Cicadulina bipunctata*	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH7-13	*Cicadulina bipunctata*	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH7-14	*Cicadulina bipunctata*	AlSalhieh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
MH8-16	*Orosius cellulosus*	AlSalhieh	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	A
MH8-17	*Orosius cellulosus*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
MH8-18	*Orosius cellulosus*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
MH8-21	*Orosius cellulosus*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
MH19-24	*Euscelidius mundus*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
MH19-25	*Euscelidius mundus*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
MH19-26	*Euscelidius mundus*	AlSalhieh	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
SUF3-1	*Laodelphax striatellus*	Alkom AlAhmar	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
SUF3-2	*Laodelphax striatellus*	Alkom AlAhmar	'*Ca*. P. solani'	99.7	XII-A (1.00)	D
SUF3-3	*Laodelphax striatellus*	Alkom AlAhmar	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
SUF5-4	*Psammotettix striatus*	Alkom AlAhmar	'*Ca*. P. asteris'	99.9	I-B (1.00)	OL873121
SUF5-5	*Psammotettix striatus*	Alkom AlAhmar	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
SUF1-6	*Cicadulina bipunctata*	Alkom AlAhmar	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
SUF1-7	*Cicadulina bipunctata*	Alkom AlAhmar	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
YM2-9	*Hyalesthes obsoletus*	Sabha	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
Z1-20	*Cicadulina bipunctata*	Kufranjeh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
Z1-40	*Cicadulina bipunctata*	Kufranjeh	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
G61-1	*Arboridia adanae*	Ain Jana	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
G61-2	*Arboridia adanae*	Ain Jana	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E
G61-3	*Arboridia adanae*	Ain Jana	'*Ca*. P. asteris'	99.6	I-R* (0.98)	E

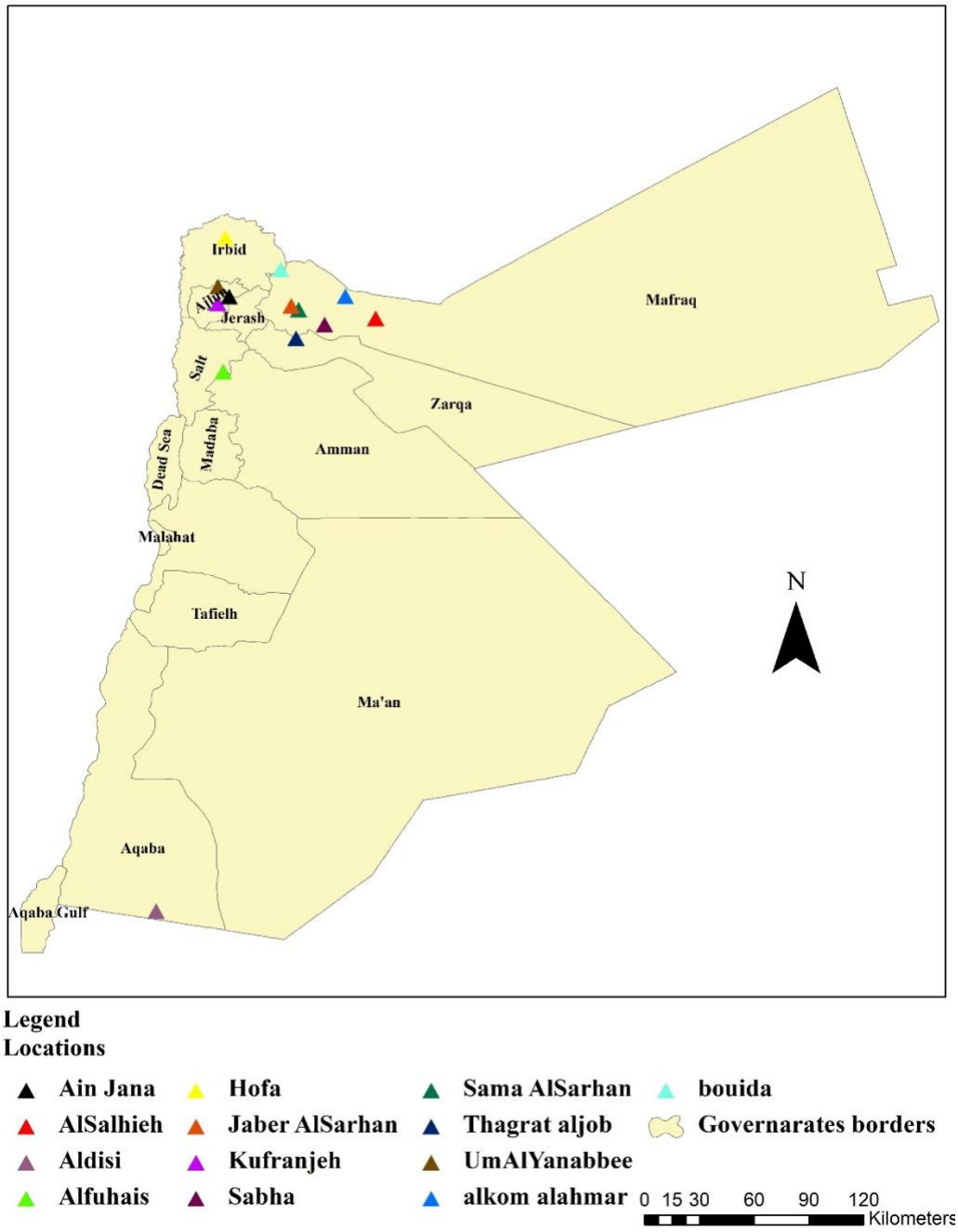

Figure 15. Maps of governorates and locations in Jordan where the surveys on GY diseases in vineyards were conducted.

Figure 16. Phytoplasma- like symptoms observed in Vitis vinifera L. in Jordan. Leaf yellowing and rolling in wine grape cultivar (A); leaf reddening and rolling in wine grape cultivar (B); leaf yellowing and rolling in table grape cultivar (C).

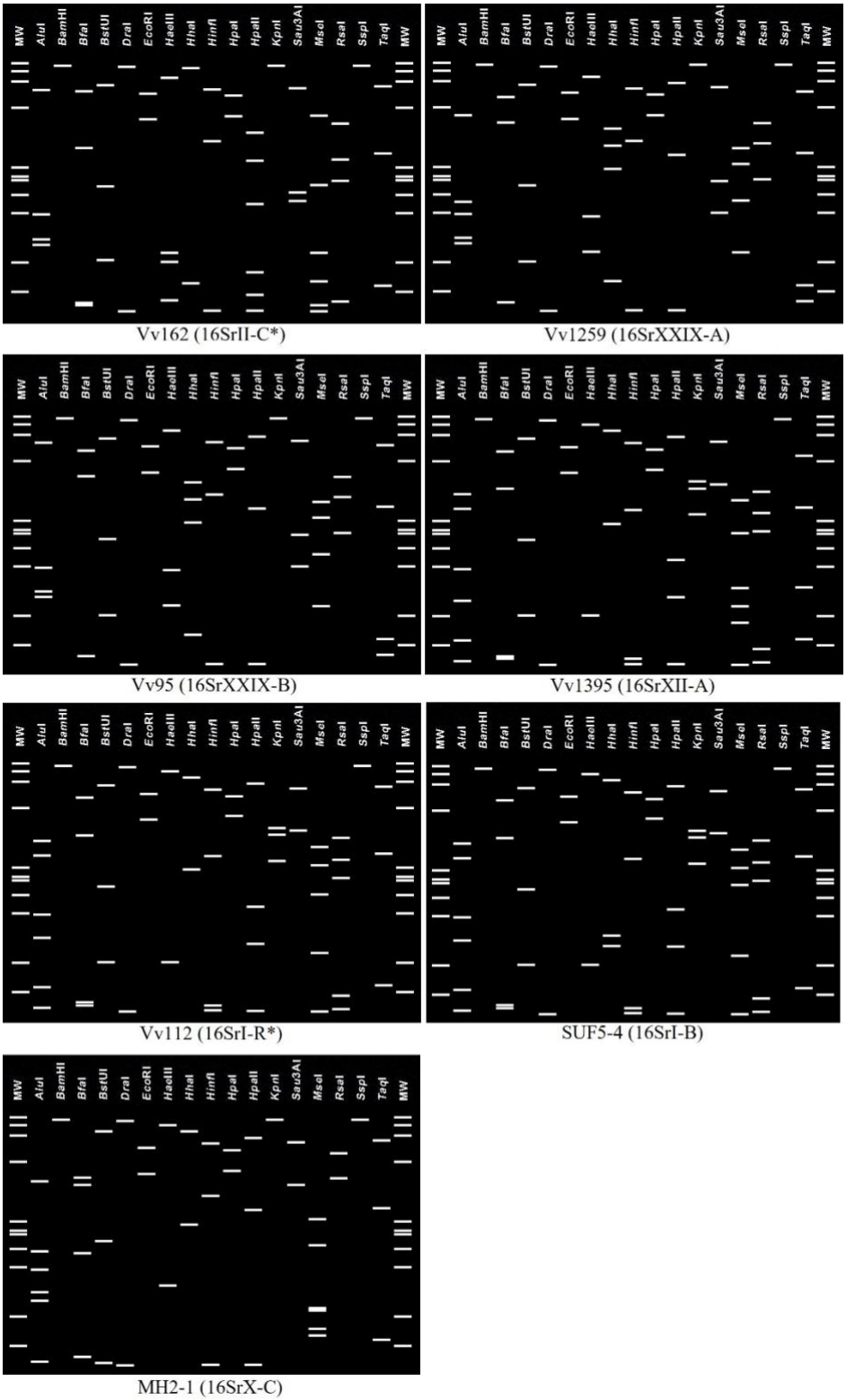

Figure 17. Virtual RFLP profiles of 16S rDNA nucleotide sequences of phytoplasma strains identified in Vitis vinifera and insects in Jordan. One strain among those sharing identical 16S rDNA sequence (Tables 2 and 4) was selected as representative strain for iPhyClassifier analyses.

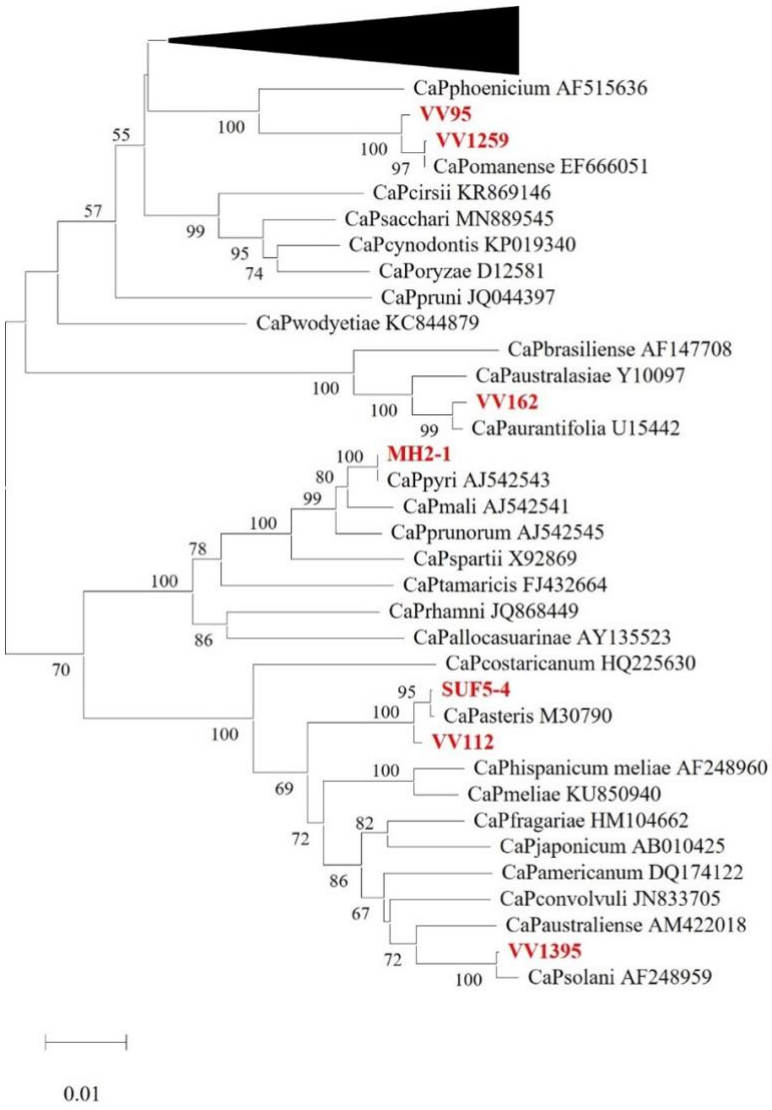

Figure 18. Phylogenetic tree based on the alignment of 16S rDNA nucleotide sequences of representative phytoplasma strains identified in *Vitis vinifera* L., putative insect vectors, and reservoir plants in Jordan (red bold characters), and reference strains of previously described '*Candidatus* Phytoplasma' species. Regarding phytoplasmas identified in this study, one strain among those sharing identical 16S rDNA sequence (Tables 2 and 4) was selected as representative strain for phylogenetic analysis. The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 0.91088584 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. This analysis involved 52 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1424 positions in the final dataset.

Figure 19. Putative insect vectors of phytoplasmas infecting *Vitis vinifera* L. in Jordan. (A) *Orosius cellulosus*, (B) *Euscelidius mundus*, (C) *Laodelphax striatellus*, (D) *Circulifer* sp., (E) *Psammotettix striatus*, (F) *Cicadulina bipunctata*, (G) *Hyalesthes obsoletus*, (H) *Empoasca* sp.

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Chapter 7. GENERAL CONCLUSIONS

The evidence that numerous yellows-type diseases of plants, believed to be caused by viruses, were associated with phloem colonization by prokaryotes morphologically resembling mycoplasmas (mycoplasma-like organisms: MLO) was first shown in 1967 (Doi *et al.*, 1967). Such great discovery has led to a huge body of phytoplasma research covering their epidemiology and achieving significant useful achievements, in terms of developing detecting methods, classification and identification systems, as well as increased the awareness about their importance, with priceless scientific products that communicated into beer journals and international symposiums. In MENA region, phytoplasma history backs to 30 years ago, with more and less attentions among the countries. Remarkably, little information is known about the epidemiology and ecological aspects in MENA. In Jordan phytoplasma is poorly understood with limited investigations. In the present PhD research project, firstly, MENA efforts in the phytoplasmas and related aspects were highlighted and reviewed. The review highlighted 12 '*Candidatus* Phytoplasma' species belonging to 14 taxonomic groups including: 16SrI, 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 6SrXIV, 16SrXV, 16SrXXIX. Several epidemic phytoplasmas diseases were reported in different MENA Countries with significant economic losses involving AlmWB, WBLD, TTB, and PD. Moreover, several potential vectors were reported. Remarkably, there are lack of studies in terms of transmission trials and phytoplasma epidemiology. Secondly, the etiology and epidemiology of phytoplasmas associated diseases with three important fruits trees including almond, pomegranate and grapevines in Jordan was investigated. Molecular-based analysis using nested PCRs with universal 16S rRNA gene evidenced the natural infection by different phytoplasma species, and the study revealed the diversity and diffusion of phytoplasmas in the Country. Seven '*Candidatus* Phytoplasma' species belonging to eight taxonomic subgroups were identified in symptomatic almond trees, four '*Ca*. phytoplasma' species belonging to five taxonomic subgroups were identified in symptomatic pomegranate trees. Moreover, subgroup 16SrI-R was reported for the first time. Concerning the grape, four '*Ca*. Phytoplasma' species belonging to four subgroups were identified in symptomatic wine making and table grape cultivars, all of them were never reported or identified within table grape. The study reported preliminary investigation on potential insect vectors in the surveyed orchards. In details, 12, 10 and 12 insect taxa were collected from almond, pomegranate, and grapevine orchards, respectively. Eight, six, and 10 insect taxa were identified

as putative vectors of '*Ca*. Phytoplasma' species infecting almond, pomegranate, and grapevine, respectively. Noteworthy, eight insect taxa were firstly reported in Jordan including *Zygina flammigera*, *Anaceratagallia frisia*, *Reptalus quinquecostatus*, *Macrosteles sexnotatus*, *Balclutha incisa*, *Eupteryx stachydearum*, *Laodelphax striatellus,* and *Orosius cellulosus.*

The study highlighted four non-crop plants as potential reservoir phytoplasma plants including *Convolvulus arvensis*, *Capsicum annuum*, *Plantago major*, and *Amaranthus* sp. In the context of epidemic phytoplasma diseases, the study reported '*Ca*. P. phoenicium', one of the most important phytoplasma in MENA region, in almond. It is well known as very epidemic and destructive for the almond trees and other stone fruits. Even with low infection rate reported in this study, its epidemical potential is extremely high. '*Ca*. P. solani' (16SrXII-A) was the most prevalent phytoplasma species identified in the symptomatic target crops. Such findings are supporting the previous reports in the country about this phytoplasmas species. '*Ca*. P. pyri', '*Ca*. P. omanense', and '*Ca*. P. ulmi' were reported for the first-time infecting almond trees.

In conclusion, this study described (i) five different phytoplasma symptoms categories associated with seven '*Ca*. Phytoplasma' species infecting almond trees in north Jordan, including one of the most epidemic stone fruits diseases; (ii) new pomegranate disease complex, including witches'-broom and leaf alteration, associated with four distinct '*Ca*. Phytopalsma' species in northern Jordan, (iii) grapevine yellows diseases associated with four distinct '*Ca*. Phytoplasma' species in different parts of the Country. Moreover, the study provided preliminary insights on their epidemiology, indicating putative insect vectors and reservoir plants potentially involved in spreading of these phytoplasmas. Results obtained by this PhD. study provide a good insight about the phytoplasmas aspects and furnished preliminary indications on the epidemiology of reported diseases in Jordan and Region. Further studies focusing on the following topics are necessary: (i) transmission trials to verify the capability of putative insect vectors, identified in the present work, to transmit various phytoplasmas species; (ii) insect population diversity and dynamics conducted throughout the whole season in the affected orchards; (iii) upscaling survey of fruits disease complex in the whole Country, and demonstrating their diffusion with more attention for the epidemic ones.