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Epidemiological and molecular study on '*Candidatus* Phytoplasma phoenicium' in Lebanon

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INDEX

1. ABSTRACT

'Candidatus Phytoplasma phoenicium', a member of the 16SrDNA phytoplasma group IX, is considered the presumptive aetiological agent of Almond witches'-broom (AlmWB) disease, which caused in Lebanon the death of more than 100,000 almond trees in the last decade. In the last few years, severe infections, frequently associated with a noticeable yield reduction, have also been observed on peach and nectarine trees.

The aim of this work is to improve the knowledge of AlmWB epidemiology through (i) the symptoms description in almond, peach and nectarine trees in order to select the most suitable period for observing the typical alterations induced by the disease and for collecting samples for a fast and effective diagnosis, (ii) the update of the data concerning the AlmWB spread in Lebanon, (iii) the molecular characterization of AlmWB phytoplasma strains isolated from different host plants and from different Lebanese regions, and (iv) a preliminary screening of the insect(s) that could be candidate vector(s) responsible for the disease transmission.

First of all, the symptom evolution was described through one-year-long observations of infected almond, peach and nectarine trees in three key-orchards located in three different Lebanese regions: Jbeil in the North, Hasbaya and Marjayoun in the South. Leaf and flower samples were collected from symptomatic and asymptomatic plants and analysed by direct and nested polymerase chain reaction (PCR) assays in order to detect AlmWB phytoplasma. Due to the importance of stone fruit in Lebanon and to the serious impact of the disease on these cultures, a national survey on AlmWB, based on the criteria derived from the symptom observation in the examined key-orchards, was carried out in 24 Lebanese districts. Leaf and flower samples were collected from 368 plants in order to detect the phytoplasma and characterize the infected regions. Moreover, molecular characterization of 24 representative *'Ca.* Phytoplasma phoenicium' strains was carried out through virtual and actual RFLP analysis of the 16S rRNA gene, in order to study the genetic variability of the pathogen and to find out possible relationships with the different hosts and the various cultivation regions. Furthermore, since the AlmWB phytoplasma insect vector(s) is(are) still unknown, a wide insect collection was carried out in two infected almond and nectarine orchards during two consecutive years in order to identify and analyze candidate phytoplasma vector(s).

The observations carried out on infected peach and nectarine trees were used to describe the symptom evolution on these two new AlmWB hosts. Even if the presence of witches'-broom is more common in almond trees than in peach/nectarine, the most important difference between peach/nectarine and almond symptoms is the development, in peach/nectarine trees, of phyllodies, never recorded on almond. They appear usually in

April/May and are easy to recognize on field. By using the specific primer pair AlWF2/AlWR2, AlmWB phytoplasma was identified in 95% of symptomatic almonds and in 100% of symptomatic peaches and nectarines selected during the national survey on AlmWB. The disease was found to be present in 16 out of 24 Lebanese districts, where it affects almond, nectarine and peach trees at different rates. A national map indicating the location of all the affected and healthy monitored villages was developed using the GIS software. Numerous meetings were held in these regions, in order to describe the disease and its possible management to the farmers.

Molecular characterization of 24 representative *'Ca.* P. phoenicium' strains by virtual RFLP assays lead to the identification of two new 16SrIX subgroups, indicated as 16SrIX-F and IX-G, distinguished by the use of *BstU*I and *Taq*I restriction enzymes. The geographical distribution of the phytoplasma subgroups here identified (IX-D, IX-F, IX-G) were also showed in the GIS map elaboration.

During a wide survey on putative AlmWB phytoplasma insect vectors, 45 species of leafhoppers, 4 genera of cixiids, and 9 species of psyllids were collected and identified. Since leafhoppers were previously investigated as AlmWB phytoplasma vectors in Lebanon, the research focused on Cixiidae and Psyllidae taxa. In detail, 64 Cixiidae and 53 Psyllidae specimens were analyzed by direct and nested PCR, using respectively the specific primers AlWF2/AlWR2 and the universal primers P1/P7 followed by F2n/R2. Whereas all the psyllids tested negatives, 11 PCR reactions on Cixiidae specimens have shown positive results using the universal primers, whereas 16 reactions gave positive results using the specific primer pair, opening new possibilities about the research of the *'Ca.* Phytoplasma phoenicium' vector(s).

Results obtained in the present study evidenced the wide diffusion of *'Ca.* P. phoenicium'-related strains in Lebanon. The pathogen affects different hosts and can be spread in territories characterised by very different climate and environmental conditions, representing a risk because of its adaptability to the neighbouring regions/Countries. The preliminary results obtained on Cixiidae analysis highlighted the presence of several phytoplasma-infected insects; their vectoring activity must be confirmed through green-house transmission assays, in order to demonstrate their role on *'Ca.* P. phoenicium' transmission. In-depth investigating on Cixiidae biology, ecology and host range will allow planning a possible management of the disease. The results obtained during the present research work suggest that regulation and control measures are urgently necessaries to limit the diffusion of Almond Witches'-broom in Lebanon but also to avoid its spread in the Middle East and in Europe.

Green almonds sold at the market (Batroun, North Lebanon).

2. INTRODUCTION

2.1 Phytoplasma main characteristics

Phytoplasmas are bacterial plant pathogens that cause economically relevant yield losses in different low- and high value, annual and perennial crops worldwide, including fruit and woody trees (Razin *et al.*, 1998; Lee *et al.*, 2000; Bertaccini *et al.*, 2007).

Phytoplasmas are wall less prokaryotes with sizes variable from 200 to 800 nm, with a single cell membrane and a very small chromosome (680-1,600 kb); they are polymorphic, and could survive and multiply only in hysotonic habitats, such as plant phloem or insect emolymph; therefore they are strictly host-dependent, but they could multiply in insect vectors and also infect their eggs.

Phytoplasmas are classified along with mycoplasmas, spiroplasmas and acholeplasmas in the class *Mollicutes* which includes bacteria with single membrane that have diverged from a gram-positive ancestor, like *Clostridium* or *Lactobacillus* spp., through genome reduction. Phylogenetic studies suggest that the common ancestor for phytoplasmas is *Acholeplasma laidlawii* Freundt in which the triplet coding for tryptophan (trp) is UGG, while in the other prokaryotes, enclosing mycoplasmas and spiroplasmas, trp is coded by UGA (Razin *et* al., 1998).

Phytoplasmas are genetically distinguishable from mycoplasmas which infect human and animal for the presence of a spacer region (about 300 bp) between 16S and 23S ribosomal regions, which codes isoleucine tRNA and part of the sequences for alanine tRNA. Moreover, phytoplasmas and acholeplasmas lack functional phosphotransferase transport system (PTS) for importing sugar (Oshima *et al.*, 2004; Bai *et al.*, 2006; Kube *et al.*, 2008; Tran-Nguyen *et al.,* 2008) whereas mycoplasmas and spiroplasmas have PTSs. Furthermore, mycoplasmas and ureaplasmas encode all eight subunits of theFoF1-type ATPase catalytic core for ATPase synthase and utilize the transmembrane potential for ATP synthesis, but all phytoplasma genomes sequenced to date lack all eight subunits.

2.2 Phytoplasma classification

The first phytoplasma identification and classification systems proposed were based on specificity of vector transmission, on range of host plants and, more recently, on symptom expression of a common host (periwinkle). In fact, because of the inability to isolate phytoplasmas in pure culture, it was not possible to apply to phytoplasmas the traditional taxonomic criteria, based on phenotypic and biochemical characters. However, experimentally the plant host and the insect vector ranges can be broader than those observed in nature, causing a considerable amount of overlaps.

Hence 1967, the term `mycoplasma like organisms' (MLOs) was used to refer to the causal agent of many plant yellows. Only the advent of molecular biology and thus the sequencing of phytoplasma 16S rRNA gene allowed classifying these pathogens as members of *Mollicutes.*

Polyclonal antisera first, and monoclonal antisera later, were used to differentiate various phytoplasma groups (Loi *et al.,* 2002, Thomas *et al.*, 2001). While polyclonal antisera have relatively low specific titres, and are not very useful for discrimination among phytoplasmas, monoclonal antisera greatly improved the reliability of immunoidentification techniques, such as ELISA, dot-blot immunoassays and immunofluorescence tests.

In 1994 the term MLOs was replaced with `phytoplasmas' by the Phytoplasma Working Team at the 10th congress of the International Organization of Mycoplasmology. This name emphasises the phylogenetic distance of these prokaryotes from some of the mycoplasmas infecting animals and humans (Gasparich *et al.*, 2004).

In 2004, the International Research Programme of Comparative Mycoplasmology (IRPCM) proposed to place phytoplasmas within the novel genus `*Candidatus* Phytoplasma' and established the rules for defining new phytoplasma species. Basically, a strain (longer than 1200 bp) can be termed new species if it shares <97.5% similarity of 16S rRNA gene sequence with previously described phytoplasma species. In the case that two phytoplasma strains share 16S rRNA gene sequence similarity >97.5% but are transmitted by different insect vectors, have different natural hosts and have significant molecular differences, the two phytoplasma strains can be described as two different phytoplasma species (IRPCM 2004).

2.3 Phytoplasma characterization

In order to achieve a general and reliable system of phytoplasma detection and identification molecular tools such as PCR/RFLP (polymerase chain reaction/restriction fragment length polymorphism) and nested-PCR on the conserved (16SrDNA) ribosomal phytoplasma region were developed and applied. This detection approach provides rapid and reliable means for preliminary classification in epidemiological studies on diseases associated with phytoplasma presence, and enables the construction of phylogenetic trees of many microorganisms especially in the *Mollicutes* taxon.

Molecular characterisation of the entire phytoplasma genome including its sequencing recently performed (Jung *et al.,* 2003) will provide, after its full annotation, more precise basis for taxonomy, but it will be necessary to do it for several other phytoplasmas in order to achieve comparative genomic that could allow a deeper understanding about physiology of these organisms.

In fact, many related phytoplasmas show clear biological differences; the study of the only 16SrDNA gene probably underestimates the biological variability of phytoplasmas closely related from a taxonomic point of view. (Kirkpatrick *et al.,* 1994; Gundersen *et al.,* 1996; Martini *et al.*, 2002; Angelini et *al.,* 2003).

For these reasons, recent studies concerning phytoplasma molecular characterization were carried out on regions less conserved than the 16SrRNA gene. The *rp* gene sequences reveal more variation than 16S rDNA (Lim *et al*., 1991), while the analyses carried out by RFLP or sequencing on *tuf* and/or *Sec*Y genes clearly show that relationships among phytoplasma strains are associated at least with their geographical distribution (Schneider *et al*., 1997; Kakizawa *et al.,* 2001; Langer *et al.,* 2004). On the contrary, the use of the 23S rDNA gene was not particularly useful since it appears more or similarly conserved as the 16S.

2.4 Phytoplasma genomes

An important contribution to a better understanding of the phytoplasma metabolism and their interaction with hosts (insects and plants) has been supplied by the complete sequencing of phytoplasma genomes.

To date, only four phytoplasma strains have been completely sequenced because molecular phytoplasma research is hindered by difficulties in obtaining high quality DNA from infected plants. These phytoplasmas are 'Ca. Phytoplasma asteris'-related strains onion yellows mutant (OY-M) (Oshima *et al.,* 2004) and aster yellows witches'-broom (AY-WB) (Bai *et al.,* 2006), 'Ca. Phytoplasma australiense' (Tran-Nguyen *et al*., 2008) and 'Ca. Phytoplasma mali' (Kube *et al*., 2008). The genomes of 'Ca. Phytoplasma asteris'-related strains OY-M and AY-WB, and 'Ca. phytoplasma australiense' include respectively a circular chromosome of 860631 bp and two plasmids, a circular chromosome of 706569 bp and four plasmids, a circular chromosome of 879324 bp and one plasmid. On the other hand, 'Ca. phytoplasma mali'-related strain AT has a linear chromosome of 601943 bp and extrachromosomal DNA was not identified. 'Ca. phytoplasma mali' is also characterized by the lowest G+C content (21.4%) in all mycoplasmas and most walled bacteria analyzed to date.

Like mycoplasmas, full-sequenced phytoplasmas lack the genes for tricarboxylic acid cycle, pentose phosphate pathway, sterol biosynthesis, fatty acid biosynthesis, *de novo* nucleotide synthesis, and biosynthesis of most amino acids. Moreover, analysis of the protein-coding genes revealed that glycolysis, the major energy-yielding pathway supposed for '*Ca*. Phytoplasma asteris', is incomplete in '*Ca.* Phytoplasma mali' and maltose and malate are probably utilized as alternative carbon and energy sources (Kube *et al*., 2008).

In general, small-genome pathogenic bacteria lost the genes for numerous biosynthetic pathways, most likely because many metabolites are available within the host cell environment, leading to a reduced selective constraint on genes for biosynthetic capabilities.

2.5 Phytoplasma life cycle

Phytoplasmas require diverse hosts, plants and insects, for their replication, survival and spread. In plant, phytoplasmas are found in phloem elements, including both mature and immature phloem cells that still have nuclei. In insect, phytoplasmas pass through insect gut cells, replicate in various body tissues, reach the salivary glands and the saliva for the subsequent introduction into plants. In plant hosts, the highest concentration of phytoplasma was found in the mature sieve tubes (Christensen *et al*., 2004). As phloem cells are considered live cells, phytoplasmas may be considered intracellular parasites.

When acquired by the insect vectors, phytoplasmas attach to the membranes of the midgut, on or between microvilli, and initiate the invasion of the midgut. Suzuki and coworkers (2006) demonstrated that the onion yellows phytoplasma (OYp) antigenic membrane protein (Amp) interacted with microfilament complexes of leafhoppers that transmit OYp but not with those of leafhoppers that do not transmit OYp. After this specific recognition, phytoplasmas invaded insect body and through haemolymph reach different tissues, including salivary glands, where they multiply.

After injection into plants, phytoplasmas negatively impact the fitness of their plant hosts. Plants are frequently stunted and may not produce normal owers, fruits and seeds.

On the contrary, in insect phytoplasmas may or may not influence fitness and survival of insect vectors that sometimes can even benefit from phytoplasma infection by living longer (Hogenhout *et al.,* 2008). For example, *DaIbulus* leafhoppers, when exposed for a long time to Maize Bushy Stunt Phytoplasma (MBSP), developed tolerance to these bacteria and well adapted to each others (Ebbert *et al.*, 2001). Beneficial symbiosis has also been observed for other leafhopper-phytoplasma association. The longevity and number of offspring of the aster yellows phytoplasma (AYp) leafhopper vector (*Macrosteles quadrilineatus* Forbes) can increase on AYp-infected China aster, lettuce, carrot and periwinkle, as compared with healthy plants (Beanland *et al.,* 2000).

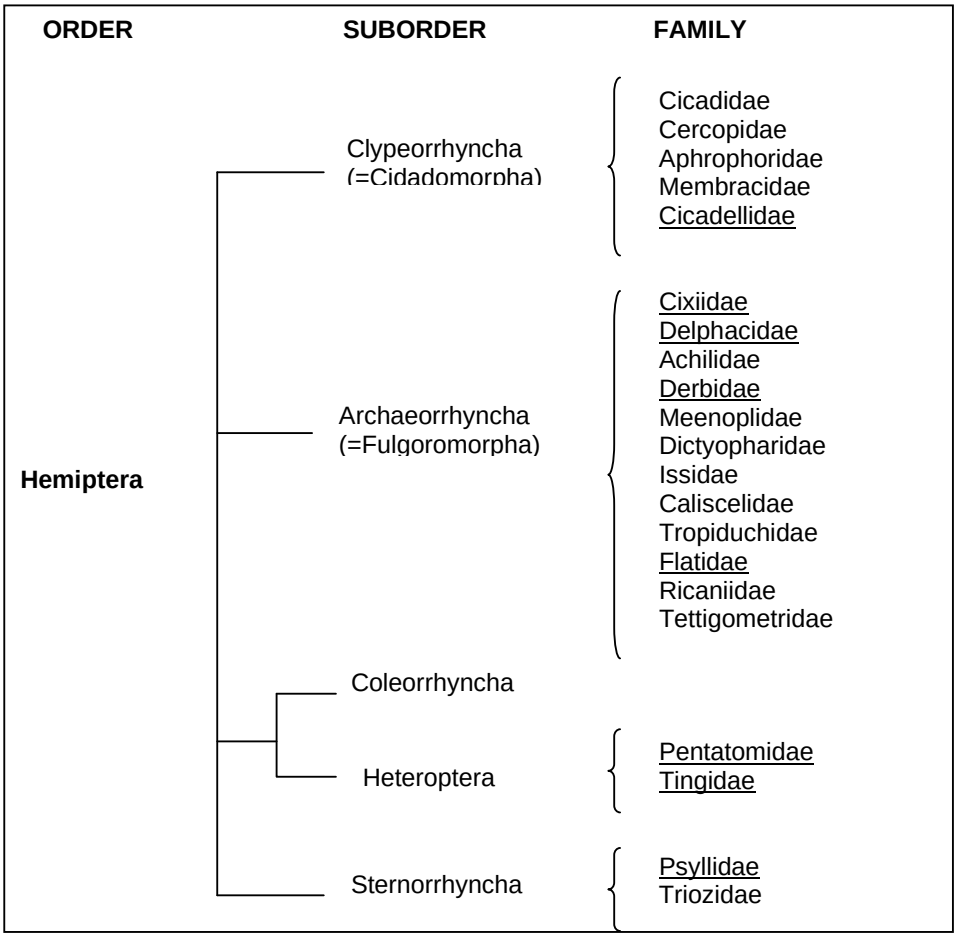
Phytoplasmas can also manipulate plants to become new hosts for leafhoppers that normally do not use these plants as hosts. The mechanisms by which phytoplasmas convert plants into more attractive hosts for insects are not yet known.

2.6 Phytoplasmas and their vectors

The single most successful order of insect vectors of phytoplasma is the Hemiptera (=Rhynchota) (Weintraub and Beanland, 2006).

Recent studies characterized within this order four suborders: i) Clypeorrhyncha (= Cicadomorpha), ii) Archaeorrhyncha (= Fulgoromorpha), iii) Prosorrhyncha (Heteroptera and Coleorrhyncha) and iv) Sternorrhyncha. Both the suborders Clypeorrhyncha and Archaeorrhyncha were former considered as Auchenorrhyncha (Biedermann and Niedringhaus, 2004). Within the order Hemiptera, the families interesting as phytoplasma vectors are highlighted in the scheme below (Fig. 1).

Figure 1. Classification of Hemiptera with particular reference to the European families of Auchenorrhyncha.



The phylogenetic relationships among these four groups are not yet clarified (Burgoin and Campbell, 2002; Sorensen *et al*., 1995).

Hemipterans differ, with few exceptions, from all other insects by their mouthparts. They have a piercing-sucking beak (the rostrum), from which the name

Rhyncota, with maxillary and labial palps totally reduced, mandibles and maxillary laciniae modified into two pairs of stylets which at rest are more or less retracted in the head with their apical parts enclosed in the grooved, segmented labium (Holzinger *et al.*, 2003).

Hemiptera collectively possess several characteristics that make its members efficient vectors of phytoplasmas:

(*a*) They are hemimetabolous; thus, nymphs and adults feed in a similar way and are in the same physical location—often both immatures and adults can transmit phytoplasmas.

(*b*) They feed specifically and selectively on certain plant tissues, which makes them efficient vectors of pathogens residing in those tissues. Furthermore, their feeding is non-destructive, promoting successful inoculation of the plant vascular system without damaging conductive tissues and eliciting defensive responses.

(*c*) They have a propagative and persistent relationship with phytoplasmas.

(*d*) They have obligate symbiotic prokaryotes that are passed to the offspring by transovarial transmission, the same mechanisms that allow the transovarial transmission of phytoplasmas (Alma *et al.*, 1997; Hanboonsong *et al.*, 2002; Tedeschi *et al.*, 2006).

Phytoplasmas are phloem-limited, therefore only phloem-feeding insects can potentially acquire and transmit the pathogen. However, within the groups of phloem-feeding insects only a small number, primarily in three taxonomic groups, have been confirmed as vectors of phytoplasmas.

i) The group containing the largest number of vector species is the suborder Clypeorrhyncha (=superfamily Membracoidea), within which all known vectors to date are confined to the family Cicadellidae (leafhoppers).

ii) The second group is Archaeorrhyncha (=Fulgoromorpha), in which four families of vector species are found (Cixiidae (planthoppers), Delphacidae, Derbidae, and Flatidae).

iii) The third group is the suborder Sternorrhyncha, in which only two genera in the family Psyllidae are confirmed vectors. (Weintraub and Beanland, 2006).

In detail, morphological and molecular evidence indicates that the Membracoidea are a monophyletic superfamily (Dietrich *et al.*, 2001); however, the phylogenetic status and relationships of the families, subfamilies, and tribes are poorly understood. The most recent analyses, based on conservative 28S ribosomal subunit DNA sequences, and in agreement with morphological analysis (Nielson, 1979), place the subfamily Deltocephalinae as the most highly derived lineage. More than 75% of all confirmed phytoplasma vector species are found in this subfamily. The feeding habits of species

within the Deltocephalinae vary from monophagous to polyphagous, and members of this group can transmit one or more different phytoplasma taxa. The subfamily containing the second largest number of confirmed vector species is the Macropsinae. Vector members of the Macropsinae can be monophagous or oligophagous, but most feed primarily on woody plants. On the basis of analysis of ribosomal DNA, the morphologically distinct membracids are part of the Cicadellidae; however, to date, no membracids have been confirmed as or are suspected of transmitting phytoplasmas. Although membracids are relatively poor transmitters of viruses compared with leafhoppers, it is unknown whether researchers have not considered membracids in phytoplasma vector studies because they appear to be a group distinct from the leafhoppers (which are known vectors) or because membracids actually do not transmit phytoplasmas. Because membracids tend to feed on woody host plants, the phytoplasma groups found primarily in woody plants, as Western-X (WX), Pear Decline (PD), Apple Proliferation (AP), or European Stone Fruit Yellows (ESFY) could be probably transmitted by them. (Weintraub and Beanland, 2006).

Vector species are found in four families of fulgorids (Archaeorrhyncha): Cixiidae, Delphacidae, Derbidae, and one species in the Flatidae. The first three families have at least one species that transmits a phytoplasma in the coconut lethal yellows group (16SrIV). Several species in these families also transmit phytoplasmas of the stolbur (Sr16XII) group. The one flatid vector, *Metcalfa pruinosa* (Say), transmits aster yellows (AY) (group Sr16I).

Two genera of psyllids (Sternorrhyncha, Psyllidae) are vectors. *Cacopsylla* spp. transmit AP group (16SrX) phytoplasmas to pome and stone fruit trees. AP phytoplasmas are the smallest, with a genome size of 630 to 690 kbp (Marcone *et al*., 1999), and it may be the case that psyllids can transmit only smaller phytoplasma genomes. The same types of trees susceptible to AP and ESFY are also susceptible to WX, which has a similar small genome size, but to date psyllids have not been implicated in WX transmission. The other psyllid genus has one vector species, *Bactericera trigonica* Hodkinson, which transmits a stolbur (Sr16XII) phytoplasma to carrots (27). It was once believed that insects can transmit phytoplasma feeding in the phloem in a non-destructive manner, but there are heteropteran vectors that have a more destructive feeding pattern (Mitchell, 2004; Okuda *et al.,* 1998).

Two heteropteran families, Pentatomidae and Tingidae, have confirmed vector species. Adults and nymphs of the brown marmorated stink bug, *Halyomorpha halys* Stål (=*H. mista* Uhler), can transmit witches' broom phytoplasma to *Paulownia* spp. trees in Asia (Hiruki, 1999). The tingid *Stephanitis typica* (Distant) transmits a root wilt to coconut palms in Southeast Asia (Mathen, 1990).

2.7 Phytoplasma acquisition and transmission

Phloem-feeding insects acquire phytoplasmas passively during feeding on the phloem of infected plants. The feeding duration necessary to acquire a sufficient title of phytoplasma is the acquisition access period (AAP). The AAP can be as short as a few minutes but is generally measured in hours, and the longer the AAP, the greater the chance of acquisition (Purcell, 1982). The AAP may also depend on the concentration of phytoplasmas in the plants. Therefore, even though the phytoplasma title could be quantified, it is unknown how its title in plants affects the AAP.

The time that elapses from initial acquisition to the ability to transmit the phytoplasmas is known as the latent period (LP) and is sometimes called the incubation period. The LP is temperature dependent and ranges from a few to 80 days (Murral *et al*., 1996). During the LP the phytoplasmas move through and replicate in the competent vector's body. Phytoplasmas can pass intracellularly through the epithelial cells of the midgut and replicate within a vesicle, or they can pass between two midgut cells and through the basement membrane to enter the hemocoel. Phytoplasmas circulate in the hemolymph, where they may infect other tissues such as the Malpighian tubules, fat bodies and brain, or reproductive organs; replication in these tissues, albeit not essential for transmission, may be indicative of a longer coevolutionary relationship between host and pathogen. Lefol and co-workers (1993) demonstrated surface protein involvement, and some level of specificity, in attachment of phytoplasma particles to insect host cells. However the molecular factors related to the movement of phytoplasmas through the various insect tissues are still unknown.

Phytoplasmas must penetrate specific cells of the salivary glands in order to be transmitted to plants and high levels must accumulate in the posterior acinar cells of the salivary gland before they can be transmitted (Kirkpatrick, 1992). At each point in this process, if the phytoplasmas fail to enter or exit a tissue, the insect would become a dead-end host and would be unable to transmit the phytoplasmas. To illustrate this point, Wayadande and co-workers (1997) showed that in the salivary glands there are three barriers that pathogens must cross before they can be ejected with the saliva: the basal lamina, the basal plasmalemma, and the apical plasmalemma. Leafhoppers can be infected by a phytoplasma and yet be unable to transmit it to healthy plants (Lefol *et al*., 1993, Vega *et al*., 1993, 1994), perhaps because of the salivary gland barriers.

For instance, during feeding on the plants, leafhoppers or planthoppers constantly secrete a small amount of sheath saliva into the leaf environment that encases and protects the delicate stylets when it solidifies. Phytoplasmas (or other circulative pathogens) are introduced into the phloem probably via watery saliva as the leafhopper stylets penetrate sieve element membranes (Lett *et al*., 2001).

Some of the same leafhopper species that are competent to transmit phytoplasmas can also transmit viruses and spiroplasmas. For example, *Circulifer tenellus* Baker transmits beet curly top hybrigeminivirus, phytoplasma (Weintraub *et al*., 2004), and *Spiroplasma citri* (Klein *et al*., 1988). It is unknown whether the receptors that allow penetration of these different pathogens into insect midgut cells are the same. Phytoplasmas cannot be cultured in vitro (Marcone *et al.,* 1999), but the closely culturable related group spiroplasmas can be used to know more about the biology of spiroplasma–insect vector interactions (Bovè *et al*., 2003, Fletcher *et al*., 1998).

2.8 Phytoplasma epidemics

The interaction between insects and phytoplasmas is complex and variable. The complex sequence of events required for an insect to acquire and subsequently transmit phytoplasmas to plants suggests a high degree of fidelity between insect vector species and the phytoplasmas that they transmit. However, numerous phytoplasmas, such as AY and WX strains in North America, are transmitted by several different insect species (Ebbert *et al*., 2001, Lee *et al.,* 1996). In addition, a single vector species may transmit two or more phytoplasmas, and an individual vector can be infected with dual or multiple phytoplasma strains (Lee *et al*., 1996; L. Beanland, unpublished data).

Vector–host plant interactions also play an important role in determining the spread of phytoplasmas. Polyphagous vectors have the potential to inoculate a wider range of plant species, depending on the resistance to infection of each host plant. Several studies (Bosco *et al.,* 1997, Marzachì *et al*., 1998**)** have shown that insects that normally do not feed on certain plant species can acquire and transmit phytoplasmas to those plants under laboratory conditions. Hence, in many cases, the host range of a vector, rather than lack of phytoplasma-specific cell membrane receptors, limits the spread of phytoplasmas by that species.

Phytoplasmas are also transmitted by the majority of the dodder species, this transmission is usually important only for research studies since it allows to transfer phytoplasmas to useful experimental plant hosts such as periwinkle (*Catharanthus roseus* G. Don.) that is the host in which the majority of reference strains for *Candidatus* species should be maintained (IRPCM, 2004).

Micro-propagation together with other agricultural practices such as grafting, cutting or other ways to propagate plant germoplasm avoiding sexual reproduction (tubers, rhizomes and bulbs) are long time known ways of phytoplasma transmission.

Recently the possibility of phytoplasma transmission by seed was also under investigation. After first suspect related to the epidemiological spreading to coconut lethal yellowing (Cordova *et al.,* 2003), other studies on Oman alfalfa (*Medicago sativa* L.)

cultivations severely affected by phytoplasma infection inducing witches' broom and loss of yield were carried out.

2.9 Spatial vector dispersal

The movement of insect vectors, and the complexities of the impact of the agrolandscape on that movement, are slowly being teased into their component parts (Uyemoto *et al*., 1998). As with any tritrophic relationship, the components of a phytoplasma disease system must overlap: vulnerable host plant in time (season) and space (geography), pathogen, and vector. Environmental conditions mediate the activity and contribution of each of the three components. At the field scale, movement of vectors can be influenced by the dispersion of host plants.

According to Power (1992), shorter distances between preferred plants increase the likelihood that an insect moves from one to the other.

As an additional layer to this complex system, there are primary and minor insect vectors; the primary vector transmits the phytoplasma to the economic crop, whereas the minor vector(s) inoculates noncrop plant hosts that serve as reservoirs of the phytoplasma. Although these two classes of vectors have seldom been identified for any crop-phytoplasma system, they are likely important in most plant diseases.

2.10 Phytoplasma and vector management

Until recently, management of plant diseases caused by phytoplasma has focused on controlling the vector by insecticides. A method to reduce within the field the alternative vector host plants and/or reservoirs of phytoplasma-infected crop plants and weeds is by roguing. Uyemoto and co-workers (1998) found that by spraying WX-infected trees with insecticide before roguing, the incidence of disease spread was significantly reduced.

Chemical control of vectors likely will continue for the foreseeable future, but vector management or management of phytoplasma spread within the plant is now slowly shifting to habitat management and the use of genetically modified crops. Habitat management can reduce pest incidence. The type of mulching materials used around coconut trees influences the abundance of the planthopper vector of lethal yellows, *Myndus crudus* van Duzee. Fewer nymphs are found around trees mulched with coarse materials such as pine bark nuggets (Howard, 1998). Although some parasitoids of leafhoppers have been identified, no studies have investigated the use of these natural enemies to effectively manage pest species. Unfortunately, the vegetation that can increase the incidence and abundance of natural enemies of vectors can also be favourable to those taxa that transmit phytoplasmas. More effort should be made to determine those

elements of the cropping environment that enhance the survival of natural enemies but do not increase vector numbers.

Genetic modifications may include enhancement of genes naturally present within the plant that code for defensive compounds or the introduction of alien genes into crop plants. The enhanced or introduced genes provide protection from the vector insect or the pathogenic phytoplasma, for example with the expression in rice of lectins highly toxic to planthoppers, that significantly reduced the survival, development, and fecundity of the planthopper *Sogatella furcifera* Horvàth and had substantial resistance against the other two planthoppers that affects rice (Naghadara *et al.,* 2004)

There is evidence that rootstock may affect vector response to plants. Annual mapping of phytoplasma infections in a vineyard in Israel led to the discovery that plants on Richter 110 rootstock had less phytoplasma incidence than did plants on Castel 216 (P.G. Weintraub, unpublished data).

Bertaccini (2007) also underlines as control of epidemic outbreak can be carried out mainly by controlling the vector, even if this protection measure resulted quite ineffective under field conditions, because it is impossible to eliminate all vectors from environments. Very important is to prevent the outbreaks of phytoplasma diseases by producing clean material or by finding phytoplasma resistant varieties or at least, tolerant but these latter can be employed only under restricted and defined environmental conditions (Carraro *et al*., 1998; Kison *et al.,* 2001).

2.11 The case study: '*Candidatus* Phytoplasma phoenicium', the causal agent of Almond Witches'-broom disease in Lebanon

2.11.1 The stone fruit production in the Mediterranean area

Almonds, in dried areas, and peaches and nectarines, in irrigated areas, are among the most important stone fruit crops grown in Mediterranean areas.

Almond is a typical Mediterranean culture, since it requires a specific climate for reliable production, as frost-free springs, reasonable rainfall in winters and springs and dry and hot summers (Ka, 1990). Almond is considered a very interesting and profitable crop, in Lebanon and neighbouring countries, because it may be harvested either in spring, to be sold as green fruit for fresh consumption, or in summer as mature nuts.

Peaches and nectarine represent an increasingly profitable crop for farmers, mainly due to low returns from other crops and their high export potential. Therefore, stone fruit production in these regions is predicted to continue to increase in the near future (USDA, 2010).

According to the Food and Agriculture Organization (FAO), the top five almond producers in 2009 were the United States with 1,162,200 metric tons (45 % of the world's production), followed by Spain (282,100 tons, 12 %), Iran (128,464 tons, 8 %), Italy (113,700 tons, 5 %), and Morocco (104,115 tons, 3 %) (Fig. 2). In the Middle East, Syria produced 97,002 tons, ranking sixth on the world production, whereas Lebanon produced 30,500 tons, ranking thirteenth on the list of producers (Faostat, 2009).

The situation on peach and nectarine production on 2009, as shown in figure 3, presents China as the first producer in the world, with 10,170,038 metric tons, followed by Italy (1,692,500 MT), USA (1,197,670 MT), Spain (1,191,300 MT) and Greece (734,000 MT). Syria ranked 26, whereas Lebanon ranked at 32nd position in the global rank per commodity (Faostat, 2009).

In Lebanon, stone fruits and almond in particular, represent the major fruit crops grown, in terms of generated income, compared with grape, olives, pome fruits and citrus (Figure 3). Within the stone fruits, almond ranks first among cherries, peaches and nectarines, apricots and plums, as reported in table 1.

Table 1. Lebanese production of fruit commodities, FAOSTAT 2009.

Rank	Commodity	Lebanese Production (Int $1000)	Production (MT)	Flag*
3	Almonds, with shell	90004	30500	F
6	Grapes	68594	120000	F
7	Olives	66858	83500	F
8	Apples	52229	126500	F
9	Lemons and limes	45595	115000	F
10	Oranges	44449	230000	F
11	Cherries	44064	34662	Im
16	Peaches and nectarines	24326	44683	Im
17	Apricots	19462	35251	Im
19	Plums and sloes	15038	25200	F

* Flags: F: FAO estimate; Im: FAO data based on imputation methodology. FAOSTAT 2009.

Fig. 2. World almond production, FAO 2009.

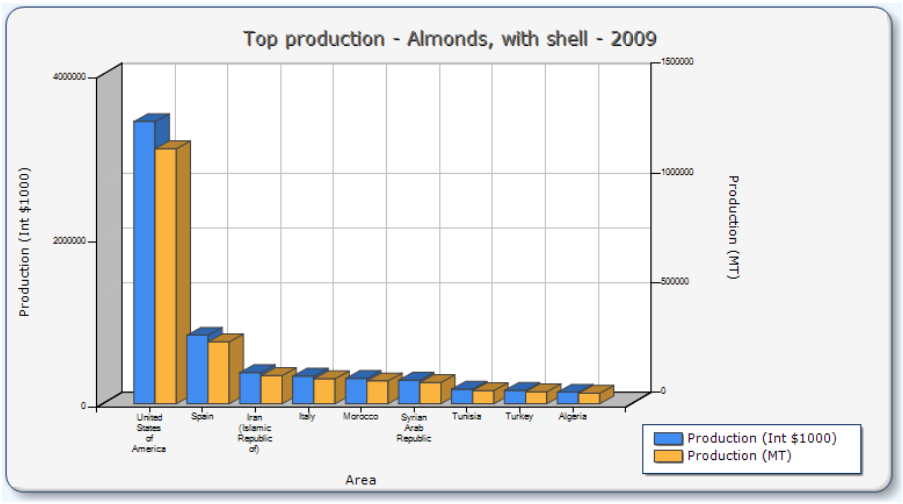


Fig. 3. World peach and nectarine production, FAO 2009.

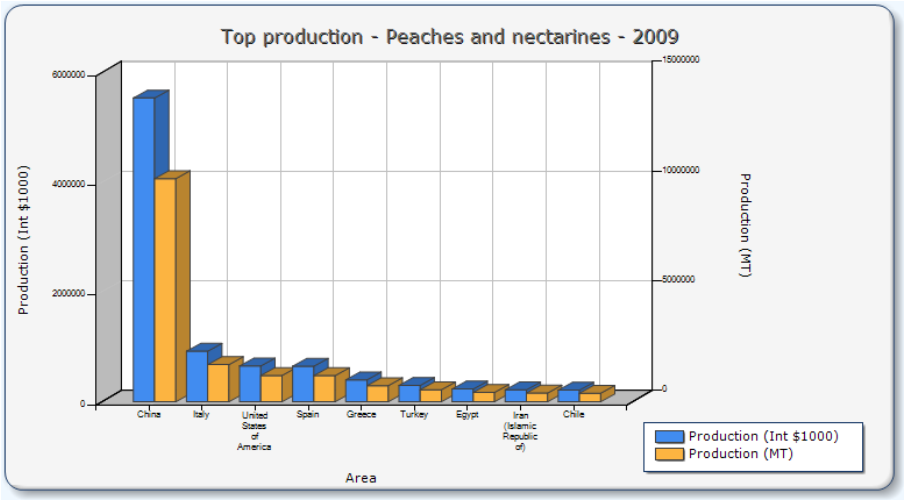
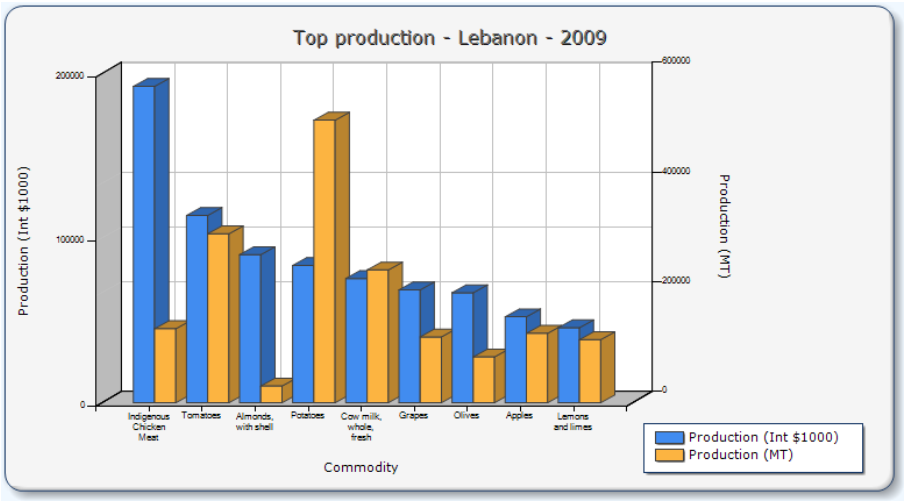


Fig. 4. Lebanon top production, FAO 2009.



2.11.2 The Almond witches'-broom disease outbreak in Lebanon

In the 1980s and 1990s, the area devoted to almond production in Lebanon increased remarkably when some growers in the Bekaa Valley preferred growing almond instead of grape, cherry, and apricot. Nevertheless, during the last decade the outbreak of an unknown disease associated with almond has led to rapid decline of almond trees in the major almond production regions.

The first epidemic occurred in the South of Lebanon in the early 1990s and it was reported in north Lebanon starting in 1995 (Abou-Jawdah *et al.*, 2002).

A survey carried out on almond trees in 1998 to 1999, using enzyme-linked immunosorbent assay (ELISA), showed that most declining trees were free from infection by six of the major stone fruit viruses, as *Apple chlorotic leaf spot virus* (ACLSV), Apple *mosaic virus* (ApMV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Tomato ringspot virus* (ToRSV) and Plum Pox Virus (PPV) (Kannaan-Atallah *et al.*, 2000). The disease rapidly spread, first in the coastal areas to an elevation up to about 500 m, where the majority of almond orchards are located. However a few trees at higher elevations (1,000 m) also showed the characteristic symptoms of witches brooms observed on the declining trees.

A phytoplasma was supposed to be the causal agent of the disease, due to the presence of witches'-broom symptoms arising from tree trunks, and readily detected from the symptomatic trees collected from three major almond growing regions: Koura and Zgharta in the North, Saida in the South and Zahlè in the Bekaa Valley. Restriction fragment length polymorphism (RFLP) analysis of PCR products amplified by the primer pair R16F2n/R16R2 revealed that the phytoplasma associated with infected almonds was similar to, but distinct from, members of the pigeon pea witches'-broom (PPWB) phytoplasma group (16SrIX) (Abou-Jawdah *et al.*, 2002). Sequencing of the amplified phytoplasma 16S rRNA gene products confirmed that almond witches'-broom (AlmWB) phytoplasma was most closely related to members of the pigeon pea witches'-broom phytoplasma group, with sequence homology ranging from 98.4 to 99.0%.

The same results were confirmed by a parallel study carried out on almond samples collected in some orchards of the Bekaa Valley in the same years, by Choueiri and co-workers (Choueiri *et al.*, 2001).

It was the first report of a phytoplasma infection in Lebanon and the first report for a phytoplasma belonging to the PPWB group infecting almond trees.

2.11.3 The Pigeon Pea Witches'-Broom group

The range of host plants of the phytoplasmas belonging to the PPWB group is quite wide and includes herbaceous plants, fruit trees as well as conifers.

The Pigeon Pea Witches-Broom (PPWB) phytoplasma, subgroup IX-A (Wei *et al.*, 2007), was firstly described by Harrison and co-workers on 1991 on pigeon pea, *Cajanus cajan*, syn. *Cajanus indicus* Spreng, an important grain legume crop of rainfed agriculture in the semi-arid tropics, where pigeon peas are both a food crop (dried peas, flour, or green vegetable peas) and a forage/cover crop.

On the same group, phytoplasmas classified in the subgroup IX-C (Khan *et al.*, 2007) affect herbaceous plants, as *Pichris echioides* L. and *Knautia arvensis* L. Coult, causing the diseases *Pichris echioides* yellows (PEY) and *Knautia arvensis* Phyllody (KAP).

A phytoplasma detected in *Dimorphotheca sinuata* DC. (Cape marigold) in southern Italy was identified by RFLP analysis as a member of the pigeon pea witches'-broom (PPWB) group and proved to be indistinguishable from the Pichris echioides yellows (PEY) reference strain (Marcone *et al.*, 2001).

Moreover, the phytoplasma causing Juniper witches'-broom on *Juniperus occidentalis* Hook., a native tree indigenous to parts of Oregon, Washington, Idaho, Nevada and California (USA), was identified and classified as 16SrDNA subgroup IX-E (Davis *et al.*, 2010).

A phytoplasma closely related to the PPWB group, causing the Little Leaf Disease (LLD), was found in *Gliricidia sepium* (Jacq.) Kunth ex Walp., a medium-sized, thornless, leguminous tree native to seasonally dry areas of Mexico and Central America, used for livestock fodder or green manure, for firewood, poles, live fences, shade (for cacao or coffee), bee forage and for rehabilitation of degraded sites, erosion control and sand dune stabilization (Kenyon *et al.*, 1998).

Moreover, on 2007 sweet orange (*Citrus sinensis* (L.) Osbeck) trees with characteristic symptoms of huanglongbing (HLB) were encountered in a region of São Paulo state, Brazil, hitherto free of HLB. These trees tested negative for the three Liberibacter species associated with HLB ('*Candidatus* Liberibacter asiaticus', '*Candidatus* Liberibacter africanus' and '*Candidatus* Liberibacter americanus') but the corresponding agent was found to have highest 16S rDNA sequence identity (99%) with the pigeon pea witches'-broom phytoplasma of group 16SrIX (Teixeira *et al.*, 2008).

Sequence homology results on BLAST search revealed also that Echinops witches' broom (EWB) phytoplasma, found in Oman from *Echinops spinosissimus* Turra, a shrub belonging to the family Asteraceae that thrives well in semi-arid habitats from the Mediterranean region to the Arabian Peninsula, shares 98% similarity with pigeon pea witches' broom (EF186825), *Lactuca serriola* phytoplasma from Iran (DQ889749), *Knautia arvensis* phyllody phytoplasma (Y18052), Iranian almond witches' broom (DQ195209) and *Pichris echioides* yellows (Y16389) phytoplasma and 98% with '*Ca*. Phytoplasma

phoenicium' (AF515636) and Honduran *Gliricidia* little leaf phytoplasma (AF361017) (Al Subhi *et al*., 2007).

In several Oman locations, again, plants of *Cassia italica* Mill. Lam. exhibiting witches' broom symptoms resulted affected by phytoplasmas whom closest phytoplasma relatives were members of the pigeon pea witches' broom phytoplasma ribosomal group (16SrIX), sharing a 93-97% sequence similarity (Khan *et al*., 2007).

2.11.4 Almond witches'-broom phytoplasma

The phytoplasma found on almond trees in Lebanon was named Almond Witches'-Broom (AlmWB) and a new subgroup, 16SrIX-B, was designated within PPWB phytoplasma group (16SrIX), based on RFLP analyses, carried out using twelve restriction enzymes: *Alu*I, *Bfa*I, *Hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Kpn*I, *Rsa*I, *Taq*I, *Tha*I, *Mse*I and *Sau*3A (Abou-Jawdah *et al.,* 2002).

In fact, the RFLP patterns of AlmWB phytoplasma strains resolved by single digestion with *Mse*I, *Hpa*II, *Tha*I, *Bfa*I, *Kpn*I, and *Sau*3A were identical to those of PPWB phytoplasma, but the patterns resolved by digestions with the other six enzymes (*Rsa*I, *Alu*I, *Hha*I, *Hae*III, *Taq*I, and *Hin*fI) were distinct from those of PPWB phytoplasma.

Phylogenetic analysis of 16S rDNA sequences from AlmWB phytoplasma and from representative phytoplasmas from GenBank confirmed that the AlmWB phytoplasma represents a distinct lineage within the pigeon pea witches'-broom subclade (Abou-Jawdah *et al.,* 2002).

In 2003, experiments were carried out grafting symptomatic almond and nectarine buds on seedlings of almond, peach, nectarine, cherry, plum and apricot. The experiments showed clearly that AlmWB can be graft transmitted to almond, nectarine, and peach seedlings (Abou-Jawdah *et al*., 2003). Over the observations, the most apparent symptom on almond, nectarine, and peach was the development of bushy growth at the base of the stem (rootstock) at or below the soil level. The stems were succulent, with shortened internodes, and the leaves were small and light green in colour. On apricot and plum, even though the grafting buds were still viable, no symptoms were observed on the parts above the grafting buds and growth was similar to the controls. The absence of AlmWB phytoplasma in these symptomless tissues was confirmed by nested-PCR analysis. On cherry, the almond and nectarine grafting buds were all dead, the growth was delayed until early May, and no differences were observed from the control.

Similar graft-inoculation experiments of infected almond shoots onto seedlings of almond (*Prunus amygdalus* (Mill.) D.A.Webb), peach (*Prunus persica* (L.) Batsch GF305) and plum (*Prunus mariana* (*P. cerasifera* x *P. munsoniana*) GF8-1) were carried out by Verdin and co-workers (2003). Symptoms developed after one month on the three

inoculated *Prunus* species, and consisted of axillary bud proliferation similar to that observed on naturally infected almond trees (Verdin *et al.,* 2003).

Moreover, the almond phytoplasma from Lebanon was shown to be identical to a phytoplasma that induces a disease called 'almond brooming' in Iran, but different from another PPWB-group phytoplasma that infects herbaceous annual plants in Lebanon (*Lactuca serriola* and *Catharanthus roseus* (L.) G.Don). In the study the complete sequence of P1/P7-amplified fragments was determined for two different Lebanese almond phytoplasma isolates, from the central Bekaa and northern Deir Amar regions, as well as for an uncharacterized phytoplasma detected in 1997 in an almond tree affected by almond brooming in the Shiraz region of Iran (Bové *et al*., 1999), and for two wild lettuce (*Lactuca serriola*) and one periwinkle (*Catharantus roseus*) plants collected in Lebanese almond orchards. The 16S rDNA sequences of the two almond phytoplasmas from Lebanon were identical and differed by only 4 nucleotides (99,7% identity) from the sequence of the phytoplasma from Iran, showing that almond brooming in Iran and almond witches'-broom in Lebanon are caused by the same phytoplasma species. The closest phytoplasma relatives were members of the pigeon pea witches'-broom (PPWB) group (Schneider *et al*., 1995): *Pichris echioides* yellows (PEY) and *Knautia arvensis* phyllody (KAP), with which they share 99% identity, and the PPWB phytoplasma, with which they share 98,5% identity. The sequences of the wild lettuce and periwinkle phytoplasmas were identical and had a higher identity (99,8%) with the sequences of the PEY and KAP phytoplasmas than with those of the almond phytoplasmas (99,3% identity). The data were confirmed also by phylogenetic analysis, that showed that the Lebanese and Iranian almond phytoplasmas cluster together and that they belong to the PPWB cluster, but that they represent a new lineage in the PPWB group defined by Schneider and co-workers in 1995 or in the 16S rIX-A group defined by Lee and co-workers (1998), and a new subclade in the '*Candidatus* Phytoplasma' phylogenetic tree (Lee *et al*., 1998; Seemüller *et al*., 1998). In contrast, the wild lettuce and periwinkle phytoplasmas cluster with all other phytoplasmas of the PPWB group. These results also indicated that the almond phytoplasmas were different from phytoplasmas infecting other *Prunus* species in Europe or the United States, such as the ESFY (90.4% identity) and Western X (94.3% identity) phytoplasmas. They were also different from phytoplasmas infecting other fruit trees, such as the apple proliferation (AP) and pear decline (PD) phytoplasmas (with 90,35 and 90,84% identity, respectively) (Verdin *et al*., 2003).

2.11.5 The new species '*Candidatus* Phytoplasma phoenicium'

Rules for the description of new phytoplasma species have been established by the International Research Program on Comparative Mycoplasmology (IRPCM, 2000). A new

species may be described when a 16S rDNA sequence (>1200 bp) has <97,5% identity with any previously described '*Candidatus* Phytoplasma' species. As yet, none of the phytoplasmas of the PPWB group has been described as '*Candidatus* Phytoplasma' species, and all of the organisms in this group had <97.5% identity with phytoplasmas in other subclade (Lee *et al.*, 1998; Seemüller *et al.*, 1998). The phytoplasma infecting the wild lettuce and periwinkle plants and the other phytoplasmas of the PPWB group had >97,5% identity with the almond phytoplasma and at this stage cannot be described as separate *Candidatus* species, even though they were found in different host plants (a condition required for species distinction of closely related phytoplasmas). Indeed, two additional properties must also be fulfilled: the phytoplasmas should be transmitted by different insect vectors and should have significant molecular or serological diversity. This has not yet been documented, as the insect vectors of phytoplasmas in the PPWB group are unknown, and genes other than ribosomal operon genes have not been isolated.

Based on its distinctive properties the phytoplasma of almond witches'-broom in Lebanon was proposed as the reference strain for the new phytoplasma subclade in the PPWB cluster and named '*Candidatus* Phytoplasma phoenicium'.

2.11.6 The disease spread in Iran

As already mentioned above, an Almond witches'-broom disease, causing severe losses on almond trees, was reported, since the years 1995, also in Iran.

Iran, in fact, was a leader in almond production. In recent years, however, production of this crop has been seriously affected by witches'-broom disease in the region of Fars and in certain other provinces in the South of the Country (Fig. 5).

Salehi and Izadpanah (1995) transmitted the almond brooming agent to almond and periwinkle. The phytoplasmal nature of the disease was established by Dienes-staining and response to tetracycline treatment (Salehi and Izadpanah, 1995, 1998). Verdin and co-workers (2003), some years later, found out that the Lebanese Almond witches'-broom phytoplasma (LalmWB) and the AlmWB phytoplasma in Iran differed only in four nucleotides in 16S rDNA. Further studies on disease symptomatology in various parts of the Fars Province coupled with molecular analyses of 16S rDNA and 16–23S rDNA spacer region (SR) sequences indicated a diversity of the phytoplasmas in Iran. For this reason, two phytoplasmas from Khafr (KAlmWB) and Neyriz (NAlmWB) in the Fars Province were compared by biological and molecular analysis.

Both infected bitter almond, wild almond, peach and nectarine but not apple and pear, by grafting. In bitter almond the symptoms induced by KAlmWB consisted of severe proliferation, internode shortening and leaf size reduction. In contrast, NalmWB caused leaf necrosis, dieback and death.

Fig. 5. Map of the Iranian provinces.

Moreover, KAlmWB was transmitted to periwinkle and eggplant and from experimentally infected periwinkle to almond by dodder. It was also transmitted from eggplant to eggplant, ornamental eggplant and tomato by grafting. Under similar test conditions, NAlmWB was not transmitted to herbaceous plants by dodder.

Phylogenetic analysis of 16S–23S rDNA spacer region (SR) sequences placed both strains in the pigeon pea witches'-broom (PPWB) group. However, based on phylogenetic and putative restriction site analyses and sequence homology, NAlmWB was identical with the Lebanese AlmWB phytoplasma, while KAlmWB was closer to the *Knautia arvensis* phyllody (KAP) agent, both of which form a new subgroup (subgroup C) in PPWB group. Clustering of KAlmWB with KAP was also confirmed by analysis of full length 16S rDNA sequence.

On the basis of host range, dodder transmission, symptomatology and molecular analyses of 16S rDNA and SR, the two different phytoplasmas related to PPWB group were associated with AlmWB disease in Iran, and KAlmWB phytoplasma was reported as a new phytoplasma of AlmWB disease (Salehi *et al.*, 2006).

Furthermore, almond trees showing other various symptoms of phytoplasma diseases such as little leaf, leaf rolling, dieback of branches, rosette and yellowing were

observed in the central regions of Iran, in Isfahan and ChaharMahal-O-Bakhtiari provinces. There regions contribute considerably to the Iranian agricultural economy where 20,930 ha of almonds are cultivated with a production of 12,187 tonnes of nuts annually (AREEO, 2008). Over four years of observation, the infected trees declined and died within 3–4 years after initiation of symptoms and all almond varieties seemed to be affected, with different degrees of susceptibility.

DNA isolated from symptomatic almond trees was used to amplify 16S rDNA and 16S-23S rDNA intergenic spacer (IS) fragments by nested PCR using phytoplasma universal primer pairs. Phytoplasmas were detected in symptomatic almonds and RFLP analyses using endonuclease enzymes *Hpa*II and *Taq*I revealed that the phytoplasmas associated with infected almonds were genetically different. Sequence analyses of these amplified fragments indicated that the studied phytoplasma strains were closely related to different phytoplasmas, as '*Candidatus* Phytoplasma aurantifolia' (more prevalent than other phytoplasmas in the central regions of Iran), '*Ca*. Phytoplasma phoenicium', '*Ca*. Phytoplasma solani' and '*Ca*. Phytoplasma trifolii' (Zirak *et al*., 2009).

Previously, '*Ca*. Phytoplasma aurantifolia' was reported as the causal agent of lime witches'-broom disease in the south of Iran (Bovè *et al*., 1999) and infected many herbaceous plants in these regions (Salehi *et al*., 2006b). The presence of wide host range of '*Ca*. Phytoplasma aurantifolia' in trees, perennials and annual plants in the south and centre of Iran suggested the involvement of common and efficient unknown insect vector(s) and the fact that it will probably create epidemics in the near future.

2.11.7 The disease spread in Lebanon

In Lebanon, even if grafting experiments under controlled conditions proved that Almond witches' broom may be transmitted to peach (*P. persica*) and nectarine (*P. persica* var*. nucipersica*), but not to apricot (*P. armeniaca* L.), only a very limited number of nectarine trees were found to be infected under natural conditions when intercropped with almond (Abou-Jawdah *et al*., 2003).

After the war of 2006, Lebanese farmers of southern Lebanon started to cultivate territories and lands that, until the year 2000, were under to Israeli control, choosing to invest in stone fruit production, due to the climate conditions and the perspectives of new markets in the region.

In 2008, during field trainings performed by the Italian NGO "Fondazione AVSI" on stone fruit Integrated Pest Management (IPM), strongly required by the farmers, interested on new and sustainable techniques, symptoms of shoot proliferation, with succulent light green leaves, characteristic of phytoplasma diseases were observed by the technicians on nectarine (*Prunus persica* var*. nucipersica*) and peach (*P. persica*) trees in some

demonstration plots in the Sarada plain (south of Lebanon). The presence of '*Candidatus* Phytoplasma phoenicium' in the two orchards was confirmed by BLAST (Basic local alignment search tool) analysis of the amplified fragment sequences.

It was the first report, published on EPPO bulletin, of a natural and epidemic spread of '*Ca.* Phytoplasma phoenicium' in peach and nectarine (Abou-Jawdah *et al*., 2009) in Lebanon.

Farmers in the region were advised to remove the infected trees immediately and indeed studies were requested, in order to better understand the disease epidemiology.

2.11.8 Specific detection of '*Ca.* Phytoplasma phoenicium'

For diagnostic purposes, rapid but sensitive and reliable tests are required. Nested PCR is costly in terms of time and reagents, whereas the development of a direct PCR (single amplification) method reduces costs and testing time.

A semispecific primer pair based on the 16S rDNA sequence of AlmWB was designed by Abou-Jawdah and co-workers on 2003, using BLAST (Altschul *et al*., 1990) analysis to confirm specificity: ALW-F2 5′-AGAGTAGCTACAACGTGAGTT-3′ and ALW-R2 5′-GAGCTATAGGCCCAGGAT-3′. The primers amplify a 390-bp fragment of the 16S rDNA.

To confirm the specificity of the new primers, simultaneous runs of nested PCR with the universal primers P1/p7 followed by F2/R2, or with the specific primers (a single PCR amplification), were carried out with samples that contained different phytoplasmas representing nine groups: tomato big bud (16SrI), peanut witches'-broom (16SrII), X-disease phytoplasma (16SrIII), elm yellows phytoplasma EY1 (16SrV), clover proliferation (16SrVI), ash yellows (16SrVII), pigeon pea witches'-broom (16SrIX), apple proliferation phytoplasma (16SrX), and Mexican periwinkle virescence (16SrXIII). Only pigeon pea witches'-broom gave a positive reaction, indicating that these primers may be specific to the pigeon pea group alone. All nine phytoplasma samples were positive in nested PCR with universal primers (Abou-Jawdah *et al*., 2003).

Verdin and co-workers (2004) designed oligonucleotides within the 16S-23S operon for specific detection of '*Ca.* P. phoenicium': the primers Alm F1 (5'-CCTTTTTCGGAAGACG-3') and AlmR1 (5'-GATAACACGCTTAAGACG-3').

2.11.9 Management of the phytoplasma

Elimination of '*Ca.* Phytoplasma phoenicium' from two infected Lebanese varieties of almond, Halwani and Khachabi, by using different tissue culture techniques was studied by Chalak and co-workers (2005). Except for the oxytetracycline treatment which totally inhibited the development of explants, stem cutting cultures associated with thermotherapy, shoot tip cultures associated or not with thermotherapy, and shoot tip

micrografting were all suitable, either for shoot regeneration or for elimination of phytoplasma from the two varieties. However, stem cutting culture coupled with thermotherapy seemed to be the most practical and effective for regeneration of phytoplasma-free plantlets and it was suggested as a routine technique for producing phytoplasma-free Lebanese almond varieties, in addition to maintaining genetic diversity (Chalak *et al*., 2005).

2.11.10 The phytoplasma transmission in field

The rapid spread of AlmWB over large geographical areas suggested the presence of an efficient vector. Most natural transmissions of phytoplasmas occur via phloem-feeding hemipteran insects, primarily leafhoppers (Sorensen *et al*., 1995; Boudon-Padieu *et al*., 1989). Within Cicadellidae, the largest number of vector genera and species occur in the subfamily Deltocephalinae, which also encompasses the greatest number of nonvector species of leafhoppers (Harris, 1979).

AlmWB represents a great threat to almond, nectarine, and peach production because it seems to be effectively transmitted by an as yet unidentified vector.

Preliminary survey on leafhoppers showed that in Lebanon the most frequently found leafhopper on stone fruits is *Asymmetrasca decedens* Paoli. Preliminary transmission tests in insectproof cages, using this leafhopper were not successful, indicating that an unknown vector may be present in low population densities or may live on other hosts and use stone fruits as a transient host (Abou-Jawdah *et al*., 2003).

Insect captures focused on psyllids and leafhoppers were also performed in and around the almond orchards in Bekaa valley and in the north Lebanon area by Verdin and co-workers. Two phytoplasma related to the *Knautia arvensis* phytoplasma (KAP), which also belongs to the PPWB cluster, were identified in leafhoppers. Other phytoplasmas, such as Aster Yellow (AY) phytoplasma and Clover Proliferation (CP) phytoplasma were also detected in leafhoppers, whereas one phytoplasma related to the Apple Proliferation phytoplasma (AP) was found in one species of psyllids. None of the insects gave positive results with the specific detection test for '*Ca.* Phytoplasma phoenicium' (Verdin *et al*., 2004).

Further studies were carried out on leafhoppers with intense collecting of insects in two infected Lebanese regions: in the north, at Bourj El Yahoudieh, and in the south, at Tamboureet regions.

The survey, carried out using yellow sticky traps, revealed that the most abundant species was *A. decedens*, which represented 82.4% of all the leafhoppers sampled. Potential phytoplasma vectors in members of the subfamilies Aphrodinae, Deltocephalinae,

and Megophthalminae were present in very low numbers including: *Aphrodes makarovi* Zachvatkin, *Cicadulina bipunctella* (Matsumura), *Euscelidius mundus* (Haupt), *Fieberiella macchiae* Linnavuori, *Allygus theryi* (Horváth), *Circulifer haematoceps* (Mulsant & Rey), *Neoaliturus transversalis* (Puton), and *Megophthalmus scabripennis* Edwards. Nested PCR analysis and sequencing showed that *Asymmetrasca decedens*, *Empoasca decipiens* Paoli, *Fieberiella macchiae*, *Euscelidius mundus*, *Thamnotettix seclusus* Linnavuori, *Balclutha* sp., *Laylatina inexpectata* Abdul-Nour, *Allygus* sp., and *Anoplotettix danutae* (Abdul-Nour) were nine potential carriers of AlmWB phytoplasma.

Although the detection of phytoplasmas in an insect does not prove a definite vector relationship, the technique can be useful in narrowing the search for potential vectors, followed by the adequate transmission tests.

2.11.11 Open possibilities

In the table 2 is reported a list of Cicadellidae species, common in Lebanon and in the Middle East regions, already known as phytoplasma vectors.

Table 2. Cicadellidae species, common in the Middle East regions, known vectors of phytoplasmas

Genus/species	Role as phytoplasma vector
Circulifer spp.	*C. haematoceps* transmits sesame phyllody in Turkey (Kersting, 1993) and Iran (Salehi and Izadpanah, 1992). *Circulifer taenellus* (Baker) transmits the beet leafhopper-transmitted virescence agent (BLTVA) phytoplasma in USA (Munyaneza *et al*, 2007).
Euscelidius spp.	*Euscelidius variegatus* Kirschbaum is known as a vector of chrysanthemum yellows phytoplasma (CYP, '*Candidatus* Phytoplasma asteris') (Bosco *et al*, 2007).
Euscelis spp.	*Euscelis incisus* Kirschbaum is known as a vector of chrysanthemum yellows phytoplasma (CYP, '*Ca.* Phytoplasma asteris') (Bosco *et al*, 2007).
Exitianus capicola Stal	*Exitianus capicola* tested positive for phytoplasma in PCR assays and transmitted the Bermuda grass white leaf (BGWL) agent to healthy Bermuda grass plants (Salehi *et al.*, 2009).
Macrosteles spp. *(Deltocephalinae)*	Many leafhopper species belonging to the genus *Macrosteles* are known vectors (Nielson, 1968; Brca, 1979). *Macrosteles quadripunctulatus* Kirschbaum transmits the AY phytoplasma in central and south European countries (Brca, 1979;

Genus/species	Role as phytoplasma vector
	Minucci *et al*., 1992) and it is known as a vector of chrysanthemum yellows phytoplasma (CYP, '*Ca.* Phytoplasma asteris') (Bosco *et al*, 2007).
Neoaliturus fenestratus Herrich-Schaffer	The leafhopper is the vector of the safflower phyllody phytoplasma (Raccah and Klein, 1982).
Orosius spp.	There are several species in the genus *Orosius*; probably all of them are vectors of phytoplasmas. *Orosius argentatus* is a very active vector in the Far East and Australia, transmitting several viruses and phytoplasmas (Nielson, 1968) and is a suspected vector of the Australian grapevine yellows phytoplasma (Osmelak *et al*., 1989). Another species, *Orosius orientalis*, found in the eastern Mediterranean and India, is the key vector for several diseases, such as the Sesamum phyllody (Vasudeva, 1961). Sesame phyllody is present in Israel (Klein, 1977) and is transmitted in laboratory by *Orosius* sp. (Klein, unpublished data). *Orosius cellulosus* transmits the pathogens of cotton phyllody and some other related diseases in Upper Volta (Laboucheix *et al*., 1973).
Psammotettix spp.	Wheat blue dwarf (WBD) phytoplasma (16SrI-C) is an important disease of winter wheat disseminated by *Psammotettix striatus* L. (Peiwen *et al*. 2004)
Recilia spp.	*Recilia banda* Kramer transmits Napier stunt phytoplasma (16SrXI strain) in western Kenya, and may be the key vector of Napier stunt disease in this region (Obura *et al*). the 16s rRNA gene of witches' broom disease of lime (WBDL) is detectable in leafhopper species (*Hishimonus phycitis*, *Recilia schmidtgeni* and *Idioscopus clypealis*) and citrus psylla (*Diaphorina citri*) in Iran (Siampour *et al*., 2006) *Rice dwarf virus* (RDV), the causal agent of rice dwarf disease. It is transmitted by leafhoppers (*Nephotettix cincticeps*, *N. nigripictus*, *Recilia dorsalis*) to rice and other gramineae plants.
Synophropsis lauri	The presence of the ESFY phytoplasma in *S. lauri* individual does not mean that it has the ability to vector phytoplasma (Jaraush *at al*, 2001)

Peach packaging in the farm of Sarada.

3. AIM OF THE STUDY

Almond witches'-broom (AlmWB), firstly described in 2001 (Verdin *et al.*, 2001), is a severe disease which affects almond, peach and nectarine trees in Lebanon and was observed, only on almond trees, also in Iran (Salehi *et al.*, 2006).

The name of the disease derives from the main typical symptom shown by the infected almond plants: the growth and development of hyper proliferated branches, called witches'-broom, caused by the simultaneous and anticipate development of the quiescent lateral buds of the branches. The disease determines on almonds the death of the trees in a few years, causing impressive economical losses for the farmers, drastically reducing the production. Moreover, since the disease is spreading also in nectarine and peach orchards, Almond witches'-broom represents a very dangerous threat not only for the Lebanese but also for all the Mediterranean cultivations of these stone fruits.

The causal agent of AlmWB is a phytoplasma, named '*Ca,* Phytoplasma phoenicium'. Phytoplasmas, originally called mycoplasma-like organisms, are nonculturable degenerate gram-positive prokaryotes, closely related to mycoplasmas and spiroplasmas, belonging to the *Mollicutes* class.

As for the other phytoplasma disease, only early detection of pathogens and prompt eradication of phytoplasma sources have been proved to be effective in disease control. The prevention strategy comprises also actions ensuring that clean planting material is used. The control of epidemic outbreaks can also be carried out by controlling the vector(s) by chemical treatments, or by endeavouring to find and/or breed varieties of crop plants that are resistant or tolerant to the phytoplasma. Nevertheless, in the Lebanese case, the mechanism of transmission of '*Ca.* Phytoplasma phoenicium' is still not well known, since up to now the insect vector(s) responsible for the phytoplasma transmission is/are unknown and it is therefore impossible to plan an efficient control strategy. The possibility to eliminate the infected trees, as a partial solution to the problem, is feasible only in presence of infection *foci*, when only few plants are infected.

The aim of the present work is to investigate the causal agent of the AlWB disease in almond, peach and nectarine trees in Lebanon and its epidemic behaviour and to gather information on the insects most probably involved in the transmission process.

The AlmWB symptoms were carefully described on almond, but are still partially unknown on peach and nectarine. Therefore a peach orchard, located at Rachaya el Foukhar, in the southern part of the Bekaa Valley, and a nectarine orchard near Sarada, in the South of the Country, where, for the first time, AlmWB was observed on nectarine trees, were selected in order to sequentially describe the phenotypic alterations induced

by the disease during the vegetative season of the host plants. The symptomatic organs were collected in the selected orchards and in an almond reference orchard located in the region of Feghal, in the North of Lebanon, well known as one of the first regions where almonds were affected by the disease 10 years ago.

Diagnostic analysis was performed on leaf and flower samples in order to detect the pathogen and to characterize its genome. Phytoplasmas, which are uncultured *in vitro*, are classified on the basis of their highly conserved ribosomal 16SrDNA gene sequences.

Since the phytoplasma genome variability may be associated to different hosts, or regions of provenience or relationship with the vectors, the genome sequence variability of the Lebanese strains of '*Ca.* Phytoplasma phoenicium' was investigated. It was interesting, in fact, to determine if the phytoplasma affecting almonds was the same infecting different plants, having different speed of spread and living in different climate and growing conditions.

According to the literature and to the preliminary observations, AlWB in Lebanon seems to occur in different areas at different altitudes and geographical conditions but the last data about its real spread dated back to the year 2000. Therefore a disease monitoring, supported by the Italian Ministry of Foreign Affairs and in particular by the Italian Cooperation Bureau, was carried out on the entire Lebanese territory, in cooperation with the NGO AVSI. The American University of Beirut, the University of Kaslik and the Lebanese Agriculture Research Institute, were also involved in the project.

Finally the present work aims also to identify the putative insect vector(s) responsible of the disease transmission. Two kinds of insect traps were placed in two of the three key-orchards studied, in order to monitor the populations of the potential insect vector(s), belonging to the phloem feeder taxa of Cicadellidae (leafhoppers), Cixiidae (planthoppers) and Psyllidae (psyllids), reported in literature as possible phytoplasma vectors. Since some preliminary data about leafhoppers have already been presented, (Dakhil *et al*., 2011), the study and molecular analyses performed in collaboration with the faculty of Science of the Lebanese University and the DIVAPRA department, "Entomologia e zoologia applicate all'ambiente – Carlo Vidano" of the University of Turin, were focused on planthopper and psyllid species, in order to verify their presence and role on '*Ca.* phytoplasma phoenicium' transmission in Lebanon.

Healthy almond blooming in Hasbaya.

4. MATERIALS AND METHODS

4.1 Map of Lebanon

The figure 6 shows the 26 Lebanese districts (Caza), divided according to three main regions: the North (beige), the South (rose) and the valley of the Bekaa (green). Moreover, the key-orchards and the insect collecting sites are indicated in the map.

Fig. 6. Map of Lebanon, showing the districts belonging to the Northern part of Lebanon (beige), as well as the Southern part (rose) and the Bekaa Valley (green). In red: the studied key-orchards; in green the collecting insect regions.

4.2 Time-course characterization of the disease symptoms

Three orchards, planted respectively with almonds, peaches and nectarines, were chosen in order to observe and describe the symptoms of Almond Witches'-broom disease throughout their vegetative season.

The monitored almond 0.2 ha orchard was located in Feghal, in the Caza (District) of Jbeil, about 165 m above mean sea level (AMSL) (Fig. 6). The trees were 10-40 years old, not irrigated and untreated with pesticides. The soil was laboured in order to control weeds. The varieties of the 72 almond trees were Helwany/Telyani (green almonds) and Khechaby (dry almonds).

The monitored 0.3 ha peach orchard was located in Rachaya el Fouchar, Caza of Hasbaya, at 700 m AMSL (Fig. 6). The 90 trees present in the orchard, belonging to the Babcock variety, were planted 8 years ago and are drip-irrigated. The orchard was conducted with traditional techniques, with pesticide treatments against fungi (tetraconazole, copper) and insects (chlorpyriphos, deltametrine, cypermetrine).

The monitored 2.4 ha nectarine orchard was located in Sarada, Caza of Marjayoun, in the South of Lebanon, at about 350 m AMSL (Fig. 6). The 10 years old trees, belonging to 4 varieties: Flankis, Jad, Fantasia, Hermosa, were drip irrigated and managed according to the IPM principles.

The three orchards were mapped and the position of each tree was characterized using a GPS device.

The vegetative development of the healthy plants in each orchard was described according to the BBCH scale for stone fruits (Meier *et al*., 1994) reported in Annex 1.

The orchards were periodically monitored from BBCH growth stage 0 (Sprouting/Bud development) until 9 (Senescence, beginning of dormancy). A total of 7, 4 and 9 checks were carried out respectively on almond, peach and nectarine trees.

All the trees belonging to each orchard were observed in order to notice the typical symptoms caused by phytoplasmas according to Bertaccini (2007):

1) virescence / phyllody (development of green leaf like structures instead of flowers)
2) sterility of flowers
3) proliferation of axillary buds resulting in a witches' broom appearance
4) abnormal internodes elongation
5) generalized stunting

The diseased trees were reported on the map which was constantly updated during the subsequent field checks, in order to evaluate the disease spread in the orchards.

The most emblematic pictures of the symptoms observed on each host were used to prepare a leaflet that was distributed to the farmers and technicians during field visits and awareness meetings carried out after the survey.

4.3 National survey on the disease diffusion

The national survey on the Almond witches'-broom diffusion was carried out on 24 Lebanese districts. Lebanon is divided in 26 districts, named Caza, but, since two of them, Beirut and Tripoli are heavily urbanised areas, characterised by the total absence of cultivated fields, the survey was restricted to the remaining 24 agricultural regions.

The almond, peach or nectarine orchards in each district were located according to two different sources: the national Census carried out in 1999 by the Lebanese Ministry of Agriculture, and the "Homogeneous zone" data, a land use study published in 2000 by the Lebanese Ministry of Agriculture, which classifies the agricultural zones according to the land use and the main cultivations in the regions.

From November 2009 to January 2010 municipalities, farmer cooperatives, the local offices of the Ministry of Agriculture, and Agricultural schools present in each Caza were informed about the survey plan and provided the information necessary to update the 11 years old Census data and to locate the almond, peach and nectarine orchards present in the areas. Taking into account the information collected in the visited villages, the number of orchards to be monitored in each district was decided according to the extent of the almond, peach and nectarine cultivation in the areas.

From February 2010 onwards, the survey was carried out in about 890 orchards, as reported in table 3. During the survey, each orchard was located by GPS, in order to record its position and to draw a regional map on the spread of the disease in the area using the GIS (Geographic Information System) software. During the visits all the threes present in each orchard were observed and monitored for Almond witches'-broom symptoms. In order to confirm the infection of symptomatic plants or to verify the presence of the pathogen in trees showing doubtful symptoms, an average of 15 leaf or flower samples were collected in each district (table 3) and analysed according to the method described in the next chapter. Symptomatic trees were preferentially sampled, in order to confirm the pathogen presence in the area.

368 samples were processed, as described later, for '*Candidatus* Phytoplasma phoenicium' identification, through the 16SrDNA gene amplification using the specific primer pair AlWF2/R2 (Abou-Jawdah *et al.*, 2003).

Table 3. Visited orchards and samples collected during the national survey in the Lebanese Caza.

	Caza (District)	Number of visited villages	Number of visited orchards	Number of collected Almond samples	Number of collected Peach / nectarine samples
Bekaa Valley	**Baalbeck**	**56**	72	24	4
	Bekaa West	**36**	77	29	15
	Hermel	**12**	14	9	0
	Rachaya	**25**	61	21	10
	Zahle	**36**	99	11	4
North	**Akkar**	**29**	30	15	1
	Baabda	**16**	8	0	5
	Batroun	**34**	41	24	0
	Becharre	**4**	4	0	0
	Donniye	**21**	35	8	8
	Jbeil	**17**	69	8	6
	Keseruan	**11**	14	1	8
	Koura	**17**	24	19	0
	Metn	**8**	5	0	6
	Zgharta	**10**	9	6	0
South	**Aley**	**36**	53	3	10
	Bent Jbail	**10**	28	10	0
	Chouf	**43**	95	18	5
	Hasbaya	**8**	29	14	8
	Jezzine	**17**	23	5	9
	Marjayoun	**12**	33	4	18
	Nabatieh	**5**	9	2	1
	Saida	**17**	34	8	4
	Sour	**15**	28	6	1
Total	**24**	**495**	**894**	**245**	**123**
Total				**368**	

On the basis of the results obtained from the molecular analysis carried out on the collected samples, a national map of the disease spread was prepared using the GIS software: the villages presenting the disease were indicated in red, while the villages not interested by the Almond witches'-broom were reported in green.

4.3.1 Percentage of infection of the orchards and index of infection within the orchards

For each district the percentage of infected orchards on the total monitored orchards was calculated and the classes of AlmWB presence frequency in the orchards were defined as follow:

0: absence of infected orchards
1: 1-10% of infected orchards
2: 10 – 25% of infected orchards
3: 25 -50% of infected orchards
4: 50 – 75% of infected orchards
5: 75 – 100% of infected orchards

The AlmWB presence frequency obtained for each Lebanese district was represented in a map.

In order to quantify the disease severity at a regional level, the percentage of infected trees within the orchards was assessed in 102 orchards located in the districts of Rachaya, Batroun and Marjayoun, chosen as representative Caza for the AlmWB spread in Lebanon. In fact, the three districts belong respectively to the three main Lebanese regions: North (Batroun), South (Marjayoun) and Bekaa Valley (Rachaya). Moreover, almonds were affected in the Caza of Batroun since at least ten years, so the region can be chosen as representative for the long period of AlmWB spread in the area; in Marjayoun, on the contrary, the disease has not been found in almond trees, but it appeared in the last four years in nectarine trees. The region of Rachaya is also characteristic because of the simultaneous presence of infected almond and nectarine trees. Twenty-five orchards in Batroun, thirty-two in Marjayoun and forty-five in Rachaya, out of the respectively visited 41, 33 and 61 orchards were selected, on the basis of the data availability and of the possibility to count all the trees in the orchards, to calculate the disease severity in the orchards.

The infection frequency, *i.e.* the percentage of infected trees was calculated and classified using the following six infection classes:

0: absence of infected trees in the orchard
1: 1-10% of infected trees
2: 11 – 25% of infected trees
3: 26 - 50% of infected trees
4: 51 – 75% of infected trees
5: 76 – 100% of infected trees.

The percentage index of infection (I%I) was calculated for each Caza according to the Townsend and Heuberger (1943) formula:

$$I\%I = [\Sigma(f \times v)/N \times (f-1)] \times 100$$

where:

I%I: percentage index of infection

f : class number

v = number of plants in a class

N = number of examined plants

4.4 Characterization of the pathogen

4.4.1 Sample collection

During the monitoring activities of the three key-orchards, 12 samples were collected from 9 trees showing AlmWB symptoms, as well as 6 samples from 3 asymptomatic trees (Table 4). Depending on the development of the trees and on the observed symptoms, leaf and/or flower samples were collected and placed in a plastic bag at 4°C, labelled and processed within 24 hours, in order to confirm the presence of '*Ca.* Phytoplasma phoenicium' in the observed trees. When both leaves and flowers were collected from a same tree, the two different organs were stored and processed separately.

Table 4. Samples collected in the three key-orchards (Feghal, Rachaya el Fouchar and Sarada).

Caza (District)	Village	Species	Variety	Age of the trees	Collected organ	Symptomatic/ asymptomatic tree	Number of samples
Jbeil	Feghal	Almond	Telyani	30	Leaves	Symptomatic	3
Hasbaya	Rachaya el Fouchar	Peach	Babcock	8	Leaves and flowers	Symptomatic	1
Marjayoun	Sarada	Nectarine	Fantasia	10	Leaves and flowers	Symptomatic	6
					Leaves and flowers	Asymptomatic	6
					Leaves	Symptomatic	2
Total samples							18

Additional samples were collected from 15 orchards, located near the observed key-orchards or in the bordering regions, such as West Bekaa, reported by local farmers to present trees apparently affected by AlmWB (Table 5). The orchards were carefully

monitored and samples were collected exclusively from those characterized by the presence of symptomatic trees, or of doubtful symptoms, in order to verify if the pathogen can induce alterations different from those observed in the key-orchards. The only exception is represented by the samplings carried out in the almond orchard located in Sarada, at the border of the monitored nectarine orchard, where all the trees were asymptomatic.

Five almond orchards, located in the village of Feghal, were visited and 11 samples were collected from 9 symptomatic and 1 doubtful trees; in one orchard monitored in Hasbaya 3 samples were collected, 2 from symptomatic and 1 from asymptomatic almond trees.

In the region of Kherbet Kanafar, 3 infected orchards and an apparently healthy one were sampled, while in the area of Marjayoun-Sarada the collection of symptomatic and asymptomatic organs was carried out in 4 nectarine and 1 almond orchards.

A total of 49 samples were collected: 24 samples from asymptomatic trees, 5 from doubtful trees, while 38 from trees showing typical AlmWB symptoms.

Table 5. Samples collected in the regions of Feghal, Hasbaya, Marjayoun-Sarada and Kerbet Kanafar.

Caza (District)	Village	Orchard	Species	Organ collected	Symptomatic or asymptomatic	Number of samples
Jbeil	Feghal	1	Almond	Leaves	Symptomatic	**2**
		2	Almond	Leaves	Symptomatic - doubt	**1**
			Almond	Leaves	Symptomatic	**1**
		3	Almond	Leaves	Symptomatic	**1**
			Peach	Leaves	Symptomatic	**2**
			Peach	Leaves and flowers	Symptomatic	**2**
		4	Almond	Leaves	Symptomatic	**1**
		5	Almond	Leaves	Symptomatic	**1**
Hasbaya	Rachaya el Fouchar	1	Almond	Leaves	Asymptomatic	**1**
			Almond	Leaves	Symptomatic	**2**
West Bekaa	Kherbet Kanafar	1	Nectarine	Leaves and flowers	Symptomatic	**4**
			Nectarine	Leaves	Symptomatic - doubt	**1**
			Nectarine	Leaves	Asymptomatic	**1**
		2	Nectarine	Leaves	Symptomatic	**2**
			Nectarine	Leaves	Asymptomatic	**1**
		3	Peach	Leaves	Asymptomatic	**2**
			Peach	Leaves	Symptomatic - doubt	**1**
		4	Peach	Leaves	Asymptomatic	**1**
			Peach	Leaves	Symptomatic	**1**
Marjayoun	Khiam	1	Nectarine	Leaves	Symptomatic	**2**
	Qlayaa	2	Nectarine	Leaves	Symptomatic	**1**
			Nectarine	Leaves	Symptomatic - doubt	**2**
	Burj el Moulouk	3	Nectarine	Leaves	Asymptomatic	**1**
			Nectarine	Leaves	Symptomatic	**3**
	Sarada	4	Peach	Leaves	Symptomatic	**1**
		5	Almond	Leaves and flowers	Asymptomatic	**8**
			Almond	Leaves	Asymptomatic	**3**
Total samples						**49**

4.4.2 DNA extraction

Leaf samples collected from almond, peach and nectarine trees were prepared for DNA extraction by cutting the veins from the leaf lamina with a sterile scalpel. Some petals and some parts of the calyx were excised from the flower samples collected from almond, peach and nectarine trees. About 100 mg of material per sample was utilised for the DNA extraction. An average of four midribs and four flowers for each sample were prepared and stored into the freezer at -20°C until DNA extraction.

DNA was extracted from mid veins of the leaf samples or from flowers using a modified Doyle and Doyle (1990) protocol.

About 100 mg of midrib or other tissue types were immersed in liquid nitrogen and ground using sterile pestles carried on the electrical drill.

CTAB (Hexadecyl trimethyl-ammonium Bromide) buffer (800 µl) and mercaptoethanol (20 µl for 1 ml CTAB) at 60°C were added to the crushed tissues, thoroughly mixed by vortex. The entire mixture was held at 60°C for 20 minutes. During the incubation the mixture was briefly vortexed several times.

After incubation, 60 µl of Iso-amylalcohol:chloroform (1:24) was added, vortexed vigorously and centrifuged at 10.000 r.p.m. for 5 minutes.

The supernatant (upper phase) was transferred to a clean microfuge tube, added of an equal volume of ice cold Iso-propanol and placed at -20°C for 20 minutes. The mixture was centrifuged at 14.000 r.p.m. for 8 minutes and the aqueous phase was discarded.

The nucleic acid pellet was washed with 75% ethanol, air-dried, suspended in 50 µl of deionized autoclaved water and maintained at –20°C until use.

4.4.3 Ribosomal RNAs gene amplification for phytoplasma identification

DNA samples obtained from veins and flowers were subjected to direct or nested polymerase chain reaction (PCR). The amplification of the ribosomal RNA gene fragments, including genes 16S rRNA, 23S rRNA and 16S-23S intergenic region, was useful to

a) detect the presence of '*Candidatus* Phytoplasma phoenicium'
b) obtain a fragment for subsequent analysis of genome characterization.

In direct PCR amplification, specific primer pair AlWF2/AlWR2 (Abu-Jawdah *et al.*, 2003) which primes a fragment of approximately 390 bp was used.

In some case, nested PCR was performed, for two reasons: in order to characterise the isolated phytoplasmas through sequencing and RFLP analysis, or in order to confirm a doubtful result.

In the first run universal primer pair P1/P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996), which primes a fragment of approximately 1800 bp was used. The obtained fragment extends from the 5'´end of the 16S rDNA to the 5´ region of the 23S rDNA.

The second run was performed with primer pair R16F2n/R16R2 (Gundersen *et al.*, 1996), which amplifies a 1200 bp fragment of the conserved region of the 16S rDNA, common to all the known phytoplasmas.

An aliquot of 2 µL of the diluted (1:40) PCR products from the first amplification was used as a template for the nested PCR.

All amplifications were performed with a thermocycler, Icycler (Bio-Rad, CA, USA) in 20µL reactions containing 200 mM each of the four dNTPs, 0.5 µM of each primer, 2 mM $MgCl_2$, 1x polymerase buffer, 1 unit Taq (ABgene) and 1-2 µL sample DNA.

Specific AlWF2/AlWR2 PCR reaction consisted of one cycle at 95° C for 2 minutes 30 seconds, 35 cycles at 94°C for 30 seconds, 44° C for 30 seconds and 72° C for 30 seconds, and a final extension step at 72° C for 7 minutes. In some case, a higher annealing temperature (50°C) was requested, in order to avoid the presence of non specific bands on the reaction.

PCR reactions carried out using the universal primers P1/P7 consisted of one cycle at 95°C for 2 minutes 30 seconds, 35 cycles at 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 2 minutes, and a final extension step at 72°C for 7 minutes.

The nested PCR reaction consisted of 1 cycle at 95°C for 2 minutes 30 seconds, 35 cycles at 95°C for 30 seconds, 44°C for 30 seconds and 72°C for 40 seconds, with a final extension step at 72°C for 7 minutes.

All the amplified products were analyzed by electrophoresis in 1.5 or 2% agarose gel, followed by staining with ethidium bromide and observed on UV transilluminator.

4.4.4 Phytoplasma characterization

The pathogen variability and the presence of polymorphisms on phytoplasma sequences was studied on 24 '*Ca.* Phytoplasma phoenicium' strains isolated from the samples previously reported in the tables 4 and 5.

The 24 amplicons obtained from F2n/R2 nested PCRs were sequenced to achieve at least 4X coverage per base position. DNA sequencing was performed in an ABI PRISM 377 automated DNA sequencer (Applied Biosystems). The nucleotide sequence data were assembled by employing the Contig Assembling program of the sequence analysis software BIOEDIT, version 7.0.0 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html).

Sequences were compared with the GenBank database by using the software BlastN (http://www.ncbi.nim.nih.gov/BLAST/) with the aim of searching possible identity. Nucleotide sequences of '*Ca.* P. phoenicium' strains identified in the present study were

deposited in the National Center of Biotechnology Information (NCBI) GenBank database at accession numbers HQ407512 to HQ407535.

The strain details are reported in the table below:

Table 6. The 24 '*Ca.* Phytoplasma phoenicium' strains chosen for Phytoplasma characterization analysis.

Caza	Region	Number of Orchards	Host	Sample	Number of strains
Jbeil	Feghal	6	Almond	Leaf	8
			Peach	Leaf	2
			Peach	Flower	1
Bekaa West	Kerbet Kanafar	2	Nectarine	Leaf	3
Marjayoun	Sarada	4	Nectarine	Flower	3
			Nectarine	Leaf	3
	Marjayoun	3	Nectarine	Leaf	4
Total strains					24

4.4.5 Virtual RFLP analysis and calculation of similarity coefficients

A total of 37 16S rRNA gene sequences of 16SrIX phytoplasma group (13 from GenBank and the 24 obtained during the orchard monitoring), plus sequences from phytoplasma strains representative of known 16Sr subgroups, were trimmed to an approximately 1.25-Kb fragment (delimited by R16F2n and R16R2 primer annealing positions), as previously described (Wei *et al.*, 2007), and exported to the program pDRAW32 (AcaClone Software, http://www.acaclone.com).

The GenBank sequences chosen were: AF248957 (PPWB), AF515637 (CPPstrain21), Y16389 (PEY), AF515636 (CPPstrainA4), GQ925918 (JunWB), AF455040 (AlmWB-P1), AF390137 (AlmWB2), AF390136 (AlmWB1), AF455038 (AlmWB3), AF455039 (AlmWB4), AF455041 (AlmWB-N1), FJ160959 (Iranian AlmWB Phytoplasma), DQ195209 (Khafr AlmWB Phytoplasma).

Each DNA sequence was analyzed through an automated *in silico* restriction assay, and digestion results were plotted on virtual gels as described by Wei and colleagues (Wei *et al.*, 2007).

In detail, each DNA fragment was digested *in silico* with 17 restriction enzymes used previously in actual enzymatic digestions by Lee and coworkers (Lee *et al.*, 1998): *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. After *in silico* restriction digestion, a virtual 3.0% agarose gel electrophoresis image was plotted and captured as a device-independent PDF file.

The virtual RFLP (Restriction Fragment Length Polymorphysm) patterns were compared and a similarity coefficient (*F*) was calculated for each pair of phytoplasma strains according to the formula described previously (Nei and Li, 1979; Lee *et al*., 1998), *F = 2Nxy /(Nx+Ny)*, in which x and y are two given strains under study; *Nx* and *Ny* are the total number of bands resulting from digestions by 17 enzymes in strains *x* and *y*, respectively; and *Nxy* is the number of bands shared by the two strains.

4.4.6 Real RFLP analysis

The DNA amplicons obtained from the 24 samples previously selected and from other 14 samples collected in the Bekaa Valley during the national survey were processed through RFLP analysis, in order to obtain electrophoretic profiles to identify the different strains of phytoplasma infecting the samples.

Amplicons were digested, *in vitro*, using the two restriction enzymes that allow the differentiation among the subgroups of 16S groupIX, such as *TaqI* for subgroup 16SrIX-G and *BstUI* for subgroup 16SrIX-F.

The restriction assays were carried out according to the following protocol: the digestion mixture was prepared for a final volume of 20 µl, as follow:

- 2 µl of buffer 10X, specific for the enzymes
- 0,6 µl of enzyme 10U/µl
- 0,2 µl of BSA 100X, only for TaqI
- 3 µl of DNA
- Sterile water, to reach the final volume of the reaction.

The reaction was incubated at 60°C for 2 hours for the enzyme *BstU*I and at 65°C for 2 hours for the enzyme *Taq*I.

The electrophoresis on 3% agarose gel was carried out for fragment separation; the gel was coloured with ethidium bromide 1µg/ml to visualize the obtained profiles on UV transilluminator. To estimate the fragment length, two different markers were used: Gene ruler for 50 bp and for 100 bp.

4.4.7 Phylogenetic analysis

Phytoplasma 16S rDNA gene sequences from this study and from GenBank were used to construct phylogenetic trees. Minimum evolution analysis was carried out using the Neighbor-Joining method and bootstrap replicated 1000 times with the software MEGA4 (http://www.megasoftware.net/index.html) (Tamura *et al.*, 2007). *Acholeplasma palmae* was used as the out-group.

4.5. Vector investigation

Phytoplasmas are transmitted in nature mainly by phloem feeder insects belonging to the order Hemiptera, mostly leafhoppers, planthoppers and psyllids. In order to screen and capture the possible vector(s) of Almond witches'-broom disease, insect traps were installed in two infected orchards over two years.

The investigation was carried out in collaboration with the University of Turin, Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali (DIVAPRA), department of "Entomologia e Zoologia applicate all'ambiente – Carlo Vidano" and with the Lebanese University, Faculty of Science.

During the first year of insect monitoring and collection, traps were installed in the key-orchards of Feghal (almond) and Sarada (nectarine). The trap located in the nectarine orchard in Sarada was installed during the second year in a nectarine orchard in the region of Kfarkela, near Sarada.

Two kinds of traps were installed in the orchards (Fig. 7, 8a and 8b):

a) 6 Double-sided, yellow sticky traps (10 cm x 30 cm)

b) 1 Malaise trap (165 cm x 115 cm x 190 cm)

Yellow sticky traps were centrally placed in the orchards, arranged in two groups of three traps, installed at 150 cm and at 30 cm from the soil level, on different contiguous trees, and replaced every 2 weeks. The two different heights were chosen in order to be able to collect flying insects as well as insects that live near to the soil level (e.g. Cixiidae which at larval instar live underground feeding on roots of their host plant).

The Malaise traps, a large tent-like structure used for trapping flying insects, even carried by the wind, is made of terylene netting, black and white coloured. Insects fly into the black tent wall and, trying to escape searching the light, are funnelled in the white part of the tent, into a collecting vessel attached to highest point of the net, full of 70% ethanol. The Malaise traps were centrally placed in the orchards, between two rows of trees. The 70% ethanol contained in the vessel was changed every 2 weeks.

In the orchards, no insecticide treatments were executed during the entire sampling period.

In 2010, the insect sampling were carried out from the beginning of February till the end of December; in 2011, from April 15 to September 6.

In addition, direct collecting was carried out by entomological sweep nets on the upper part of the plant and on the grass, with the aim to find insects that may be excluded by the static sampling methods and also in order to find spontaneous weeds, possible alternative host plants for the insects. Field collecting was carried out in March 2010 visiting not only the key-orchards of Feghal and Sarada, but also different areas, in the surrounding of the orchards: the coastal region of Barbara, near Feghal, 10 m on the sea

level, the cave of Feghal, at 150 meters altitude, as well as the village of Ouyoun el Ghazlen, in the northern region of Akkar, severely affected by the disease. In the South, collecting was focused on the border of the orchard of Sarada and in one nectarine small orchard in Wadi Khansa, some kilometres far from Sarada. In May 2010, 3 other visits to the key-orchard of Feghal were executed.

In June and September 2011 field collections were focused on the Cixiidae presence in and near the key-orchards of Feghal and Kfarkela (Caza of Marjayoun), and in three other localities, at higher altitudes, in order to find the possible habitat of the species. In fact the movement of vectors from forest habitat to crop is important in the incidence and spread of phytoplasma diseases. Supposing that the most wild and rich in biodiversity regions could be interesting habitats for Cixiidae, visits were carried out in the area of Tannourine-Kartaba-Laqlouk (1200-1500 m), Becharre (1400-1600 m) and the surroundings of the natural reserve of Jabal Moussa (1600 m).

4.5.1 Insect identification

Every 15 days the yellow sticky traps were examined in the laboratory of the Lebanese University of Beirut under a stereo-microscope and a preliminary sorting was carried out on the insects captured into the plastic bottles of Malaise traps, because of the non-specificity of the traps. Leafhoppers and psyllids were identified in Beirut, at the Lebanese University laboratories, whereas all the cixiids found on the alcohol of Malaise traps were preserved in alcohol (ethanol) into vials and then sent to the DIVAPRA department of the Faculty of Agriculture of Turin, to be identified.

The samples on the sticky traps were removed using a drop of toluene, in order to identify the species they belong to, whereas the samples in alcohol were dried, then the males were dissected and their genitalia examined.

Since preliminary studies have already been carried out on the Cicadellidae species as putative vectors of the phytoplasma (Dakhil *et al.*, 2011), whereas no information is available up to now on Psyllidae and Cixiidae specimens, molecular analysis for phytoplasma identification were performed on these two groups.

Fig. 7. Malaise trap installed in the key orchard of Kfarkela.

Fig. 8a and 8b. Yellow sticky traps installed at 30 and 150 cm in the key-orchard of Feghal.

4.5.2 DNA extraction from insects

Insects collected on Malaise trap vessels or with sweep nets were processed through molecular analysis. Insects captured on yellow sticky traps were too dry to perform DNA extraction and were excluded from the molecular analysis. Insect samples were prepared for DNA extraction putting 1-5 insect per tube, depending on insect size.

The DNA was extracted using a modified procedure of Marzachì and co-workers (1998). One to five insects were crushed with pestles in a 1.5 ml Eppendorf tube, in the presence of 500 µl of extraction buffer (2% cethyltrimethyl ammonium bromide (CTAB), 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 0, 2% ß -mercaptoethanol) heated at 60°C, mixed and then incubated at 60°C for 30 min. Tubes were centrifuged at room temperature (13.000 r.p.m., 8 min).

Supernatant was collected and mixed with an equal volume of chloroform isoamylalcohol (24:1) and centrifuged again (13.000 r.p.m., 8 min).

Cold isopropanol was added to the upper phase (1:1) and then precipitated by centrifugation (13.000 r.p.m., 20 min) at 4°C.

The pellet was washed twice with cold 70% ethanol, mixed and centrifuged in refrigerate centrifuge (4°C) at 13.000 rpm for 5 minutes, then dried in a speed-vac and finally resuspended in 50 µl TE (10 mm Tris, pH 8.0, 1 mm EDTA) or sterile water and incubated at 60°C for 1 hour.

Samples were centrifuged at room temperature (spin) and then frozen until use.

4.5.3 Phytoplasma identification in DNA extracted from the insects

The identification of phytoplasmas extracted from insects was carried out as already described, through direct and nested PCR, using respectively the specific primer pair AlWF2/AlWR2 (Abu-Jawdah *et al*., 2003) and the primer pairs P1/P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996) followed by the primers R16F2n/R16R2 (Gundersen *et al.*, 1996).

A total of 53 specimen belonging to the family of Psyllidae and 64 specimens belonging to the family Cixiidae were processed as previously described.

Nectarine elimination in the key-orchard of Sarada (South Lebanon).

5. RESULTS

5.1 Symptom observation

5.1.1 Description of the symptoms on almond

On almonds, as described by Abou-Jawdah and colleagues (2002), the most characteristic symptom found is the proliferation of shoots at several points on the main trunk with an appearance of witches'-broom. The name "almond witches'-broom" (AlmWB) for the disease was chosen because of the main symptom observed on the infected plants.

One of the early symptom observed in the key-orchard in Feghal (February) is an anticipated flowering, 20 to 30 days before the appearance of the first open flowers on the healthy plants. Moreover, the flower peduncle in the infected trees was usually longer than the peduncle of healthy flowers (Fig. 9).

During the spring time (March, April) witches'-brooms and proliferation appeared on the main trunk (Fig. 10) and on branches (Fig. 11).

In May leaves started to yellowing and dry (Figs.12 and 13). A continuous development of witches'-broom from the trunk and the main branches was observed during the entire year (from spring to winter).

Trees declined rapidly and died within 3 to 4 years after the appearance of the first symptoms (Figs. 14 and 15). Generally in the first year of symptom appearance, only some branches showed the typical alteration, whereas the other branches maintained the normal development and presented dark green leaves. During the second year, symptoms appeared in the entire canopy; the lower branches showed extensive proliferation with smaller leaves, light green in colour, and shoots, developed perpendicularly to the main branches, become stunted with short internodes (rosetting) (Fig.16).

All cultivated varieties (Helwani, Talyani, Khechaby) seemed equally susceptible to the disease. Many trees showed stunted growth with short internodes and small leaves; others showed proliferation of several lateral individual slender branches, mostly with an upright growth but without witches'-broom. In wild almond (*Prunus orientalis* Miller) which can also be severely infected, a delay in the appearance of symptoms is often observed.

During particularly warm summers, as the summer 2010, in the district of Jbeil and Koura, early senescence and reddening of the entire canopy was observed, conferring a "burning" aspect to the trees (Fig. 17).

The fruit yield on infected almond branches was greatly reduced. During the first symptomatic year, trees produced just a few small and dark fruits, with shrivelled or sour almonds. After the second year, no fruits were observed on the infected plants.

In general, a severe susceptibility of infected trees to powdery mildew was recorded during the entire observation period.

In the regions where the disease is spread since many years, the landscape shows the heavy impact of the disease (Figs. 18 and 19).

In the table 7 the main symptoms observed during the disease monitoring were reported.

Table 7. Symptom observation on almond trees.

Principal growth stage *	Description of the growth stage *	Symptom observation
0: Sprouting/Bud development	00: Dormancy	Early flowering; elongated peduncles; development of proliferated shoots
6: Flowering	69: End of flowering: all petals fallen	Extensive proliferation, with smaller and light green leaves
7: Development of fruit	71: Ovary growing	Perpendicularly developed shoots, stunted, with short internodes (rosetting)
7: Development of fruit	73: Second fruit fall	Dry shoots, development of witches'-broom from the trunk and the main branches
7: Development of fruit	76: Fruit about 60% of final size	Dry shoots, development of witches'-broom from the trunk and the main branches
7: Development of fruit	77: Fruit about 70% of final size	Dry shoots, development of witches'-broom from the trunk and the main branches
9: Senescence, beginning of dormancy	93: Beginning of leaf fall	Early senescence and reddening of the entire canopy

Notes: *: Principal growth stages according to the BBCH stone fruit scale, Meier *et al*., 1994.

5.1.2 Description of the symptoms on peach

On diseased peaches, the first symptom observed is the presence of witches'-brooms on the branches not pruned (Fig. 20) and the proliferation of shoots from the collar of the trunk (Fig. 21). During the season, an early development of buds and a precocious flowering, about one month before the occurrence of the same phenological stages on healthy plants, were observed (Figs. 22 and 23). At the beginning of the disease appearance, only some branches of the infected trees showed these symptoms, whereas the other branches had a normal development. In April, during the normal development of shoots, after flowering, the development of proliferated shoots, with thin leaves and very short internodes was observed (Figs. 24 and 25).

In May, more than two months after flowering, phyllody and flower deformation appeared on the diseased trees. Pink and purple flowers (Fig. 26) were observed on some branches, which showed well developed leaves; sepals reached a length of 3-5 cm, were coloured and serrate; petals were thick, irregular, with bright colours. Sometimes pistil and stamens were absent or modified, and leaves and small shoots could be found frequently inside the flower (Figs. 27 and 28).

The proliferated shoots developed from the collar of the infected trees were easily infected by the powdery mildew agent (Fig. 29).

The few fruits produced by the symptomatic plants were abnormal, generally elongated and curved. Moreover these fruits were small, sour and could not be sold (Fig. 30).

The infected trees lacking phyllody were similar to healthy peaches, except from the colour of leaves, lighter than the healthy ones, and their lamina margin, that was serrate (Fig. 31).

In October, an early senescence of trees was observed: the reddening of the leaves, as well as their early fall was recorded (Figs. 32, 33 and 34).

In the table 8 the main symptoms observed during the disease monitoring were reported.

Table 8. Symptom observation on peach trees.

Principal growth stage *	Description of the growth stage *	Symptom observation
0: Sprouting/Bud development	00: Dormancy	Early development of buds, early flowering
7: Development of fruit	71 Ovary growing; fruit fall after flowering	Proliferated shoots, with thin leaves and very short internodes
7: Development of fruit	75: Fruit about half final size	Phyllody, flower deformation, proliferation from collar, abnormal fruits
9: Senescence, beginning of dormancy	91: Shoot growth completed; foliage still fully green	Early senescence

Notes: *: Principal growth stages according to the BBCH stone fruit scale, Meier *et al*., 1994.

5.1.3 Description of the symptoms on nectarine

The observation on nectarine trees was performed on an early variety, Flankis. The first symptom observed in February was an early flowering, occurring 15 to 20 days earlier than normal, followed by the earlier development of all the dormant buds of the branches (Figs. 35, 36 and 37). In recently infected plants only few branches showed the symptoms, whereas other branches were dormant and developed normally. During the following years, disease symptoms were observed on all the branches (Figs.38, 39 and 40).

In March, lateral buds developed simultaneously young twigs with smaller leaves (Fig. 41); tip leaves were reddish (Figs. 42 and 43). In April-May some phyllodies were visible on infected trees (Fig. 44): big flowers, deep coloured appeared on the branches (Fig. 45, showing very long (2 to 5 cm) red or purple and serrate sepals (Fig. 46) and pink, thick and irregular petals. Phyllodic flowers did not completely develop all the reproductive organs and formed vegetative structures in place of stamens and pistils

(Fig.47). Sometimes, twig and leaves developed inside the flower (Figs. 48, 49 and 50); twigs were green, thick, carrying some serrate little leaves.

Leaves on affected branches were serrate, slim and light green in colour (Fig. 51).

During the first two years after the symptom appearance, altered branches showed abnormal elongated fruits (Fig. 52, 53, 54 and 55); starting from the third year, no fruits were produced anymore.

Proliferating shoots showing witches' brooms usually developed from the collar of the trees. From May to August-September the infected plants were less easily identified: the only symptoms, sometimes very mild, were some yellowish, slim and succulent shoots and suckers, developed perpendicularly on main branches (Fig.56).

Typical symptoms appeared during fall in September: in fact diseased trees were characterized by early leaf senescence, reddish leaves and lignified witches' broom on the top of the branches (Figs.58 and 59). Moreover, during summer and fall, symptomatic trees were more severely affected by powdery mildew than others (Fig. 57). The disease is not leading infected peaches to the dieback as fast as on almond infected trees.

During winter, the lignified witches'-broom could be easily detected at the top of the branches (Fig. 60).

In the table 9 the main symptoms observed during the disease monitoring were reported.

Table 9. Symptom observation on nectarine trees.

Principal growth stage *	Description of the growth stage *	Symptom observation
6: Flowering	64: About 40% of flowers open	Early flowering, earlier development of all the dormant buds
6: Flowering	67: Flowers fading: majority of petals fallen	lateral buds develop simultaneously young twigs with smaller leaves
7: Development of fruit	72: Green ovary surrounded by dying sepal crown, sepals beginning to fall	Red tip leaves, serrate leaves, perpendicular development of twigs.
7: Development of fruit	75: Fruit about half final size	serrate leaves
7: Development of fruit	76: Fruit about 60% of final size	Flower phyllody, flower deformation, abnormal elongated fruits.
7: Development of fruit	77: Fruit about 70% of final size	Flower phyllody, serrate leaves, proliferation from the collar
8: Maturity of fruit and seed	87: Fruit ripe for picking	Witches'-broom of the suckers inside the canopy
9: Senescence, beginning of dormancy	91: Shoot growth completed; foliage still fully green	Early senescence, leaf yellowing or reddening before the healthy plants
0: Sprouting/Bud development	00: Dormancy	Lignified witches'-broom at the top of the branches

Notes: *: Principal growth stages according to the BBCH stone fruit scale, Meier *et al.*, 1994.

Symptom observation on almond trees — Key orchard of Feghal

Fig.9 Elongated flower peduncule

Fig.10 Witches'-broom

Fig.11 Proliferation from the trunk

Fig.12 Perpendicular drying shoots

Symptom observation on almond trees — Key orchard of Feghal

Fig.13 Witches'-broom development

Fig.14 Tree decline

Fig.15 Tree decline

Fig.16 Shoot rosetting

Symptom observation on almond trees — Key orchard of Feghal

Fig.17 Reddening of the canopy

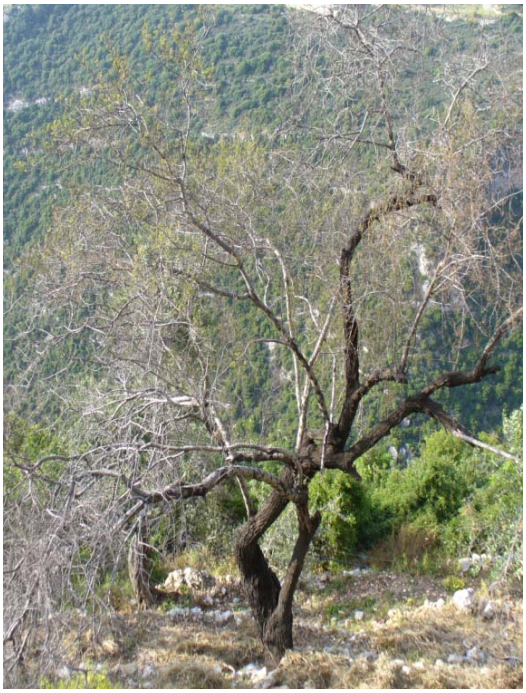


Fig.18 dead tree

Fig.19 Effect of the disease on the landscape

Symptom observation on peach trees — Key orchard of Rachaya el Fouhar

Fig. 20. Winter witches'-broom

Fig 21. Proliferation from the collar

Fig. 22 and 23. Early flowering and bud development

Fig. 24 and 25. Proliferated shoots

Symptom observation on peach trees — Key orchard of Rachaya el Fouhar

Fig. 26. Flower phyllody

Fig. 27. Phyllody, with development of twigs

Fig. 28. Phyllodiy; sepals transformed in leaves

Fig. 29. Proliferation from the collar, affected by powdery mildew agent

Fig 30. abnormal fruit development

Fig. 31. Light green leaves (right)

Symptom observation on peach trees — Key orchard of Rachaya el Fouhar

Fig. 32 and 33. Early senescence and reddening of the proliferated shoots

Fig. 34. Early senescence of the canopy (left: healthy tree, right: diseased tree)

Symptom observation on nectarine trees — Key orchard of Sarada (Marjayoun)



Fig. 35, 36 and 37. early flowering and early bud development

Fig 38. Eearly bud development (red arrow) (right: healthy plant)


Fig 39 and 40. Early bud development left:infected; right: healthy pant

Symptom observation on nectarine trees — Key orchard of Sarada (Marjayoun)

Fig. 41. lateral shoot development.

Fig. 42 and 43. Perpendicular shoot development, with red tips. Left: infected tree; right: healthy tree.

Fig. 44. Infected tree with phillodies.

Fig. 45. Floral phillody.

Symptom observation on nectarine trees — Key orchard of Sarada (Marjayoun)

Fig. 46. Floral phillody with purple elongated sepals.

Fig. 47. Floral phillody which didn't develop the reproductive organs.

Fig. 48 and 49. Floral phillodies which develop twigs inside the flowers.

Fig. 50. Floral phillodies which develop twigs inside the flowers.

Fig. 51. Infected tree, showing slim and light green leaves.

Symptom observation on nectarine trees — Key orchard of Sarada (Marjayoun)

Fig. 52. Abnormal fruit development . Left: infected tree; right: healthy tree. (May)

Fig. 53. Abnormal shoot development.

Fig. 54 and 55. Abnormal fruit development. Left: infected tree; right: healthy tree.

Fig. 56 Slim and succulent perpendicular shoots.

Fig. 57 witches'-broom affected by powdery mildew (left: healthy; right: infected tree)

Symptom observation on nectarine trees — Key orchard of Sarada (Marjayoun)

Fig. 58. Early leaf senescence.

Fig. 59. Early senescence of the infected tree, showing witches'-broom (right); left: healthy tree.

Fig. 60. Witches'-broom on the wintering branches.

5.2 Distribution of the disease in the key-orchards

Symptomatic and asymptomatic plants, as well as the sampled trees collected in order to confirm the phytoplasma presence in the symptomatic trees were localized in the map of the monitored key-orchards.

The disease spread was followed for two consecutive years, during which the maps were updated, the new infected as well as the eradicated trees were recorded and the incidence of the disease in the orchard, as the percentage of the total plants showing AlmWB symptoms was calculated.

In the almond key-orchard of Feghal (Fig. 61), in 2009 the incidence of the disease was 79.17% that, added to the 15.28% of dead trees, reaches the 94.44% of trees affected by the disease. Only 5.56% of the trees did not show any symptoms of the disease.

In 2010, only 61 trees out of 72 were still present in the orchard (Fig. 62). The incidence of the disease, calculated on the left trees, increased: 90.16% of the trees showed AlmWB symptoms; 6.56% of new died trees were recorded, and therefore the total incidence of the disease was 96.72%. Two of the four non symptomatic trees showed the symptoms, leaving only 3.28% of the orchard without symptoms.

As reported on the maps (Figs. 61 and 62), the infected trees were distributed in the entire orchard. The four healthy trees were randomly located within the central rows. After one year, only two plants were still healthy, in the upper part of the orchard. The dead trees were close to the already dead trees.

In the peach key-orchard of Rachaya el Fouchar, the incidence of the disease during the first monitoring (January 2009) was 21.88% and no dead plants were observed (Fig. 63). All the 21 symptomatic trees were eliminated together with 2 asymptomatic trees, located near the symptomatic ones. On 2010 symptoms of the disease were newly observed on 8.22% of the orchard plants, with the left 91.78% of the left trees asymptomatic (Fig. 64). The distribution of the infected trees is random within the orchard; the trees newly affected in 2010 were located near the eliminated ones.

Moreover, nearby the orchard some almond trees were observed. On 2009 all the almond trees were asymptomatic, whereas on 2010 two of them showed severe symptoms (witches'-broom and proliferation). It is a rare case of copresence of almond and peach trees infected in the same place.

In the nectarine key-orchard of Sarada, 30 and 80 symptomatic plants were observed respectively in 2007 and 2008 and eliminated. Only 8 symptomatic trees were observed and eliminated in 2009 (Fig. 65), while no new symptomatic trees were recorded in 2010. The distribution of the symptomatic plants was random. The trees weren't close but, generally, located near the orchard borders.

In the table 10 the percentages of the incidence of the disease in the three key-orchards are resumed.

Table 10. Observation of symptomatic, dead and asymptomatic trees in the three key-orchards on 2009 and 2010.

Year 2009								
Orchard	**Species**	**Number of trees**	**Number of sympt. trees**	**% of sympt. trees**	**Number of dead trees**	**% of dead trees**	**Number of asympt. trees**	**% of asympt. trees**
Feghal	Almond	72	57	**79.17**	11	**15.28**	4	**5.56**
Rachaya el Fouchar	Peach	96	21	**21.88**	0	**0.00**	75	**78.13**
Sarada	Nectarine	2846	8	**0.28**	0	**0.00**	2838	**99.72**
Year 2010								
Orchard	**Species**	**Number of trees**	**Number of sympt. trees**	**% of sympt. trees**	**Number of dead trees**	**% of dead trees**	**Number of asympt. trees**	**% of asympt. trees**
Feghal	Almond	61	55	**90.16**	4	**6.56**	2	**3.28**
Rachaya el Fouchar	Peach	73	6	**8.22**	0	**0.00**	67	**91.78**
Sarada	Nectarine	2838	0	**0.00**	0	**0.00**	2838	**100.00**

Fig. 61. Map of the key-orchard in Feghal (Caza of Jbeil), 2009.

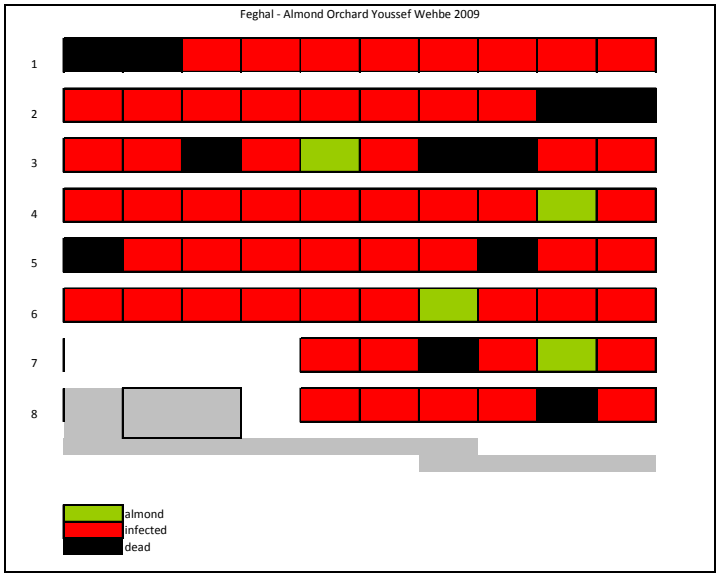


Fig. 62. Map of the key-orchard in Feghal (Caza of Jbeil), 2010.

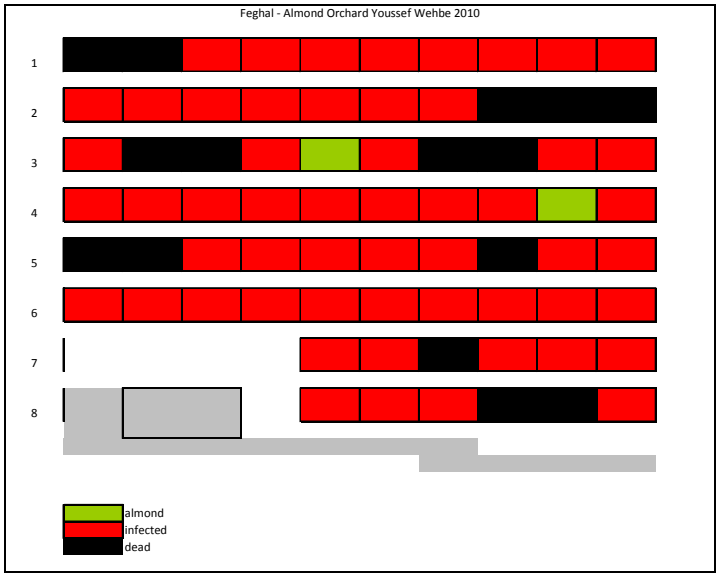


Fig. 63. Map of the key-orchard in Rachaya el Fouchar (Caza of Hasbaya), 2009.

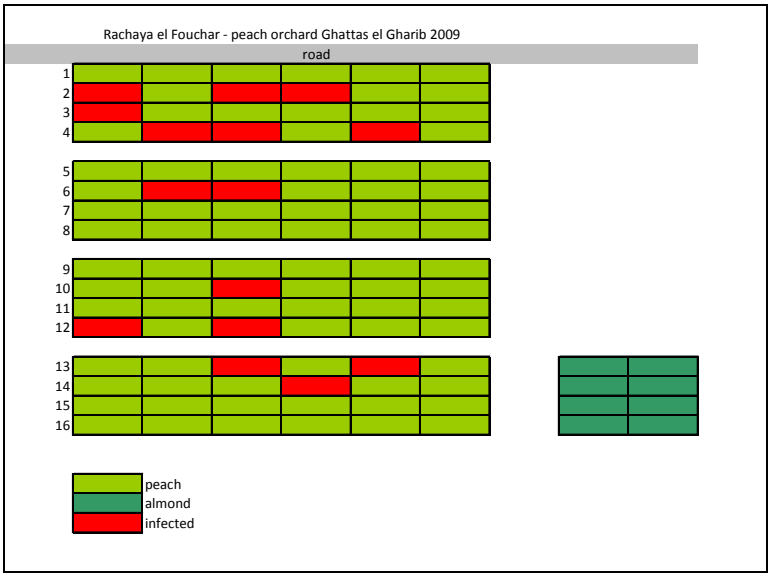


Fig. 64. Map of the key-orchard in Rachaya el Fouchar (Caza of Hasbaya), 2010.

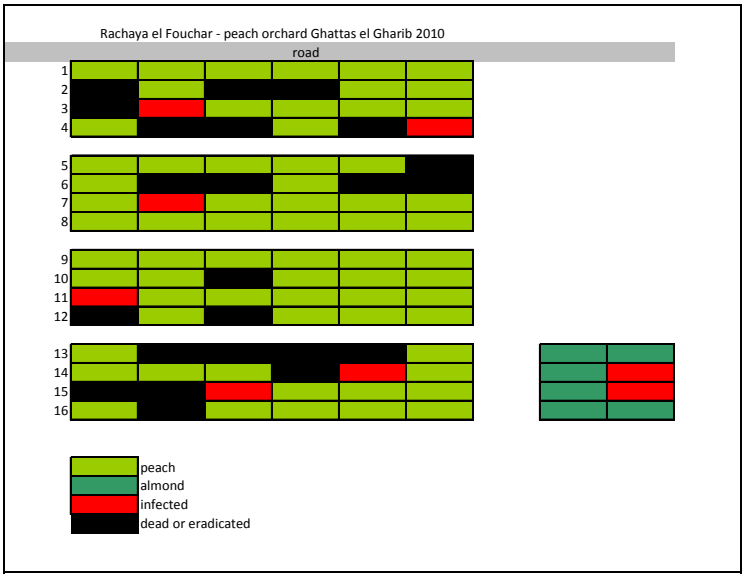
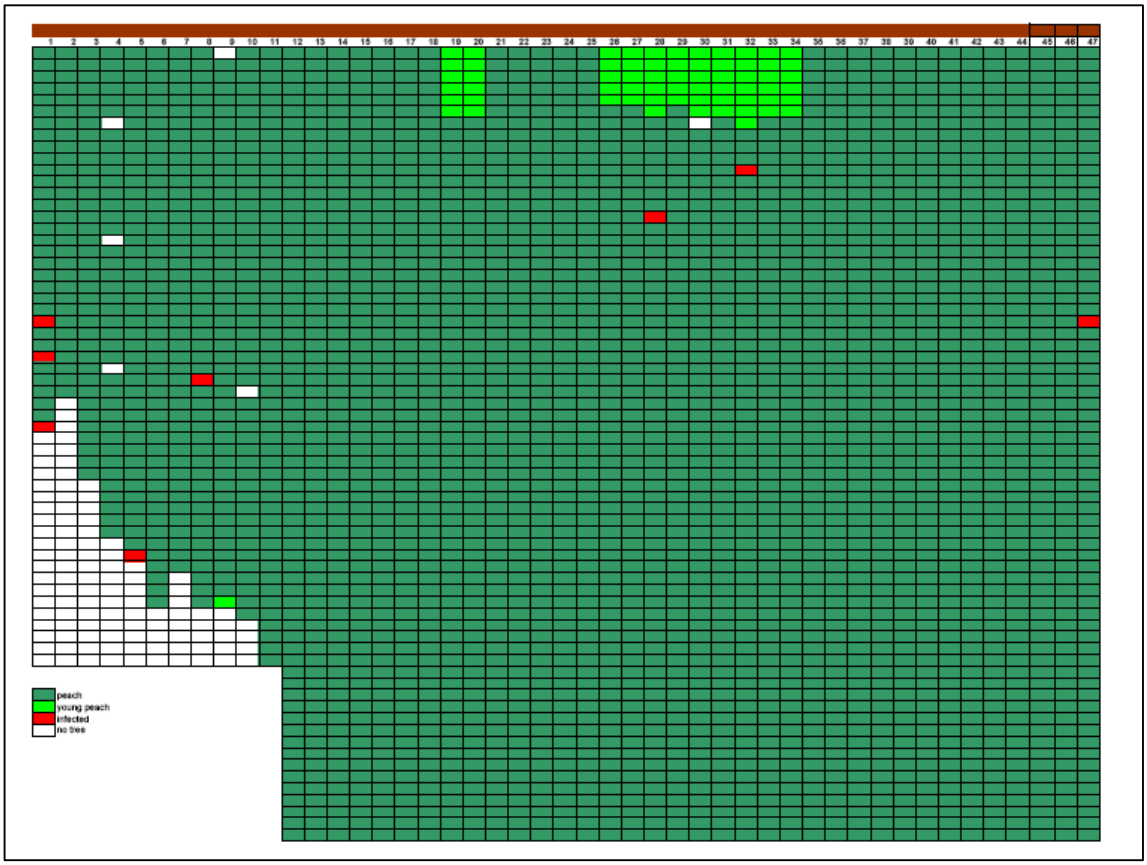


Fig. 65. Map of the key-orchard in Sarada (Caza of Marjayoun), 2009.



5.3 The AlmWB diffusion in Lebanon

The national survey concerning the AlmWB diffusion in Lebanon was carried out in about 890 orchards located in 490 villages of the 24 Lebanese Cazas (fig. 5)

In each orchard the symptomatic trees were identified and in some selected sites some of them were sampled in order to verify the disease aetiology. Some samples were also collected from trees showing doubtful symptoms, especially in areas characterized by a sporadic presence of the disease. Finally, samples were collected also from asymptomatic trees.

The results of the amplification analysis carried out on the 368 samples collected during the national survey (Table 11) are reported in the table 12.

A total of 245 almond samples (66.6 % of the total samples) were collected and processed: 94 from the Bekaa Valley, 81 from the ten Cazas of the Northern part of Lebanon and 70 from the nine Cazas of the Southern part of Lebanon. One hundred and twenty one out of 127 symptomatic samples (32.9% of the total samples) resulted to be infected by the AlmWB phytoplasma, while all the asymptomatic samples tested negatives, except for one sample collected in Saida.

Moreover, among the 123 peach/nectarine examined samples (33.4% of the total samples), 33 were collected in the Bekaa Valley, 34 in the ten Cazas of the Northern part of Lebanon and 56 in the nine Cazas of the Southern part of Lebanon: in particular no symptomatic trees were found and sampled in the Northern Lebanon. All the 40 symptomatic samples (10.9% of the total samples) coming from the Bekaa valley and the Southern part of Lebanon gave positive results when tested with the above mentioned primer. All the 83 asymptomatic samples tested negatives to the analysis.

Table 11. Description of the 368 samples collected during the national survey on the AlmWB distribution in Lebanon.

	Caza (District)	Almond samples			Peach/nectarine samples		
		Number	Symptomatic	Asymptomatic	Number	symptomatic	Asymptomatic
Bekaa Valley	Baalbeck	24	9	15	4	1	3
	Békaa West	29	20	9	15	12	3
	Hermel	9	3	6	0	0	0
	Rachaya	21	20	1	10	10	0
	Zahle	11	1	10	4	0	4
North	Akkar	15	9	6	1	0	1
	Baabda	0	0	0	5	0	5
	Batroun	24	16	8	0	0	0
	Becharre	0	0	0	0	0	0
	Donniye	8	7	1	8	0	8
	Jbeil	8	3	5	6	0	6
	Keseruan	1	0	1	8	0	8
	Koura	19	18	1	0	0	0
	Metn	0	0	0	6	0	6
	Zgharta	6	3	3	0	0	0
South	Aley	3	0	3	10	0	10
	Bent Jbail	10	0	10	0	0	0
	Chouf	18	9	9	5	0	5
	Hasbaya	14	4	10	8	7	1
	Jezzine	5	3	2	9	1	8
	Marjayoun	4	1	3	18	8	10
	Nabatieh	2	0	2	1	0	1
	Saida	8	1	7	4	1	3
	Sour	6	0	6	1	0	1
Total	**24**	245	**127**	**118**	**123**	**40**	**83**

Table 12. Results of the AlWF2/R2 amplification of the 368 samples collected during the national survey.

	Number of collected samples	Symptomatic	positive	Asymptomatic	positive
Almond					
Bekaa Valley	**94**	**53**	**51/53**	**41**	**0/41**
Northern Lebanon	**81**	**56**	**53/56**	**25**	**0/25**
Southern Lebanon	**70**	**18**	**17/18**	**52**	**1/52**
Tot	**245**	**127**	**121/127**	**118**	**1/118**
Peach and Nectarine					
Bekaa Valley	**33**	**23**	**23/23**	**10**	**0/10**
Northern Lebanon	**34**	**0**	**-**	**34**	**0/34**
Southern Lebanon	**56**	**17**	**17/17**	**39**	**0/39**
Tot	**123**	**40**	**40/40**	**83**	**0/83**

The disease appeared widely distributed in different regions, at different rate. Sixteen out of the 24 visited districts were affected by the disease, which is present in all the 5 Cazas of the Bekaa Valley (Baalbeck, Bekaa West, Hermel, Rachaya and Zahle), in 6 out of 10 Cazas in the North (Akkar, Batroun, Donniye, Jbeil, Koura and Zgharta) and in 5 out of the 9 Cazas in the South (Chouf, Hasbaya, Jezzine, Marjayoun and Saida).

A total of about 40,000 newly infected trees was counted during the survey, taking records of all the infected trees found during the monitoring and reported by the Municipalities and/or the farmers in the endemic regions.

5.3.1 The detailed distribution of the disease in the Lebanese regions

The percentage of the infected orchards in each Caza was calculated (Table 13) and classified in the 6 classes reported in the Materials and Methods. The classification was used to draw a national map of frequency of infected orchards in each Caza (Fig. 66).

In the Bekaa valley, the northern region of Hermel was characterised by the presence of few almond orchards, 35 % of which was affected by the disease. Only in the 20 % of the orchards located in the wide district of Baalbeck trees clearly showing the disease symptoms were observed. The region of Zahle, rich in nurseries and stone fruit orchards, presented only a few disease *foci*, which were quickly eradicated. On the contrary, the Cazas of Bekaa West and Rachaya were heavily interested by the disease, observed on the 38% almond and the 68 % nectarine visited orchards.

The almond orchards of the northern Cazas, Akkar, Donniye, Zgharta, Koura, Batroun and Jbeil, were frequently affected by Almond witches'-broom. The majority of the visited almond orchards were infected by the phytoplasma: in the district of Koura the disease was observed in the 95 % of the visited orchards.

The disease appeared in these regions many years before the year 2000, as reported by Abou-Jawdah in 2002, and is still having a serious impact on the cultivation. It is common to find almond orchards totally destroyed and neglected, completely abandoned by the growers.

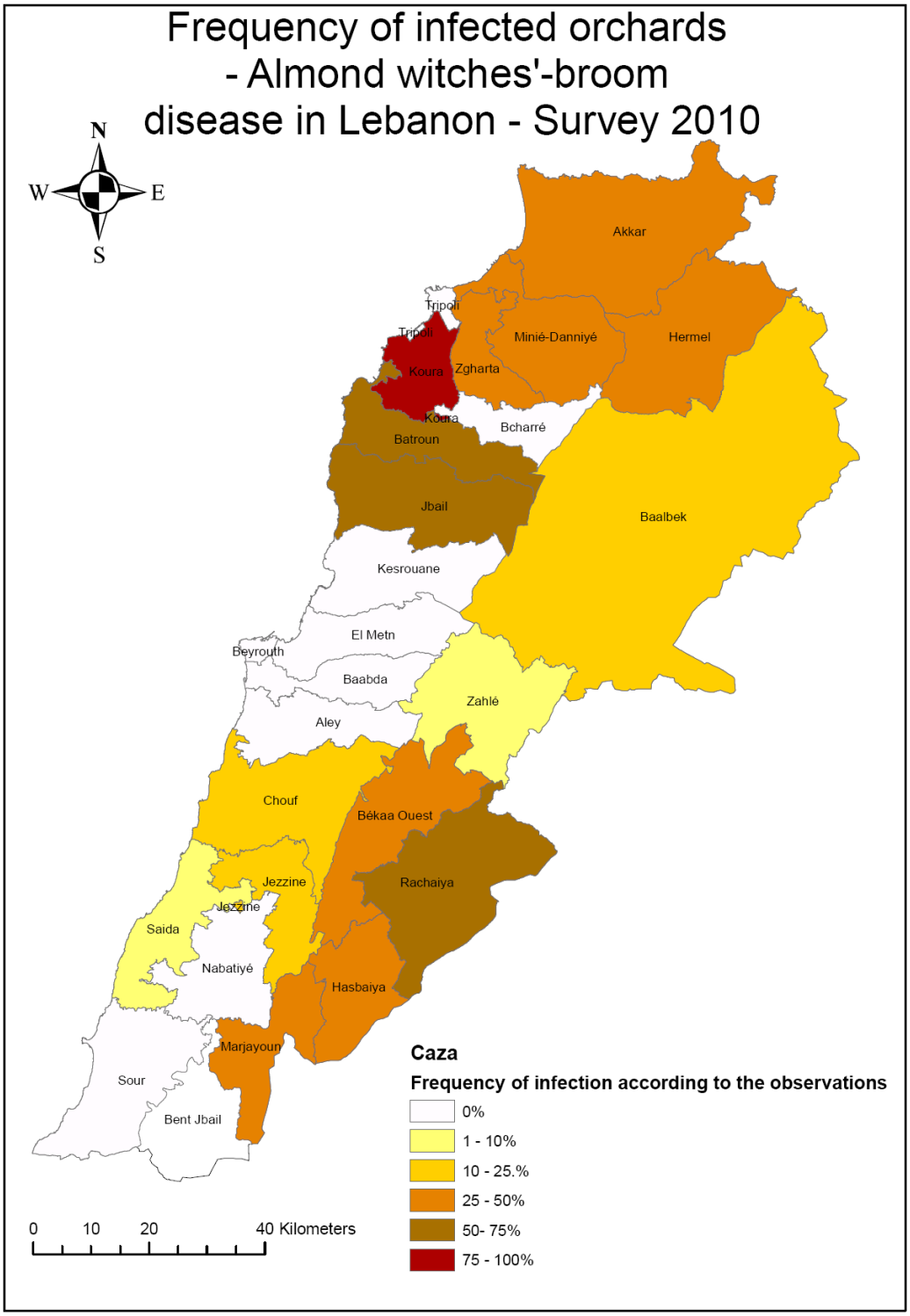
In the northern Cazas peach or nectarine orchards were very rare, due to the limited water resources. Therefore only some peach/nectarine trees were found near the houses, just for family consumption. No symptomatic nectarine or peach trees were found in these areas, apart from in Feghal, where the incidence of the disease on almond is very high. In the only 4 gardens with almond or peach monitored in the region of Becharre, totally planted with apples, no symptomatic trees were observed. Similar results were obtained in the few orchards examined in the region of Jbeil and in the districts of Keseruan, Metn and Baabda.

In the Southern Lebanon, numerous nectarine orchards were recently planted in the Cazas of Aley and Chouf: while no infected trees were found in the Aley area. In the Chouf regions some infected almond trees, planted near or in nectarine orchards were detected. In the Cazas of Jezzine, Marjayoun and Hasbaya, the frequency of infected orchards, planted with both nectarine/peach and almond trees, ranged from 21 and 34%. In these regions, some infected plants were found in numerous orchards and rapidly eliminated. In the region of Saida, near the village of Tamboureet, where on 2002 the disease was observed for the first time (Abou-Jawdah *et al.,* 2002), some old trees still showed witches'-broom, but the majority of the symptomatic plants were eliminated years ago. No infected trees were observed in the regions of Sour, Nabatieh and Bent Jbeil, where however stone fruits are rarely cultivated.

Table 13. Percentage of the infected orchards on the total of the visited orchards.

	Caza (District)	Number of visited orchards	number of infected orchards	**Percentage of infected orchards (%)**
Bekaa	Baalbeck	72	15	20.83
	Bekaa West	77	30	38.96
	Hermel	14	5	35.71
	Rachaya	61	42	68.85
	Zahle	99	4	4.04
North	Akkar	30	13	43.33
	Batroun	41	27	65.85
	Donniye	35	13	37.14
	Jbeil	69	48	69.57
	Koura	24	23	95.83
	Zgharta	9	4	44.44
South	Chouf	95	10	10.53
	Hasbaya	29	10	34.48
	Jezzine	23	5	21.74
	Marjayoun	33	10	30.30
	Saida	34	1	2.94

Fig 66. Frequency of infected orchards on the total of the monitored orchards. Survey 2010.



5.3.2 Disease severity

The percentage of infected trees per orchards was assessed in 102 orchards located in the Batroun, Marjayoun and Rachaya districts (Table 14) and classified according to the infection classes reported in the "Materials and methods" (Chapter 4.3).

Moreover, the infection frequency and the percentage index of infection were calculated (Table 15).

Table 14. Classification of the monitored orchards in Batroun, Marjayoun and Rachaya based on the percentage of infected trees.

Caza	Batroun		Marjayoun		Rachaya	
Infection class	Almond	peach/ nectarine	Almond	peach/ nectarine	Almond	peach/ nectarine
0 : 0%	7	1	6	15	4	5
1: 1 -10%	1	0	0	11	7	8
2: 11- 25%	0	0	0	0	4	0
3: 26 -50%	0	0	0	0	5	0
4: 51 – 75%	0	0	0	0	1	0
5: 76 - 100	16	0	0	0	9	2
Total	24	1	6	26	30	15

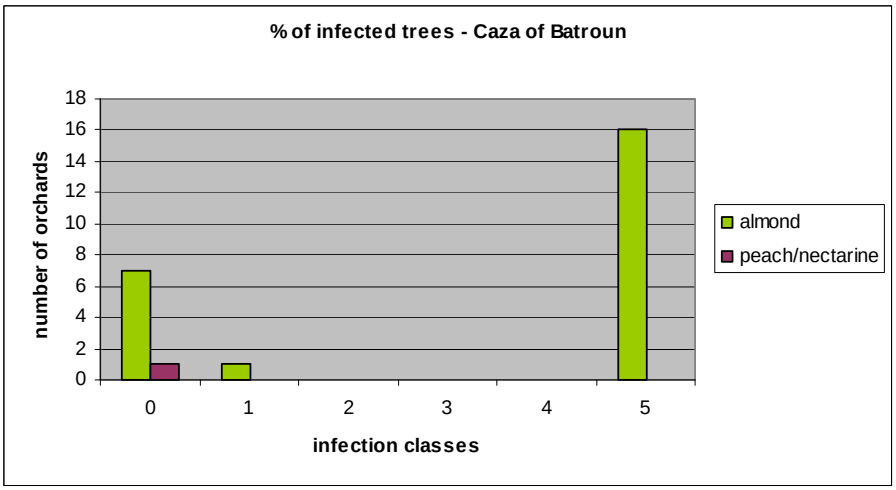
Table 15. Distribution of the infection frequency and percentage index of infection in the districts of Batroun, Marjayoun and Rachaya.

	Batroun		Marjayoun		Rachaya	
Infection class (% of observation)	almond	peach	almond	peach/ nectarine	almond	peach/ nectarine
0	29.17	100.00	100.00	57.69	13.33	33.33
1	4.17	0.00	0.00	42.31	23.33	53.33
2	0.00	0.00	0.00	0.00	13.33	0.00
3	0.00	0.00	0.00	0.00	16.67	0.00
4	0.00	0.00	0.00	0.00	3.33	0.00
5	66.67	0.00	0.00	0.00	30.00	13.33
Cumulative infection frequency	**70.83**	**0.00**	**0.00**	**42.31**	**86.67**	**66.67**
Percentage Index of Infection (I%I)	**67.50**	**0.00**	**0.00**	**8.46**	**52.67**	**24.00**

In the Caza of Batroun, 25 orchards were monitored in order to assess the percentage of infected trees. The Batroun district is characterized by the typical presence of non irrigated almond orchards on the coastal areas and the almost total absence of peach or nectarine orchards. No infected trees were observed in a peach and seven almond orchards. AlmWB symptoms were observed on the 71 % of the almond orchards, in particular 4.17% of the orchards showed an infection frequency ranging from 0.1 to 10 %,

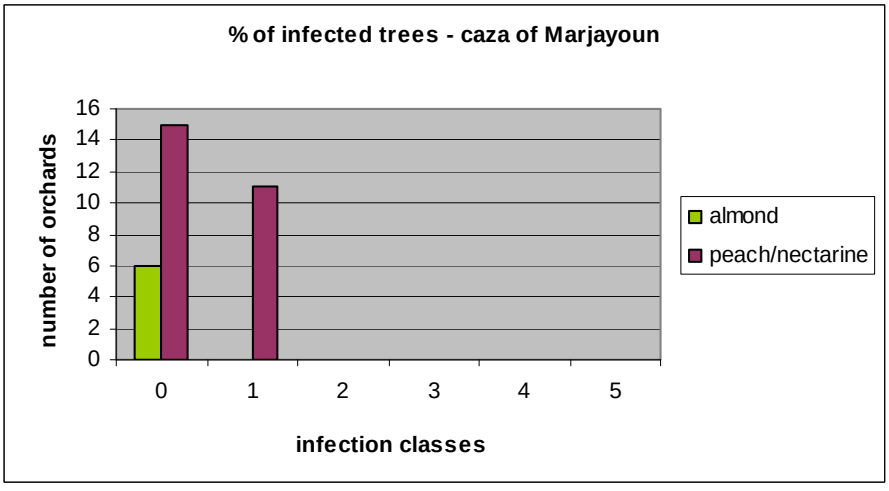
while 66.67 % of the orchards showed a percentage of infected trees ranging from 75 and 100 %. (Fig. 67). The percentage index of infection in the Batroun Caza was 67.5%, entirely due to the almond orchards

Fig. 67. Distribution of the infection frequency, Caza of Batroun.



In the Caza of Marjayoun, no symptomatic trees were found in the 6 visited almond orchards. No disease symptoms were observed in 15 peach/nectarine orchards, while a maximum of 10 % infected trees were detected in the remaining 11 cultivations (Fig. 68). The 42 % of the peach/nectarine orchards showed the disease symptom, but the percentage index of infection was lower than 10 %.

Fig. 68. Distribution of the infection frequency, Caza of Marjayoun.

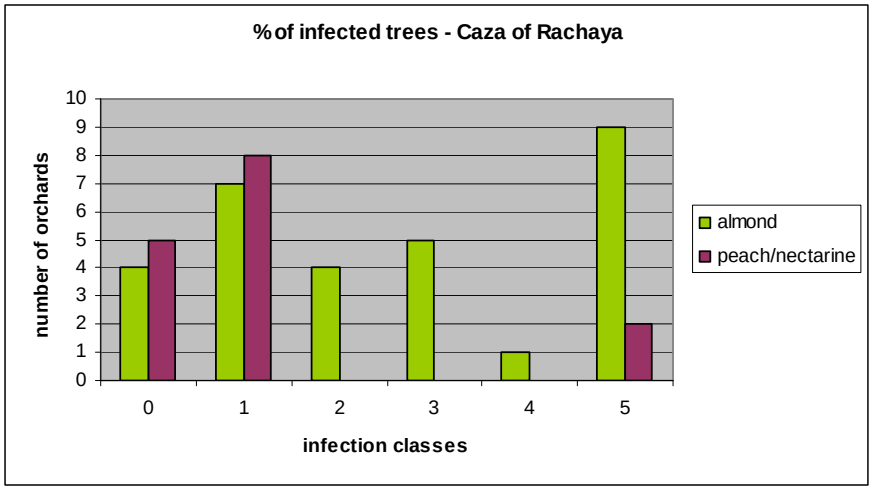


In the Caza of Rachaya, 45 orchards were monitored; in the 30 almond orchards, the disease severity greatly varied ranging from class 0 and class 5, as shown in figure 69.

The disease symptoms were not observed in 5 peach orchards, affected 10 % and more than 75 % of the trees respectively in 8 and 2 cultivations.

The cumulative infection varied from 66.67% in the peach/nectarine orchards to 86.67% in almond orchards. The percentage index of infection reached 24 % on peach/nectarine and 52.67 % on almond.

Fig. 69. Distribution of the infection frequency, Caza of Rachaya.



5.3.3 The national map of AlmWB distribution in Lebanon

The national map of the AlmWB distribution in Lebanon (Fig. 70) was obtained by combining the symptom observations carried out in the almond and peach/nectarine orchards and the results of the molecular assays executed on the samples collected during the field survey.

The GPS coordinates taken for each monitored orchard were reported into the GIS software, related to the phytosanitary status of the orchard, according to the molecular analysis results.

This elaboration allowed showing the spread of the disease in the visited Lebanese villages.

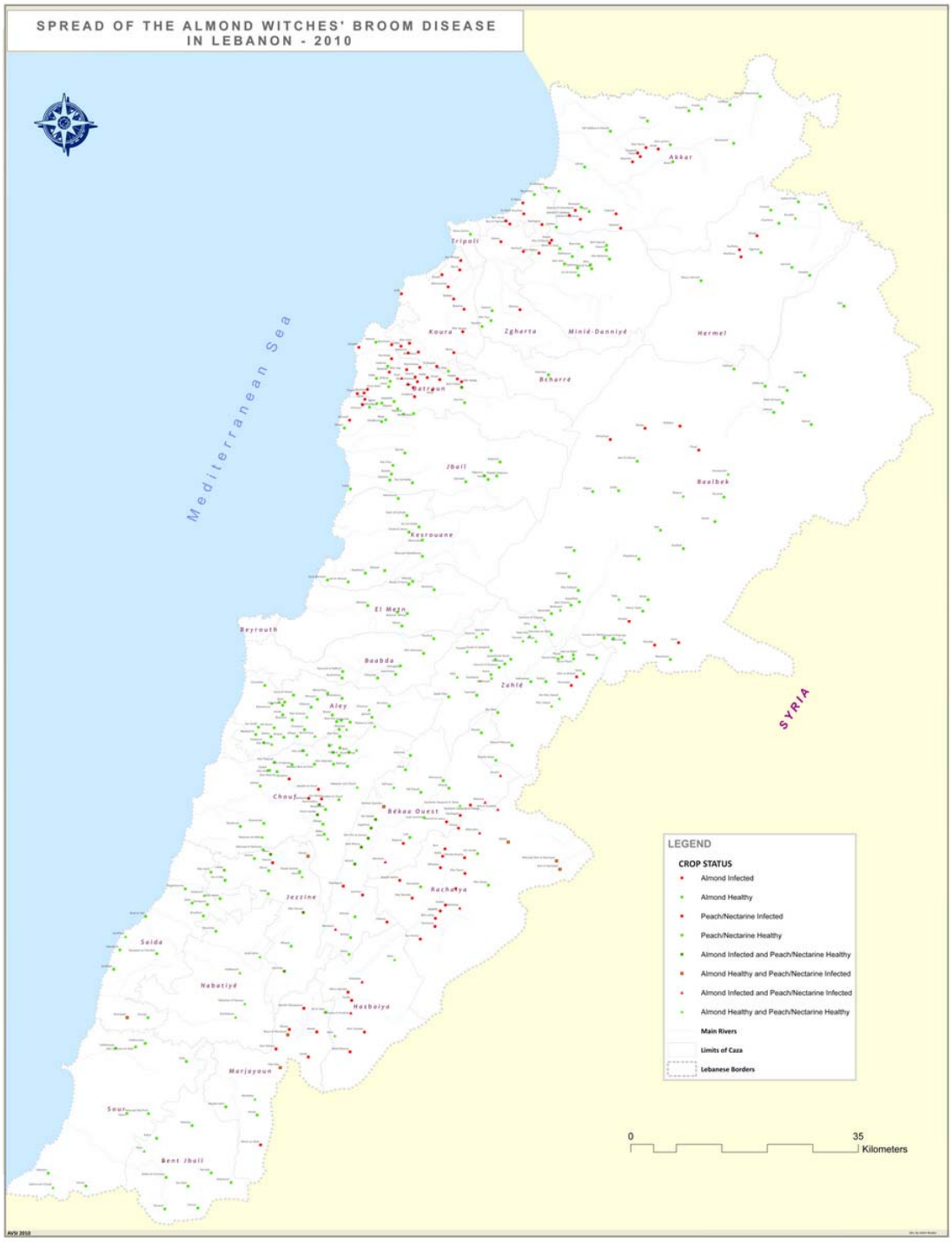
The map clearly shows that the disease is present in 16 out of 24 districts of Lebanon, with different incidence. The Northern part of the Country is heavily affected by the disease, from the villages of Akkar to the regions of Feghal, Chmout and Rachana, which represent the border between the infected and the healthy coastal areas of Lebanon. Only one infected tree was found in the village of Monsef, grafted with infected buds.

Some *foci* of infection, where the disease spread recently, are distributed randomly in the Southern regions, as in the central area of the Chouf, in the villages of Baaqline, Jdeidet el Chouf, Kahlouniyeh, Ain Qeni, Aammatour and Aret Jandal, where only the almond trees were infected. Other foci were located in the Caza of Jezzine (Bizri, Aazour, Aariye, Kfar Houne and Aaichiye), where almond or nectarine were infected. An infected nectarine sample was collected also in the south of Saida, in Kharayeb.

In the northern regions, in Hermel, the disease was spread on almond trees in three villages, in the upper part of the hills, whereas all the other orchards on the plains were not affected by the disease. In Baalbeck and Zahle, just some orchards were affected by Almond witches'-broom, on both almond and peach/nectarine trees. The regions of West Bekaa, Rachaya, Hasbaya and Marjayoun were heavily affected by the disease, and some infected orchards were located near the borders with Syria and Israel.

The map has been presented to the Lebanese Ministry of Agriculture, in order to show the disease distribution and incidence and to discuss the urgent control management of AlmWB at a national level.

Fig. 70. Map of the Almond Witches'-broom spread in the Lebanese regions.



5.4 Characterization of the pathogen

5.4.1 Identification of '*Candidatus* Phytoplasma phoenicium' on samples

The 16S rRNA phytoplasma gene amplification was carried out on the 18 leaves and flower samples collected in the three key orchards and on the 49 samples collected in the neighbouring regions (tables 4 and 5).

All the 67 collected samples were analysed using the universal primer pair P1/P7 followed by the nested PCR using the primer pair F2n/R2.

All the 38 symptomatic samples gave positive results, except for one sample collected from plants showing witches'-brooms, in the orchard in Burj el Moulouk,in August, late in the season, and consisting of very old leaves, probably insuitable for the molecular assay.

The 5 samples labelled as "Symptomatic – doubt" were collected from trees showing symptoms not typically observed on the AlmWB affected trees, such as yellow or silver leaves, or proliferations different from the observed ones. All these samples tested negatives.

All the 24 asymptomatic samples gave negative results to the amplification. Control PCRs containing water instead of DNA yielded no visible DNA amplification.

In general, the DNA extraction and fragment amplification from flowers gave better results than from the leaves, since it was unnecessary to repeat the extraction because of the viscosity of the leaf tissues. Since the universal primers used in PCR analysis amplified a 16S rDNA fragment that is common to all the described '*Candidatus* Phytoplasma' species, the identification of the phytoplasma associated with the observed symptoms was carried out by sequencing twenty-four strains from the positive samples (reported in the tables 15 and 16).

The 16SrDNA sequences of these 24 strains, compared to the Gene bank accessions revealed that phytoplasma strains present in the selected samples identified shared 99-100% of sequence identity with '*Ca.* Phytoplasma phoenicium' (accession number AF515636), ribosomal group IX.

These results confirmed that the symptoms observed during the season and described as the AlmWB symptom expression in almond, peach and nectarine hosts can be utilized for the identification of AlmWB infected trees, even if molecular analysis is always necessary as the final confirmation of the infection.

Table 15. Results of 16SrDNA fragment amplification of the key-orchards' samples.

Caza (District)	Village	Species	Variety	Sample collected	Sympt. or asympt. sample	Sample code	Sample code – strain	PCR analysis P1/P7 and nested F2n/R2
Jbeil	Feghal	Almond	Telyani	Leaves	Sympt	**A1**	**A1-1**	**+**
		Almond	Telyani	Leaves	Sympt	**A9**	**A9-1**	**+**
		Almond	Telyani	Leaves	Sympt	**A16**	**A16-4**	**+**
Hasbaya	Rachaya el Fouchar	Peach	Babcock	Leaves	Sympt	**SP1**	**SP1-1 (l)**	**+**
Marjayoun	Sarada	Nectarine	Fantasia	Leaves and flowers	Sympt.	**N1**	**N1-1 (l)**	**+**
							N1-2 (f)	**+**
		Nectarine	Fantasia	Leaves and flowers	Asympt	**N2**	**N2-1 (f)**	**-**
							N2-2 (l)	**-**
		Nectarine	Fantasia	Leaves and flowers	Asympt	**N3**	**N3-1 (f)**	**-**
							N3-3 (l)	**-**
		Nectarine	Fantasia	Leaves and flowers	Asympt	**N4**	**N4-1 (f)**	**-**
							N4-2 (l)	**-**
		Nectarine	Fantasia	leaves	Sympt.	**N5**	**N5-1**	**+**
		Nectarine	Fantasia	Leaves	Sympt	**N8**	**N8-1**	**+**
		Nectarine	Fantasia	Leaves and flowers	Sympt	**N9**	**N9-1 (l)**	**+**
							N9-7 (f)	**+**
		Nectarine	Fantasia	Leaves and flowers	Sympt	**N10**	**N10-1 (l)**	**+**
							N10-8 (f)	**+**

Table 16. Results of 16SrDNA gene of the samples collected in the orchards of Feghal, Hasbaya, Kherbet Kanafar and Marjayoun-Sarada.

Caza (District)	Village	Orchard	Species	Sample collected	Sympt. or asympt. Sample	Sample code	Sample code – strain	PCR analysis P1/P7 and nested F2n/R2
Jbeil	Feghal	Orchard 1	Almond	Leaves	Sympt.	**A11**	A11-4	+
			Almond	Leaves	Sympt.	**A13**	A13-1	+
		Orchard 2	Almond	Leaves	Sympt – doubt	**A17**	A17-1	-
			Almond	Leaves	Sympt.	**A18**	A18-1	+
		Orchard 3	Almond	Leaves	Sympt.	**APl3**	APl3-1	+
			Peach	Leaves	Sympt.	**P1**	P1-2	+
			Peach	Leaves and flowers	Sympt.	**P2**	P2-1 (l)	+
							P2-6 (f)	+
			Peach	Leaves	Sympt.	**P3**	P3-1	+
		Orchard 4	Almond	Leaves	Sympt.	**A3**	A3-1	+
		Orchard 5	Almond	Leaves	Sympt.	**A4**	A4-1	+
Hasbaya	Rachaya el Fouchar	Orchard 1	Almond	Leaves	Asympt	**SA1**	**SA1-1**	-
			Almond	Leaves	Sympt	**SA3**	**SA3-1**	+
			Almond	Leaves	Sympt	**SA4**	**SA4-1**	+
West Bekaa	Kherbet Kanafar	Orchard 1	Nectarine	Leaves and flowers	Sympt.	**N18**	N18-1 (l)	+
							N18-7 (f)	+
			Nectarine	Leaves and flowers	Sympt.	**N19**	N19-1 (l)	+
							N19-7 (f)	+
			Nectarine	Leaves	Sympt. – doubt	**N20**	N20-2	-
			Nectarine	Leaves	Asympt.	**N21**	N21-2	-
		Orchard 2	Nectarine	Leaves	Sympt.	**N29**	N29-1	+
			Nectarine	Leaves	Sympt.	**N30**	N30-1	+
			Nectarine	Leaves	Asympt.	**N31**	N31-1	-
		Orchard 3	Peach	Leaves	Sympt. – doubt	**P5**	P5-1	-
			Peach	Leaves	Asympt.	**P6**	P6-1	-
			Peach	Leaves	Asympt.	**P7**	P7-1	-
		Orchard 4	Peach	Leaves	Asympt.	**P8**	P8-1	-
			Peach	Leaves	Sympt.	**P9**	P9-1	+
Marjayoun	Khiam	Orchard 1	nectarine	Leaves	Sympt.	**N12**	N12-1	+
			nectarine	Leaves	Sympt.	**N14**	N14-1	+
	Qlayaa	Orchard 2	nectarine	Leaves	Sympt.	**N13**	N13-1	+
			nectarine	Leaves	Sympt. – doubt	**N15**	N15-1	-

Caza (District)	Village	Orchard	Species	Sample collected	Sympt. or asympt. Sample	Sample code	Sample code – strain	PCR analysis P1/P7 and nested F2n/R2
			nectarine	Leaves	Sympt. – doubt	**N16**	N16-1	**-**
	Burj el Moulouk	Orchard 3	nectarine	Leaves	Asympt.	**N25**	N25-1	**-**
			nectarine	Leaves	Sympt.	**N26**	N26-1	**-**
			nectarine	Leaves	Sympt.	**N27**	N27-2	**+**
			nectarine	Leaves	Sympt.	**N28**	N28-1	**+**
	Sarada	Orchard 4	Peach	Leaves	Sympt.	**P10**	P10-1	**+**
		Orchard 5	Almond	Leaves and flowers	Asympt	**A2**	**A2-1 (f)**	**-**
							A2-2 (l)	**-**
			Almond	Leaves and flowers	Asympt	**A3**	**A3-1 (f)**	**-**
							A3-3 (l)	**-**
			Almond	Leaves and flowers	Asympt	**A4**	**A4-1 (f)**	**-**
							A4-3 (l)	**-**
			Almond	Leaves and flowers	Asympt	**A5**	**A5-1 (f)**	**-**
							A5-3 (l)	**-**
			Almond	Leaves	Asympt	**A6**	**A6-1**	**-**
			Almond	Leaves	Asympt	**A7**	**A7-1**	**-**
			Almond	Leaves	Asympt.	**A8**	**A8-1**	**-**

Examples of gels concerning the electrophoresis runs of the fragments amplified using the nested primer pair R16F2n/R16R2 (F2n/R2) are shown in the figures 71 and 72. Phytoplasma reference strain STOL (stolbur group, subgroup 16SrXII-A) was used as a positive control, whereas W1 and W2 were reaction mixtures without DNA template used as negative controls.

Fig.71. Electroforetic gel of the amplified fragment F2n/R2 carried out on the samples coming from Feghal, Hasbaya, West Bekaa and Marjayoun-Sarada regions.

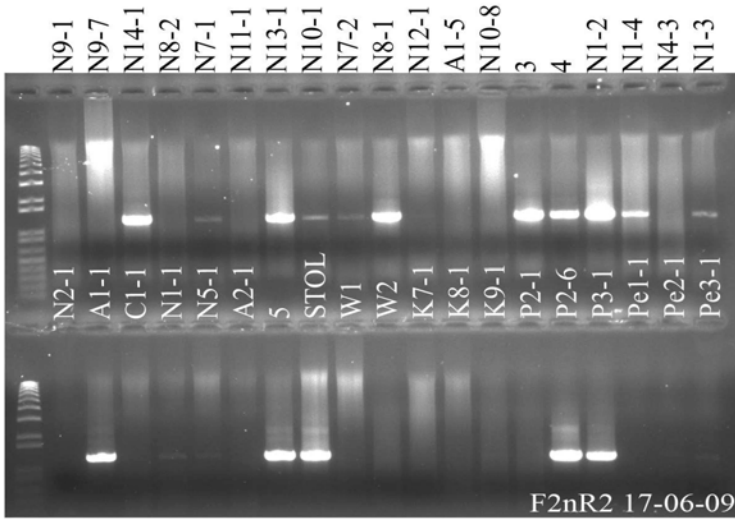
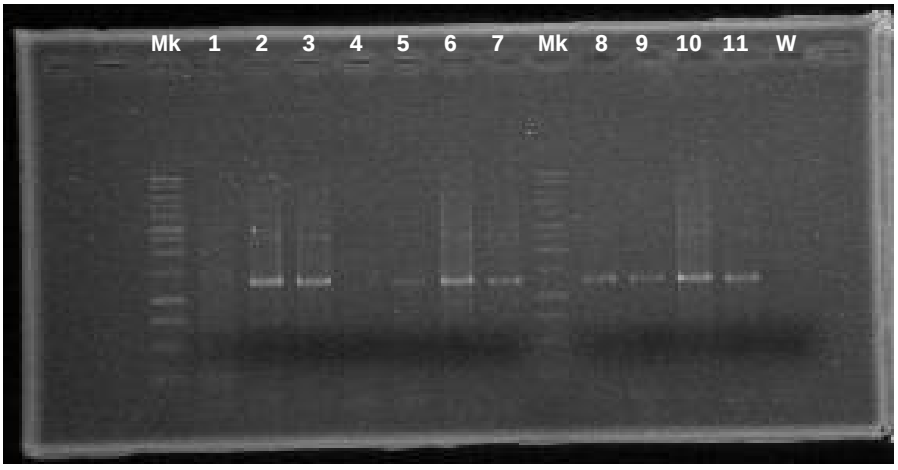


Fig.72. Electroforetic gel of the amplified fragment F2n/R2 carried out on the samples collected in Feghal, Hasbaya, West Bekaa and Marjayoun-Sarada regions. Mk: marker; 1-10: samples A1-1, A18-1, A3-1, SA1-1, SA3-1, N18-1, N18-7, N19-1, N19-7, N29-1; 11: positive control; W: water.



5.4.2 New subgroups in group 16SrIX determined by virtual RFLP analyses

The 24 strains sequenced were processed in order to indeed characterize the pathogen variability.

RFLP analysis was carried out in order to determine the subgroup of '*Ca*. P. phoenicium' strains identified on the samples, by computer-simulated restriction analyses, executed on R16F2n/R16R2 sequences from the 24 '*Ca.* P. phoenicium' strains previously selected, collected in the regions of Feghal, Kherbet Kanafar and Marjayoun-Sarada.

Visualization and comparison of virtual gel plotted images (fig. 73) revealed three different RFLP patterns, indicating genetic diversity among '*Ca.* P. phoenicium' strains in Lebanon (Table 18).

The pattern exhibited by DNAs from 15 '*Ca.* P. phoenicium' strains was indistinguishable from that characteristic of strains classified in the '*Ca.* P. phoenicium', subgroup IX-D. The remaining two virtual RFLP patterns differed from the pattern of the previously described subgroup IX-D and shared similarity coefficients ranging from 93 to 97%, confirming their affiliation with group IX; according to Wei and co-workers (2007) and Lee and co-workers (2007), each of the two new RFLP patterns possibly identifies a new subgroup in group IX.

The 16S rDNAs from '*Ca.* P. phoenicium' strains N5-1 and N27-2, collected in the orchards of Sarada and Marjayoun, exhibited identical virtual RFLP patterns using 17 restriction enzymes. Since the *Bst*UI RFLP pattern distinguished (similarity coefficient ≤97%) strains N5-1 and N27-2 from strains in all previously described subgroups in group IX, these two strains are classified in new subgroup IX-F.

The 16S rDNAs from '*Ca.* P. phoenicium' strains N1-2, A1-1, A13-1, A18-1, P3-1, A3-1, and A4-1 exhibited identical virtual RFLP patterns, which distinguished (similarity coefficient ≤97%) these strains from the individuals belonging to all previously described subgroups, including new subgroup IX-F, on the basis of digestion with *Taq*I. Hence, '*Ca.* P. phoenicium' strains N1-2, A1-1, A13-1, A18-1, P3-1, A3-1, and A4-1 are placed in a new subgroup, IX-G (Figure 74; Table 17).

The AlmWB-associated Lebanese '*Ca*. P. phoenicium' strains, whose sequences were retrieved from the GenBank, shared a virtual RFLP similarity coefficient > 98% with '*Ca*. P. phoenicium' strains of subgroup IX-D, while the Iranian phytoplasma strains associated with AlmWB and almond broomings shared a similarity coefficient > 99% with phytoplasmas of subgroup IX-C (Table 17).

Table 17. Similarity coefficient obtained through virtual RFLP analysis of 16S r DNA sequences from 24 '*Ca*. P. phoenicium' strains and from representative strains of group 16SrIX.

	Strain	Acc.No.	Subgr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	PPWB	AF248957	-A	100																												
2	CPP[a] strain 21	AF515637	-B	76	100																											
3	PEY	Y16389	-C[b]	89	87	100																										
4	CPP[a] strain A4	AF515636	-D[b]	77	97	86	100																									
5	JunWB	GQ925918	-E	89	85	96	84	100																								
6	**N1-2**	HQ407512	**-G**	75	95	84	97	82	100																							
7	N14-1	HQ407513	-D	75	93	84	97	82	93	100																						
8	**A1-1**	HQ407514	**-G**	75	95	84	97	82	100	93	100																					
9	**A13-1**	HQ407515	**-G**	75	95	84	97	82	100	93	100	100																				
10	**A18-1**	HQ407516	**-G**	75	95	84	97	82	100	93	100	100	100																			
11	P2-6	HQ407517	-D	77	97	86	100	84	97	97	97	97	97	100																		
12	**P3-1**	HQ407518	**-G**	75	95	84	97	82	100	93	100	100	100	97	100																	
13	**A3-1**	HQ407519	**-G**	75	95	84	97	82	100	93	100	100	100	97	100	100																
14	N19-1	HQ407520	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100															
15	**A4-1**	HQ407521	**-G**	75	95	84	97	82	100	93	100	100	100	97	100	100	97	100														
16	**N5-1**	HQ407522	**-F**	74	93	83	97	80	93	93	93	93	93	97	93	93	97	93	100													
17	A11-4	HQ407523	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100												
18	N10-8	HQ407524	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100											
19	N8-1	HQ407525	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100										
20	N28-1	HQ407526	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100									
21	P1-2	HQ407527	-D	73	90	82	93	80	90	90	90	90	90	93	90	90	93	90	90	93	93	93	93	100								
22	N9-7	HQ407528	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100							
23	N29-1	HQ407529	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100						
24	N18-1	HQ407530	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100	100					
25	A16-4	HQ407531	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100	100	100				
26	**N27-2**	HQ407532	**-F**	74	93	83	97	80	93	93	93	93	93	97	93	93	97	93	100	97	97	97	97	90	97	97	97	97	100			
27	PL3-1	HQ407533	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100	100	100	97	100		
28	P10(297)	HQ407534	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100	100	100	97	100	100	
29	N13-1	HQ407535	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100	100	100	97	100	100	100

In detail, thirty-seven percent (9/24) of '*Ca.* P. phoenicium' strains exhibited virtual RFLP patterns distinct from those of IX known subgroups. In particular 33% (2/6) of the strains from Sarada region belongs to subgroups IX-F and -G; 25% (1/4) of the strains from Marjayoun region belongs to subgroup IX-F, and 54% (6/11) of the strains from Feghal belongs to the subgroup IX-G, whereas all the strains from Kerbet Kanafar (3/3) belong to the already described subgroup IX-D.

Table 18. Occurrence of '*Ca.* P. phoenicium' strains belonging to distinct 16SrIX subgroups in orchards of Lebanese regions.

Sample code or Strain	Caza	Region	Orchard No.	Host	Sample	Subgroup IX
A1-1	Jbeil	Feghal	8	Almond	Leaf	**-G**
A16-4				Almond	Leaf	**-D**
A13-1			9	Almond	Leaf	**-G**
A11-4				Almond	Leaf	**-D**
A18-1			10	Almond	Leaf	**-G**
P1-2			11	Peach	Leaf	**-D**
P2-6				Peach	Flower	**-D**
P3-1				Peach	Leaf	**-G**
PL3-1				Almond	Leaf	**-D**
A3-1			12	Almond	Leaf	**-G**
Al4-1			13	Almond	Leaf	**-G**
N18-1	Bekaa West	Kerbet Kanafar	14	Nectarine	Leaf	**-D**
N19-1				Nectarine	Leaf	**-D**
N29-1			15	Nectarine	Leaf	**-D**
N1-2	Marjayoun	Sarada	1	Nectarine	Flower	**-G**
N5-1				Nectarine	Leaf	**-F**
N9-7				Nectarine	Flower	**-D**
N8-1			2	Nectarine	Leaf	**-D**
N10-8			3	Nectarine	Flower	**-D**
P10(297)			4	Peach	Leaf	**-D**
N13-1		Marjayoun	5	Nectarine	Leaf	**-D**
N14-1			6	Nectarine	Leaf	**-D**
N27-2			7	Nectarine	Leaf	**-F**
N28-1				Nectarine	Leaf	**-D**

Fig.73. Virtual RFLP patterns of representative strains of 16SrIX subgroups. 17 restriction enzymes were used in order to characterize the subgroup rIX strains.

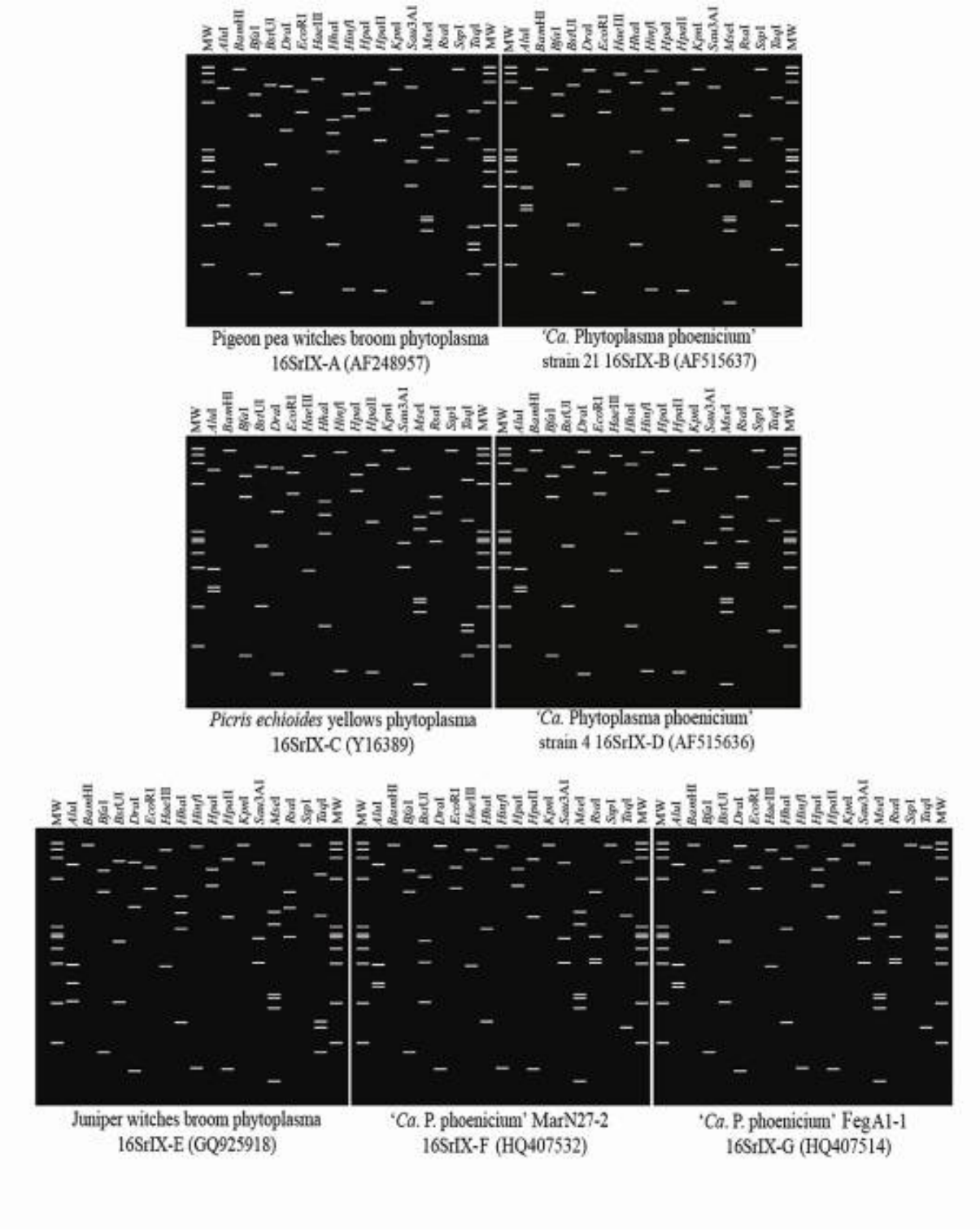
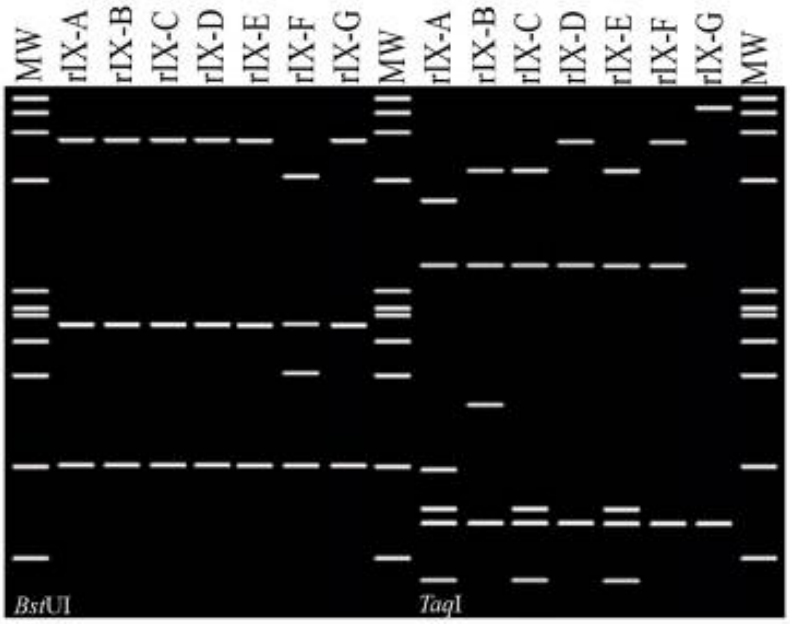


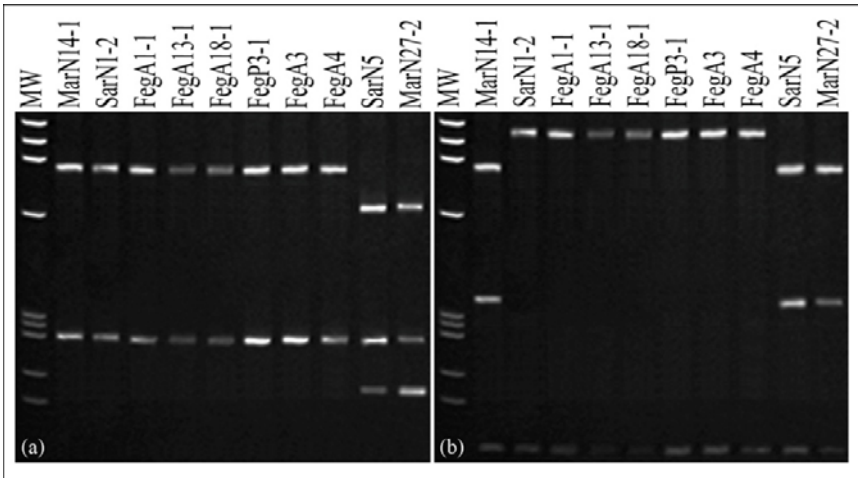
Fig. 74. Virtual R16F2nR2 RFLP patterns by key enzymes *BstU*I and *Taq*I for distinguishing among 16SrIX subgroups.



5.4.3 Real RFLP analyses

Actual gel electrophoresis-RFLP analyses, carried out using the distinguishing enzymes *Bst*UI and *Taq*I on R16F2n/R16R2 PCR products from strains N27-2, N5-1, N1-2, A1-1, A13-1, A18-1, P3-1, A3-1, and A4-1 confirmed the virtual RFLP patterns (Figure 75).

Fig. 75. Actual gel-showing the *BstU*I and *Taq*I RFLP patterns of R16F2nR2 amplicons from '*Ca.* Phytoplasma phoenicium' strains.



Moreover, 14 strains collected in 2010 in the Bekaa Valley, in the regions of Baalbeck, Bekaa West and Rachaya, were processed through real RFLP analysis (table 19).

All the 14 strains, digested with the enzymes *Taq*I and *BstU*I, showed the typical 16SrIX-D profile (Figs 76 and 77).

Table 19. Strains of '*Ca.* Phytoplasma phoenicium' collected in the regions of Baalbeck, West Bekaa and Rachaya processed with real RFLP analyses.

Caza (District)	Region	Host	Sample collected	Number of samples	Subgroup IX
Baalbeck	Mchaytiye	Nectarine	Leaf	1	**-D**
Bekaa West	Qaraoun	Peach	Leaf	2	**-D**
	Sahbine-Akabe	Peach	Leaf	2	**-D**
	Ayn el Jawzi	Peach	Leaf	1	**-D**
Rachaya	Bakka	Peach	Leaf	1	**-D**
	Mazraait deir el aachayer	Peach	Leaf	1	**-D**
		Nectarine	Leaf	1	**-D**
	Deir el aachayer	Nectarine	Leaf	3	**-D**
	Mdoukha	Nectarine	Leaf	1	**-D**
	Rachaya	Peach	Leaf	1	**-D**

Fig. 76. Polyacrylamide gel showing the RFLP patterns of phytoplasma F2n/R2 DNA fragments digested with *BstU*I. MK: marker; 1-14 samples from Baalbeck, Bekaa West and Rachaya); 15: DNA from subgroupIX-C (Naxos) as a control.

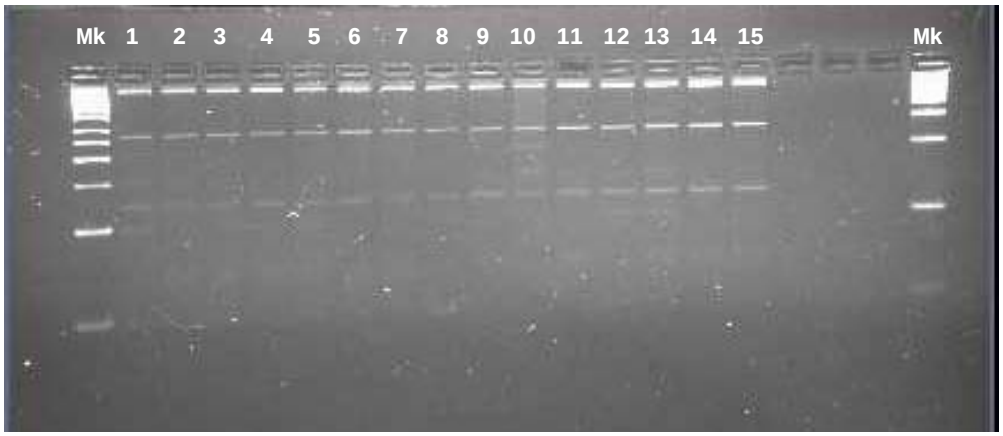
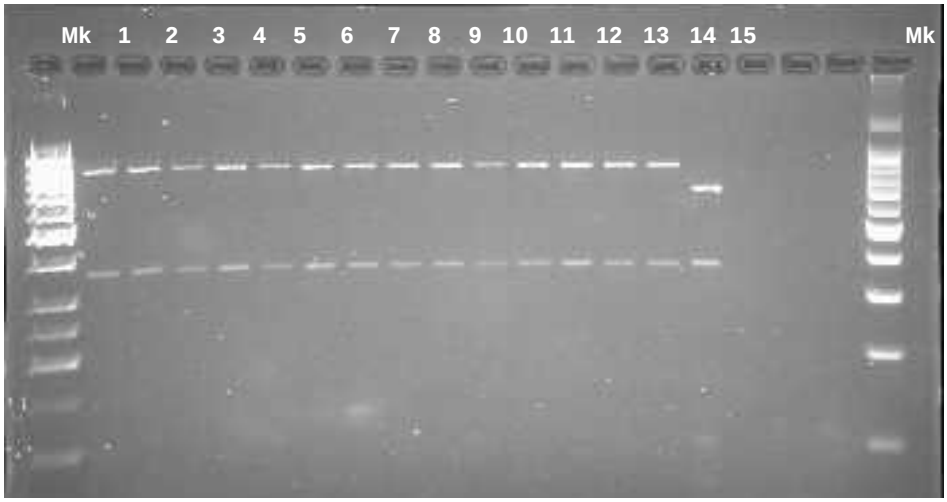


Fig. 77. Polyacrylamide gel showing the RFLP patterns of phytoplasma F2nR2 DNA fragments digested with *TaqI*. MK: marker; 1-14 samples from Baalbeck, Bekaa West and Rachaya; 15: DNA from subgroupIX-C (Naxos) as a control.



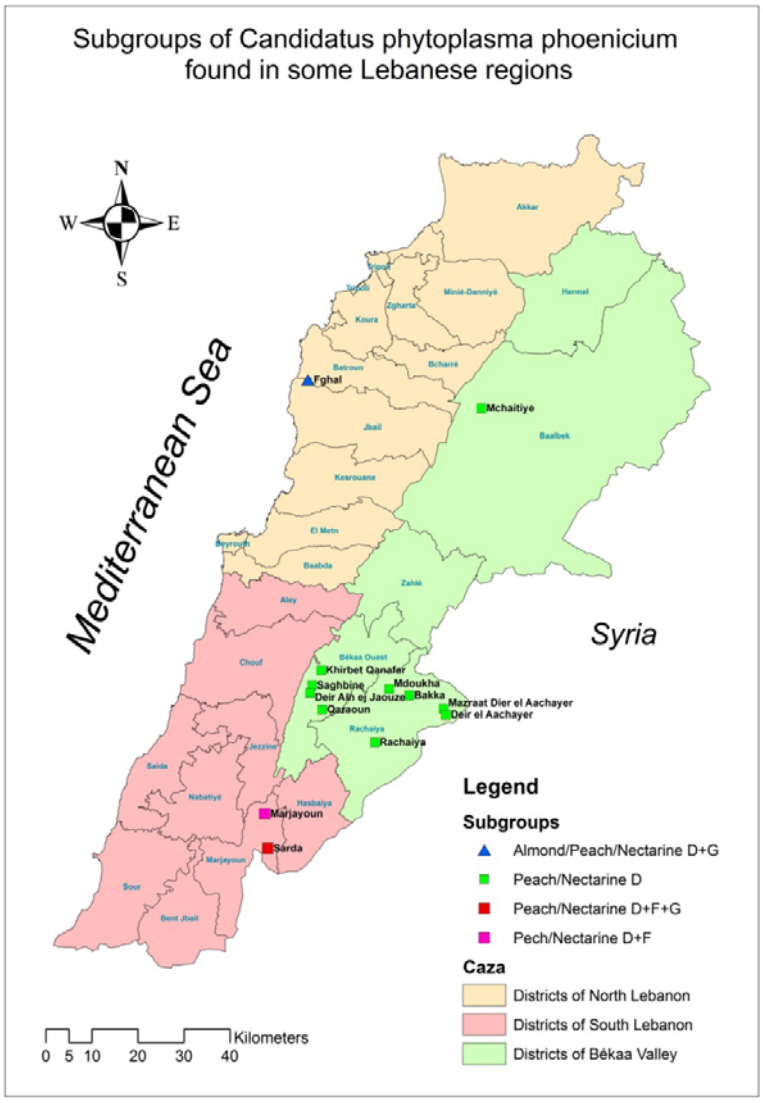
5.4.4 The subgroup distribution

The subgroup distribution was represented in the figure 78, showing that the '*Ca.* Phytoplasma phoenicium' strains characterized by the new subgroup 16Sr IX-F are present in two Lebanese southern regions, namely Sarada and Marjayoun (red and pink dots).

The '*Ca.* Phytoplasma phoenicium' strains characterized by the new subgroup 16Sr IX-G are present both in the northern regions, as Feghal (blue triangle), and in the South, in Sarada (red dot). All the '*Ca.* Phytoplasma phoenicium' strains collected in West Bekaa and in the Bekaa Valley belong to the subgroup 16Sr IX-D (green dots), that is present in the North and in the South of Lebanon as well.

In the same orchard, as in the orchard 1 in Sarada, the orchard 7 in Marjayoun and the orchards 9 and 11 in Feghal, there is the co-presence of different subgroup strains.

Fig. 78. Distribution of the '*Ca.* Phytoplasma phoenicium' subgroups in Lebanon.



5.4.5 Phylogenetic relationships

The selected twenty-four phytoplasma 16SrDNA sequences were processed in BLAST searches, in order to verify their sequence similarity with the reference strains and all the sequences yielded best hits with '*Ca.* Phytoplasma phoenicium', subgroup IX-D.

A minimum-evolution (ME) phylogenetic analysis of 16S rRNA gene sequences showed that phytoplasma strains of all 16SrIX subgroups cluster together on a separate tree branch within the same group (Figure 79).

Inside the group IX branch, the 24 '*Ca*. P. phoenicium' strains clustered along with previously characterized Lebanese '*Ca*. P. phoenicium' strains, associated with AlmWB, in a phylogenetic subclade with the representative '*Ca*. P. phoenicium' strain A4, subgroup IX-D (Figure 80).

'*Ca.* P. phoenicium' strains of new confirmed subgroup IX-G clustered together in a separate subclade within that of '*Ca*. P. phoenicium' (subgroup IX-D).

On the other hand, Iranian phytoplasma strains associated with AlmWB clustered in a separate subclade with the representative strain of the subgroup IX-C.

A phylogenetic tree based on 16S rDNA sequences from previously characterized phytoplasma strains, 24 '*Ca*. P. phoenicium' strains from this work, and *A. palmae* is shown in Figure 79. A focus on the 24 '*Ca*. P. phoenicium' strains is drawn in figure 80.

Fig. 79. Phylogenetic tree inferred from phytoplasmal 16S rDNA F2nR2 sequences. *Acholeplasma palmae* was used to root the tree. Bootstrap values are displayed at tree nodes. GenBank accession numbers of nucleotide sequences are shown along with the name of phytoplasma strains.

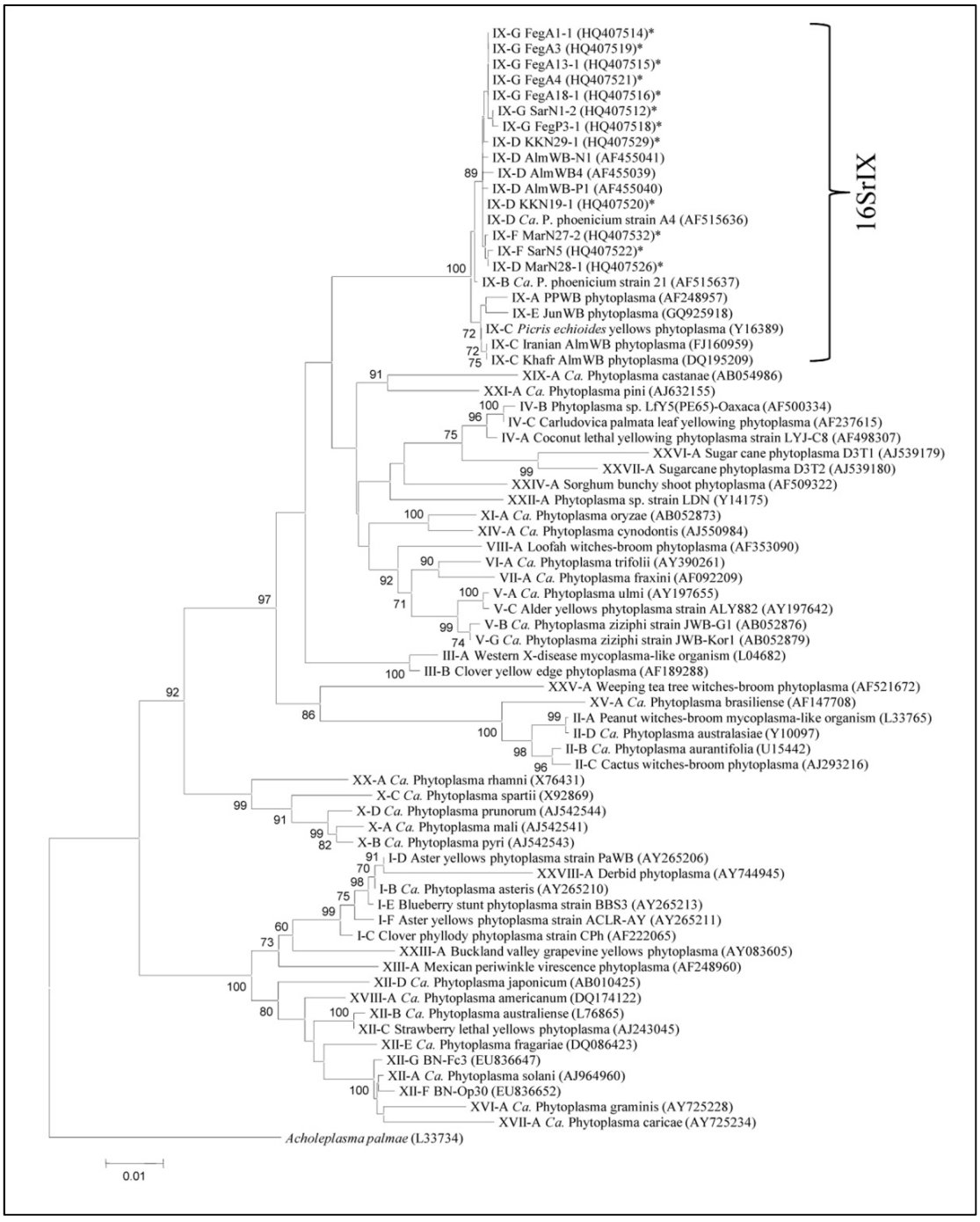
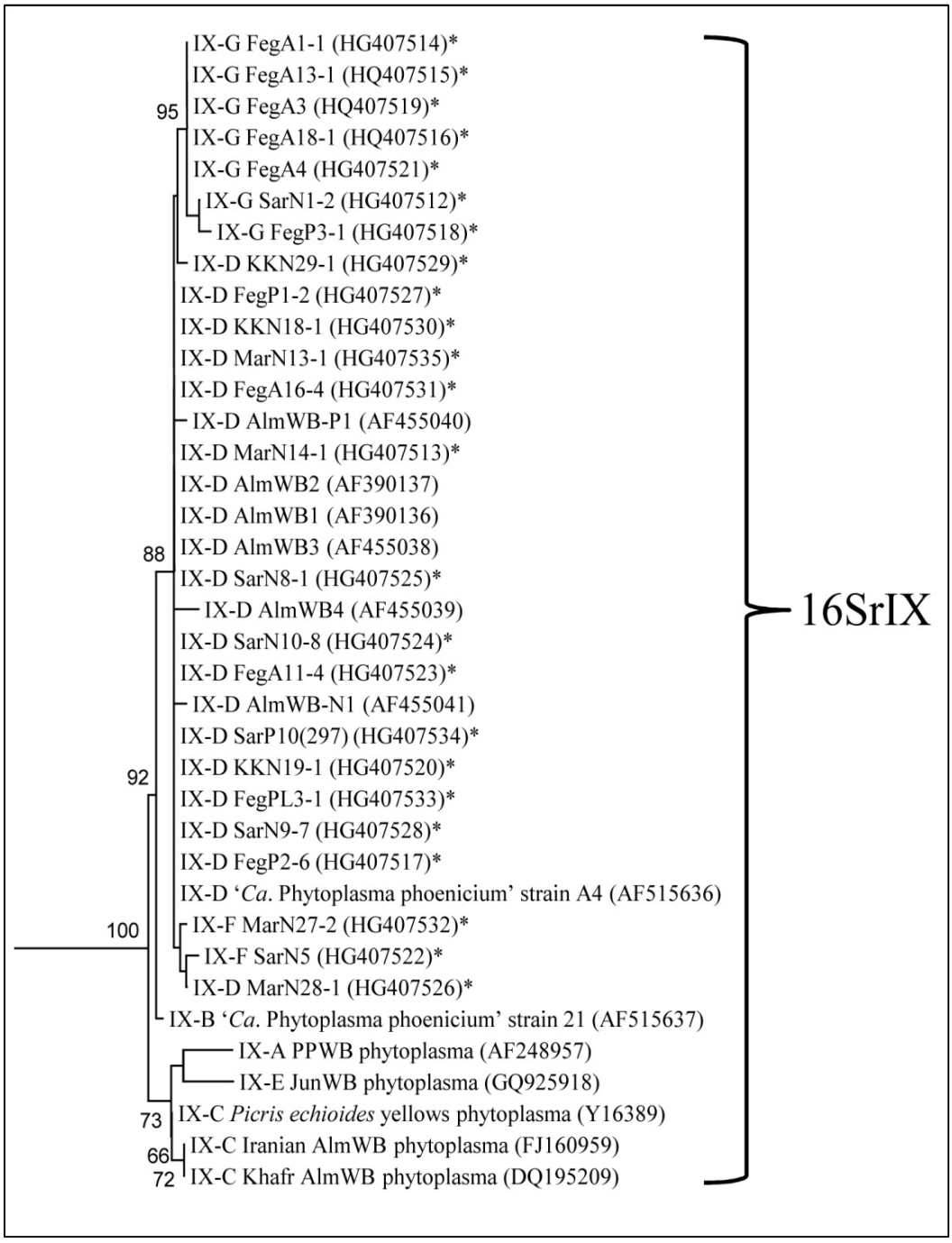


Fig. 80. Group 16SrIX branch in phylogenetic tree inferred from phytoplasma 16SrDNA R16F2nR2 sequences. Bootstrap values are displayed at tree node.



5.5 Insect identification

5.5.1 Cicadellidae

a. On traps

Three genera and 45 species belonging to the family Cicadellidae were identified in the traps installed in Sarada and Feghal in 2010 (Table 20). Eleven species were found exclusively in the orchard of Feghal. Three genera and 12 species were found only in the orchard of Sarada, whereas 22 species were common to both areas.

The most abundant species collected in Feghal were *Zyginidia sohrab* Zachvatkin, (1,482 specimens) and *A. decedens (*1,062 specimens). In the orchard of Sarada, *A. decedens* was particularly abundant: a total of 12,647 adults were counted in the traps where 306 specimens of *Z. sohrab* were also found.

Some specimens belonging to families of the taxon Auchenorryncha, other than Cicadellidae, were also found and in particular Aphrophoridae *(Mesoptyelus impictifrons* (Horvath)) in Feghal; Caliscelidae (unidentified) and Flatidae (*Phantia subquadrata* (Herrich-Schäffert)) in Sarada. Some Delphacidae and Issidae were found in both localities.

In 2011 the collections obtained in Feghal and Kfarkela are presented in table 22. Within the family of Cicadellidae 1 genus and 44 species were identified. In details, 13 species were found only in the traps installed in Kfarkela; 3 species were found in Feghal, whereas 1 genus and 28 species were found in both orchards.

A. decedens was the most abundant species collected in both the localities: 3,669 specimens in Feghal and 6,385 in Kfarkela. The second most abundant species in Feghal was *Z. sohrab* (974 i.), and *Megophthalmus scabripennis* Edwards (2,048 i.) in Kfarkela.

In the Auchenorrhyncha taxon, insects belonging to five families, different from Cicadellidae, were also found, as Aphrophoridae, Cercopidae, Delphacidae, Dictiopharidae and Issidae.

Among the species found in the traps, a difference was observed between the years 2010 and 2011. In fact, 15 species, *Arocephalus lakonicus* Dlabola, *Balclutha punctata* Fabricius, *Circulifer haematoceps* (Mulsant and Rey), *Doratura homophyla* Flor, *Euscelis lineolatus* Brulle, *Exitianus nanus* Distant, *Jubrinia distircta* Linnavouri, *Macrosteles sexnotatus* Fallen, *Iassus* sp., *Paradorydium* sp., *Macropsis* sp., *Ribautiana tenerrima* Herrich-Schaeffer, *Zyginella pulchra* Low, *Asyraca claviformis* Saludos, and *Phantia subquadrata* (Herrich-Scaffer), characterised by a low presence of specimens, collected during the year 2010 were not found in 2011.

On the contrary, 8 species *Aphrodes makarovi* Zachvatkin, *Agallia brachyptera* Boheman, *Allygus mixtus* Fabricius, *Jassargus kurdicus* Remane and Schultz, *Thamnotettix agilis*, *H ecalus eximius* Kirschbaum, *Batracomorphus irroratus* Lewis and *Balcanocerus*

balcanicus Horvat, never collected in 2010 were found in 2011, mainly in the orchard of Kfarkela, showing a great biodiversity in the region.

b. In field

Cicadellidae and other Auchenorryncha were collected by using sweep nets and the subsequent identification was mainly focused on the species previously indicated as more interesting in the role of possible phytoplasma vectors (Abdul-Nour, personal communication).

In 2010, in the region of Feghal and near the key orchard (the cave of Feghal and Barbara), 29 specimens of Deltocephalinae and one or few individuals belonging to Megophtalminae (1 specimen), Typhlocybinae (76 sp.), and other families as Delphacidae (1 sp.), Issidae (2 sp.), Aphrophoridae (15 sp.), Cercopidae (4 sp.) were found.

In the region of Akkar, at Oyoun el Ghazlen, only 10 specimens of *A. decedens* were captured, whereas in the South of Lebanon, at the key orchard of Sarada, 29 specimen of Cicadellidae (subfamilies Aphrodinae, Deltocephalinae, Hecalinae, Megophtalminae and Typhlocybinae) were found as well as one Delphacidae and one Issidae. In the orchard of Wadi Khansa, 13 Cicadellidae (belonging to the subfamilies Megophtalminae and Typhlocibinae) and one Aphrophoridae were collected.

In 2011 the field collecting weren't focused on Cicadellidae, but on the Cixiidae specimens, explaining the absence, on table 2, of other data about the collecting by using sweep nets.

Table 20. Cicadellidae collected in the key-orchards of Feghal and Sarada (Malaise and sticky traps) and in the fields of Wadi Khansa, Barbara and Oyoun el Ghezlane, 2010.

Family Cicadellidae		Sarada			Wadi Khansa	Feghal			Feghal, near the cave	Barbara	Ouyoun el ghezlane (Akkar)	Total
Ssubfamily	species	Malaise trap	Sticky traps	sweep nets		Malaise trap	sticky traps	sweep nets				
Family Cicadellidae												
Aphrodinae	*Anoscopus albifrons* (Linnaeus)			1								1
Agallinae	*Anaceratagallia laevis* (Ribaut)	6	2			0	0					8
Deltocephalinae	*Anoplotettix eckerleini* Dlabola	0	0			0	1			2		3
	Arocephalus lakonicus Dlabola	1	0			0	0					1
	Balclutha punctata (Fabricius)	1	0			1	0					2
	Balclutha sp.	2	0			1	0			1		4
	Cicadulina bipunctata (Melichar)	7	0			0	0					7
	Circulifer haematoceps (Mulsant & Rey)	1	0			0	0					1
	Deltocephalinae unID	4	0			0	0					4
	Doratura homophyla (Flor)	2	4			0	0					6
	Euscelidius mundus (Haupt)	0	0			16	3	2	3	2		26
	Euscelis alsius Ribaut	6	0			0	0					6
	Euscelis incises (Kirschbaum)			3					1	2		6
	Euscelis lineolatus Brulle	0	2			0	1					3
	Euscelis sp.	0	0			1	0					1
	Exitianus capicola (Stal)	3	1			1	0					5
	Exitianus nanus (Distant)	0	0			1	0					1
	Exitianus sp.	3	0			0	0					3
	Fieberiella macchiae Linnavuori	2	0			3	0					5
	Goniagnathus brevis (HerrichSchäffer)	3	0			0	0					3

Family Cicadellidae		Sarada			Wadi Khansa	Feghal			Feghal, near the cave	Barbara	Ouyounel ghezlane (Akkar)	Total
Ssubfamily	species	Malaise trap	Sticky traps	sweep nets		Malaise trap	sticky traps	sweep nets				
	Grammacephalus pugio (Noualhier)	0	0			2	0					2
	Jubrinia distircta Linnavuori	1	0			0	0					1
	Laylatina inexpectata Abdul-Nour	0	0			1	0					1
	Macrosteles sexnotatus (Fallen)	1	0			0	0					1
	Macrosteles sp.	2	0			0	0					2
	Neoaliturus fenestratus (Herrich-Schaffer)	3	4			0	0	1				8
	Phlepsius intricatus (Herrich-Schaffer)	7	1			1	0					9
	Phlepsius sp.	1	0			0	0					1
	Proceps acicularis Mulsant & Rey	0	0			4	1					5
	Psammotettix gr. Provincialis (Ribaut)	15	4			3	0		2	1		25
	Recilia schmidtgeni (Wagner)	67	9			3	0					79
	Synophropsis lauri (Horvath)	1	0			36	12			1		50
	Thamnotettix klapperichi Dlabola	0	0			0	0	3				3
	Thamnotettix seclusus Linnavuori	0	0			5	1		1	2		9
	Thamnotettix sp.	0	0			1	0	3				4
	Thamnotettix wittmeri Dlabola	1	1			51	1	2				56
Hecalinae	*Hecalus glaucescens* (Fieber)			1								1
Iassinae	*Iassus* sp.	0	1			0	0					1
Idiocerinae	*Balcanocerus ramallahicus* (Dlabola)	0	0			5	8					13
	Hespericerus brusinae (Horvath)	1	0			6	1					8

Family Cicadellidae		Sarada			Wadi Khansa	Feghal			Feghal, near the cave	Barbara	Ouyounel ghezlane (Akkar)	Total
Ssubfamily	species	Malaise trap	Sticky traps	sweep nets		Malaise trap	sticky traps	sweep nets				
Dorycephalinae	*Paradorydium* sp.	0	1			0	0					1
Macropsinae	*Macropsis* sp.	1	0			0	0					1
Megophthalminae	*Megophthalmus scabripennis* Edwards	1	12	5	3	5	3	1				30
Typhlocibinae	*Asymmetrasca decedens* (Paoli)	427	12040	19	10	144	918	75			10	13643
	Edwardsiana rosae (Linnaeus)	0	0			0	2					2
	Edwardsiana sp.	2	0			3	5					10
	Empoasca decipiens Paoli	15	21			2	2					40
	Empoasca solani Delong	0	13			0	0					13
	Eupteryx gyaurdagica Dlabola	2	2			21	0					25
	Eupteryx sp.	0	5			0	0					5
	Eupteryx stachydearum (Hardy)	7	5			3	1					16
	Eurhadina angulata Linnavuori	0	0			3	11					14
	Ficocyba ficaria (Horvath)	22	40			11	53					126
	Frutioidia bisignata (Mulsant & Rey)	14	12			52	11					89
	Frutioidia divina Logvinenko	1	0			7	0					8
	Hauptidia ecbalii Linnavuori	59	62			125	56					302
	Lindbergina cretica Asche	0	3			33	6					42
	Ribautiana (Typhlocyba) tenerrima Herrich-Schaeffer	1	0			0	0					1
	Typhlocybinae unID	120	367			120	108					715
	Zygina gr. Flammigera (Fourcroy)	122	89			117	88					416

Family Cicadellidae		Sarada			Wadi Khansa	Feghal			Feghal, near the cave	Barbara	Ouyoun el ghezlane (Akkar)	Total
Ssubfamily	species	Malaise trap	Sticky traps	sweep nets		Malaise trap	sticky traps	sweep nets				
	Zyginella pulchra Low	0	0			0	19					19
	Zyginidia sohrab Zachvatkin	285	21			1362	120	1				1789

Table 21. Auchenorrhyncha families, Cicadellidae excluded, found in the key-orchards of Feghal and Sarada (Malaise and sticky traps) and in the fields of Wadi Khansa, Barbara and Oyoun el Ghezlane, 2010.

Family	species	Sarada			Wadi Khansa	Feghal			Feghal, near the cave	Barbara	Ouyoun el ghezlane (Akkar)	Total
		Malaise trap	Sticky traps	sweep nets		Malaise trap	sticky traps	sweep nets				
Aphrophoridae	*Aphrophoridae* unID	0	0			1	0		2			3
	Mesoptyelus impictifrons Horvath	0	0		1	16	1	3	2	8		31
Caliscelidae	*Caliscelidae* unID	1	0			0	0					1
Cercopidae	*Cercopis intermedia* Walker	0	0			0	0		2	1		3
	Neophylenus sp.								1			1
Delphacidae	*Asiraca claviformis* Saludos	1	0			0	0	1				2
	Delphacidae unID	21	4	1		3	1					30
Flatidae	*Phantia subquadrata* (Herrich-Schaffer)	1	0			0	0					1
Issidae	*Issidae* unID	2	1	1		8	0	1		1		14

Table 22. Cicadellidae and other Auchenorrhyncha collected in the key-orchards of Feghal and Kfarkela (Malaise and sticky trap), 2011.

Family and subfamily		Kfarkela		Feghal		Total
	species	Malaise trap	Sticky traps	Malaise trap	sticky traps	
Family Cicadellidae						
Sub. Aphrodinae	*Anoscopus albifrons* (Linnaeus)	2	13	1	0	16
	Aphrodes makarovi Zachvatkin	1	22	0	0	23
Sub. Agallinae	*Agallia brachyptera* Boheman	2	0	4	0	6
	Anaceratagallia laevis (Ribaut)	18	3	3		24
Subfamily Deltocephalinae	*Allygus mixtus* Fabricius	3	2	0	0	5
	Anoplotettix eckerleini Dlabola	0	0	5	20	25
	Balclutha sp.	1	2	1	2	6
	Cicadulina bipunctata (Melichar)	0	3	0	1	4
	Euscelidius mundus (Haupt)	6	5	9	19	39
	Euscelis alsius Ribaut	3	1	0	0	4
	Euscelis sp.	0	2	0	0	2
	Exitianus capicola (Stal)	1	1	0	0	2
	Fieberiella macchiae Linnavuori	1	0	5	10	16
	Grammacephalus pugio (Noualhier)	0	2	0	2	4
	Jassargus kurdicus Remane & Schulz	2	0	0	0	2
	Laylatina inexpectata Abdul-Nour	47	21	2	2	72
	Neoaliturus fenestratus (Herrich-Schaffer)	2	25	0	0	27
	Phlepsius intricatus (Herrich-Schaffer)	2	1	0	0	3
	Proceps acicularis Mulsant & Rey	1	0	0	0	1

Family and subfamily		Kfarkela		Feghal		Total
	species	Malaise trap	Sticky traps	Malaise trap	sticky traps	
	Psammotettix gr. Provincialis (Ribaut)	9	8	0	0	17
	Recilia schmidtgeni (Wagner)	3	6	4	1	14
	Synophropsis lauri (Horvath)	0	0	24	17	41
	Thamnotettix agilis	0	1	0	0	1
	Thamnotettix seclusus Linnavuori	1	4	7	8	20
	Thamnotettix wittmeri Dlabola	12	8	40	25	85
Sub. Hecalinae	*Hecalus glaucescens* (Fieber)	1	0	0	0	1
	Hecalus eximius Kirschbaum	2	0	3	0	5
sub. Iassinae	*Batracomorphus irroratus* Lewis	0	1	0	0	1
Sub. Idiocerinae	*Balcanocerus balcanicus* Horvat	0	0	0	2	2
	Balcanocerus ramallahicus (Dlabola)	2	0	1	11	14
	Hespericerus brusinae (Horvath)	0	3	0	43	46
Sub. Mego-phthal-minae	*Megophthalmus scabripennis* Edwards	11	2037	5	2	2055
Sub. Typhlocibinae	*Asymmetrasca decedens* (Paoli)	121	6264	992	2677	10054
	Edwardsiana rosae (Linnaeus)	32	1	2	5	40
	Empoasca decipiens Paoli	14	117	1	24	156
	Empoasca solani Delong	1	1	0	0	2
	Eupteryx gyaurdagica Dlabola	10	22	2	0	34
	Eupteryx stachydearum (Hardy)	13	17	1	0	31
	Eurhadina angulata Linnavuori	0	1	0	9	10

Family and subfamily		Kfarkela		Feghal		Total
	species	Malaise trap	Sticky traps	Malaise trap	sticky traps	
	Ficocyba ficaria (Horvath)	2	8	5	2	17
	Frutioidia bisignata (Mulsant & Rey)	59	20	17	16	112
	Frutioidia divina Logvinenko	12	2	9	0	23
	Hauptidia ecbalii Linnavuori	29	46	21	56	152
	Lindbergina cretica Asche	3	1	2	4	10
	Typhlocybinae unID	101	328	112	157	698
	Zygina gr. Flammigera (Fourcroy)	27	245	259	147	678
	Zygina sp.	82	161	85	77	405
	Zyginidia sohrab Zachvatkin	23	19	848	126	1016
Other families						
Aphrophoridae	*Mesoptyelus impictifrons* Horvath	2	7	9	7	25
Cercopidae	*Cercopis intermedia* Walker	0	2	0	0	2
Delphacidae	*Delphacidae* unID	31	35	5	1	72
Dictiopharidae	*dictiopharidae* unID	0	0	1	0	1
Issidae	*Issidae* unID	6	1	2	2	11
	Issus abdulnouri Dlabola	0	0	1	1	2

5.5.2 Cixiidae

a. On traps

Among the Cixiidae, 88 specimens were collected in Feghal and 26 in Sarada in 2010, as reported in table 23.

In Feghal the highest insect number was observed in April-May (1/04/2010 - 3/05/2010), in July (29/06/2010 – 20/07/2010) and in October (14/10/2010 – 1/11/2010), whereas in Sarada there is a first, weak pick in May and a second, more evident, in October (14/10/2010 – 1/11/2010) (fig. 81).

The collected Cixiidae belong to 3 different genera, *Cixius*, *Tachycixius* and *Hyalesthes*.

At least one new species belonging to the genus *Cixius* and 4 new species belonging to the genus of *Tachycixius* were observed and identified for the first time at the Museum für Naturkunde of Berlin.

All the insects belonging to the genus *Hyalesthes* were identified as *Hyalesthes obsoletus* Signoret.

Since the identification mainly rely on the male genitalia, all the female specimens were not identified at species level and were indicated as "sp.".

In 2011, the specimens collected in the orchards of Feghal and Kfarkela since April till September were respectively 75 (57in the Malaise trap and 18 in the sticky traps) and 58 (29 in the malaise traps and the other 29 in the sticky traps) (tab. 24).

It is possible to recognise the presence of two picks in both the localities: in Feghal a first pick from the end of April and the beginning of May, and a second pick during the month of August (08/08/2011 – 22/08/2011), whereas in Kfarkela the first pick appeared at the end of May (16/05/2011 – 30/05/2011) and the second pick started at the beginning of September (22/08/2011 – 06/09/2011) (fig. 82).

In 2010 the pick periods in Feghal and in Sarada were quite contemporaneous. Even if the southern regions are characterised by a warmer climate, in comparison with the weather data of the coastal regions of Feghal, the expected early development of the insects in this areas didn't happened. In 2011, the picks of Cixiidae presence in the orchards in Kfarkela were quite delayed instead.

Table 23. Cixiidae collected by Malaise and yellow sticky traps in Feghal and Sarada, 2010.

Installation date	05/02/2010	18/02/2010	03/03/2010	17/03/2010	01/04/2010	19/04/2010	03/05/2010	19/05/2010	31/05/2010	11/06/2010	29/06/2010	20/07/2010	02/08/2010	17/08/2010	30/08/2010	30/9/2010	14/10/2010	01/11/2010	18/11/2010	08/12/2010	23/12/2010	total
Collecting date	18/02/2010	03/03/2010	17/03/2010	01/04/2010	19/04/2010	03/05/2010	19/05/2010	31/05/2010	11/06/2010	29/06/2010	20/07/2010	02/08/2010	17/08/2010	30/08/2010	16/09/2010	14/10/2010	01/11/2010	18/11/2010	08/12/2010	23/12/2010	01/04/2011	
Feghal	**0**	**1**	**1**	**3**	**6**	**6**	**3**	**0**	**0**	**0**	**11**	**0**	**0**	**1**	**2**	**14**	**29**	**5**	**5**	**0**	**1**	**88**
Sarada	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**2**	**1**	**2**	**10**	**6**	**2**	**0**	**0**	**26**

Fig. 81. Cixiidae collected by Malaise and yellow sticky traps in Feghal and Sarada, 2010.

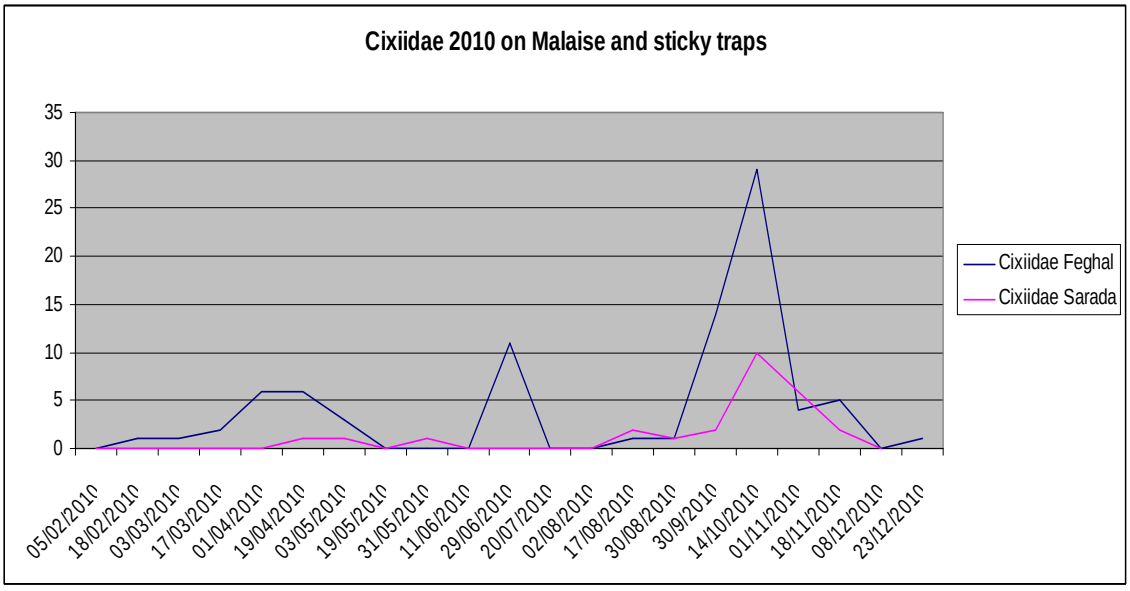
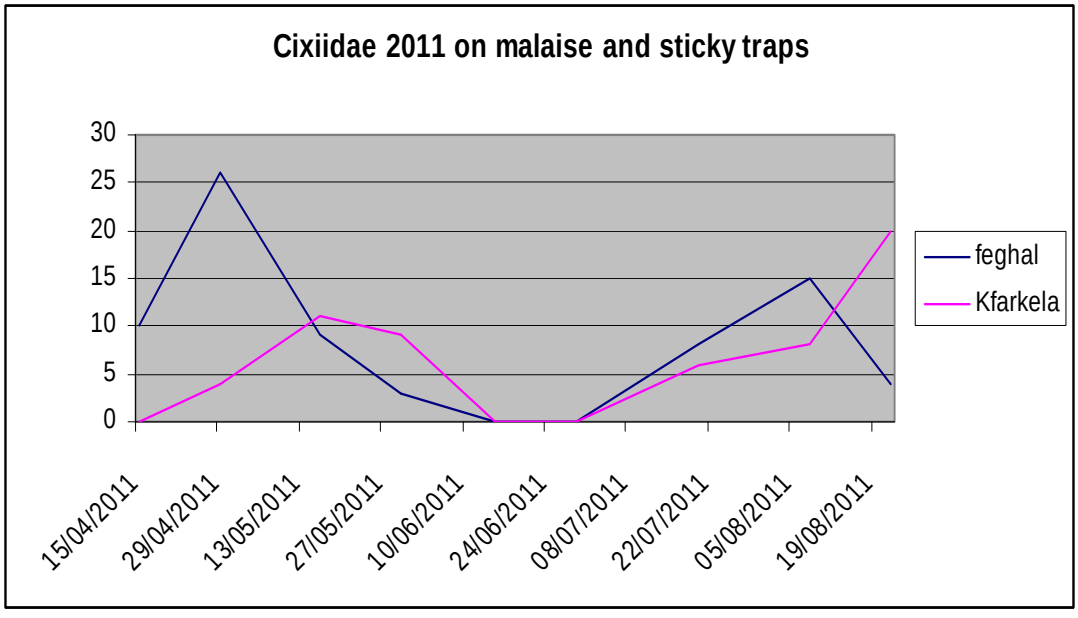


Table 24 Cixiidae collected by Malaise and yellow sticky traps in Feghal and Kfarkela, 2011.

installation date	15/04/2011	29/04/2011	16/05/2011	30/05/2011	15/06/2011	29/06/2011	20/07/2011	08/08/2011	22/08/2011	Total
collection date	29/04/2011	16/05/2011	30/05/2011	15/06/2011	29/06/2011	20/07/2011	08/08/2011	22/08/2011	06/09/2011	
Feghal	**10**	**26**	**9**	**3**	**0**	**0**	**8**	**15**	**4**	**75**
Kfarkela	**0**	**4**	**11**	**9**	**0**	**0**	**6**	**8**	**20**	**58**

Fig 82. Cixiidae collected by Malaise and yellow sticky traps in Feghal and Kfarkela, 2011.



b. In field

Cixiidae field collection is hampered by the total lack of information about the natural habitat of these insects in Lebanon, and their life cycle.

Only one specimen of Cixiidae belonging to the genus *Tachycixius* was found in March 2010, on nectarine trees, in the infected orchard of Wadi Khansa (Marjayoun) (table 25).

In 2011, during the field monitoring carried out in June two cixiids specimens, belonging to the species *H. obsoletus* , were found in the key-orchard of Feghal, on olive trees and one female belonging to the genus *Reptalus* was found in Becharre (table 25).

In September, no insects were found neither in the key orchards nor in the regions of Becharre, Laqlouk and Kartaba.

Table 25. Cixiidae specimens collected during the field visits in March 2010 and June 2011.

Locality	Sweep net collection – Cixiidae	
Wadi Khansa (Marjayoun)	**On nectarine**	30/03/2010
	Tachycixius sp.	1
Feghal	**On olive trees**	07/06/2011
	Hyalesthes obsoletus Signoret	2
Becharre	**On weeds**	09/06/2011
	Reptalus sp.	1

5.5.3 Psylloidea

a. On traps

Within the superfamily Psylloidea, only few specimens were collected in 2010 in the Malaise trap vessels and subsequently identified (Table 26). In the yellow sticky traps no specimens were found.

The most abundant species in both localities was *Cacopsylla myrthi* Puton, with 13 adults in Sarada and 26 in Feghal, collected in the middle of March. Afterwards no other specimens were captured.

In 2011, since their presence in the orchards was very rare and the preliminary results on PCR amplification have given all negative results, psyllids were not monitored anymore.

Table 26. Psylloidea collected on 2010 in the orchards of Feghal and Sarada (Malaise trap).

Locality		M	F
Sarada	**Malaise traps**	**03/03/2010 - 17/03/2010**	
	Acizzia hollisi Burckhardt	1	0
	Agonoscena cisti (Puton)	2	0
	Arytainilla cytisi (Puton)	0	1
	Cacopsylla myrthi (Puton)	6	7
Feghal	**Malaise traps**	**03/03/2010 - 17/03/2010**	
	Cacopsylla pulchella (Löw)	0	1
	Cacopsylla myrthi (Puton)	0	1
	Cacopsylla cfr. hippophaes labelled as *C. melanoneura (*then *C. Myrthi)*	16	9

b. In field

During field collecting, 35 specimens of Psylloidea were collected, as reported in detail on table 27.

The most abundant, *Arytainilla cytisi* Puton, were found exclusively on *Calycotoma* sp., a broom typical of the Lebanese landscape that was blooming during the collecting period.

Table 27. Psylloidea specimens collected during the field visits in March 2010.

Locality	Sweep net collection – Psylloidea	M	F
Barbara (near Feghal)	**on weeds**	24/03/2010	
	Livilla spectabilis (Flor)	1	1
	Arytainilla cytisi (Puton)	3	7
Ouyoun el Ghazlen (Caza of Akkar, North Lebanon)	**on *Calycotoma* sp.**	27/03/2010	
	Arytainilla cytisi (Puton)	4	5
Wadi Khansa (Marjayoun)	**on nectarine**	30/03/2010	
	Cacopsylla cfr. Hippophaes	1	1
Sarada (Marjayoun)	**on peach tree**	30/03/2010	
	Cacopsylla myrthi (Puton)	1	0
	Trioza urticae (Linnaeus)	0	1
	on weeds	30/03/2010	
	Cacopsylla bidens (Sulc)	0	1
	Cacopsylla myrthi (Puton)	1	8

5.6 Phytoplasma identification in insect samples

Some specimens belonging to the new species were not processed through molecular analysis, in order to keep some insects for the identification and description of the species.

5.6.1 Cixiidae

The molecular analyses were performed on the Cixiidae specimens collected on Malaise trap in 2010. 43 specimens collected in the Malaise trap in Feghal and 21 collected in the orchard of Sarada were tested using both the nested PCR with P1/P7 and F2n/R2 primer pairs and the specific AlmWF2/R2 primer pair.

Five Cixiidae from Feghal tested positive for both the amplification; 5 tested positives only with the nested PCR and 8 with the specific primers. Among the insects collected in Sarada, one tested positive for the amplification of the 16S gene whereas 3 tested positive for the specific AlW F2/R2 amplification (tables 28 and 29).

5.6.2 Psyllidae

Thirty-four specimens of *C. myrthi*, collected from the Malaise traps in the orchards of Feghal and Sarada and 19 collected on fields with sweep nets were analysed in order to find the presence of '*Candidatus* Phytoplasma phoenicium' in their body.

All the specimens gave negative results on PCR amplification, using both nested and direct PCR, respectively using the P1/P7 and F2n/R2 primer pairs and the specific AlmWF2/R2 primer pair (Tables 30 and 31).

Table 28. PCR results of the Cixiidae specimens collected on Malaise trap in Feghal, 2010.

Locality	Species	collection on Malaise Trap	male / female	F2n/R2	ALW F2/ALW R2
Feghal	*Cixius* sp. n. 1	1-14/04/2010	1m	+	+
	Cixius sp. n. 1	1-14/04/2010	1m	+/-	+
	Cixius sp. n. 1	1-14/04/2010	1f	-	-
	Cixius sp. n. 1	1-14/04/2010	1f	+	+
	Cixius sp. n. 1	1-14/04/2010	1m	+/-	-
	Tachycixius sp. n. 2	1-14/04/2010	1f	+	-
	Tachycixius sp. n. 2	1-14/04/2010	1m	+	-
	Tachycixius sp. n. 2	1-14/04/2010	1m	+/-	-
	Cixius sp. n. 1	01/04/2010	1f	-	-
	Tachycixius sp. n. 2	19/05/2010	1m	-	-
	Tachycixius sp.	30/09-14/10/10	1f	-	+
	Tachycixius sp.	30/09-14/10/10	1f	+	+
	Tachycixius sp.	30/09-14/10/10	5f	-	-

Locality	Species	collection on Malaise Trap	male / female	F2n/R2	ALW F2/ALW R2
	Tachycixius sp.	30/09-14/10/10	1f	+	-
	Tachycixius sp.	14/10-01/11/10	11 f	-	-
	Tachycixius sp.	14/10-01/11/10	1f	-	+/-
	Tachycixius sp.	14/10-01/11/10	1f	-	+
	Cixius sp.n.1	14/10-01/11/10	3f	-	+
	Cixius sp.n.1	14/10-01/11/10	1f	-	-
	Tachycixius sp.n.6	01/11 - 18/12/2010	2 m	-	-
	Tachycixius sp.	01/11 - 18/12/2010	1f	-	-
	Cixius sp.1	18/11 - 08/12/2010	1f	+	+
	Cixius sp.1	18/11 - 08/12/2010	1f	-	+
	Cixius sp.1	18/11 - 08/12/2010	1m	-	+
	Tachycixius sp.n.6	18/11 - 08/12/2010	1m	-	-
	Tachycixius sp.n.6	08/12 - 23/12/2010	1m	-	-

Table 29. PCR results of the Cixiidae specimens collected on Malaise trap in Sarada, 2010.

Locality	Species	collection on Malaise Trap	male / female	F2n/R2	ALW F2/ALW R2
Sarada	*H. obsoletus*	28/09 - 20/10/2010	1m	-	-
	Tachycixius sp.	28/09 - 20/10/2010	4 f	-	-
	Tachycixius sp.n.4	28/09 - 20/10/2010	1m	-	-
	Tachycixius sp.n.6	28/09 - 20/10/2010	3 m	-	-
	H. obsoletus	02/11 - 18/11/2010	1f	-	+
	Tachycixius sp.	02/11 - 18/11/2010	2 f	-	-
	Tachycixius sp.	02/11 - 18/11/2010	1f	-	+
	Tachycixius sp.n.3	02/11 - 18/11/2010	1m	-	+
	Tachycixius sp.n.3	02/11 - 18/11/2010	1m	-	-
	Tachycixius sp.n.6	02/11 - 18/11/2010	1m	-	-
	H. obsoletus	September 2010	1m	-	n.t.
	H. obsoletus	September 2010	1m	-	n.t.
	H. obsoletus	September 2010	1m	-	-
	H. obsoletus	September 2010	1m	+	-
	Cixius (*Orinocixius*)	September 2010	1f	-	-

Table 30. PCR results of the Psyllidae specimens collected on Malaise trap in Feghal and Sarada in 2010.

Locality		M	F	F2n/R2	ALW F2/ALW R2
Sarada	**Malaise traps**	18/03/2010			
	Cacopsylla myrthi (Puton)	4		-	-
	C. myrthi		3	-	-
	C. myrthi	2		-	-
	Cacopsylla myrthi (Puton)		4	-	-
Feghal	**Malaise traps**	17/03/2010			
	C. myrthi		3	-	-
	C. myrthi		3	-	-
	C. myrthi	1	2	-	-
	C. myrthi	3		-	-
	C. myrthi	3		-	-
	C. myrthi	3		-	-
	C. myrthi	3		-	-

Table 31. PCR results of the Psyllidae specimens collected with sweep nets in Barbara, Oyoun el Ghazlen and Sarada in March 2010.

Locality	Sweep net collection	M	F	F2n/R2	ALW F2/ALW R2
Barbara (near Feghal)	**on weeds**	**24/03/2010**			
	Arytainilla cytisi Puton	3		-	-
	A. cytisi Puton		3	-	-
	A. cytisi Puton		2	-	-
Ouyoun el Ghazlen (Caza of Akkar, North Lebanon)	**on Calicotoma**	**27/03/2010**			
	Arytainilla cytisi Puton	1	2	-	-
	Arytainilla cytisi Puton		3	-	-
Sarada (Marjayoun)	**on weeds**	**30/03/2010**			
	Cacopsylla myrthi (Puton)		3	-	-
	Cacopsylla myrthi (Puton)		2	-	-

5.7 **Extension services**

Since molecular analysis confirmed the '*Ca.* P. phoenicium' presence in samples collected from apparently diseased trees, the symptoms observed on the selected plants were considered as the reference symptoms for the Almond witches'-broom disease.

Other symptoms, as silver or yellow leaves, branch and shoot shrivelling, atypical bud proliferation, reduction of the lamina surface were attributed to other physiological, chemicals or phytopathological problems, since the molecular assays did not confirm the infection by '*Ca.* P. phoenicium'.

Both the symptom description and numerous pictures of each morphological alteration observed on the diseased trees were presented in a booklet which included the symptom evolution on different organs (buds, flowers, branches, fruits) during the period most suitable in order to recognize the disease and a few information about the disease management, as the use of certified trees, when a new orchard is planted, and the elimination of the infected trees, if present in a reduced percentage in the orchard. A poster which summarized the most important information about the disease was also prepared.

The booklet and the poster were distributed to both agricultural technicians and stone fruit producers in different moments:

1) during field monitoring in 2009, 2010 and 2011 at farmer meetings usually organized before the beginning of the orchard survey;

2) during the training organized to inform Lebanese technicians about the disease (Jihad el Binaa Cooperative, Municipality of Kab Elias, LARI – department of Akkar staff)

3) during the meetings with technicians and farmers at the end of the survey in all the Lebanese districts

4) during the Ministry of Agriculture conferences about stone fruit diseases

5) The farmers were provided with a dedicated phone number that they could call in order to give and receive information about the disease and to require a monitoring visit to their orchards.

The booklet and the poster were also presented to the Lebanese Ministry of Agriculture and the FAO offices.

Moreover 10 farmer meetings were organized in 9 districts (Akkar, Baalbeck, Batroun, West Bekaa, Hasbaya, Jbeil, Jezzine; Rachaya and Zahle) at the end of the national survey in order to show to the 230 participants the map of the disease diffusion and to present the preliminary control measures.

All the prepared material is added as Annex 2 (publication of AlmWB symptoms) and Annex 3 (distribution of AlmWB disease in Lebanon).

Lebanese sweets made of pistachios and almonds.

6. DISCUSSION AND PERSPECTIVES

Disease observation and identification

The description of symptoms evolution in infected peach and nectarine trees was based on observations performed during an entire vegetative season on these two new hosts of Almond witches'-broom.

Even if the presence of witches'-broom is more common in almond trees than in peach/nectarine, the most important difference between the symptoms observed on these different hosts are the phyllody, never recorded so far on almond. Moreover, the disease seems to lead the almond trees to a fast death (maximum 4 years after the first symptom appearance), whereas infected peach/nectarine trees can survive longer in the orchards.

Phyllody and flower malformation appear usually in April/May and are easy to recognize on field. In the contrary, over the season, farmers can observe shoot proliferation or light green leaf development, but they normally do not associate such a symptom with a disease that cannot be controlled by using pesticides. In fact the farmers frequently treat the phytoplasma infected trees with numerous active substances such as fosetyl – aluminium, copper or winter oil which are totally ineffective against the causal agent of AlmWB.

The pictures and the description contained in the booklets or reported in the posters, which show to the farmer the disease symptoms, provided the information for an early and fast diagnosis on field. This material is, in the meantime, very useful also for updating the knowledge of Lebanese technicians, nurserymen and engineers.

In fact, knowledge and awareness are generally considered prerequisites to the adoption of new technologies in agriculture, including IPM (Rogers, 1995) and are strongly needed for a conscious management of pests and diseases.

Both participatory approaches and farmer field schools have been used to develop science based knowledge and to increase farmer knowledge and awareness of IPM and impact evaluations have shown that such approaches can significantly improve farmers' knowledge (WDR, 2008; Rola *et al*., 2002, Hashemia *et al*., 2008).

The accurate description of the symptoms of a new or unknown disease provides a sound basis for locating the infected trees during the most suitable period to monitor the orchards. Effective disease control depends primarily on early, accurate identification of the disease and its causal agents.

Since Almond Witches'-broom is spreading in Lebanon and it is not still clearly known by the growers, quick and reliable identification of the infected trees is necessary to plan adequate strategies for the disease containment. When such information lacks excessive number of insecticide treatments are applied, often suggested by technicians or

agriculture engineers. The impact on environment is detrimental and, often, such arbitrary practises facilitated the disease spread when infected plants are left on the fields instead.

Disease severity

The data collected about the AlmWB spread in the Country showed the progressive extent of the disease in the Lebanese regions. Certainly the time allowed the disease spreading in the almond growing regions very fast, together with the lack of any action taken by the farmers or by the local/national offices of the Ministry of Agriculture in order to stop the disease, producing a devastating impact on the economy of the almond producers. The disease, after at least a decade of years, has reached very high value of frequency of infection and severity in the almond orchards, e.g. reaching the 96% in the Koura district, entirely cultivated with almonds.

The severity of the disease in the almond orchards, observed in the Batroun district, chosen as representative of the almond growing regions, reaches 67.5%. On the contrary, the disease presence in the nectarine and peach orchards seems to be considerably lower than the rate in the almond orchards. In fact, in the Marjayoun district, that can be representative of the national situation, in the regions where only peach and nectarine are affected by the disease, the index of infection has been measured at 8.5%. In this region the disease was never observed in almond trees.

Interestingly, in the Rachaya district, where for the first time during the survey the disease has been observed both in almond and peach/nectarine orchards, the index of infection changes: we observed a high index of infection in almond, about 53%, but also a high index in peach/nectarine orchards: 24%.

The difference between the two situations (almond vs peach/nectarine) may be due to the host plant, if peach/nectarine trees are less adapted as a phytoplasma hosts. Other explication can come from differences in the orchards environment that can contribute to the features of the pathogen, or can influence the presence and the behavior of the insect vector(s).

Phytoplasma characterization

Despite the differences in symptom expression and in the infection severity, the molecular analyses performed on the samples collected from almond, peach and nectarine infected trees showed a high genetic homogeneity of the phytoplasma strains and therefore ruled out the possibility of the existence of two different host-specific pathogens.

On the other hand, '*Ca.* Phytoplasma phoenicium' populations showed a certain internal variability. In fact, the molecular characterization of representative phytoplasma

strains allowed the identification of two new 16SrIX subgroups (IX-F and IX-G). Such evidences opened new opportunities for in-depth studies of the distribution of '*Ca*. P. phoenicium' subgroups (16SrIX-B, -D, -F, -G) in weeds, insect vector populations, and plant hosts from orchards located in different geographic areas in order to investigate possible differences in biological properties among AlmWB phytoplasma strains.

The application of automated virtual restriction analysis should facilitate such studies as in previous works. For example, a high degree of genetic heterogeneity among 'bois noir' (BN) phytoplasma strains infecting *Vitis vinifera* L. in Italy was described through automated virtual RFLP analysis of 16S rDNA (Quaglino *et al.*, 2009).

Regional subgroup distribution

Until this study, two 16Sr subgroups, IX-B and -D, were described within '*Ca.* Phytoplasma phoenicium' (Verdin *et al.*, 2003, Abou-Jawdah *et al.*, 2003). Such subgroups were isolated from Northern Lebanon and the Bekaa valley. In the present study, two new 16SrIX subgroups were found, the IX-F and IX-G. In details, the strains of subgroup IX-F were found only in nectarine plants, while the strains of subgroup IX-G were identified in almond, nectarine, and peach plants. Co-presence of '*Ca*. P. phoenicium' strains of diverse 16SrIX subgroups was found in individual orchards both in the North and in the South of Lebanon (i.e., orchards No. 1, 7, 8, 9, and 11, Table 18). In particular, three 16SrIX subgroups (IX-D, IX-F, and IX-G) co-exist and infect nectarine plants in Sarada regions (South Lebanon). On the other hand, peaches and nectarines in the Bekaa valley were infected only by subgroup 16SrIX-D. The result is interesting because in the North and in the South of Lebanon, where geographical and agricultural features are quite homogeneous, AlmWB phytoplasma populations showed a higher genetic diversity than in comparison with those identified in the Bekaa valley, a wide and intensively cultivated plain where different geo-ecological niches coexist. On the basis of these results, it is not possible to clearly determine a significant relationship between the AlmWB phytoplasma subgroups and the plant hosts, as well as between the phytoplasma subgroups and the geographic origins, except for the Bekaa Valley, where only the phytoplasma subgroup 16SrIX-D were identified.

This preliminary information needs to be confirmed increasing the number of plant samples and accessions considered. Investigation of whether particular '*Ca*. P. phoenicium' subgroup(s) (i) are correlated with symptom severity, (ii) are associated with specific plant hosts, (iii) are specifically transmitted by insect vector(s), (iv) are present in Lebanese regions and in neighboring countries, must be carried out in order to provide further valuable information on epidemiology of AlmWB.

The Phytoplasma in the Middle East area

Apart from Lebanon, '*Ca.* Phytoplasma phoenicium' has been signaled also in Iran (Salehi *et al.*, 2006). Further studies revealed the presence of '*Ca*. Phytoplasma aurantifolia' associated with "Almond brooming" disease in Iran (Salehi *et al.*, 2009). '*Ca*. Phytoplasma aurantifolia' and '*Ca.* Phytoplasma phoenicium' induce clearly distinct symptoms on the same plant host.

According to the little information given by the scientific literature (Salehi *et al.,* 2009; Verdin *et al.,* 2004; Zirak *et al.*, 2009) AlmWB appeared in both the Countries in the same period and, due to the distance between Lebanon and Iran, it is unlikely that the transmission took place through insect vectors. Unfortunately, no information is available on the disease diffusion in the two countries located between Lebanon and Iran, namely Syria, where almond are intensively cultivated, and Iraq. It seems more likely that humans played a role in the disease diffusion, even if trades of plants or seedlings from Lebanon and Iran are not reported. However, as underlined also by Verdin and colleagues (2004), the disease might be present and spreading in additional areas of the Middle East.

Virtual RFLP-based molecular characterization and phylogenetic analyses performed in this study demonstrated divergence between Lebanese (subgroups 16SrIX-B, -D, -F, -G) and Iranian (subgroup 16SrIX-C) '*Ca*. P. phoenicium' strains, both associated with AlmWB. This genetic diversity among '*Ca*. P. phoenicium' strains suggests possible influence of different ecological and/or climatic niches on phytoplasma population composition. This hypothesis was suggested by Cai and co-workers (2008), who found out that genetic heterogeneity among cactus witches'-broom (CaWB) phytoplasma strains in China was correlated to environmental conditions.

The insect vector(s)

Over two years, numerous insect species were collected in two regions (Feghal and Marjayoun), chosen for their incidence of AlmWB. The region of Feghal, in northern Lebanon, was one of the first regions affected by the disease. Farmers told that the disease came from the Northern regions, from the Syrian border until Feghal, where its diffusion ended. In fact, this area represents a border, in the coastal area, between the endemic regions and the healthy ones. The region of Marjayoun, where the Sarada and Kfarkela key-orchards are located, is the region where, on 2008, for the first time the AlmWB symptoms were observed in nectarine orchards. The plain of Marjayoun is directly adjacent to the Israeli territory, where stone fruits are likewise cultivated. Until now, no information is available about the disease presence in Israel, even if it is likely that the vector(s) can easily cross the borders and spread in both Countries.

Which insect species is/are involved in the '*Ca*. P. phoenicium' transmission is still unknown, but the data collected are useful to narrow next searches.

The trap collecting of Cicadellidae, Cixiidae and Psyllidae over two seasons was useful as a first screening among all the species present in the Lebanese orchards. A wide identification of the Lebanese Cicadellidae specimens has been already performed by Abdul-Nour (1986; 1987; 1988; 2001), but not focused on phytoplasma transmission and/or stone fruit orchard biodiversity; moreover, a first screening of Cicadellidae as possible vector of '*Ca.* Phytoplasma phoenicium' using molecular tools was carried out by Dakhil and colleagues (2011).

The present work confirms the data about the wide range of Cicadellidae species present in the stone fruit orchards and was focused on molecular investigation on Cixiidae and Psyllidae species, never studied before as possible vectors of '*Ca*. P. phoenicium'.

It is well known that the relationship of species incidence and abundance with the incidence of disease can provide clues to the identity of vector species (Weintraub *et al*., 2006). From this point of view, the results obtained by Dakhil and colleagues (2011) in Lebanon, specifically on *A. decedens*, are interesting because the present study confirms that *A. decedens* was one of the most abundant species found in both the localities.

As far as the other 8 Cicadellidae species found positive to '*Ca*. P. phoenicium' by Dakhil and colleagues (2011) are concerned, the presence of these insects in the orchards during our researches was so rare that it is an open question how they could be involved in the pathogen transmission. In the table below (table 32), the total number of collected individuals of such insect species are reported. It is evident the difference between the population of *A. decedens* and the other eight species. It is clear the difference between the population of *A. decedens* and the other eight species, in both the localities. Moreover, a certain difference in the presence of some species in the almond or the peach/nectarine orchards has been evidenced, as for *Empoasca decipiens* or *Euscelidius mundus*, more present in the almond orchards than in the nectarine one, or *Laylatina inexpectata,* more present (during the second year of collecting) in the nectarine orchard in Kfarkela. All these aspects must be deeper investigated, in order to find their roles and implication in the '*Ca*. P. phoenicium' epidemiology.

However, also the number of Cixiidae collected in the orchards over the two years of search is quite small, and their role in vectoring the phytoplasma is still doubtful. In fact, even if the molecular analysis performed on Cixiidae evidenced that certain insect species were infected by AlmWB phytoplasma, only transmission experiments will provide the definitive evidence of phytoplasma-vectoring capacity, as previously reported for other insect vectors (Tedeschi *et al*., 2002).

Table 32. Number of specimens belonging to the nine potential Cicadellidae vectors of '*Ca*. P. phoenicium' (Dakhil *et al*., 2011) collected over two years in Feghal, Sarada and Kfarkela, Lebanon.

Species	Total number of adults collected in 2010 (February-December) in Feghal ALMOND.	Total number of adults collected in 2010 (February-December) in Sarada NECTARINE.	Total number of adults collected in 2011 (April – September) in Feghal ALMOND.	Total number of adults collected in 2011 (April – September) in Kfarkela NECTARINE.
Allygus sp.	0	0	0	5
A. danutae	0	0	20	5
A. decedens	1,137	12,489	3,670	6,385
Bachlutha sp.	1	2	3	3
Em. Decipiens	4	36	25	131
Eu. Mundus	21	0	28	11
F. macchiae	3	2	15	1
L. inexpectata	1	0	4	68
T. seclusus	6	0	15	5

During the present study, it was difficult to find a sufficient number of adults in the orchards, as well as in the highest regions of Mount Lebanon. In fact, neither during spring time, nor during the autumn, where according to the trap data the insects were particularly abundant in the fields, only 2-3 specimens were collected. The reduced number of insects caught in the field prevents the execution of the transmission assays, where a certain number of adults is required. Knowing that an abundant population of insect vector of phytoplasma is usually found near or on the host plant, demonstrating that the transmission of pathogens by insects depends on the abundance of the vector(s) and their interplant movement in the orchards (Irwin and Ruesink, 1986; Power, 1987, 1992), the research of the '*Ca*. P. phoenicium' vector(s) still needs a lot of information.

Moreover, Cixiidae field collection was hampered by the total lack of information about the natural habitat of these insects and their life cycle in Lebanon, or in the neighbouring regions. So far, information is available on the planthoppers vectors of grapevine yellows in the Israeli vineyards. According to these studies, *Hyalesthes obsoletus* Signoret, in vineyard, has two generation per year. The overwintering nymphs emerge around the end of May, and adults can be captured until the first part of June. Eggs are

laid, and the summer generation lasts from about mid-June until the middle of September when adults are again captured on sticky traps. This summer generation develops much more quickly than the winter generation (about twice as fast) and is much larger (Klein *et al*, 2001). Orenstein and co-workers (2003), on the other hand, found out the first adults only at the beginning of September. Later investigations found out that *Vitex agnus-castus* L., a shrub belonging to the *Verbenaceae,* is a preferred host plant for *H. obsoletus* (Sharon *et al*., 2005) even if *Olea europaea* L. also served as a host for adults. In Lebanon, the presence of *V. agnus-castus* is very rare, mostly in the nearby of the cultivated areas, and there is a complete absence of other information about the planthoppers in general.

Even if the planthopper presence in traps and in orchards is very low, they can be very efficient vectors, transmitting phytoplasmas when, occasionally, they are carried by the wind in the orchards. They seem do not feed preferentially on almond and nectarines, so maybe it is necessary to focus on other possible host plants, such as weeds within and around the crop area, as reported for many phytoplasma diseases such as those associated with Ca. Phytoplasma asteris or stolbur phytoplasmas. If confirmed, almond and probably peach species might be considered as dead-end hosts of the disease. Dead-end hosts, in fact, are defined as plants that can be inoculated and subsequently become infected with phytoplasma, but from which insects cannot acquire phytoplasma (Weintraub, 2006). Several other dead-end hosts have been identified, e.g. *Cyclamen persicum* L. for Aster Yellow (AY) phytoplasma (Alma *et al*., 2000) or grapevine for stolbur (Stol) phytoplasma associated with bois noir grapevine yellows (GY), transmitted by the planthopper *Hyalesthes obsoletus* (E. Boudon-Padieu and M. Maixner, personal communication, in Weintraub and Beanland, 2006). This can explain why the population of Cixiidae is so scarce in the orchards, confirming that these insects are only occasional feeders on stone fruits and live in other preferred niches, on other host plants. The presence of other disease reservoirs, probably asymptomatic, where Cixiidae live and/or from where Cixiidae can acquire the phytoplasma must be identified, as a necessary step for the understanding of AlmWB epidemiology.

Moreover, temperature as well as other climatic factors could strongly influence the life cycle and behavior of the insect vectors (Johannesen *et al.* 2008), consistently altering their host plant feeding preferences and, eventually, selection of '*Ca*. P. phoenicium' strains. These factors could also influence the variety and number of weeds hosting the phytoplasmas in and around orchards.

In this complex system, where numerous factors of this tritrophic relationship are still unknown, it is possible that more than one vector is involved in AlWB transmission. In fact, vegetation composition, habitat diversity, and the nature of ecotones in and near a phytoplasma-vulnerable crop can have important effects on the presence and dispersal of

vectors, their natural enemies, and other insects. The Lebanese environmental conditions where the disease is present, as well as the field distribution of the disease, sometimes just on the border of the orchards, sometimes in the middle of it, could suggest that one species is responsible for the long distance dispersal of the phytoplasma and other specie(s) for the transmission into the fields.

Final considerations

AlmWB phytoplasma is a very dangerous pathogen for almonds, due to the rapid decline of affected trees, to its ability to be rapidly spread by a yet unknown vector, to its high pathogenicity to young and old trees, and to its adaptation to several microclimates, from the coastal region up to an elevation of about 1,000 m. Moreover, it represents a great threat also for peach and nectarine trees, wherever they are grown, from the plains to the highs, in irrigated and non irrigated areas.

In Lebanon, after ten-fifteen years since its appearance, the disease became endemic in the north of the Country and very difficult to manage and control. On the other hand, when farmers are well informed about the disease and its risks, a rapid elimination of the infection *foci* represented the only way to successfully control the disease spread. An important work still needs to be done, in order to inform all farmers about the disease, to improve the role of Lebanese extension services and to support the Ministry of Agriculture in defining the necessary control strategies.

Moreover, even though wild almonds are native to Lebanon and stone-fruit cultivation started over 100 years ago, AlmWB was introduced only recently. Therefore, more strict certification and quarantine measures on the movement of stone fruit germoplasm should be imposed. The rapid increase in the importance of phytoplasma diseases makes it imperative to standardize also the diagnostic techniques that can be used routinely in certification of stone fruits for phytoplasma detection.

In fact, the risk of the disease spread concerns not only Lebanon or Middle East countries, but all the Mediterranean Countries where stone fruits trees are cultivated.

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9. ANNEX 1

Phenological growth stages and BBCH-identification keys of stone fruit. Meier *et al.*, 1994

Stone fruits: cherry = *Prunus cerasus* L., plum = *Prunus domestica* L. ssp. *domestica*, peach = *Prunus persica* Batsch., apricot = *Prunus ameriaca* L.

Code Description

Principal growth stage 0: Sprouting/Bud development

00 Dormancy: leaf buds and the thicker inflorescence buds closed and covered by dark brown scales
01 Beginning of bud swelling (leaf buds); light brown scales visible, scales with light coloured edges
03 End of leaf bud swelling: scales separated, light green bud sections visible
09 Green leaf tips visible: brown scales fallen, buds enclosed by light green scales

Principal growth stage 1: Leaf development

10 First leaves separating: green scales slightly open, leaves emerging
11 First leaves unfolded, axis of developing shoot visible
19 First leaves fully expanded

Principal growth stage 3: Shoot development 1

31 Beginning of shoot growth: axes of developing shoots visible
32 Shoots about 20% of final length
33 Shoots about 30% of final length
3 . Stages continuous till . . .
39 Shoots about 90% of final length

Principal growth stage 5: Inflorescence emergence

51 Inflorescence buds swelling: buds closed, light brown scales visible
53 Bud burst: scales separated, light green bud sections visible
54 Inflorescence enclosed by light green scales, if such scales are formed (not all cultivars)
55 Single flower buds visible (still closed) borne on short stalks, green scales slightly open
56 Flower pedicel elongating; sepals closed; single flowers separating
57 Sepals open: petal tips visible; single flowers with white or pink petals (still closed)
59 Most flowers with petals forming a hollow ball

Principal growth stage 6: Flowering

60 First flowers open
61 Beginning of flowering: about 10% of flowers open
62 About 20% of flowers open
63 About 30% of flowers open
64 About 40% of flowers open
65 Full flowering: at least 50% of flowers open, first petals falling
67 Flowers fading: majority of petals fallen
69 End of flowering: all petals fallen

Principal growth stage 7: Development of fruit

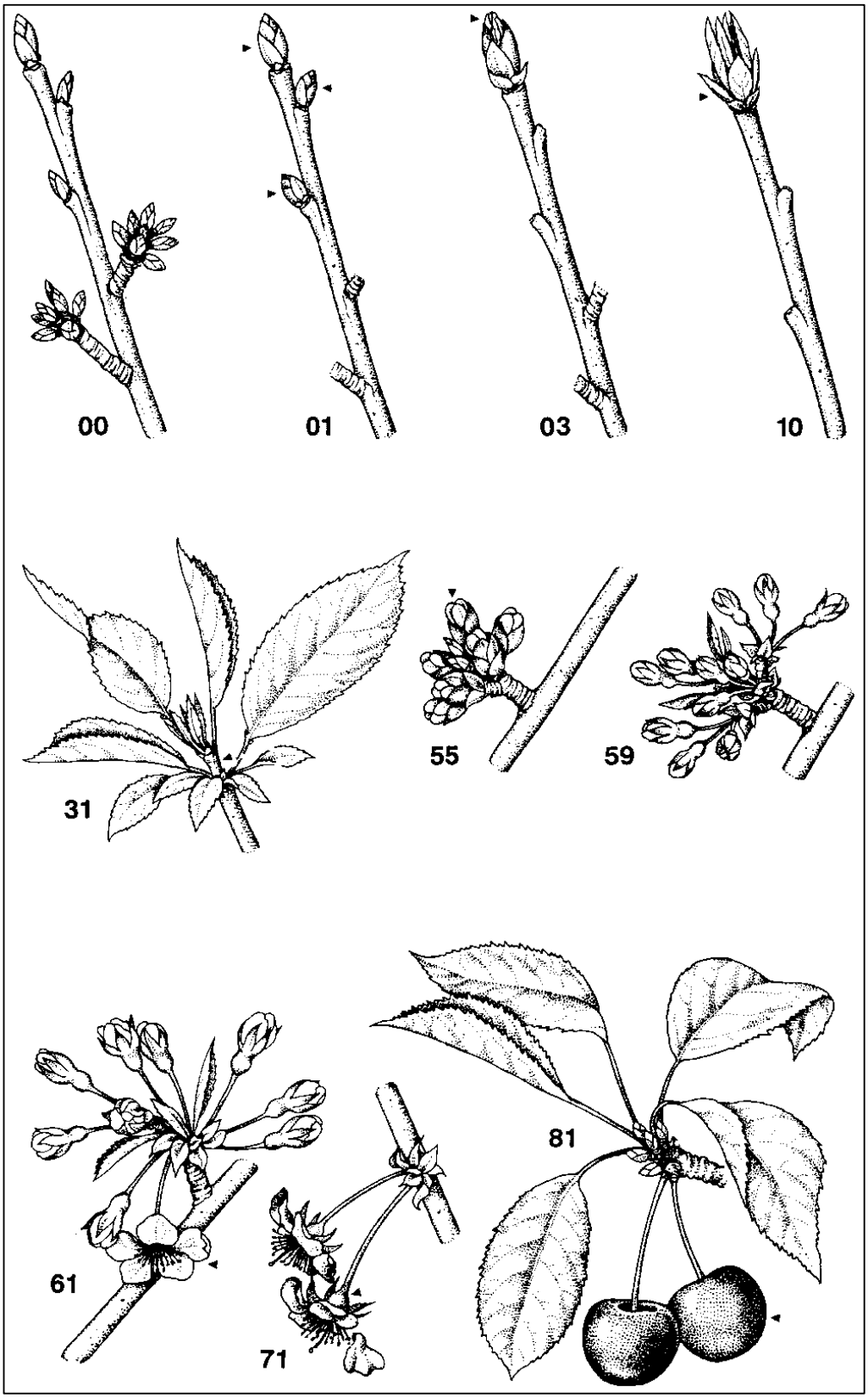
71 Ovary growing; fruit fall after flowering
72 Green ovary surrounded by dying sepal crown, sepals beginning to fall
73 Second fruit fall
75 Fruit about half final size
76 Fruit about 60% of final size
77 Fruit about 70% of final size
78 Fruit about 80% of final size
79 Fruit about 90% of final size

Principal growth stage 8: Maturity of fruit and seed

81 Beginning of fruit colouring
85 Colouring advanced
87 Fruit ripe for picking
89 Fruit ripe for consumption: fruit have typical taste and firmness

Principal growth stage 9: Senescence, beginning of dormancy

91 Shoot growth completed; foliage still fully green
92 Leaves begin to discolour
93 Beginning of leaf fall
95 50% of leaves discoloured or fallen
97 All leaves fallen
99 Harvested product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