Asymmetrasca decedens, a vector of '*Candidatus* Phytoplasma phoenicium' associated with almond witches' broom disease

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Abstract

'*Candidatus* Phytoplasma phoenicium' is associated with a lethal disease of almond, peach and nectarine named almond witches' broom disease. The disease spreads rapidly in Lebanon from the coastal areas to elevations exceeding 1000m killing over 150,000 trees within two decades. The mode of spread suggested the involvement of efficient vector(s). *Asymmetrasca decedens* (~~order~~ Hemiptera ~~and family~~ Cicadellidae) is the most abundant leafhopper species present in Lebanese stonefruit orchards. Living *A. decedens* were collected from fields heavily infested by AlmWB and followed by an inoculation-access period of 30 days on healthy GF-677 and GF-

plant ? plant ?

real-PCR results not shown

305 seedlings using 25 leafhoppers per seedling. PCR analysis supported by sequencing showed that *A. decedens* is a carrier of the phytoplasma, ~~and that the phytoplasma~~ accumulates ~~in the salivary glands~~. Transmission trials in insect-proof cages, showed a transmission efficiency of ?? 32%. One year post-inoculation, '*Ca.* P. phoenicium' was detected in the newly emerged leaves of inoculated seedlings that were not exposed to leafhopper feeding; however, the characteristic symptoms of witches' broom were not observed. All phytoplasma-positive seedlings and *A. decedens* samples showed 99·99% nucleotide identity in their 16S/23S spacer-region and had *in silico* identical RFLP patterns similar to 16SrIX-D. This manuscript represents the first report for a leafhopper ? vector of '*Ca.* P. phoenicium' and shows that the incubation period of the disease is longer than one year. The importance of phytosanitary control measures, the adoption of a national strategy and a regional cooperation in order to contain the further spread of the disease are discussed.

Keywords: phytoplasma, witches' broom, transmission, *A. decedens*, vector

Introduction

In the 1990's a devastating disease on almond trees appeared in Lebanon characterized by proliferation, small yellowish leaves, bushy growth, dieback and appearance of witches' broom on the stems. Infected trees either did not produce any fruits, or produced a limited number of deformed fruits, resulting in practically 100% marketable yield loss. The disease was named almond witches' broom (AlmWB), it spread rapidly and killed about one hundred thousand trees over a period of ten years (Abou-Jawdah *et al*., 2002). The disease was associated with a phytoplasma that belongs to the pigeon pea witches'-broom (PPWB) group (16SrIX)(Abou-see lee et al. 2012

Jawdah *et al.*, 2002). Later on it was designated formally as '*Candidatus* Phytoplasma phoenicium' (Verdin *et al.*, 2003). The 16SrIX- D subgroups are the most predominant in Lebanon followed by subgroups G and F (Molino Lova *et al.*, 2011). More recent surveys identified over 40,000 new trees of almond, peach and nectarine infected with AlmWB (Molino Lova *et al.*, 2011). the same The disease epidemic spread rapidly from coastal areas to high mountainous areas (>1200 m), crossing different ecological niches. In addition, AlmWB spread in properly managed orchards, in neglected orchards and to isolated wild ~~trees~~ growing wild. reference? These observations suggested the presence of one or more efficient aerial vectors.

Phytoplasmas are prokaryotes similar to bacteria, but deprived of a cell wall and their growth is exclusively limited to the plant phloem tissue. Insect transmission is the most important and efficient factor in phytoplasma epidemiology. Only selected species can act as vectors since this involves elements of vector-pathogen-host specificity (Bosco & D'Amelio, 2010). not in references Phytoplasmas are ~~mainly~~ essentially transmitted by phloem-feeding insects ~~which belong to the families Cicadellidea, Cixiidae, Psyllidae, Cerocopidae, Delphacidae, Derbidae, Meenoplidae, and Flatidae in the~~ order ~~Hemiptera (Rojas-Martinez, 1995). The most common vectors are~~ leafhoppers (Cicadellidae), planthoppers (Cixiidae) and psyllids (Psyllidae) (Weintraub & Gross, 2013).For example, in Europe, ~~the~~ two ~~most~~ important fruit tree phytoplasma diseases are transmitted by psyllids (Hemiptera, Psyllidae). ~~Two different *Cacopsylla* species:~~ *Cacopsylla picta* (Förster) and *Cacopsylla melanoneura* (Förster) were reported to transmit '*Ca.* Phytoplasma mali'(16SrX-A), the causal agent of the apple proliferation phytoplasma. While *Cacopsylla pruni* (Scopoli) transmits '*Ca.* Phytoplasma prunorum' (16SrX-B), ~~an~~ the agent of European stone fruit yellows (ESFY).

Field surveys conducted in two AlmWB-infected almond orchards, one located in South Lebanon and the second in the North, *Asymmetrasca decedens* [~~order~~ Hemiptera, ~~family~~ Cicadellidae, ~~and subfamily~~ Typhlocybinae(Allegro *et al.*, 2011)] was the most abundant hemiptera species ~~present;~~ representing over 82% of total leafhoppers caught in sticky yellow traps and in malaise traps (Dakhil *et al.*, 2011). *A. decedens* is a polyphagous species which may feed on a wide variety of economic crops such as peach, almond, citrus, grapevine, beans, beet, cotton, lucerne and potatoes (Jacas *et al.*, 1997). PCR tests conducted using a semi-specific primer pair, which detects phytoplasmas belonging to the 16SrRNA-IX group, showed that *A. decedens* along with ~~six~~ eight other leafhopper species carry 16Sr IX phytoplasma and may be potential vectors (Dakhil *et al.*, 2011). However, phytoplasmas may be acquired by insects but may not be transmitted during feeding (Marzachì *et al.*, 2004). Phytoplasmas are transmitted in a persistent propagative manner (Marzachì *et al.*, 2004). For an insect carrier to become a vector, an intimate association with the phytoplasma is required. The phytoplasma must be able to multiply in the vector, circulate in the hemolymph, accumulate in the salivary glands and be secreted with the saliva upon feeding on plant phloem cells. Such a cycle may take (Hogenhout et al. 2008) (several days even more several months.. For example in the case of C. pruni most transmissions occur only after an effective latency of 8 months (Thébaud et al. 2009)) ~~two to several weeks; for example, in the case of *C. pruni* transmission of the ESFY phytoplasma, the minimum acquisition access period is 2-4 days; the minimum latent period is 2-3 weeks; and the minimum inoculation period is 1-2 days. The retention of infectivity lasts through the winter until the following spring (Carraro *et al.*, 2001~~). Therefore, only appropriate transmission tests will provide the definite evidence of the role of an insect as a vector, while the detection of a phytoplasma in an insect is just considered as a preliminary step. However, controlled transmission tests are not always straight forward; many vectors do not survive easily in captivity. The different life stages may have differences in the efficiency of transmission,

useful !

not in references

→ idea? host symptomless → interest of molecular techniques: give more details

symptom development on the inoculated plants, and the incubation period,(which may take between one week and 18 months). In the case of ESFY it may take 4-5 months and some hosts may remain symptomless (Carraro *et al.* 1998b). Hence, molecular techniques may play an important role in phytoplasma detection in asymptomatic and susceptible hosts during the incubation or latent period (Mehle *et al.*, 2010).

why b?

Not a objective → discussion

The identification of the vector (s) of AlmWB phytoplasma would be of great interest for the development of an integrated management program to contain the further spread of the disease and to reduce its negative impact on the stone fruit industry. The major objective of this work was to investigate the ~~role of *A. decedens* in transmission of AlmWB~~ phytoplasma under controlled experimental conditions.

capacity of A. decedens to transmit AlmWB phytoplasma

almond = Prunus dulcis (Mill.) D.A. Webb
syn. P. amygdalus

Materials and methods

Plant material

Certified tissue culture seedlings of two stone fruit rootstocks were imported from Italy, peach almond hybrid "GF-677" rootstock (*Prunus persica* × *P. amygdalus*) and the peach seedling"GF-305". The seedlings were transplanted into 25 cm diameter pots containing a mixture of potting soil, sand and perlite (2:1:1) and maintained in insect-proof cages, within an insect-proof net house.

Leafhoppers and transmission

Field visits were conducted to stone fruit orchards infected with AlmWB located in the North and South of Lebanon. A special hand-held mechanical aspirator (D-Vac Vacuum Insect Net-Model 122, Rincon-Vitova Insectaries, Ventura, CA, USA) was used to collect insects from

	small cages	bigger cages
GF677	?	?
GF305	?	?

number seedlings?

why 25?

AlmWB-infected trees. The *A. decedens* leafhoppers were sorted out by mouth aspirator and brought to a cold room where they were counted and dispensed into falcon tubes. Transmission trials were initiated in the same day of insect collection. Collected insects were released either in small insect-proof cages containing a single seedling or in bigger cages that can accommodate six seedlings. An average of 25 leafhoppers per seedling was used. The leafhoppers were allowed an inoculation access feeding on stone fruit seedlings (GF-677 and GF-305) for 30 days. Afterwards, the insects were sprayed with insecticides at 5-day intervals (spinosad and acetamiprid, in alternation). A total of 34 seedlings were inoculated in these tests. Two controls were used, healthy seedlings maintained in insect-proof cages and healthy seedlings subjected to feeding by leafhoppers collected from a nectarine orchard in Wata Al Jawz, an AlmWB-free region.

beginning ?

?

Observations on symptom development were recorded at weekly intervals. Leaf samples were collected periodically from inoculated seedlings and tested by polymerase chain reaction (PCR) for the presence of '*Ca.* P. phoenicium'. Samples of leafhoppers that were collected from AlmWB-infested almond orchard or from AlmWB free regions were also tested by PCR. Using a stereoscope, from three batches each consisting of three leafhoppers, the heads were removed from the rest of insect body, the salivary glands were dissected, transferred into a microfuge tube (1.5mL) containing 25µl STE buffer and their DNA extracted separately and used for phytoplasma detection by PCR.

??

all?

→ Why? objective?

Molecular diagnosis

Total Nucleic Acid extraction.

For plant samples, the total nucleic acids (TNA) were extracted from 100mg of leaf midribs following the CTAB protocol as described previously (Abou-Jawdah *et al.*, 2002). For the leafhoppers, groups of five *A. decedens* ?? insects were put in a 1.5 ml Eppendorf tube and the TNAs were extracted according to the procedure described by Marzachi *et al* (2012) references !. The final TNA precipitate was suspended in 50 µl of TE buffer (10mM trisHCl pH 8, 1 mM EDTA) or distilled sterile water. TNA extracts were analyzed in a 1 % agarose gel electrophoresis to determine their quality results. Total DNA were quantified using a NanoDrop results ? 2000c (NanoDrop Technologies, USA) and stored at -20 °C.

Phytoplasma Detection by Polymerase chain reaction

The semi-specific primer pair, ALW-F2/ALW-R2, which amplifies a DNA fragment of 390 bp from 16SrRNA group-IX (16 SrIX) phytoplasmas, was used in PCR assays as described previously (Abou-Jawdah *et al.*, 2003). Each amplification reaction was performed in 20µl reaction mixture containing 2µl of template DNA, 10µl of REDTaq® ReadyMixTM PCR Reaction Mix (Sigma-Aldrich, MO, USA), 0.25µM of each primer and 7µl of sterile water. Amplification was done with a Bio-Rad ThermalCycler 1000 (Bio-Rad Laboratories, Hercules, CA, USA). For selected leafhoppers or inoculated plant samples that were positive with the previous test, another test using nested PCR was performed. as previously described (Abou-Jawdah 2003) ~~In the first run the universal primer pairP1/P7 (Schneider *et al.*, 1995) which amplifies a DNA fragment of about 1800 bp was used. The second run was performed with primer pair R16F2n/R16R2, which amplifies a 1200 bp fragment (Gundersen & Lee, 1996).~~ The PCR products were purified with the Illustra™ GFX PCR DNA and Gel Band Purification kit (GE Healthcare, UK) and cloned using the pGEM-T Easy Vector System II (Promega, USA). Sequencing of PCR products ~~or~~ of the cloned inserts was performed ~~at~~ by in Macrogen ~~sequencing facility~~ (~~Macrogen Inc.~~ Inc., Seoul, Korea). The

insects ⊕
plants ⊕
all have been cloned ? how many ?

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 using the software BlastN (http://www.ncbi.nim.nih.gov/BLAST/)

16S rRNA gene analysis

interest ?

Virtual RFLP analyses of the 16S rRNA gene sequences (1253bp) were performed using the *i*PhyClassifier (Zhao *et al*., 2009). Collective RFLP patterns were based on analysis with 17 restriction enzymes*:* *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. *In silico* restriction digestion and pairwise virtual RFLP pattern comparison were performed. Virtual gel images were generated for the 17 enzymes or the *Taq*I for distinguishing among 16SrIX subgroups. The sequences analyzed included two from insect bodies, two from salivary glands, and four from inoculated seedlings. no more ? why ?

Results

Symptom Development

The transmission trials using leafhoppers were initiated on May 2012, and symptoms were monitored at weekly intervals. Symptoms started to develop on 16 inoculated seedlings within 25 days post-inoculation ? (dpi). By 30dpi, four out of 15 GF-305 seedlings and 12 or 11 ? see line 188 out of 19 GF- 677 seedlings developed symptoms (Table 1). The observed symptoms were not typical of AlmWB phytoplasma; they consisted mainly of downward leaf curling or rolling and proliferation of new growth at the leaf axils. The curled leaves were smaller than normal leaves but were not chlorotic; moreover, many growing tips were burned. Since more or less not correct similar symptoms were still not carriers of phytoplasma ?

observed on some of the control plants which were inoculated with leafhoppers originating from an area free of AlmWB phytoplasma, it was concluded that the symptoms may have been largely induced by leafhopper feeding.

In August, a new flush of growth appeared which looked normal. During winter, the leaves dropped and in early March the new growth looked normal and was similar to that of non-inoculated plants.

Molecular Diagnosis

At 30 and 60 dpi, PCR tests showed that all the inoculated symptomatic plants gave positive results using the AlmWB semi-specific primers, AlwF2/R2. When the new summer flush appeared in August (about three months post-inoculation), the new growth looked normal. New leaf samples were collected and the PCR results showed that only three samples of the GF-305 seedlings were positive out of the four that were positive at 60 days dpi. Similar results were obtained with the GF-677 seedlings where only 8 seedlings tested positive out of the 11 ?12? seedlings that were previously positive. During winter, all the leaves dropped. In the following spring season, new growth emerged which appeared normal. PCR tests were repeated and all the seedlings, whose summer flush tested positive, were also positive with the new spring growth (Table 2, Figure 1). Therefore out of a total of 34 inoculated seedlings only 11 seedlings got infected as revealed by PCR tests about one year post-inoculation; however, none developed AlmWB-associated symptoms.

young ?

not coherent

The leafhoppers collected from Wata Al Jawz, an AlmWB-free area, gave negative PCR results using the semi-specific primer pair, ALW-F2/ALW-R2. Five representative batches of *A. decedens* leafhoppers used in the inoculation tests were tested by PCR and all batches tested

how many have been tested? enough to conclude all leafhoppers at the region are (-) ?

what's means ?

positive (Figure 2). When the salivary glands were dissected and tested by PCR, they were also positive (Figure 2).

Sequences of the amplified products from four samples, one sample each from the insect body (GenBank Accession: KF359551) , the salivary glands (GenBank Accession: KF488577) , the inoculated GF-677 seedlings (GenBank Accession: KF500029) and from GF-305 (GenBank Accession: KF500030) were deposited at the GenBank. Blast analysis showed 99.9% identity with '*Ca*. P. phoenicium'. The virtual RFLP patterns of the sequences derived from eight queries of 16S rDNA F2nR2 fragments were identical (similarity coefficient 1.00) to the reference pattern of 16Sr group IX, subgroup D (GenBank accession: AF515636) using either the 17 restriction enzymes (Fig. 3) or *Taq*I (Fig. 4). Therefore, phytoplasmas present in the insects and in the inoculated seedlings are all similar and members of 16SrIX-D '*Ca*. P. phoenicium'.

interest comparing sequences?

Real-time quantitative PCR (qPCR) results showed that the phytoplasma concentration in the salivary gland per unit of DNA is about double that found in the remaining parts of the body (data not shown).

It's an essential result => authors must describe the M&M and comment the results in details

Discussion

'*Candidatus* Phytoplasma phoenicium' is associated with a devastating and lethal disease of almond, peach and nectarine that has been reported so far only in Lebanon and Iran (Abou Jawdah *et al*, 2002, Verdin *et al*., 2003). '*Ca* P. phoenicium' has all the characteristics of a dreadful quarantine pest. It is a lethal disease of three major stone fruit crops; cannot be controlled by classical control measures, has the potential to occupy different ecological niches, and its unaided transmission across natural barriers seems limited since it has been reported in only two countries. Therefore, the Lebanese authorities took official phytosanitary measures trying to mitigate the further spread of AlmWB disease. The rapid spread of AlmWB in Lebanon suggested the presence of one or more efficient vectors. A previous survey showed that several leafhopper species are carriers and potential vectors of the disease of which ***A. decedens***, being the most dominant leafhopper detected in stone fruit orchards (Dakhil *et al*., 2011). Therefore, ~~the first~~ transmission trials were initiated with this potential vector.

The leafhoppers used in these trials were carriers of '*Ca.* P. phoenicium', as evidenced by PCR tests and sequence analysis. Moreover, '*Ca* P. phoenicium' was detected in the salivary glands of *A. decedens* ~~at relatively higher concentrations than in the remaining parts of the body (data not shown)~~, giving further evidence of a potential role in AlmWB transmission.

yes but it's not a prove

The initial symptoms which were observed one month post-inoculation were not attributed to phytoplasma infection, they were correlated with leafhopper feeding, since leafhoppers feed mainly on leaves, and cause a symptom known as the "hopperburn" (~~Allegro *et al*., 2011~~ Backus et al. 2005). In eastern Spain, a high infestation of *A. decedens* in almond orchards induced stunted shoots with small curled leaves that were only observed on young flush. The damage was mainly destructive to nursery seedlings, young non-bearing trees, and over-grafted plants (Jacas *et al*., 1997).

So 25 was too much. suggestions to reduce this number?

The transmission trials conducted in this study showed that ? 11 out of 34 inoculated stone fruit seedlings got infected with '*Ca.* P. phoenicium', as evidenced by its PCR detection in the new growth that emerged 1, 2 or 3 months p.i. also! one year post-inoculation. The PCR data were confirmed by sequencing of the PCR products and detection of '*Ca.* P. phoenicium' in the inoculated seedlings. Using the iPhyclassifier, the same phytoplasma subgroup 16SrIX-D was detected in the leafhoppers used for phytoplasma transmission and in the inoculated seedlings. The detection of '*Ca.* P.

not all

false! not all the leafhoppers have been tested by PCR.

how it has been estimated?

phoenicium' in the leafhoppers and in the inoculated ? ~~certified~~ seedlings provides a strong evidence for the role of *A. decedens* as a vector of '*Ca.* P. phoenicium'.

Two important features resulting from transmission experiments are discussed: the efficiency of transmission and the long incubation period. First, transmission efficiency of 32% may be considered ? acceptable even if a ? relatively high 10? 100? number of insects were used per seedling. This may be justified by the large numbers of *A. decedens* detected early in the spring season when the new seedling growth was still succulent. In general, vector abundance is documented as a determinant of disease risk (Girod *et al*., 2011). no! see comments This species was the most abundant and 544, 2760 and 3901 insects were collected on six yellow sticky traps during the months of March, April and May 2002, respectively (Dakhil *et al*., 2011).These results were confirmed in a recent survey with a slight difference in timing, whereby 3800, 11,700 and 7200, were trapped in May, June and July 2012 (Abdul-Nour personal communication). In this experiment, several factors may have affected the observed transmission efficiency. The experiment was conducted in insect-proof cages under greenhouse conditions, and a large number of leafhoppers died within two weeks of transfer to the insect-proof cages, suggesting that the survival potential of *A. decedens* under the experimental conditions was limited. Moreover, several attempts failed to rear this leafhopper in insect-proof cages in an effort to study the transmission characteristics, mainly the latency period.

Even though several leafhopper species belonging to the Cicadellidae family and sub-families were reported to transmit phytoplasmas, only one report mentions *Asymmetrasca decedens* as a potential phytoplasma vector (Pastore *et al*., 2006 2004). Moreover, most leafhoppers in the subfamily Typhlocybinae are reported to be mesophyll feeders (Nault & Rodriguez 1985).This characteristic reduces their potential to act as phytoplasma vectors. However, *Asymmetrasca*

Abou-Jawdah 2011
A. decedens
Euscelidius sp.
Fieberiella sp
PCR⊕

decedens and its close relative, *Empoasca decipiens*, the two predominant species in stone fruit orchards in Lebanon, were found to be carriers of AlmWBphytoplasma (Dakhil *et al.*, 2011). In Italy, these two ~~genera~~ species were also found to be positive for ESFY in PCR assays, and *E. decedens* (a synonym to *A. decedens)* was shown to transmit ESFY from really ? *Prunus armeniaca* L. to *P. armeniaca* L.(Pastore *et al.*~~2004~~ 2006). no access to the article In Cuba, 67 *Empoasca* spp. samples were examined by PCR and 63 were found carrying '*Candidatus* Phytoplasma aurantifolia' (Arocha *et al.*2006). Normally insects with piercing sucking mouthparts try several probes with their stylet before starting to feed, and there is a probability that in Typhlocybinae, even though the largest number of thrusts occurs in the mesophyll, a low percentage may reach the phloem tissue. Accordingly, a low percentage of transmission may occur by the cell rupture feeding strategy (formerly known as lacerate-and-flush), whereby the insect feeds primarily in the mesophyll cells (Backus *et al.*, 2005). Several cells are punctured by the insect stylets, saliva is released and then the insect ingests the liquefied cells. During this process some phloem cells are sometimes hit and their contents are ingested (Backus *et al.*, 2005).

~~Second, the long incubation period observed following this transmission. Phytoplasma symptoms can start to appear on plants as soon as 7 days after the insect has introduced the phytoplasma, but this is not always the case since the symptoms may also take 6 to 24 months to develop depending on both the phytoplasma and the plant host species (Hogenhout *et al.*, 2003). Even in grafting experiments, symptoms may take a long time to appear, for example, it took around 18 months for the ESFY symptoms to appear on patch grafted three-year old plum and peach seedlings (Pastore *et al.*, 2001). Flavescence dorée of grapevine is symptomless in some cultivars, and it also has a long (up to 3 year) latent period before symptoms can be seen. These data may be explained by the fact that phytoplasmas live inside plants as parasites but they can~~

→ rhabdovirus / out of subject

? ? 2008?

not in references

no link with results

~~become pathogens in later stages when suitable conditions occur such as special weather conditions or changes in the production practices (Mehle *et al.*, 2010).The long incubation period poses a problem in early visual disease detection, and may have played a role in the spread of the AlmWB disease to distantly isolated regions in Lebanon, through the production, in nurseries, of AlmWB- infected asymptomatic seedlings. This observation necessitates stricter phytosanitary control measures on stone fruit nurseries and mother stock plants. For this reason, specific AlmWB detection methods based on PCR and qPCR are being developed. In Lebanon, over the last two years, all stone fruit nurseries have been surveyed using PCR based methods.~~

The rapid spread of the disease over distantly located regions, and the detection of AlmWB phytoplasma in some other leafhopper species may indirectly represent a hypothesis that other potential vectors for AlmWB phytoplasma may be present. Effectively, for many phytoplasma diseases more than one vector was reported. For example, '*Candidatus* Phytoplasma solani' (16SrXII-A) agent of the bois noir (BN) disease of grapevine is transmitted by two genera of Cixiidae , *Hyalesthes obsoletus* is the major reported vector but recently *Reptalus panzeri* was reported also as a natural host and several other vectors are suspected (Cvrkovic *et al.*, 2013).The other potential vector(s) of AlmWB phytoplasma may not be a normal pest on stone fruits but attacks stone fruits only during part of its life cycle or only occasionally when its natural hosts become limited. For example, even though the vector of "bois noir" (BN) *Hyalesthes obsoletus* cannot live on grapevines, it accidentally feeds on different crops and has been proved to transmit the phytoplasma from weeds to grapevine (Maixner, 1994; Weintraub & Wilson, 2010). Therefore, the preferred host(s) for some suspected vectors of the grapevine bois noir may be weeds or other plants. Effectively, investigations concerning '*Ca.* P. solani' confirmed that the epidemiology of this type of phytoplasma is very dependent on herbaceous weeds that act as

no comments on the Dakhill results? and potential vectors of AlmWB?

natural hosts for the vector, *Hyalesthes obsoletus,* nymphs and as natural reservoirs for this phytoplasma (Maniyar *et al*., 2013).

In conclusion, the detection of the '*Ca*. P. phoenicium' in the salivary glands of *Asymmetrasca decedens* in addition to the transmission trials confirm that this leafhopper is a vector of '*Ca*. Phytoplasma phoenicium', the suspected causal agent of AlmWB. This constitutes the first report of vector transmission of the phytoplasma associated with AlmWB disease and the second experimental proof that *A. decedens* may act as a phytoplasma vector in stone fruits (~~Pastore *et al*., 2004~~). Further research is needed on the modality of transmission (efficiency of different life stages, latency period,…), and the possibility of the occurrence of other potential vectors. Furthermore, *A. decedens* has a wide host range and can feed on a variety of cultivated and wild plants, trees, shrubs and herbaceous plants. Therefore, further studies must be conducted on the epidemiology of the disease including its alternative hosts and their relative importance in disease spread. Screening for resistant germplasms may also represent a possible option, although all the almond varieties present in Lebanon are susceptible. In view of the importance and severity of AlmWB disease, regional and international cooperation should be established in order to develop an integrated pest management approach to contain the disease, prevent its further spread and to reduce its negative impact on the stone fruit industry.

no, it's not a prove

So this result is not original?

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Acknowledgements

The AUB team is greatly thankful to his Excellency the Minister of Agriculture for his personal follow up on this important problem and to the Ministry Officers, Mrs. Lama Haidar and Mrs.Rola Al-Achi for their devotion and cooperation. Our thanks are also extended to the AVSI

Co-Author

officers, mainly Dr. Marina Molino Lova, Mrs. Chantal Mahfoud and Mrs. Nadine Hanna, who greatly facilitated the progress of these activities. This research was partially funded by a grant from the Italian cooperation and the Association of Volunteers in International Service (AVSI) as well as by the National Program for the Improvement of Olive Oil's Quality and Actions against the Diffusion of Stone Fruit Phytoplasma "The Project" (Project No. AID 9627) implemented by the Lebanese Ministry of Agriculture

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Figure legends

Figure 1: Agarose gel electrophoresis of PCR products obtained from DNA samples extracted at 12 months post-inoculation, from 16 seedlings inoculated with *A. decedens* carrying '*Ca.* P. phoenicium'. AK1-AK9 and AF1-AF7, represent different inoculated seedlings. M: 1 Kbp Ladder , (A): healthy seedling, (B): positive control

Figure 2: Agarose gel electrophoresis of PCR products obtained from: (A) salivary glands and(B) body of *A. decedens* collected from AlmWB-infected orchard, (C) *A. decedens* collected from healthy orchard, (D) healthy control, (E) AlmWB positive control. (M) 1Kbp ladder.

Figure 3: Collective virtual RFLP patterns with 17 restriction enzymes derived from *in silico* analysis, using *i*PhyClassifier, of 16S rRNA gene (R16F2n/R2 amplicon) fragments obtained from four representative samples extracted from *A. decedens* body *or* salivary glands, and from inoculated seedlings, all four samples showed the same pattern. The restriction fragments were resolved by *in silico* electrophoresis through 3□% agarose gel. MW, marker øX174 RFI DNA *Hae*III digest.

Figure 4: Virtual RFLP patterns derived from *in silico* RFLP, using pDRAW32, with the key restriction enzymes *Taq*I distinguishing "*Ca.* P. phonicium" subgroup D from representative strains of different subgroups in group IX phytoplasma. The pattern obtained from insect bodies, insect salivary glands and inoculated seedlings were similar to that represented in lane A. The 16S subgroups are represented by the following strains (GenBank accession numbers): IX-A (EF193383, PPWB), IX-B (AF390136,AlmWB), IX-C (HQ589191, NaxY), IX-D (AF515636, AlmWB), IX-E (GQ925919, JunWB). The restriction fragments were resolved by *in silico* electrophoresis through 3□% agarose gel. MW, marker øX174 RFI DNA *Hae*III digest.

Table 1 : Results of AlmWB transmission trials on two stone fruit rootstocks (GF-305 and GF-677) using *A. decedens* as vector at two months post inoculation

or 30 days?

Region of insect collection	GF-305		GF-677	
	Symptoms per total inoculated	PCR positive per symptomatic seedlings	Symptoms per total inoculated	PCR positive per symptomatic seedlings
Kfarkela			1/1	1/1
Kfarkela	2/3	2/2	2/3	2/2
Feghal	0/6	0/0	2/6	2/2
Kfarkela			1/1	1/1
Kfarkela			1/1	1/1
Kfarkela			1/1	1/1
Feghal	2/6	2/2	4/6	4/4
Total	4/15	4/4	12/19	12/12

difference?

difference?

in the text: all plants with symptoms were confirmed positive by PCR

Table 2: PCR detection of '*Ca.* P .phoenicium' in seedlings that developed symptoms following inoculation by *A. decedens.* Results taken 1, 2, 3 and 12 months post-inoculation

Variety	Seedling Code	1 months post-inoculation	2 months post-inoculation	3 months post-inoculation [a]	12 months post-inoculation [a]	Gel Fig 1
GF-305	AF3	+	+	-	-	← ? → +
	AF7	+	+	+	+	+
	AK4	+	+	+	+	+
	AK5	+	+	+	+	← ? → ⊖
GF-677	AF1	+	+	+	+	← ? → ⊖
	AF2	+	+	+	+	+
	AF4	+	+	+	+	← ? → ⊖
	AF5	+	+	-	-	← ? → +
	AF6	+	+	+	+	← ? → ⊖
	AK1	+	+	+	+	+
	AK2	+	+	+	+	+
	AK3	+	+	-	-	⊖
	AK6	+	+	-	-	← ? → +
	AK7	+	+	+	+	+
	AK8	+	+	-	-	← ? → +
	AK9	+	+	+	+	+
Total		16/16	16/16	11/16	11/16	

[a] Results for 3 and 12 months post-inoculation are for leaf samples collected from new growths that were not subjected to direct leafhopper feeding

to precise in M&M