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ALMOND WITCHES' BROOM PHYTOPLASMA: DEVELOPMENT OF DETECTION METHODS, EPIDEMIOLOGY AND MANAGEMENT OF THE DISEASE

by
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AN ABSTRACT OF THE THESIS OF

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In Lebanon since the early 1990s, a severe disease of stone fruits characterized by proliferation and appearance of witches' broom symptoms devastated almond, peach and nectarine plants; over 200,000 trees were affected. The disease was named almond witches' broom (AlmWB) and the causal agent was identified as "*Candidatus* Phytoplasma phoenicium", a phytoplasma belonging to sub-group 16SrIX-D. So far, pathogen detection relied on PCR and management relied mainly on eradication of infected trees. This research focused on development of serological detection methods which are normally less expensive and require less experience than PCR methods. Phytoplasma are phloem limited and cannot be cultured in vitro; therefore, recombinant DNA technology was successfully used to amplify, clone and express two integral membrane protein genes of "*Ca.* P. phoenicium". However, the levels of expression are low and optimizations of protein expression protocols are in progress in order to produce enough antigen for antibody production. Development of rapid, sensitive and specific serological detection method will provide an efficient tool in surveys aiming at early detection of the pathogen for eradication purposes. Since no resistant almond cultivars have been identified, grafting experiments were conducted in field and in greenhouse trials. The two grafting trials showed promising results. In the field trial, the growth from apricot or plum scions grafted on AlmWB-infected almond trees was symptomless for over a year. Similarly grafting AlmWB-infected scions on seedlings of plum and apricot, grown in the greenhouse, resulted in growth of symptomless shoots. An interesting recovery phenomenon was observed with three varieties; shoots developing from apricot Farclo grafted on AlmWB-infected trees in the field showed severe symptoms two months post-grafting but recovered three months later and remained symptomless to present; about two and a half years. in the greenhouse trial, the growth of AlmWB-infected scions grafted on Early blush apricot developed symptoms two months post grafting and recovered three months later. Quantitative real time PCR analysis confirmed recovery and reduction of phytoplasma concentration from 44 GU/ng DNA to null upon recovery. Understanding the recovery phenomenon may help developing a curative control measure for the disease. Five months post-grafting in the greenhouse the phytoplasma was not detected by PCR in the following treatments; Angelino plum, Red plum and Early blush apricot; while it was detected at low level in Farclo apricot and Jawhara plum, even though their growth was symptomless. If these data are confirmed in long term field trials, and the horticultural characteristics are maintained, replanting AlmWB infested regions with almond would become possible. A marker based on cleaved amplified polymorphic sequences (CAPS) was used to evaluate the biodiversity of "*Ca.* P. phoenicium" in four regions in North Lebanon. The results showed that only one type was present; this may be related to the recent introduction of the disease to Lebanon from a single source of origin.

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ABBREVIATIONS

%	Percent of a Hundred
°C	Degree Celsius
µg	Microgram(s)
µM	Micromolar
µL	Micloliter(s)
L	Liters(s)
mL	Milliliter(s)
AlmWB	Almond witches'-broom
AMP	Antigenic membrane protein
ANOVA	Analysis of Variance
AP	Apple proliferation
ATP	Adenosine triphosphate.
AYWB	Aster yellows witches' broom
Bp	Base pair(s)
CBWB	Chinaberry witches' broom
Cq	Quantitation cycle
CTAB	Cetyl trimethyl ammonium bromide
DBIA	Dot blot immunoassay
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme Linked Immunosrbent Assay

ESFY	European stone fruit yellows
et al.	*Et al* (and others)
E. coli	*Escherichia coli*
FAO	Food and Agricultural Organization
GU/ µl	Genomic unit per microliter
Ha	Hectare
HCl	Hydrochloric acid
IAA	Indole-acetic acid
IMP	Immuno-dominant membrane protein
Inmp	Integral membrane protein
IPTG	Isopropyl β-D-1- thiogalactopyranoside
ISEM	Immuno-sorbent electron microscopy
Kb	Kilo base pair
KDa	Kilo daltons
LWB	Witches' broom of lime
M	Molar
mM	Millimolar
MLO	Mycoplasma-like organism
MSC	Multiple cloning site
NaCl	Sodium chloride
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
POX	Peroxidase
pH	Hydrogen potential
PPO	Polyphenol oxidase
PPWB	pigeon pea witches'-broom

qPCR	quantitative PCR
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolution per minute
®	Registered trade mark
SDS	Sodium dodecyl sulfate
SLY	Strawberry lethal yellows
sp.	Species
SSR	Single sequence repeats
SOD	Superoxide dismustase
Taq	Thermus aquaticus
TEM	Transmission electron microscopy
TM	Trade mark
TPIA	Tissue print immunoassay
TSWV	*Tomato spotted wilt virus*
tuf	Gene encoding elongation factor
UNIMI	Università degli Studi di Milano
USA	United States of America
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Phytoplasmas are disease causing plant pathogens which act as obligate parasites belonging to class mollicutes. They have been reported to cause infections in several economically important crops worldwide through inhabiting the sieve tubes of the host and causing their collapse within a few years of infection. In Lebanon, phytoplasma symptoms on almond trees were first detected in the 1990s in South of Lebanon followed by detection of the disease in North of Lebanon in 1995. The disease was named as Almond witches' broom (AlmWB) due to the characteristic witch broom symptom exhibited on almond trees. During the first year of infection the symptoms appeared as early flowering, small light green leaves, stunted growth, or decrease in internode elongation followed by witches' broom appearance on main branches during the second year. Trees showing symptoms normally do not set fruits or set only few dark colored and small fruits, leading to total loss of marketable yield (Abou-Jawdah, Karakashian, Sobh, Martini, & Lee, 2002). Stone fruit production in Lebanon occupies large acreage of 23,000 Ha out of which almond production in 1998 has covered 6,800 Ha; 29.5% of the total area of stone fruit production (Faostat, 2013). In 1996, almond production in Lebanon reached to 37,385 tons followed by decrease in production to 23,000 tons in 2002; probably due to the spread of AlmWB on almond orchards. The latest statistics show that in 2012 production of almond has increased to 26,000 tons (Faostat, 2013). Molecular diagnostic techniques allowed the classification of AlmWB into the pigeon pea witches' broom group 16SrIX and subgroup 16SrIX-B, then

corrected as 16SrIX-D under the scientific name of "*Ca.* P. phoenicium" (Quaglino et al., 2015).

Almost 200,000 AlmWB infected trees have been infected in Lebanon. Several thousand AlmWB-infected trees have been eradicated in different regions because of the difficulty in controlling the spread of the disease and its management. Proper management measures coupled with a rapid and inexpensive detection method are required for success in re-cultivating almond trees in Lebanon.

This study aims at:

1. Development of serological detection methods for the specific detection and or quantitation of "*Ca*. P. phoenicium".
2. Disease management: Evaluation of the potential of grafting on resistant rootstocks for management of almond witches' broom disease through greenhouse and field trials
3. Epidemiology and strain identification: Infected almond samples collected from different regions in Lebanon will be tested by cleaved amplified polymorphic sequences a (CAPS) marker to identify the strains of the AlmWB phytoplasma present.

CHAPTER II

LITERATURE REVIEW

A. Definition of Phytoplasma

Phytoplasmas are known to be obligate parasites inhabiting plant phloem tissue and insect haemolymph. Upon their discovery they were classified as mycoplasma-like organisms and put under the order Acholeplasmatales, class Mollicutes, the closest being to the genus *Acholeplasma* (Kube, Mitrovic, Duduk, Rabus, & Seemüller, 2012). Current classification states that phytoplasmas belong to Superkingdom Prokaryota; Kingdom Bacteria; Phylum Tenericutes; Class Mollicutes; Genus *Candidatus* (Ca.) Phytoplasma (Hogenhout *et al*., 2008). Being part of the class Mollicutes, phytoplasmas are characterized by the lack of a rigid cell wall which is replaced by a single-unit membrane. They appear round, filamentous and pleomorphic in shape and range between 200-800 nm in size (Firrao et al., 2004). Phytoplasmas inhabit nutrient-rich plant phloem tissue as well as the haemolymph, gut lumen and saliva of phloem feeding insects rendering their main mode of transmission by leafhoppers, plant-hoppers and psyllids (Bertaccini & Duduk, 2010).

B. History of Phytoplasma

Throughout history numerous crops showed symptoms of disease such as the mulberry dwarf disease in Japan in 1602, and the sugarcane-silver leaf disease in Taiwan in 1958; due to their symptoms and mode of transmission they were thought to be viral diseases (Shikata, Teng, & Matsumoto, 1969). In 1967, Japanese researchers

discovered the presence of mycoplasma-like organisms (MLOs) in an ultrathin leaf section of a mulberry plant exhibiting leaf yellowing symptoms thus categorizing the disease causing agent for the first time as MLOs rather than viruses (Doi, Teranaka, Yora, & Asuyama, 1967). Following this discovery, many other reports of MLOs associated with yellows diseases were announced on a variety of crops (Bowyer, Atherton, Teakle, & Ahern, 1969; Davis & Whitcomb, 1970; Ulrychová, Jokeš, & Kynčlová, 1980). Due to the inability of *in vitro* culture of phytoplasmas researchers have used mono and polyclonal antibodies and DNA probes along with restriction fragment length polymorphism (RFLP) for the detection and identification of phytoplasmas. In 1994, the word "mycoplasma-like organisms" was replaced by "phytoplasma" at the 10th congress of the International Organization of Mycoplasmology (Lee, Gundersen-Rindal, & Bertaccini, 1998). Further molecular studies on the phylogeny of phytoplasma resulted in the designation of a new taxon which was named "*Candidatus* phytoplasma" in the year 2004 by the International Organization of Mycoplasmology (Duduk & Bertaccini, 2011).

C. Characteristics of Phytoplasma

Phytoplasmas have unique features that allow their separation from other disease causing agents. Their size, absence of a cell wall, small genome and lack of certain metabolic pathways distinguish them from other bacteria (Hogenhout & Segura, 2010).

1. Phytoplasma genome

Phytoplasma genome usually consists of one chromosome with a size ranging from 600-1150 kb and several small plasmids; it is characterized by having low G+C content , 23-26.2% (Oshima, Maejima, & Namba, 2013; Seemüller, Marcone, Lauer, Ragozzino, & Göschl, 1998). Certain metabolic pathways that are required for phytoplasma survival outside a host have been lost such as: tricarboxylic acid cycle, sterol biosynthesis, fatty acid biosynthesis, *de novo* nucleotide synthesis, biosynthesis of most amino acids and metabolic genes for the pentose phosphate pathway. It was also found that phytoplasmas have multiple copies of transporter-related genes. Phytoplasmas, unlike mycoplasmas and bacteria, lack F_1F_0-type ATP synthase encoding gene which is responsible for synthesis and hydrolysis of ATP (Oshima et al., 2013). It was proven that phytoplasma genome consists of a high number of simple sequence repeats (SSRs) which are mainly responsible for their ability of adapting to different environments such as inhabiting plants then moving to insect haemolymph, gut tissue and salivary glands to reach back to the phloem of other host plants (Wei, Davis, Suo, & Zhao, 2015).

Based on the 16S rRNA sequence, phytoplasmas have been classified into 37 species of '*Candidatus* phytoplasma' and into 32 groups. To date five complete genomes of phytoplasma have been sequenced '*Candidatus* phytoplasma asteris' strains OYM and AYWB, '*Candidatus* phytoplasma australiense' strains CBWB and SLY and '*Candidatus* phytoplasma mali' strain AP-AT (Wei et al., 2015).

2. Phytoplasma movement in plants

Phytoplasmas are proven to be obligate parasites for both plants and insect vectors since they lack specific metabolic pathways that allow their growth outside a host. Inside plants they replicate and are found to be highly concentrated in the mature sieve tube cells that lack nuclei and the immature phloem cells. Recent studies have also proven that they are found in the parenchyma cells located near the sieve tube (Hogenhout et al., 2008). Inside plants, phytoplasmas move through the sieve plate pores causing damage to cells of the sieve tube through forming cavities which allow their horizontal movement within the plant cells (Rudzińska-Langwald & Kamińska, 1999).

3. Host range

Worldwide, more than 300 plant species have been reported to be infected by phytoplasmas, many of which have caused devastating economic losses to the agricultural sector such as coconut lethal yellowing disease, grapevine yellows, apple proliferation disease European stone fruit yellows (ESFY) (Bertaccini & Duduk, 2010), and almond witches' broom (Abou-Jawdah et al., 2002). The host range of phytoplasmas varies greatly among phytoplasma, it depends on its strain and vectors host range.

4. Physiological effects of phytoplasma infection on plants

When infected by phytoplasma, plants exhibit physiological changes starting from decrease in chlorophyll and carotenoid contents in leaves leading to chlorosis which negatively affects photosynthesis especially photosystem II along with an increase in carbohydrate content in mature leaves and a decrease in starch content of roots and other sink tissues (Bertaccini & Duduk, 2010).

Phytoplasma triggers an imbalance in phyto-hormone concentration of infected plants such as Indole 3- acetic acid (IAA) known as auxin which is responsible for cell division and elongation; decrease in IAA concentration is responsible for symptoms such as stunting and witches' broom. Infected plants also exhibit an increase in anti-oxidative enzymes such as polyphenol oxidase (PPO), peroxidase (POX) and superoxide dismustase (SOD) and a decrease in soluble protein content as compared to healthy control hypothesizing an acceleration in proteolysis and/or a decrease in amino acid and protein synthesis (Zafari, Niknam, Musetti, & Noorbakhsh, 2012).

5. Symptoms caused by phytoplasma

Phytoplasmas are responsible for several diseases on crops worldwide and symptoms of the disease vary according to the crop; its variety and its age, strain of phytoplasma and environmental conditions (Dickinson, Tuffen, & Hodgetts, 2013).

As mentioned above, in plants, phytoplasmas interfere with balance of growth regulators and hormones causing abnormalities in development and certain metabolic processes leading to a certain group of characteristic symptoms such as witches' broom

due to proliferation of axillary buds, phyllody where the floral organs develop into leafy structures, and virescence which is caused by the development of chloroplasts in flower petals. Other common symptoms of phytoplasma infection are yellowing of the leaves, stunting of plants, phloem necrosis, sterility of flowers and abnormal internode elongation (Bertaccini & Duduk, 2010; Hogenhout et al., 2008).

In insects, phytoplasmas usually do not have a negative impact, but some leafhopper species exhibit shorter life span due to phytoplasma infection, while other species have higher fecundity rate. Phytoplasma infection can trigger a change in the plants to make them more attractive to some insect vectors broadening their host range (Hogenhout et al., 2008).

D. Phytoplasma Transmission

Being obligate parasites, phytoplasmas are transmitted to plant hosts through several ways, the major one being through sap-sucking insects followed by vegetative propagation such as cuttings and grafting, dodder transmission and possibly through seed transmission (Bertaccini, Duduk, Paltrinieri, & Contaldo, 2014).

1. Insect transmission

The main mode of transmission of phytoplasmas is by insect vectors belonging to the families Cicadellidae, Cixiidae and Psyllidae. Some phytoplasma strains can be transmitted by several insect species such as, aster yellows phytoplasma (AYP) sub-clade *Candidatus* (Ca.) phytoplasma asteris which is transmitted by 30 different insect

species, while others require a specific insect species for transmission (Hogenhout et al., 2008).

Not all insects that acquire phytoplasma have the ability to transmit it to host plants thus some insects can acquire the disease but can not act as a vector of the disease (Abou-Jawdah et al., 2014). Once insects start feeding on infected plants; also known as acquisition feeding, phytoplasma moves through the stylet reaching the intestine where passage to the circulatory system of the haemolymph occurs. Phytoplasmas colonize and multiply inside the salivary glands of insects prior to infection of other plants; the period between the colonization and spread of infection is called latency period. Once insects acquire the phytoplasma they remain infectious throughout their entire life. Certain studies have revealed the presence of phytoplasma in the nymph and eggs of infected insects but it is still not confirmed if all phytoplasma strains can be transmitted transoverially (Nynne M Christensen, Axelsen, Nicolaisen, & Schulz, 2005).

2. Transmission through vegetative parts and grafts

Since axenic culture of phytoplasmas has failed, their maintenance in living hosts for scientific research has been successfully performed through grafting of infected scions on healthy rootstocks. Success rate of grafting depends on scion and rootstock quality, grafting method, strain of phytoplasma and type of crop; herbaceous or woody plant. A transmission trial was conducted using four different grafting methods; whip graft, bark graft, budding and chip budding, on MM106 apple rootstocks with AP-infected scions. During the first year post grafting, the bark grafted and whip grafted seedlings showed symptoms with 100% efficiency while budding and chip

budding exhibited 34% and 54% efficiencies respectively, during the second year more than 50% of the trees showed symptoms with bud grafting technique (Aldaghi, Massart, Steyer, Lateur, & Jijakli, 2007).

Transmission of phytoplasma through grafting depends on seasonal changes and temperature variations within different geographical areas. During winter, some phytoplasmas in central European regions overwinter in roots of woody plant hosts and spread to the aerial parts during spring; while others, due to warmer climates in Mediterranean regions, overwinter not only in the roots of their hosts but also in the sieve tubes of stems that develop late in the season (Garcia-Chapa, Medina, Viruel, Lavina, & Batlle, 2003).

3. Dodder transmission

Plant parasitic dodder (*Cuscuta* sp) plays an important role in phytoplasma transmission to different hosts being woody plants or herbaceous plants. Once dodder germinates, haustoria grow within the host vascular tissue and forms direct connections with the host phloem and plasmodesmata. Once the connection is made with a phytoplasma infected host, phytoplasma cells move through the haustoria of the dodder and multiply inside it until they move to other healthy hosts (Přibylová & Špak, 2013).

E. Diagnostic Techniques of Phytoplasma

Over the years detection of phytoplasma has been performed using diverse methods such as electron microscopy, serological techniques such as Enzyme Linked Immuno-sorbent Assay (ELISA), polymerase chain reaction (PCR) and real-time PCR also known as quantitative PCR (qPCR).

1. Histological techniques

Study conducted by (Doi et al., 1967) using transmission electron microscopy was the first to associate disease causing "yellows" symptom to MLOs, which lead many other researchers to conduct studies using light microscopy, electron and transmission microscopy along with advanced staining techniques to detect and diagnose phytoplasmas.

Light microscopy has been used for preliminary detection of phytoplasmas using staining techniques such as methyl green, Feulgen stain and Dienes' stain. Certain stains that allow the detection of histological changes in infected hosts allowed the detection of European stone fruit yellows and Apple proliferation phytoplasmas by light microscopy; this method is quick, less expensive but less reliable than electron microscopy. Transmission electron microscopy (TEM) has been widely used for the detection of phytoplasma through preparing thin sections of infected plants sieve tube and visualizing phytoplasma cells; but sometimes phytoplasma infection can cause damage to the cell organelles and collapse of the sieve tube making its detection time consuming and difficult. To make TEM more specific, researchers developed Immuno-Sorbent Electron Microscopy (ISEM) where specific antiserum against a certain

pathogen is bound to the EM grid making the detection of the pathogen more specific. Using this method, the detection of Aster Yellows phytoplasma from infected aster plants was made possible (Musetti & Favali, 2004).

2. Serological techniques

Prior to the use of advanced and highly sensitive molecular techniques for phytoplasma detection, researchers used immunological techniques known as serological techniques to detect and differentiate phytoplasmas. Primarily immuno-diffusion tests were used to determine the antigenicity of membrane proteins such as the case of phytoplasma associated with aster-yellows disease. With the advance of science, researchers started making use of more sensitive, reliable and rapid serological techniques such as Enzyme Linked Immunosrobent Assay (ELISA), Tissue print immunoassay (TPIA) and Dot blot immunoassay (DBIA) (Maramorosch & Raychaudhuri, 2013).

a. Development of Enzyme Linked Immunosorbent Assay (ELISA) kit

For large scale phytoplasma detection surveys molecular techniques become expensive, laborious and time consuming. In such cases, Enzyme linked Immmuno-sorbent assay (ELISA) is economical, rapid and reliable. Over the years polyclonal antibodies for phytoplasma detection have been successfully produced for clover

phyllody, aster yellows (AY), ash yellows and many other phytoplasmas (Shahryari, Shams-Bakhsh, Safarnejad, Safaie, & Kachoiee, 2013).

i. Phytoplasma membrane proteins

Due to the lack of a cell wall, phytoplasma cell membrane is directly exposed to the host environment, whether plant or insect. On the surface of the cell membrane, there are several membrane proteins; immunodominant membrane proteins (IDPs), which are thought to have a role in host-phytoplasma interaction. These proteins are divided into three groups: immuno-dominant membrane protein (IMP), immuno-dominant membrane protein A (IdpA) and antigenic membrane protein (AMP) occupying different regions in the phytoplasma genome (Kakizawa, Oshima, & Namba, 2006).

Partial sequencing of "*Ca.* P. phoenicium", revealed the presence of 333 protein sequences out of which 69 are membrane proteins, 19 of which are AlmWB-unique membrane proteins. The integral membrane proteins (*inmp)* in AlmWB not only play a role in host-phytoplasma interaction, pathogenicity and transmission but are also important in AlmWB strain differentiation of different hosts such as almond, peach and nectarine. Multiple sequence analysis using housekeeping genes *tufB* and *groEL* showed 100% similarity between AlmWB infecting all three previously mentioned hosts but by the use of *inmp* gene, difference was detected between AlmWB infecting almond and nectarine from that infecting peach (Quaglino et al., 2015).

ii. Antibody production and Enzyme Linked Immuno-sorbent Assay (ELISA)

Over the past years, phytoplasma membrane proteins have been used in the process of polyclonal and monoclonal antibody production for use in serological techniques which facilitate large scale detection surveys.

An example is the antibody production of "*Ca.* P. aurantifolia"; causal agent of witches'-broom of lime (LWB), using recombinant IMP (Shahryari et al., 2013). The membrane protein was amplified using primers containing restriction sites of *SalI* and *NotI* enzymes and ligated in plasmid vector pTZ57R/T (InsTAcloneTM PCR Cloning Kit, Fermentas, Vilnius, Lithuania) which was in turn transformed in *Escherichia coli* (*E. coli*) strain DH5α. Positive clones were selected, purified and sub-cloned in expression vector pET28a downstream a 6xHis tag sequence and transformed in *E. coli* strain BL21 (D3). Induction of the transformed cells was performed using 1 mM Isopropyl β-D-1- thiogalactopyranoside (IPTG). After cell lysis using ultrasonic waves, the protein produced was purified using column containing Ni-NTA agarose beads (Qiagen, Hilden, Germany) and analyzed on sodium dodecyl sulfate -polyacrylamide gel (SDS-PAGE). Two rabbits were immunized with the purified protein, five intra-muscular injections were administered at two week intervals, then 14 days after the fifth injection, blood was collected from the rabbits and antibody purification from the serum was performed. An indirect ELISA was performed at a dilution rate of 1:500 (v/v) using healthy plant as negative control and LWB infected plant as a positive control along with three different phytoplasmas infected plants one of them being "*Ca.* P. phoenicium" on almond from Fars and Birjand, Iran which positively reacted with the produced antibodies against LWB.

One of the disadvantages of using bacterial cell expression systems is that some membrane proteins are toxic to the cell and during expression cause bacterial cell death leading to failure in protein expression. To solve this problem, a new system has been designed for protein expression in cell free systems using bacterial cell extract, wheat germ and rarely rabbit reticulocyte extracts allowing protein expression outside bacterial cells in open medium. The system contains a reaction mixture (RM) chamber where amino acids or labeled derivatives are provided and the feeding mixture (FM) chamber containing low molecular weight precursors, these chambers are separated by a semi-permeable membrane ensuring the exchange of substrates during the reaction (Schneider et al., 2010).

Transcription in cell free systems is based on bacteriophage T7 RNA polymerase thus cloning of the gene is required in a specialized cell free expression vector of the family pIVEX which contains T7 promoter, prokaryotic Shine-Dalgarno sequence, multiple cloning site (MSC) and a T7 terminator. The transformation efficiency of the vectors in competent cells have been studied by (Rogé & Betton, 2005) in the aim of using these vectors for in vivo protein expression. Transforming pIVEX vector in *E. coli* strain BL21 (λDE3) was not successful due to the absence of *lacI* gene which codes for the lactose repressor that controls the basal protein expression of the vector which appeared to be incompatible with the competent cells. These findings lead to transformation inside BL21 (λDE3) *E. coli* strain containing plasmids such as pLysS encoding a T7 lysozyme which naturally inhibits the activity of bacterial T7 RNA polymerase, and pDIA17 plasmid containing *lacI* gene both carrying the p15A origin of replication. The results showed that highest transformation efficiency was when transformed in BL21 (λDE3) containing pDIA17 plasmid which resulted in $7.5x10^8$

transformants/ µg of DNA; while in the case of BL21 (λDE3) alone and BL21 (λDE3) containing pLysS plasmid, the efficiency was $3.1x10^4$ and $4.5x10^4$ transformants/ µg of DNA respectively. Based on these results, it was concluded that pIVEX can be transformed in competent cells exhibiting sufficient amounts of T7 RNA polymerase repressors controlling the basal protein expression of pIVEX.

To test the expression of protein in vivo using pIVEX vectors, maltose-binding protein (MalE) was ligated to different sets of pIVEX vectors and transformed in BL21 (λDE3) containing pDIA17 plasmid previously proven to tolerate the presence of this vector, induction of bacterial cells was performed using IPTG at final concentration of 500 µM. The same was applied using RTS500 *E.coli* HY with the RTS ProteoMaster instrument provided by Roche the resulting proteins were run on a SDS_PAGE gel. Treatments of *in-vivo* system allowed protein expression in all plasmid combinations; while *in-vitro,* three out of four treatments expressed the protein of interest showing that with the appropriate plasmid combination and host suitability pIVEX vectors can be used for *in-vivo* protein expression trials (Rogé & Betton, 2005).

Protein expression using pIVEX vector *in-vivo* has also been conducted by (Ishido, Yamazaki, Ishikawa, & Hirano, 2011) using *E. coli* strain KRX whose T7 RNA polymerase is controlled by a rhamnose promoter (rhaBAD). Addition of rhamnose to the transformants induces RhaR which is an activator triggering the production of RhaS that binds to rhamnose leading to the transcription of rhaBAD. Consequently in the absence of rhamnose T7 RNA polymerase can't be transcribed thus controlling the basal protein expression of pIVEX.

To date, expression of phytoplasma membrane proteins has been accomplished using the traditional method. But based on recent studies, protein expression might be

possible using pIVEX vector inside competent cells such as KRX and M15 which contain the pREP4 plasmid harboring the *lacI* gene.

b. Dot Blot and tissue print Immunoassays (DBIA & TBIA)

Dot blot Immunoassay and TPIA are used for detection of plant pathogens such as viruses, bacteria and phytoplasma. The two methods follow the same protocol except that in DBIA the solution containing the antigen is spotted on a nitrocellulose membrane (1-10µl) which usually has a porosity of 0.2 µm; while TPIA is based on cutting a cross section of fresh plant tissue such as the stem or leaf petiole and pressing them over nitrocellulose membrane or nylon membrane. The membrane is then immersed in a solution of bovine serum albumin to block the remaining active sites. After blocking the active sites the membrane is washed with wash buffer and incubated with the primary antibody followed by incubation with the secondary antibody conjugated with alkaline phosphatase. Once incubation is over the membrane is washed and substrate buffer containing Nitroblue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) is added. A positive reaction shows a dark blue precipitate, This method was used to detect apple proliferation (AP) phytoplasma, apricot chlorotic leaf roll and aster yellows groups (Ploaie, Chireceanu, Tatu, & Fatu, 2008). The major two advantage of TPIA over dot blot are that no sample extraction is needed and TPIA allows localization of the pathogen in the plant tissue (Lin, Hsu, & Hsu, 1990).

3. Molecular techniques

In the past, the only methods to detect and identify phytoplasma diseases were through monitoring symptoms and using microscopy to visualize phytoplasma cells within the tissue of the infected plant these methods are unreliable, time consuming, laborious and inconsistent. During the 1980s scientists started making use of molecular techniques such as development of molecular probes and performing DNA hybridization assays and later using polymerase chain reaction techniques which provided rapid, sensitive and reliable results for disease identification (Lee, Davis, & Gundersen-Rindal, 2000).

a. Polymerase chain reaction

Phytoplasma detection using PCR-based assays began in late 1980s and early 1990s using primers designed according to cloned phytoplasma DNA sequences which allowed specific detection of low titer phytoplasmas that had failed to be detected using DNA-hybridization and serology (Lee et al., 2000).

In order to increase sensitivity and accuracy in detection of phytoplasma, universal primer pairs were developed which target the 16S rRNA gene sequence of phytoplasma. For example, the P1/P7 primers amplify a 1,800 bp product that contains the 16S rRNA region along with 16/23S spacer region allowing the detection of a wide variety of phytoplasmas. In addition, the resulting amplicons may be used for nested PCR runs with other group or phytoplasma specific primers (Green, Thompson, & MacKenzie, 1999). To detect phytoplasma with low titer especially in woody plants, nested-PCR technique was adopted where the PCR product of the primary PCR run

using universal primer pairs P1/P7 are diluted and used in another PCR run with primers such as, R16F2n/R16R2 which targets a region of the amplicon produced during the first PCR run. This method increases the specificity of detection, detects low titer phytoplasma that are unevenly distributed inside woody plants and aids in performing restriction fragment length polymorphism (RFLP) for phytoplasma classification (Gundersen & Lee, 1996).

In Lebanon, phytoplasma infection of almond, peach and nectarine was detected using universal primer pairs P1/P7 and R16mF2/R16mR1 followed by nested PCR using R16F2n/R16R2 primers. After RFLP analysis it was designated as Almond witches'-broom (AlmWB) and classified under subgroup 16SrIX-B in pigeon pea witches'-broom group (16SrIX) (Abou-Jawdah et al., 2002). To facilitate detection of "*Candidatus* phytoplasma phoenicium" causal agent of AlmWB and increase specificity, the primer pair Alm-F2/R-2 which amplifies a 390 bp region was designed (Abou-Jawdah, Dakhil, El-Mehtar, & Lee, 2003) but it was proven to be semi-specific or group specific, since it allowed detection of phytoplasmas from group 16SrIX. Consequently, primer pair AW16sF/AW23sR amplifying a 492 bp DNA fragment was designed for specific detection of "*Ca.* P. phoenicium" (Jawhari et al., 2015).

b. Real-time PCR (qPCR)

Phytoplasmas are usually unevenly distributed inside the host and their titer changes during seasons; decreasing to low levels where conventional PCR may not detect their presence. Real time PCR or quantitative PCR (qPCR) is the most advanced and sensitive molecular technique for phytoplasma detection and quantitation. The

qPCR rely on two detection systems, first being the use of intercalating dyes for fluorophore detection such as SYBR green and the other being fluorogenic probes such as TaqMan probes. The cycle by which the target gene reaches above the threshold level and emits fluorescence is known as the quantitation cycle (Cq) previously known as threshold cycle (Ct). This technique allows quantitation of phytoplasma inside host tissue aiding in studies of host resistance and of phytoplasma distribution (Nynne Meyn Christensen, Nyskjold, & Nicolaisen, 2013).

For the detection and quantitation of "*Ca.* P. phoenicium" in different tissues of almond and nectarine plants, a qPCR assay was developed using newly designed AlmWB specific primers AWsF/AWsR which amplify a 132 bp region covering the intergenic spacer region along with the 23SrRNA region of AlmWB phytoplasma and a TaqMan labeled probe. The study showed that AlmWB phytoplasma titer is highest in phloem and root tissue of almond, nectarine and peach and it also showed that the designed qPCR technique was highly specific to AlmWB phytoplasma and not to other phytoplasmas that were tested (Jawhari et al., 2015).

c. Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism RFLP technique uses restriction enzymes discovered in a group of bacteria in 1960. Initially this technique was used to study the pattern of plant chromosomal DNA by cutting the DNA at specific restriction sites and running it on a gel using electrophoresis which separated the individual fragments by size and allowed comparison between different plants (Bernatzky, 1989).

RFLP of 16S rRNA PCR products resulted in the classification of phytoplasmas into more than 30 major 16S rDNA groups and more than 90 sub-groups (Valiunas, D., Jomantiene, R., Ivanauskas, A., Urbonaite, I., Sneideris, D., & Davis, R. E. 2015)

Phytoplasma infected almond, peach and nectarine samples were collected from different regions of Lebanon and RFLP analysis of the 16s rDNA sequence was performed, the analysis showed that AlmWB is caused by a new phytoplasma related to pigeon pea witches' broom phytoplasma (PPWB), group 16SrIX. Consequently, AlmWB was classified in a new sub-group 16SrIX-B or 16SrIX-D within the PPWB group.

F. "*Candidatus* Phytoplasma phoenicium" in Lebanon

In the early 1990s, a disease associated with almond was discovered causing rapid decline of almond trees in different regions of almond production in Lebanon. Based on the symptoms exhibited; proliferation and witches' broom, it was suspected that the causal agent was a phytoplasma. Surveys conducted on almond orchards in Lebanon showed that the disease had spread in three main regions: North Lebanon, South Lebanon and Bekaa valley. Molecular techniques such as PCR and RFLP conducted on infected almond trees confirmed that the disease causal agent is a phytoplasma. Studies on the 16S rRNA gene showed that it is closely related to PPWB group with 98.4% identity and distantly related (having less than 87% identity) to previously described stone fruit infecting phytoplasmas such as European stone fruit yellows (ESFY), peach X-disease and peach yellow leaf roll (Abou-Jawdah et al., 2002).

Usually phytoplasmas have more than one host, such as ESFY which has been proven to infect peach, apricot, cherry, plum and almond (Abou-Jawdah et al., 2002). This encouraged conduction of surveys on other stone fruits and symptoms of phytoplasma were observed on peach and nectarine. Molecular detection methods confirmed that peach and nectarine were infected with "*Ca.* P. phoenicium" causal agent of AlmWB of almond increasing the host range of this phytoplasma to almond, peach and nectarine in Lebanon (Abou-Jawdah, Sobh, & Akkary, 2009).

Rapid spread of AlmWB in Lebanon indicated the presence of insect vector that allowed large scale dissemination of the phytoplasma to different regions. A survey conducted in mid-November 2001 until end of May 2002 in two AlmWB infected areas in Lebanon showed the presence of a high number of leafhoppers. About 12,756 leafhoppers were trapped, representing 27 genera belonging to 4 subfamilies. The largest population, 82.4%, was composed of *Asymmetrasca decedens*; which belongs to Typhlocybinae subfamily order Hemiptera. Detection of phytoplasma inside insects was carried out using PCR and nested PCR. Sequencing confirmed the presence of "*Ca.* P. phoenicium" by 99% identity in *A. decedens, Empoasca decipiens, Euscelidius mundus* and *Fieberiella macchiae* indicating that these insects can be possible vectors of the disease (Dakhil, Hammad, El-Mohtar, & Abou-Jawdah, 2011). Recently, it was proven that *A. decedens* is a vector of AlmWB through inoculating healthy seedlings (GF677 and GF305) with *A. decedens* collected from almond orchards infected with AlmWB (Abou-Jawdah et al., 2014).

G. Disease Management

Disease management caused by phytoplasmas is difficult because they inhabit the phloem tissue of their host and curative chemical control measures are not cost effective or environmentally safe. There isn't a single effective control measure of phytoplasmas; so adoption and integration of different control measures are recommended in order to decrease the spread and the incidence of the disease.

1. Preventive measures

Preventing disease introduction into a geographic area or a country is the first step in proper management because it would eliminate the chances of disease occurrence in the field. Preventive measures include using certified disease free seedlings, grafting on clean rootstocks, proper insect scouting and control measures, use clean agricultural tools and keeping the orchard free of weeds that can act as alternative hosts of phytoplasmas.

2. Tissue culture technique

Tissue culture technique has been widely used for production of virus free seedlings through shoot tip culture which can be coupled with the use of heat treatment. This technique has been adopted for regenerating seedlings from infected AlmWB infected Halwani and Khachabi almond varieties (Chalak et al., 2005) where shoot tip culture or stem cutting culture coupled with or without thermotherapy, and shoot tip

micro-grafting showed promising results in generation of plants that were free of phytoplasma from both varieties of almonds tested.

3. Use of antibiotics

Antibiotics such as tetracycline have been used for the control of phytoplasmas in fruit trees but they are not widely used because the application is not economically feasible and the effect is short termed. Antibiotic introduction has been through sprays, root dips, soil drenches, tissue wicks and direct injection into the trunk which is the most effective method of control. Oxytetracyline (OTC), a naturally produced tetracycline antibiotic, inhibits the multiplication of bacterial cells, has high thermo-stability and can be injected inside tree trunks thus making it a good candidate for use in phytoplasma control. However, its application is expensive, laborious and requires several injections since its effect does not last over a long period of time. Moreover its agriculture use may be prohibited in several countries and the probability of occurrence/ development of resistant phytoplasma strains may not be excluded (Stockwell & Duffy, 2012).

4. Grafting on resistant rootstocks

Control of phytoplasma has been carried out by decreasing the population of vectors through the use of pesticides which are expensive and do not always decrease the incidence of infection. More sustainable methods of control are required such as planting resistant seedlings or grafting on resistant rootstocks that can tolerate the

disease and/or induce recovery of the infected scions. Breeding of a resistant rootstock to apple proliferation phytoplasma (AP) has been conducted through crossing *M. seiboldii*; exhibiting high tolerance to AP, with M9 stock which provides favorable agronomic traits such as yield, size and quality to apple trees. The obtained recombinant progenies were inoculated with "*Ca.* P. mali" infected scions of cv. Golden Delicious and M9 rootstocks were also inoculated as a positive control and they were monitored over a period of eight years for development of disease, qPCR from the recombinant progenies showed that phytoplasma was present in the phloem at a rate of 10^4 to 10^6 cells/g while in the positive control it was 1,000 times higher. Eight years post-grafting the scions grafted on resistant rootstocks gave PCR-negative results confirming recovery after AP infection in apples (Jarausch et al., 2011).

Grafting experiments have been conducted in Lebanon on AlmWB by grafting infected almond and nectarine buds in mid-June 2001 on almond, plum, cherry, apricot, peach and nectarine seedlings. Symptom appearance was recorded and starting November of the same year till August of 2002. PCR and nested PCR were conducted at monthly intervals using phytoplasma universal primer pairs P1/P7 followed by R16F2n/R16R2. Buds grafted on almond, peach and nectarine exhibited symptoms starting February of 2002 from the scion and rootstock level which showed bush-like growth from the base and weak light green leaves with short internodes. The presence of AlmWB was confirmed by PCR and nested PCR followed by RFLP. While buds grafted on seedlings of apricot and plum did not develop symptoms and were negative with PCR and nested PCR until 14 months post inoculation (Abou-Jawdah et al., 2003).

Based on the results obtained, it was hypothesized that AlmWB may be controlled by grafting buds on rootstocks which exhibit tolerance or resistance against

the phytoplasma allowing the cultivation of almond in AlmWB phytoplasma infested areas.

5. Induced plant resistance

A method to decrease disease incidence is to trigger the systemic acquired resistance (SAR) of the host through applying SAR inducers on plants. An example of this technique was the application of benzothiadiazole (BTH) on *Arabidopsis thaliana* plants under controlled conditions prior to exposure to the vector of X-disease in fruit trees; *Colladonus montanus* commonly known as mountain leafhopper. An average of 74% of the non-treated plants acquired the disease from the vector while 35% of the treated plants acquired the disease; additionally the rate of vector survival on BTH treated plants was lower than that of non-treated plants (Weintraub, 2007).

6. ***Weed control***

Phytoplasmas are transmitted by insects that are often polyphagous, which can feed and transmit the disease to a number of plant species including weeds. Therefore, efficient weed control in a field can decrease disease incidence in trees or herbaceous hosts (Hogenhout et al., 2008).

CHAPTER III

MATERIALS AND METHODS

A. Development of serological detection methods

In order to develop serological detection methods such as ELISA, TPIA and DBIA, AlmWB membrane protein sequences were kindly provided by Dr. Fabio Quaglino, University of Milan, designated as AlmWB_1160 peptidase S24 like *inmp* (appendix I, A) and AlmWB_1850 hypothetical *inmp* (appendix I, B).

1. AlmWB membrane proteins and primer design

Primers for membrane proteins detection of AlmWB were designed based on the obtained membrane protein sequences and sequence of the expression vector to be used; pIVEX 1.3 wheat germ (wg) vector with a C terminal 6xhistidine tag (appendix I, C). Primers were designed to contain restriction sites that do not target the membrane protein but are necessary for ligation in the MCS of the expression vector. Restriction sites were *NcoI;* sticky end cutting restriction enzyme, cutting at C^CATG_G site located on the forward primer, and *SmaI;* a blunt end restriction enzyme cutting a CCC^GGG site located on the reverse primer. On the 5' end of the forward primer, 6 nucleotides were added for the proper cleavage of the restriction site during digestion and ATG coding for methionine start codon was included inside the restriction site of *NcoI.* From the reverse primer on the 3' strand the stop codon; TAA, was removed in order not to stop protein expression and allow successful purification of protein (Table 1).

Based on AlmWB membrane protein sequences, primers were designed for cloning and protein expression in pQE-32 vector (appendix I, D) taking into consideration that pQE-32 vector has N- terminal 6xhistidine sequence and two stop codons TAG and TGA at the C-terminal after the MCS. The primers were designed to contain *SphI* restriction enzyme site GCATG^C in the forward primer and *PstI* restriction enzyme site CTGCA^G in the reverse primer. The pQE-32 vector has an additional nucleotide G before the MCS, consequently one nucleotide A was removed from both membrane protein sequences at the 5' end to keep the sequence in frame and at the 3' end stop codon was kept in order to ensure the stop of protein expression.

Upon delivery the primers were re-suspended with Ultra pure^{TM} distilled DNAse, RNAse free water to a final concentration of 100 pmol/µL and some tubes were diluted to a final concentration 10 pmol/µL and stored at -20 ºC (Table 1).

Table 1: Locally designed Primers for cloning in pIVEX 1.3 wg and pQE-32 expression vectors

Primer	Sequence*	*inmp* gene	Size (bp)
Cloning in pIVEX 1.3 wg vector			
FepAlmWB-1160	5'TACATGCC**ATGG**ATCGAAACGTATGGCCA3'	AlmWB-1160	578
RepAlmWB-1160	5'TAGTCCCCCGGG**AAGAATAATATTTTTCGGATCATT**3'		
FepAlmWB-1850	5'TACATGCC**ATGG**CAGTTATGTTAAAATTAAATAA3'	AlmWB-1850	728
RepAlmWB-1850	5'TAGTCCCCCGGG**TTTAACACTTTGAATTTCAGG**3'		
Cloning in pQE-32 vector			
FAlm	5'*ACAT*GCATGC**TGAATCGAAACGTATGGCCAA**3'	AlmW	584

WB-1160		B-1160	
RAlm WB-1160	5’*AA*CTGCAG**TTAAAGAATAATATTTTTCGGATCATTTTTAACATA**3’		
FAlm WB-1850	5’*ACAT*GCATGC**TGACAGTTATGTTAAAATTAAATAAAGATAAAC**3’	AlmWB-1850	734
RAlm WB-1850	5’*AA*CTGCAG**TTATTTAACACTTTGAATTTCAGGAG**3’		

* The underlined regions correspond to the restriction sites of the enzymes and membrane protein genes are represented by bold letters.

2. Gene amplification

a. DNA extraction

AlmWB-infected almond samples were collected from orchards located in Feghal region, north of Jbeil district. Samples of 150 mg of leaf midrib and phloem tissues from AlmWB- infected trees were placed in 1.5 mL Eppendorf tube and placed in liquid nitrogen to aid in the grinding process using a pestle attached to an electric drill. Total DNA extraction was carried out using cetyl-trimethyl ammonium bromide CTAB extraction protocol, where 800 µL of autoclaved CTAB buffer (appendix II, A) along with 20 µL of β-mercaptoethanol, an antioxidant, were added to each tube and incubated at 60 ºC for 20 minutes shaking the mixture every five minutes. After incubation, 600 µL of isoamyl alcohol-chloroform (1:24 v/v) were added to each sample followed by vigorous vortexing and centrifugation at 10,000 rpm for five minutes. The supernatant was placed in new Eppendorf tube and 600 µL of ice cold isopropanol were added and the mixture was held at -20 ºC for 60 minutes followed by centrifugation at

14,000 rpm for 8 minutes. The aqueous phase was discarded and the pellet rinsed with 600 µL of 70% ethanol followed by centrifugation at 14,000 rpm for 5 minutes and air drying.

The pellet was re-suspended with 50 µL of DNAse and RNAse free water and the nucleic acid quality was assessed by mixing 2 µL of DNA with 2 µL of 6x loading dye (appendix II, B), the mixture wasd loaded in 1% Agarose gel (appendix II, B) along with Bench top 1 Kb DNA ladder (Promega, Wisconsin, USA). The gels were run at 100V for 35 min and stained with ethidium bromide (0.5g/L) for 30 min followed by rinsing with distilled water for 5 mins.

b. PCR and gel electrophoresis

Using the designed primers PCR was performed on the extracted DNA using 5x FIREPol® Master Mix (Solis BioDyne) containing FIREPol® DNA polymerase, 5x reaction buffer B (0.4M Tris-HCl, 0.1M $(NH_4)_2SO_4$, 0.1% w/v Tween-20, 7.5 mM $MgCl_2$ 1mM dNTPs, blue and yellow dye). Each 20 µL PCR reaction mix contained 4 µL from the 5x master mix, 0.5 µL of forward primer (10 pmol/µL), 0.5 µL of reverse primer (10 pmol/µL), 2 µL DNA and 13 µL DNAse, RNAse free water. Two negative controls were included in the PCR run: Only DNAse, RNAse free water or DNA from healthy almond trees.

All the PCR runs were conducted in C1000 Thermal Cycler (Bio-Rad, USA). The run using primers FepAlmWB1160/RepAlmWB1160 consisted of denaturation step at 95°C for 3 min, 40 cycles of 95°C for 30s, 58°C for 30s (primer annealing), 72°C for 1 min (extension) and 72°C for 7 min for final extension. Run using primers

FepAlmWB1850/RepAlmWB1850 consisted of denaturation step at 95°C for 3 min, 40 cycles of 95°C for 30s, 54°C for 30s (primer annealing), 72°C for 1 min (extension) and 72°C for 7 min for final extension.

PCR runs using primer FAlmWB1160/RAlmWB1160 and FAlmWB1850/RAlmWB1850 were performed using the same protocol which is as follows denaturation step at 95°C for 3 min, 40 cycles of 95°C for 30s, 55°C for 30s (primer annealing), 72°C for 1 min (extension) and 72°C for 7 min for final extension.

The PCR products were analyzed using wide mini-sub cell GT electrophoresis machine attached to Power PAC 300 (Bio-Rad Laboratories, Hercules, USA) power outlet. The runs were performed using 1% agarose gels (Bio-Rad Laboratories, Hercules, USA) in 1x TAE buffer (appendix II, B) at 100V for 30 min and stained in ethidium bromide for 30 min and rinsed in distilled water. The gels were visualized under UV and photographed by Gel Doc XR+ system (Bio-Rad Laboratories, Hercules, USA).

The PCR products were purified using peqGOLD gel extraction kit (peqlab, Germany) by adding equal volume of binding buffer and vortexing for few minutes followed by loading it into perfectbind DNA column and centrifuging. After centrifugation the flow through was discarded and the column was washed with wash buffer containing 100% ethanol after that the column was dried by centrifuging for 7 min at 11,000 rpm and elution buffer was added to elute the PCR product which was collected by centrifugation.

3. Cloning in pGEM-T easy vector

The same protocol of cloning was applied for both genes amplified by both designed primers.

a. Ligation

PCR and ligation in pGEM-T easy vector should be performed in the same day because the polymerase used during the PCR reaction adds A residues at the end of the amplicon which in turn binds to the complementary T residues located on the pGEM-T easy vector (appendix I, D). Loss of A residues might impair ligation process inside the vector leading to absence of colonies on selective media.

Ligation was performed using Ligate-IT Rapid Ligation Kit (USB products, Affymetrix). The components of the reaction were: 4 µL of 5x Ligate-IT reaction buffer, 1 µL of Ligate-IT T4 DNA ligase, 2 µL of 100 ng PCR products, 1 µL of pGEM-T easy vector (a vector to insert molar ratio of 1:3) and 12 µL of DNAse, RNAse free water. The entire mixture was centrifuged and held at room temperature (24°C) for 15 min followed by direct transformation in competent cells or storage at -20°C.

b. Transformation

Following ligation the plasmid was transformed in *E. coli* bacterial strain XL1Blue used to produce high plasmid copy number for sub-cloning. An aliquot of 150 µL from the competent cells was thawed on ice for 15 min, 10 µL from the plasmid were added to 50 µL of XL1Blue, gently mixed and incubated on ice for 20 additional min followed by heat shock at 42 ºC for 30 sec using the Thermomixer comfort

(Eppendorf, Germany) and directly transferring the cells in the tube on ice for 2 min. This process allows the plasmid to enter into the competent cell. After incubation on ice, 1 mL of LB broth (USB) (appendix II, C) was added to each tube and incubated for 1 h at 37 ºC followed by centrifugation for 2 min at 8,000 rpm.

Since pGEM-T easy vector harbors ampicillin resistance gene aiding the selection process, cells were streaked on LB plates containing 50 mg/mL ampicillin and coated with 60 µL X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (appendix II, D), 10 µL of IPTG (isopropyl-thio-β-D-galactopyranoside) (appendix II, D) along with additional 30 µL of LB+Amp. The streaked plates were covered by aluminum foil and incubated at 37 ºC overnight. After incubation the plates were checked for development of blue and white colonies. The white colonies were selected and added to LB+Amp broth and incubated overnight at 37 ºC on a bench top shaker (Ecotorn, Infors HT) at 250 rpm.

c. Miniprep and DNA sequencing

In order to extract the plasmid from the competent cells, miniprep was performed using Qiaprep® Spin Miniprep kit (Qiagen, Germany) according to manufacturer's instructions. From the overnight cultures, 3 mL were taken and centrifuged at 8,000 rpm for 5 min, the pellet was re-suspend in 250 µL of buffer P1, containing RNase A, this was followed by addition of 250 µL of buffer P2 mix and incubation for 5 min. After incubation, 350 µL of buffer N3 were added and the mixture centrifuged for 10 min at 13,000 rpm. The supernatant was then transferred to QIAprep spin column and centrifuged at maximum speed for 1 min, followed by washing with

750 µL of buffer PE; containing 100% ethanol, followed by centrifugation at 11,000 rpm for 7 min to remove ethanol. To elute the plasmid, 50µL DNAse and RNAse free water were added and incubated for 2 min followed by centrifugation at maximum speed for 2 min. The plasmids were used to transform competent cells or stored in -20 ºC for sequencing. Purified plasmids were sent to sequencing at the High Throughput Genomics Center (Seattle, Washington, USA). Sequencing was performed using an automated DNA sequencer and the primers used were M13 forward and reverse primers targeting strands from pGEM-T easy vector and its MCS which contains the gene/insert of interest.

4. Ligation in expression vector

Since pGEM-T easy vector is a cloning vector it is not efficient in protein expression, therefore the plasmids obtained from miniprep were digested using restriction digestion enzymes and the inserts were ligated in two expression vectors, pIVEX 1.3 wg, an in vitro expression vector, and pQE-32, a bacterial cell expression vector.

a. Restriction digestion

The genes cloned in pGEM_T easy vector along with their corresponding expression vectors were excised by restriction enzymes *NcoI* and *SmaI* (Thermo-scientific, Massachusetts, USA) for cloning in pIVEX 1.3 wg vector and *SphI* and *PstI* (Thermo-scientific, Massachusetts, USA) for cloning in pQE-32 vector. Digestion was

conducted in the same reaction tube using both enzymes due to having compatible reaction buffer and thermal activity.

The reaction for cutting the inserted gene from pGEM_T easy vector in order to sub-clone in pIVEX 1.3 wg and pQE-32 vectors consisted of: 2 µL 10x buffer Tango (33mM Tris-acetate, 10mM magnesium acetate, 66mM potassium acetate and 0.1 mg/mL BSA), 1 µL of each *NcoI* and *SmaI* enzymes for sub-cloning in pIVEX 1.3 wg vector and *SphI* and *PstI* for sub-cloning in pQE-32 vector, 6 µL of pGEM-T easy vector + gene of interest and 10 µL DNAse and RNAse free water. Simultaneously, both vectors were digested with their corresponding restriction enzymes with 2 µL 10x buffer Tango, 1 µL of each of the restriction enzymes, 4 µL of vector and 12 µL of DNAse and RNAse free water. Both reactions were incubated at 37ºC overnight followed by enzyme inactivation at 65ºC for 20 min.

The digestion products were mixed with 5 µL of 3x loading dye and loaded onto 1% Agraose gel with 1 Kb DNA ladder and electrophoresis was conducted at 100V for 35 min. Staining was performed after electrophoresis by placing the gel in ethidium bromide for 30 min followed by de-staining for 5 min in distilled water.

Gels were visualized on UV transilluminator 2000 (Bio-Rad Laboratories, Hercules, USA) and gel purified using peqGOLD gel extraction kit (peqlab- Germany) following manufacturers' instructions. The bands of interest along with that of the vector were excised from the electrophoresis gel and weighed about 0.3 g of each equal volume (V/W) of binding buffer was added (about 300 µL) and held at 60 ºC for 20 min until the gel gets fully dissolved, the mixture was loaded onto the spin column and centrifuged at maximum speed for 1 min and the flow through was discarded. The spin column was washed with wash buffer (to which 24 mL of 100% ethanol were added

before use). The wash buffer was removed by centrifuging at 11,000 rpm for 7 minutes. The nucleic acids were eluted with DNAse and RNAse free water and centrifuged at maximum speed for 1 minute.

b. Ligation in protein expression vectors

Purified digests/ inserts were ligated in the corresponding protein expression vectors using Ligate-IT Rapid Ligation Kit (USB products, Affymetrix) by adding 4 µL of 5x Ligate-IT reaction to 7 µL of 25 ng/µL of purified gene product, 3 µL of purified pIVEX 1.3 wg vector, 1 µL of Ligate-IT T4 DNA ligase and 5 µL of DNAse and RNAse free water. The entire mixture was held at 24ºC for 15 min and directly transformed in competent cells or stored at -20ºC. The same technique was used for ligation in pQE-32 vector.

c. DNA sequencing

Plasmids were sent to the University of Saint Joseph, Lebanon for sequencing using automated DNA sequencer. The plasmids were sent with M13 forward and reverse primers targeting part of the expression vector and the MCS containing the gene of interest.

d. Transformation

After receiving the sequences, positive plasmids were transformed in competent cells that have the ability to express protein rather than producing large copy numbers of the plasmid.

Transformation was performed in *E. coli* strains M15 and KRX single step competent cells (Promega, Wisconsin, USA) for both *inmp* genes, AlmWB_1160 and AlmWB_1850.

M15 and KRX cells were thawed on ice for 15 minutes and 5 µL of 50 ng/µL plasmid were added to 50 µL of the competent cells. They were mixed gently and incubated on ice for 20 minutes followed by heat shock at 42ºC for 30 seconds followed by direct exposure to ice for 2 min. To each tube of transformed cells 1 mL LB was added and incubated in water bath at 37ºC for an hour after which they were harvested by centrifugation. pIVEX 1.3 wg vector contains a gene of ampicillin resistance and M15 bacterial cells contain pREP4 plasmid which has resistance gene against Kanamycin; therefore, those transformed in M15 were streaked on plates containing LB+Amp and kanamycin 25mg/mL, while those transformed in KRX competent cells were streaked on plates containing LB+Amp. The plates were covered with aluminum foil and incubated in an inverted position at 37ºC overnight. Untransformed competent cells, M15 and KRX, were used as negative controls and were streaked on plates with antibiotics and incubated overnight at 37 ºC.

The following day, positive colonies from M15 cells were selected and added to a tube containing LB+Amp+Kan solution, while colonies from KRX were added to a tube containnig LB+Amp , and incubated at 37ºC in a shaker at 250rpm.

One mL from each culture was transferred to an Eppendorf tube and 220 µL of 80% glycerol was added to it. The tubes were labeled and stored at -80ºC for future use.

5. Protein expression

Protein expression of AlmWB-1160 *inmp* was performed in two *E. coli* strains, M15 and KRX, in order to select for higher levels of expression.

a. Optimization of induction process

Cell cultures of M15 and KRX; containing the expression plasmid, that were stored at -80 ºC were thawed on ice, and 100 µL was inoculated in 15 mL LB+Amp+Kan and LB+Amp, respectively, and activated overnight at 250 rpm and 37ºC. Untransformed M15 and KRX cells were also inoculated in LB+Kan and LB broth, respectively, to check for expression of bacterial proteins.

The growth rate of bacteria was monitored using spectrophotometry, once their optical density at 600 nm reached 0.7-0.9, protein expression was induced. Transformed M15 and untransformed M15 were induced with 1mM IPTG and those transformed in KRX and untransformed KRX were induced 0.1% L- rhamnose monohydrate (L5701 Promega, Wisconsin, USA). The cells were incubated at 30ºC. Aliquots of 3 mL of culture were removed just prior to induction and at 1, 3 and 4 hours and overnight after induction, centrifuged at 5,000 rpm for 5 minutes and stored at -80ºC for one day to aid the process of chemical cell lysis.

b. Bacterial cell lysis and protein purification

For bacterial cell lysis two methods were implemented; one being lysis under native conditions (chemical cell lysis) and the other by denaturing conditions. Protein purification was performed by using affinity chromatography matrix.

i. Native conditions (Chemical cell lysis)

The cell pellets obtained from 100 mL cell cultures and stored at -80ºC were thawed on ice for 15 minutes and to each tube 6 mL of lysis buffer (appendix II, E) was added and mixed by vortexing until fully dissolved. Then 300 µL of freshly prepared lysozyme from chicken egg white (L-6876 Sigma-Aldrich, Missouri, USA) were added along with 166 µL of protease inhibitor cocktail (P8340 Sigma-Aldrich, Missouri, USA) and 1 mL of 0.1 M phenylmethanesulfonyl fluoride (PMSF) (P7626 Sigma-Aldrich, Missouri, USA); the mixture was incubated on ice for 1 hour after which freshly prepared deoxycholic acid (appendix II, D) was added at a final concentration of 0.5 mM (D-6750 Sigma-Aldrich, Missouri, USA) along with Deoxyribonuclease I from bovine pancreas, DNase I (D5025, sigma) and $MgCl_2$ at a final concentration of 15 mM; precursor for the activity of DNase I enzyme (appendix II, D). The entire mixture was incubated on ice for an additional 15 minutes and centrifuged at 8,200 rpm for 30 minutes. The Supernatant was recovered and 60 µL of Ni-NTA agarose (Qiagen, Germany) matrix was added to each tube and incubated on ice with gentle shaking for 3 hr. After incubation beads were collected by centrifugation at 2000 rpm for 3 min and supernatant was discarded. Beads were washed 3 times with 2 mL wash buffer (appendix II, E), followed by elution step performed at 1400 rpm for 20 min using 50

µL elution buffer (appendix II, E). Samples were centrifuged at 2000 rpm for 3 min and supernatant was saved for SDS-PAGE run.

ii. Denaturing conditions

Cell pellets obtained from 150 mL of M15 and KRX competent cells induced with 1 mM IPTG or 0.1% rhamnose, respectively, were thawed on ice for 15 min followed by resuspension in 5 mL of 6M Guanidine Hydrochloride (GuHCl) buffer (appendix II, E) at a rate of 5 mL/g wet weight, cell lysis was performed at room temperature for 1 hour at 150 rpm. Soluble protein was separated from cellular debris by centrifugation at 15,000 rcf for 30 min, supernatant was recovered and 60 µL Ni-NTA matrix was added and incubated at room temperature for 3 hr. After incubation samples were centrifuged at 2000 rpm for 3 min and washed twice with wash buffer C pH= 6.3 (appendix II, E). Elution was accomplished by adding 100 µL of buffer E pH= 4.5 (appendix II, E) and incubated for 20 min at 1400 rpm.

After cell lysis and purification using both methods, samples were analyzed by SDS-polyacrylamide gel electrophoresis. The gels were prepared by using 30% bisacrylamide solution containing 2.7% bis-acrylamide (Bio Rad Laboratories, Hercules, USA) with separating gel (12%) and the stacking gel (4%) (Appendix II, F). Aliquots of 15 µL from the lysates were mixed with 15 µL of 2x loading buffer containing dithiothreitol (DTT) (Bio-Rad Laboratories, Hercules, USA) and incubated at 95ºC for 5 minutes (appendix II, E). The samples along with Precision Plus protein Kaleidoscope pre-stained Ladder (Bio-Rad Laboratories, Hercules, USA) were loaded

on the gels and electrophoresis was run at 110V for 2 hours using 0.5x running buffer (Appendix II, E). Once run time was over the gels were stained in Coomassie brilliant blue staining dye (Appendix II, E) for 30 minutes followed by de-staining in destaining buffer (appendix II, E) for 2 hours, gels were visualized using White light 2000 (Bio-Rad Laboratories, Hercules, USA).

B. Disease management through grafting

Aiming at establishing a proper disease management technique, three grafting trials were conducted: a greenhouse trial and two field trials. Visual observation on disease development was complemented by PCR and/or qPCR analysis for detection and quantitation of the pathogen.

1. Grafting on resistant rootstocks, greenhouse trials

a. Grafting of infected almond scions

A total of 38 two-year old seedlings were provided by a nursery in Bekaa region consisting of 8 almond seedlings, 6 plum variety Jawhara, 6 plum variety Angelino, 6 Red plum, 6 apricot variety Early Blush and 6 apricot variety Farclo.

Bud grafting of AlmWB-infected almond scions was performed on April 13, 2015 on at least 4 seedlings of each variety. Seedlings that were not grafted with infected scions were considered as negative controls for each treatment and grafted almond seedlings were considered as positive controls.

Seedlings were monitored for symptom development periodically; phytoplasma detection and quantitation were performed by PCR and qPCR on a monthly basis starting from July, 2015

b. Grafting infected almond trees with scions from tolerant varieties long term field trial 1

On April 13, 2013 a grafting experiment was conducted on almond trees located in Feghal area by grafting scions from different plum and apricot varieties on almond trees infected with AlmWB phytoplasma and exhibiting severe disease symptoms.

Grafting was carried out in five treatments where each treatment contained two AlmWB-infected almond trees grafted with one of the following scion varieties: Plum Abou-riha, Janarek Plum, Plum Fortune, Plum Santarosa and Apricot Farclo.

c. Grafting of AlmWB-infected almond scions on tolerant rootstock varieties, long term field trial 2

For future long term grafting experiments; in February 2015, more than 100 plum seedlings along with 50 almond seedlings as positive control were distributed to farmers in Feghal area. The plum seedlings will be grafted in early spring 2016 with scions of the variety Halawani, the most commercially appreciated variety (Appendix IV).

2. Phytoplasma detection and quantitation

a. Disease detection by PCR

For disease detection from samples grafted in field total nucleic acids were nextracted from 150 mg of plant midrib tissue from rootstock and scions of each treatment. PCR was performed on DNA extracted from 2, 5 and 6 months post grafting followed by yearly PCR runs in 2014 and 2015. PCR was carried out using AlmWB semi-specific primer pairs AlmF2/AlmR2 amplifying a 390 bp region. The reaction mixture consisted of 2x REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich, Missouri, USA) containing (20mM Tris-HCl, 100 mM KCl, 0.002% gelatin, 3 mM $MgCl_2$, 0.4 mM dNTPs mix and 0.06 unit/µL *Taq* DNA polymerase). Each 20 µL PCR reaction mix contained 10 µL from the 2x master mix, 0.5 µL of forward primer (10pmol/µL), 0.5 µL of reverse primer (10pmol/µL), 2 µL of 10x diluted DNA and 7 µL DNAse, RNAse free water. The cycling conditions consisted of: 95ºC for 2 min, 35 cycles at 94ºC for 30 sec, 44ºC for 30 sec and 72ºC for 1:30 min followed by final extension at 72ºC for 7min. PCR results were visualized on 1% agarose gel after electrophoresis.

For disease detection in greenhouse grafting trials, total nucleic acid was extracted from 150 mg of leaf midrib from scions of each treatment in July, August and September using cetyl-trimethyl ammonium bromide CTAB extraction protocol as previously described. Detection of "*Ca.* P. phoenicium" in conventional PCR AlmWB specific primer pairs AW16sF/AW23sR were used which amplify a 492 bp region. The reaction mixture consisted of 5x FIREPol® Master Mix (Solis BioDyne) containing

FIREPol® DNA polymerase, 5x reaction buffer B (0.4M Tris-HCl, 0.1M $(NH_4)_2SO_4$, 0.1% w/v Tween-20, 7.5 mM $MgCl_2$, 1mM dNTPs, blue and yellow dye). Each 20 µL PCR reaction mix contained 4 µL from the 5x master mix, 0.5 µL of forward primer (10pmol/µL), 0.5 µL of reverse primer (10pmol/µL), 2 µL (120 ng/µL) of 10x diluted DNA and 13 µL DNAse, RNAse free water.

The cycling conditions were as follows: 94ºC for 3 min, 35 cycles at 94ºC for 30 sec, 52ºC for 30 sec and 72ºC for 1 min followed by final extension at 72ºC for 7min. DNA from healthy plants of each treatment was considered as negative control and DNA from grafted almond scions was considered as positive control for the PCR assays.

Amplified PCR products were run on a 1% agarose gel by electrophoresis and stained in ethidium bromide (0.5g/L) for 30 min followed by destaining in distilled water, visualization was carried out by Gel Doc XR+ system (Bio-Rad Laboratories, Hercules, USA).

b. Phytoplasma quantitation using qPCR (greenhouse grafting trail)

A real time PCR (qPCR) assay was carried for quantitation of phytoplasma titer in AlmWB-infected plant tissue coming from treatments that exhibited positive reactions in conventional PCR.

qPCR assays were carried out using primers and probes previously reported by (Jawhari et al., 2015). For specific detection of AlmWB, AWsF/AWsR primers targeting 132 bp region were used along with a TaqMan® probe; AW23plus, which was labeled with a Black Hole Quencher plus (BHQplus™) at the 3’ end , and 6-carboxyfluorescein (FAM) at the 5’ end. Normalization of phytoplasma titer was

performed using primer pair Prun18S-F/Prun18S-R amplifying a 109 bp amplicon of the 18S rRNA gene of almond along with Prun18S-TaqM probe labeled with Black Hole Quencher plus at the 3' end and 6-carboxyfluorescein (FAM) at the 5' end.

i. Real time PCR protocol

Real time PCR runs were performed in Hard-Shell® 96-Well PCR plates (Bio-Rad Laboratories, Hercules, USA) in CFX96 Touch thermal cycler (Bio-Rad Laboratories, Hercules, USA). For detection of "*Ca.* P. phoenicium" each 20 µL qPCR reaction contained 2 µL of DNA template, 10 µL of 2x iQ^{TM} Supermix (Bio-Rad Laboratories, Hercules, USA), 0.3 µM; 0.6 µL, of each forward and reverse primers (AWsF/AWsR), 0.2 µM; 0.4 µL, of AWs23plus probe and 6.4 µL DNAse and RNAse free water. The cycling conditions were: 95ºC for 3 min, followed by 40 cycles of 95ºC for 10 s, 63ºC for 15s and 72ºC for 15s. As for the internal control reaction, 20 µL qPCR reaction consisted of 2 µL of healthy DNA template, 10 µL of 2x iQ^{TM} Supermix (Bio-Rad Laboratories, Hercules, USA), 0.4 µM; 0.8 µL, of each forward and reverse primers (Prun18S-F/Prun18S-R), 0.2 µM; 0.4 µL, of Prun18S-TaqM probe and 6 µL DNAse and RNAse free water. Cycling conditions were as follows: 95ºC for 3 min, followed by 40 cycles of 95ºC for 10s and 65ºC for 30s.

ii. Generation of standard curves

In order to generate a standard curve of AlmWB, plant DNA infected with "*Ca.* P. phoenicium" was subjected to PCR using AlmF2 and P7 primers that amplify a 579

bp amplicon containing the target region of "*Ca.* P. phoenicium" qPCR primers and probe. The PCR product was purified using peqexGOLD gel traction kit (peqlab, Germany) and cloned into pGEM-T easy vector system II (promega, Wisconsin, USA) and transformed in XL1Blue cells. Selected colonies were subjected to purification using Qiaprep® Spin Miniprep kit (Qiagen, Germany) and the resulting plasmids were quantified with the ND-1000 spectrophotometer (NanoDrop Technologies, USA) and serially diluted. Plasmid copy number calculation was performed using the following formula: copy number = concentration of plasmid/ [(size of insert + size of vector) x 660)/ (Avogadro's number)], where size of insert= 579bp, size of vector= 3015 bp and Avogadro's number= 6.022×10^{23}, 127.8 ng of pGEM-T easy vector (3,015+579) consists of 3.7×10^{10} plasmids.

For normalization of phytoplasma titer a standard curve of plant internal control was generated from healthy almond phloem DNA. Quantitation was carried out with the ND-1000 spectrophotometer (NanoDrop Technologies, USA) and DNA was serially diluted from a starting quantity of 295ng.

Copy number calculation of phytoplasma was performed using the following formula $Nt=10^{\frac{Cq-b}{a}}$, where Nt is the target copy number, Cq or Ct the quantitation cycle of each sample, a and b the slope and the intercept of each standard curve respectively. Efficiencies of the standard curves were calculated by the Bio-Rad CFX Manager software through adopting the following formula $E = [10^{(\frac{-1}{slope})} - 1] * [100]$.

iii. Statistical analysis

Statistical analysis was performed by IBM SPSS statistics 21. For study of almond scion recovery from phytoplasma infection one way ANOVA was followed by Tukeys HSD test for multiple comparisons was performed

C. Epidemiology and strain identification of *"Ca.* P. phoenicium"

For the study of "*Ca.* P. phoenicium" strains in different regions, almond phloem samples were collected from almond trees exhibiting disease symptoms from Feghal in Jbeil district and Thoum and Rashana in Batroun district.

After C-TAB DNA extraction, samples were subjected to PCR using primer pair gep F1/R1 amplifying a 990bp amplicon. The cycling conditions were as follows: 95ºC for 5 min, 35 cycles of 95ºC for 1 min and 50ºC for 2 min and an extension step at 72ºC for 2.5 min followed by final extension at 72ºC for 7 min. The direct PCR products were diluted 30x and used as DNA template for nested PCR using primer pair gep F2/R2 amplifying a 690bp amplicon. The cycling conditions were as follows: 95ºC for 5 min, 35 cycles of 95ºC for 1 min, 52ºC for 2 min and an extension step at 72ºC for 2.5 min followed by final extension at 72ºC for 7 min. PCR products were electrophoresed and visualized by Gel Doc XR+ system Bio-Rad Laboratories, Hercules, USA)

For RFLP, the obtained nested PCR amplicons were used as DNA templates and total DNA extract from infected plants was used as positive control for restriction digestion by Hpy188III restriction enzyme having TCNNGA restriction site. Restriction digestion mixture included 6 µL of nested PCR product, 2 µL of 10x NE buffer 4

(containing 50mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate and 1mM dithiothreitol) 0.2 µL of BSA supplied with the restriction enzyme, 1 µL of Hpy188III (BioLabs®, New England) and 10.8 µL of DNAse and RNAse free water. The entire mixture was held at 37ºC overnight and enzyme activity was stopped by heat inactivation at 65ºC for 20 min. To each 20 µL reaction tube 5 µL of 3x loading dye was added and run on 3% agarose gel (Appendix II, B) and electrophoresed using 0.5x TBE buffer. Gel was incubated in ethidium bromide for 30 min and washed with distilled water followed by UV visualization on Gel Doc XR+ system (Bio-Rad Laboratories, Hercules, USA**).**

CHAPTER IV

RESULTS

A. Antibody Production against Phytoplasma Membrane Proteins

1. Membrane protein amplification

DNA samples from AlmWB-infected and healthy almond trees were subjected to four PCR protocols in order to amplify the genomes of two membrane proteins to be cloned in two protein expression vectors. For protein expression in pIVEX 1.3 wg vector, PCR conducted using Fep/RepAlmWB-1160 primers amplified a 578 bp DNA amplicon from AlmWB infected samples, but not from healthy controls (Figure 1). Similarly, PCR using Fep/RepAlmWB-1850 primers yielded an amplicon of 728 bp (Figure 2). The two amplicons were of the expected size. On the other hand, for expression in pQE-32 vector, PCR conducted using F/RAlmWB-1160 primers and F/RAlmWB-1850 primers amplified a 584 bp and a 734 bp amplicon respectively (Figure 3). Healthy almond DNA samples used as negative controls in the PCR runs showed no amplification products when using any of the primers proving their specificity to AlmWB phytoplasma.

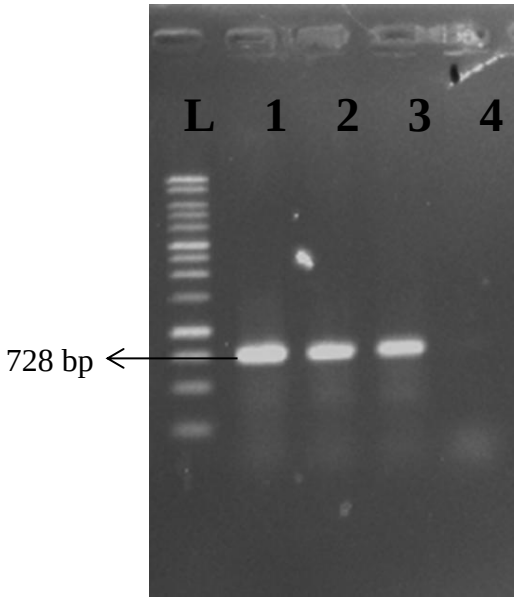


Figure 1: Agarose gel electrophoresis of PCR products using Fep/RepAlmWB-1850 primers. L= 1 Kb ladder; 1, 2 and 3: 728 bp amplicon from AlmWB-infected samples; 4: healthy control

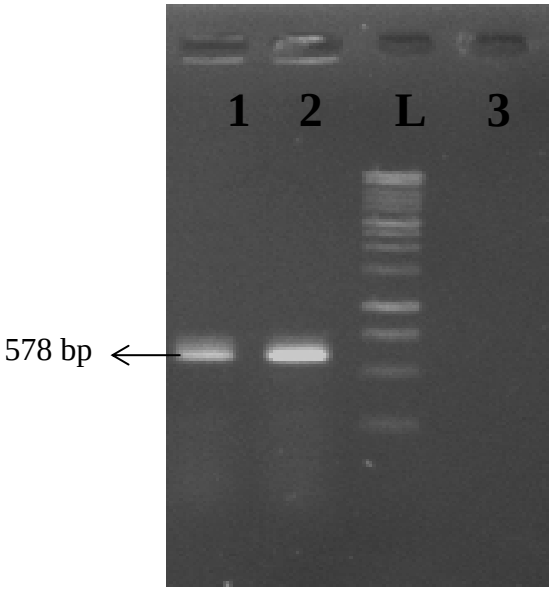


Figure 2: Agarose gel electrophoresis of PCR products using Fep/RepAlmWB-1160 primers. L= 1 Kb ladder; 1and 2: 578 bp amplicon from AlmWB-infected samples; 3: healthy control

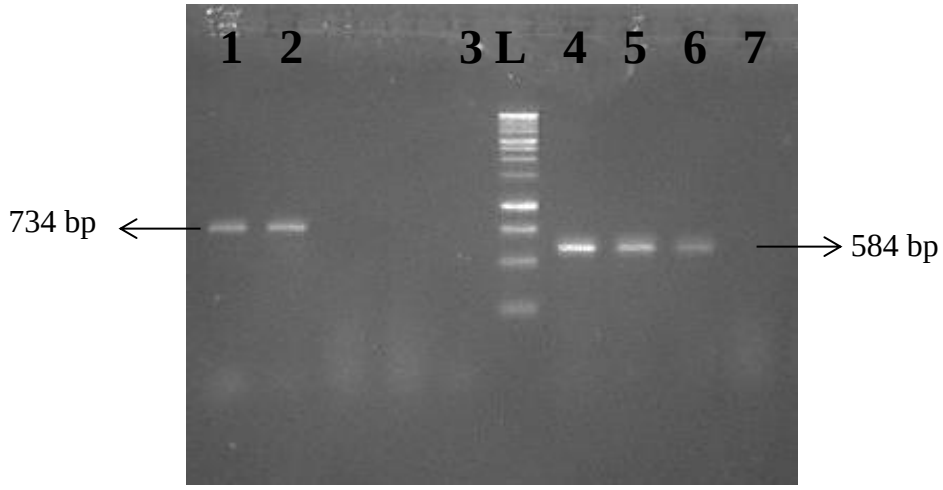


Figure 3: Agarose gel electrophoresis of PCR products from DNA of healthy almond (lanes 3 &7) and AlmWB-infected samples (lanes 1, 2, 4, 5& 6). 1, 2 and 3= using F/RAlmWB-1850 primers, L= 1 Kb ladder, 4, 5 and 6= using F/RAlmWB-1160 primers

2. Cloning of membrane proteins in cloning vectors

PCR products that were amplified by Fep/RepAlmWB-1160 and Fep/RepAlmWB-1850 were directly purified and ligated into pGEM-T easy cloning vector and transformed in competent cells of XL1Blue strain of *E.coli* and streaked on LB+Amp. White and blue colonies were observed after one day of incubation (Figure 4). The white colonies, successfully transformed, were selected for recombinant plasmid purification.

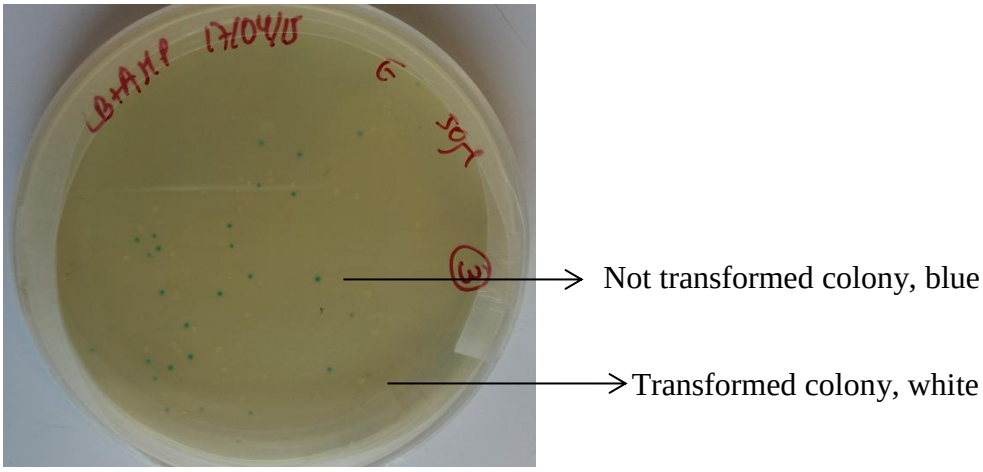


Figure 4: XL1Blue competent cells transformed with pGEM-T easy vector + AlmWB-1160 gene and streaked on LB+Amp plates coated with IPTG and X-Gal. The inserted amplicons were amplified using primers Fep/RepAlmWB-1160 and Fep/RepAlmWB-1850

Similarly, white and blue colonies were observed when PCR products of primers F/RAlmWB-1160 and F/RAlmWB-1850 were ligated into pGEM-T easy vector followed by transformation in XL1Blue. The cells were streaked on LB+Amp plates and incubated overnight at 37ºC (Figure 5, 6).

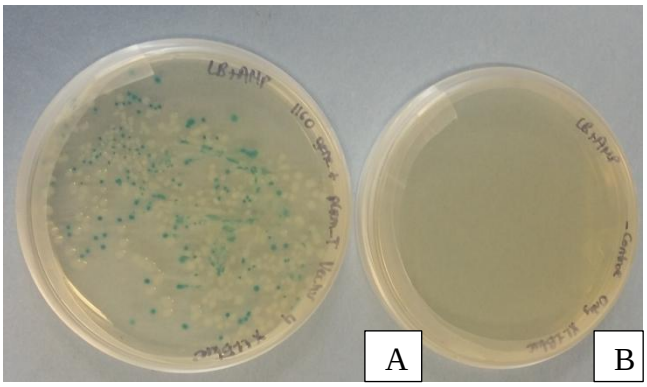


Figure 5: XL1Blue competent cells transformed with pGEM-T easy vector + AlmWB-1160 membrane protein gene and streaked on LB+Amp plates coated with IPTG and X-

Gal (A). The white colonies represent successfully transformed bacterial cells. Plates inoculated with non-transformed bacteria used as negative controls (B). The inserted amplicons were amplified using primers F/RAlmWB-1160 and F/RAlmWB-1850

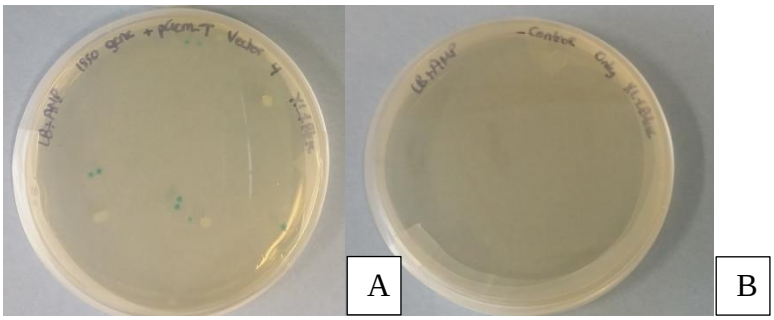


Figure 6: XL1Blue competent cells transformed with pGEM-T easy vector + AlmWB-1850 gene and streaked on LB+Amp plates coated with IPTG and X-Gal (A). Plates inoculated with non-transformed bacteria used as negative controls (B)

3. Sub-cloning in expression vectors

a. Sub-cloning in pIVEX 1.3 wg vector

Recombinant PGEM-T easy plasmids subjected to restriction digestion using *NcoI* and *SmaI* restriction enzymes yielded two bands, one representing pGEM-T easy vector (3,015 bp) and the second either the 578 or the 728bp inserts (Figure 7). The purified inserts were ligated into pIVEX 1.3 wg expression vector (3,236 bp) which was digested with the same restriction enzymes.

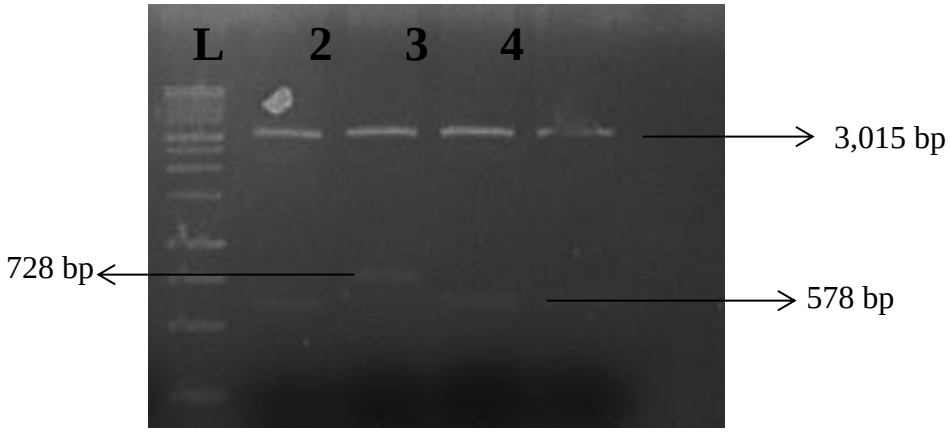


Figure 7: Agarose gel electrophoresis of recombinant pGEM-T easy vector + membrane proteins after restriction digestion with *NcoI* and *SmaI*. L= 1 Kb ladder; 2 and 4: pGEM-T easy vector+AlmWB-1160; 3: pGEM-T easy vector+AlmWB-1850

Restriction digestion products were gel purified and in turn each gene was ligated into the expression vector. Transformation of the recombinant pIVEX 1.3 wg plasmids containing AlmWB-1160 gene were performed in *E. coli* strains M15 and KRX; while recombinant plasmids containing AlmWB-1850 gene were performed only in *E. coli* KRX strain since transformation in M15 competent cells was not successful (Figures 8, 9). Non-transformed bacterial cells were considered as negative controls.

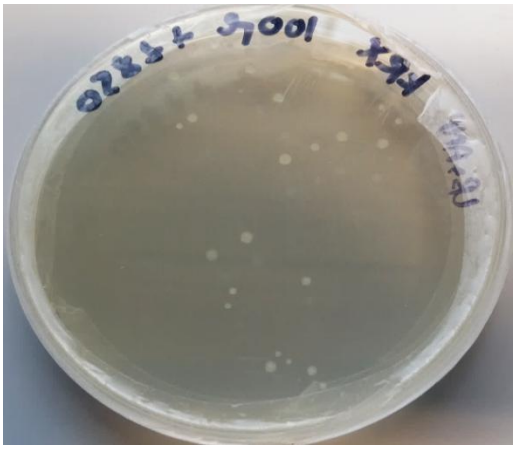


Figure 8: White colonies originating from KRX competent cells transformed with pIVEX 1.3 wg vector + AlmWB-1850 gene and streaked on LB+Amp plates

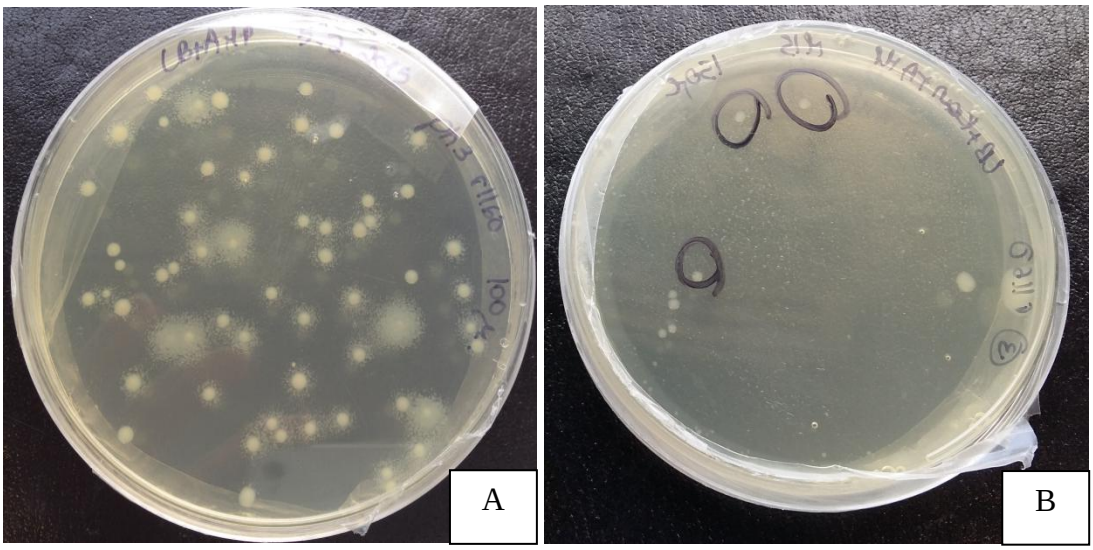


Figure 9: White colonies originating from KRX competent cells transformed with pIVEX 1.3 wg vector + AlmWB-1160 gene and streaked on LB+Amp plates (A). White colonies originating from M15 competent cells transformed with pIVEX 1.3 wg vector + AlmWB-1160 gene and streaked on LB+Amp+Kan plates (B)

b. Sub-cloning in pQE-32 vector

For sub-cloning in pQE-32 protein expression vector, both pGEM-T (3,015 bp) easy vector containing either AlmWB-1160 gene (584 bp) or AlmWB-1850 gene (734 bp) and pQE-32 vector (3,462 bp) were subjected to restriction digestion using *SphI* and *PstI* restriction enzymes (Figure 10).

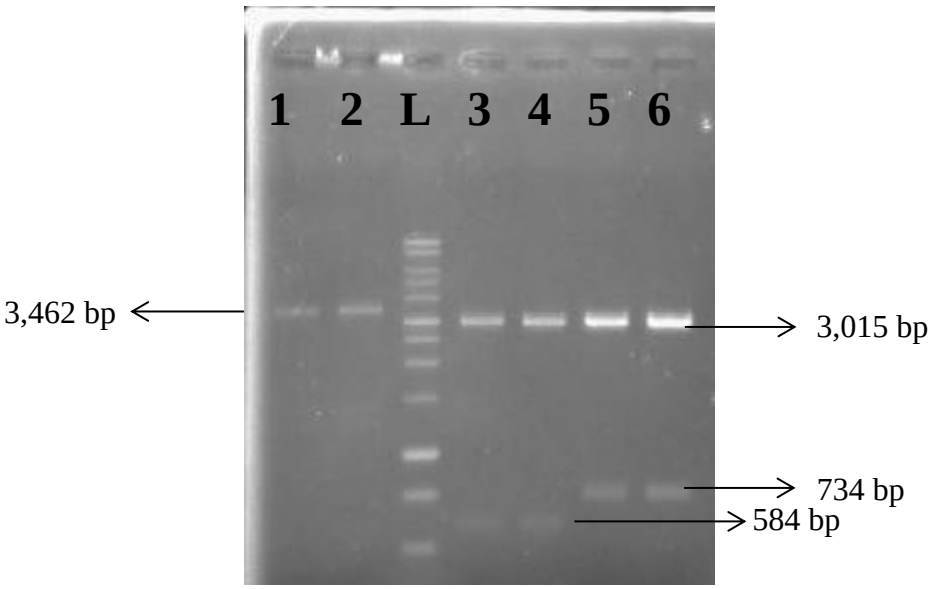


Figure 10: Agarose gel electrophoresis of recombinant pGEM-T easy vector + membrane proteins and pQE-32 expression vector after restriction digestion with *SphI* and *PstI*. L= 1 Kb ladder; 1 and 2: pQE-32 vector; 3 and 4: pGEM-T easy vector+AlmWB-1160; 5 and 6: pGEM-T easy vector+AlmWB-1850

After restriction digestion, gel purification was performed. PQE-32 linearized expression vector was ligated with AlmWB-1160 gene and AlmWB-1850 gene

followed by transformation in both M15 and KRX *E. coli* competent cells and streaked on LB+Amp+Kan and LB+Amp plates, respectively (Figure 11, 12).

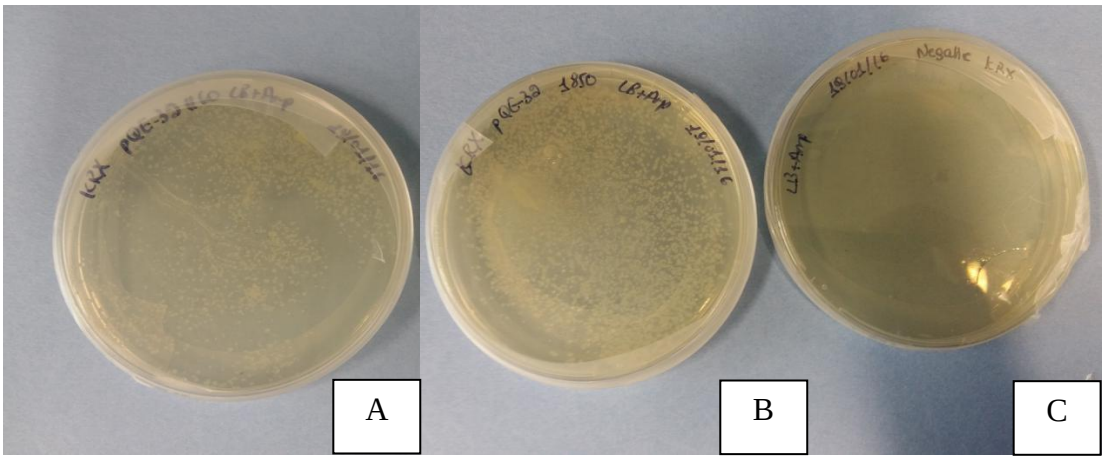


Figure 11: White colonies originating from KRX competent cells transformed with pQE-32 vector + AlmWB-1160 gene and streaked on LB+Amp plates (A). White colonies originating from KRX competent cells transformed with pQE-32 vector + AlmWB-1850 gene and streaked on LB+Amp plates (B). Plates inoculated with non-transformed bacteria used as negative controls (C)

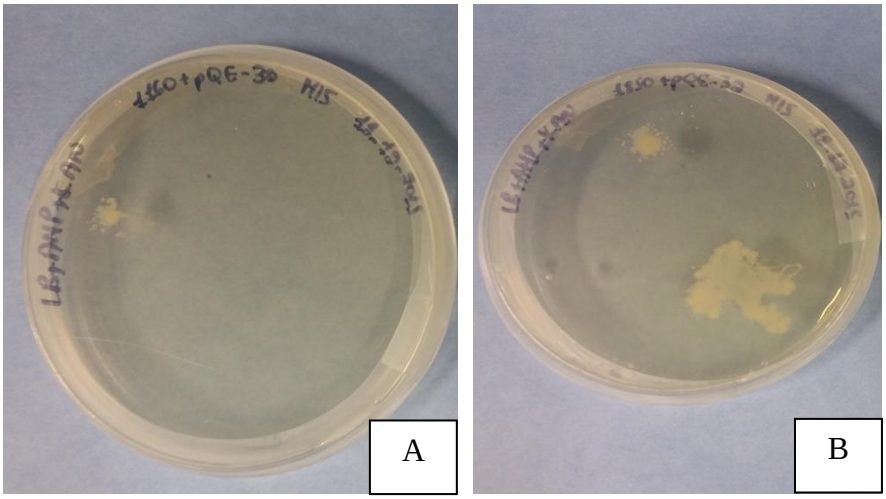


Figure 12: White colonies originating from M15 competent cells transformed with pQE-32 vector + AlmWB-1160 gene and streaked on LB+Amp+Kan plates (A). White

colonies originating from M15 competent cells transformed with pQE-32 vector + AlmWB-1850 gene and streaked on LB+Amp+Kan plates (B)

4. Protein expression

Based on sequencing results, positively transformed colonies were used for protein expression, while non-transformed M15 and KRX cells were used as negative controls.

When aliquots of 3 ml M15 competent cells transformed with recombinant pIVEX 1.3 wg expression cassette containing AlmWB-1160 gene having a molecular weight of 24.45 kDa and M15 negative control were induced with 1 mM IPTG for 1, 3 and 4 hrs and overnight a protein band of the expected size, about 24 kDa, was observed following SDS-PAGE gel electrophoresis in the protein pattern of transformed, cells but not in the transformed cells (Figure 13). The same procedure was conducted in KRX competent cells using 0.1% rhamnose for initiation of protein induction.

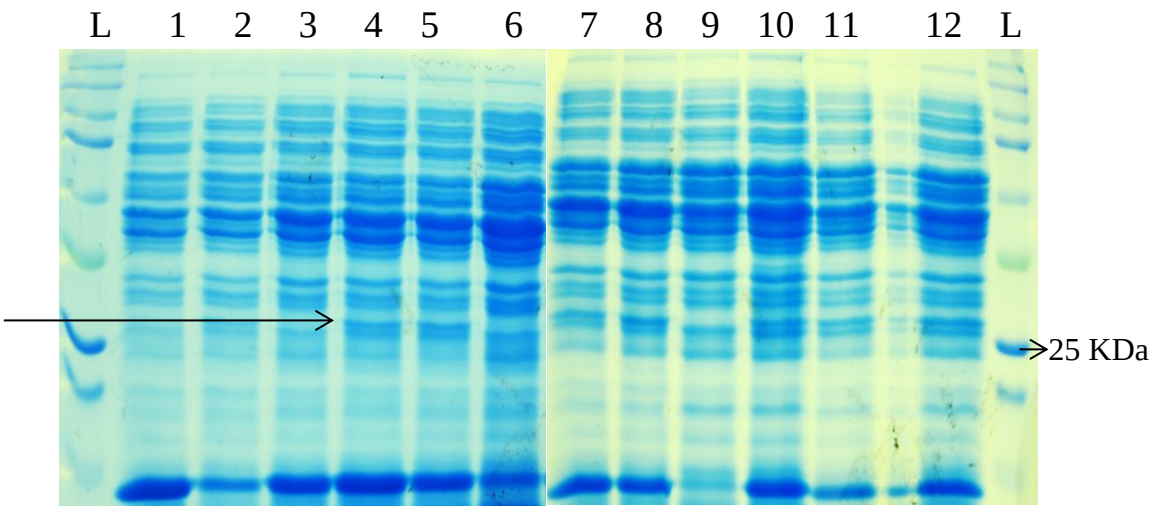


Figure 13: SDS-PAGE gel stained with Coomassie blue showing protein expression of 24.45 kDa: 3,6 and 9 = non transformed M15 cells induced with IPTG for 1 and 3 hrs and overnight; 1-2, 4-5, 7-8 and 10-11-12: M15 cells transformed with IPTG 1 mM for 1, 3, 4hrs and overnight, respectively; L= Protein Ladder.

5. Protein purification

For the purpose of protein purification, large scale protein expression was performed. Cell lysis was conducted using the two previously described methods, native and denaturing, for both expression systems.

a. Protein purification from pIVEX 1.3 wg expression system

Since transformation of recombinant plasmid pIVEX 1.3 wg + AlmWB-1850 was not successful in M15 cells, protein purification was performed for M15 competent cells harboring AlmWB-1160 gene. For transformed KRX cells, purification was performed for cells harboring AlmWB-1160 and AlmWB-1850 genes. Purification was performed under both native (chemical lysis) and denaturing conditions (Figure 14). Untransformed M15 and KRX cells were used as negative controls while recombinant CYSDV CP (35 kDa) was used as positive control.

Recombinant protein expression was successful using KRX cells transformed with pIVEX 1.3 wg expression cassettes of the two membrane genes. The two membrane proteins were expressed and successfully isolated under both native and denaturing conditions AlmWB-1160 24.45 KDa while AlmWB 1850 29.09 KDa. Under native conditions, more than one protein band appeared that were absent in the negative

control; however, under denaturing conditions the most prominent band was that correlated with the monomers of the membrane proteins, while the other bands were very faint. These results would have been very promising. However, the bands observed in the negative controls used with the same *E. coli* strains using pQE32 vector showed a somewhat similar protein pattern to that observed in the transformed and induced cells transformed with pIVEX 1.3 wg expression cassettes. Therefore, it is necessary to repeat the experiment to make sure that nothing wrong occurred with the negative control.

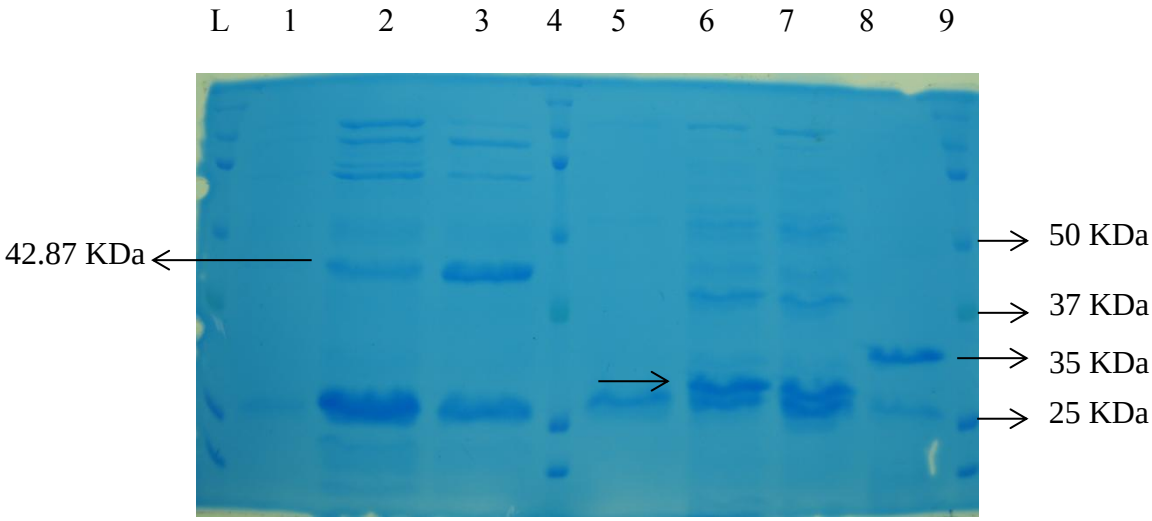


Figure 14: SDS-PAGE gel stained with Coomassie blue after Ni-NTA purification of pIVEX 1.3 wg+AlmWB-1160 and pIVEX 1.3 wg+AlmWB-1850 recombinant plasmids transformed in KRX competent cells; L: Protein ladder; 1: Untransformed KRX cell considered as negative control under native conditions; 2: KRX transformed with recombinant pIVEX 1.3 wg+AlmWB-1160 lysed under native conditions (24.45 KDa); 3: KRX transformed with recombinant pIVEX 1.3 wg+AlmWB-1850 lysed under natuve conditions (30.05 KDa); 4: Protein ladder; 5: Untransformed KRX cell considered as negative control under denaturing conditions; 6: KRX transformed with recombinant pIVEX 1.3 wg+AlmWB-1160 lysed under denaturing conditions (24.45 KDa); 7: KRX transformed with recombinant pIVEX 1.3 wg+AlmWB-1850 lysed under denaturing conditions (30.05 KDa); 8: recombinant CYSDV coat protein (35 KDa) under denaturing conditions; 9: Protein Ladder

Both recombinant membrane proteins of "Ca. P. phoenicium" were successfully expressed in bacterial cells (two in KRX and one in M15). The recombinant membrane proteins were successfully purified under native or denaturing extraction conditions. Under native conditions, monomers, dimers and polymers were observed in gels, while under denaturing conditions, mainly monomers were observed.

b. Protein purification from pQE-32 expression system

Attempts to express the two AlmWB membrane proteins using pQE-32 vector were not successful in both *E. coli* strains, M15 and KRX. No specific bands of the expected sizes were observed in polyacrylamide gels; the bands observed from cultures induced by IPTG or rhamnose, respectively, did not differ from those obtained from the negative control using protein extraction under native or denaturing conditions (Figure 15).

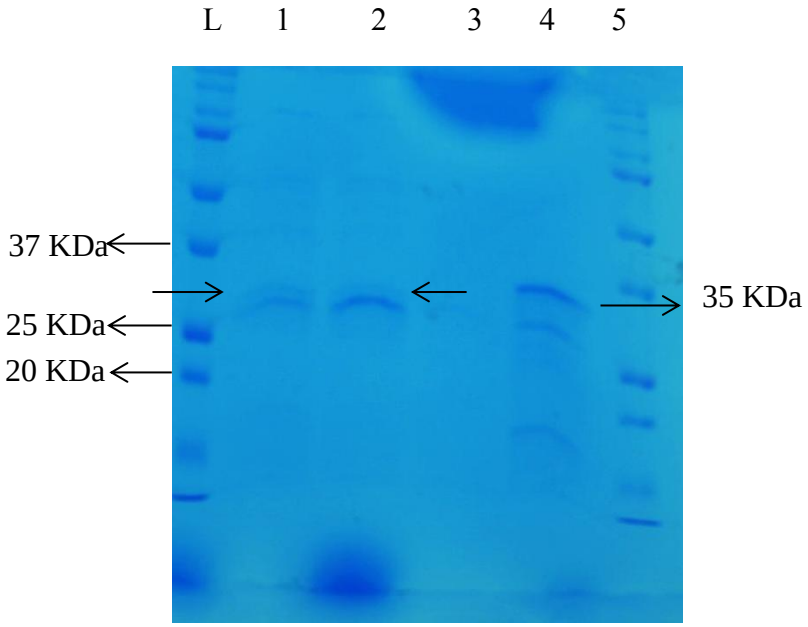


Figure 15: SDS-PAGE gel stained with Coomassie blue after Ni-NTA purification of pQE-32+AlmWB-1160 and pQE-32+AlmWB-1850 recombinant plasmids transformed in KRX competent cells; L: Protein ladder; 1: Untransformed KRX cell considered as negative control under denaturing conditions; 2: KRX transformed with recombinant pQE-32+AlmWB-1160 lysed under denaturing conditions (25.1 KDa); 3: KRX transformed with recombinant pQE-32+AlmWB-1850 lysed under denaturing conditions (30.73 KDa); 4: recombinant CYSDV coat protein (35 KDa) under denaturing conditions; 5: Protein Ladder

B. Disease management through grafting on resistant/tolerant rootstocks

1. AUB greenhouse experiment: Grafting of AlmWB-infected almond scions on different rootstocks

a. Scion survival rate and symptom development

A total of 38 stone fruit seedlings were used in this trial out of which 25 were inoculated with scions originating from AlmWB- infected trees and 13 served as negative controls. The scion survival rate ranged between 50 and 80%. In almond used as positive control survival was 4 out 5; in plum, varieties Jawhara, Angelino and red plum, the survival was 2, 3 and 2 out of 4, respectively; and in apricot, varieties Early blush and Farclo, the survivals were 2 and 3 out of 4, respectively (Table 2).

Table 2: Greenhouse grafting trial: number of AlmWB-infected scions that successfully grew after grafting on seedlings of different stone fruits varieties, grafting on 13-04-2015

	Treatments	Number of seedlings	Negative control	Grafted	Scion survival
1	**Almond (Halawani)**	8	3	5	4/5
2	**Plum (Jawhara)**	6	2	4	2/4

3	**Plum (Angelino)**	6	2	4	3/4
4	**Plum (Red)**	6	2	4	2/4
5	**Apricot (Early Blush)**	6	2	4	2/4
6	**Apricot (Farclo)**	6	2	4	3/4

Symptom development started two months post grafting; in June, one seedling from the positive control treatment showed smaller leaves. While in July, three months post grafting, one seedling from the positive controls showed typical symptoms of AlmWB by developing witches' broom symptoms from the rootstock and not from the newly emerged scion (Figure 16).

Two months post grafting one seedling from the apricot Early blush treatment the almond scion developed bush like growth with stems having short internodes or witches broom like symptoms; but three months post grafting, the same seedling showed recovery and exhibited development of normal stems with normal internodes and leaves (Figure 17). Similarly, two months post grafting, one seedling from the red plum treatment showed smaller leaves but without proliferation of witches' broom symptoms; however, three months post grafting, the almond growth grafted on Red plum recovered and developed normal growth. In August and September, about five months post-grafting, all shoots that developed from infected almond scions grafted on plum and apricot showed normal growth. On the other hand, one of the positive controls showed disease symptoms.

Figure 16: Positive control almond exhibiting proliferation from the base of the plant with witches' broom symptoms, light green leaves and short internodes

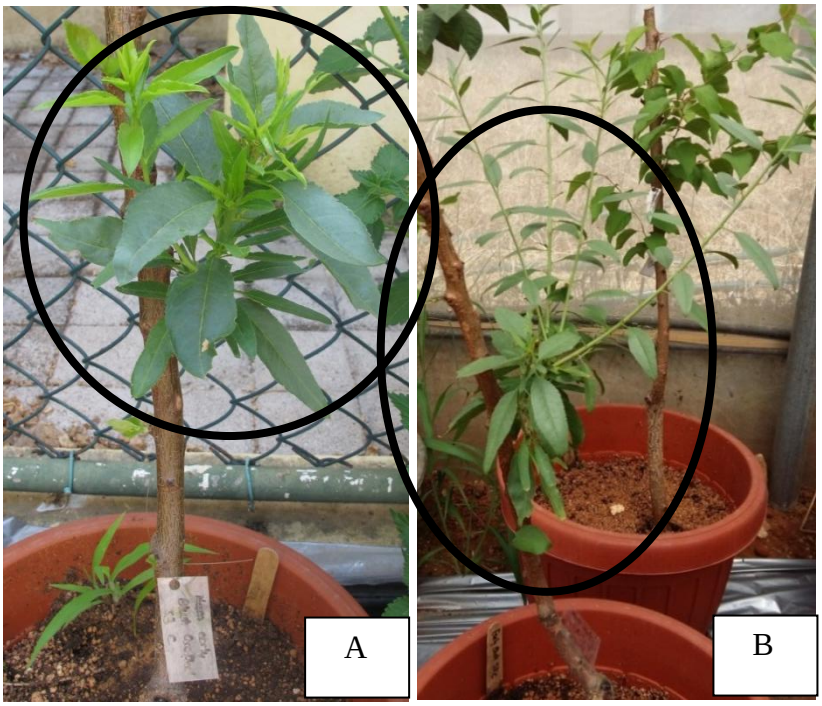


Figure 17: Apricot variety Early blush, 2 months post grafting, showing early development of witches' broom symptom (A). Apricot variety early blush, 3 months post grafting, showing recovery with development of healthy shoots from the same shoots that showed witches' broom symptoms earlier (B)

b. Detection of "*Ca.* P. phoenicium" using conventional PCR

AlmWB detection was carried out on monthly intervals starting from three months post grafting, July, August and September. Alm16sF/Alm23sR specific primers were used in detection amplifying a 492 bp region (Table 3).

In July, PCR tests gave positive results for infection of the new growth by "*Ca.* P. phoenicium" on two of the positive controls, on one scion of Early blush apricot, and on one Red plum.

In August, both apricot varieties, Early blush and Red plum did not exhibit a positive result with PCR, while two samples of the positive controls and one sample plum variety Jawhara exhibited positive reaction with PCR.

In September, three out of four positive controls gave positive result by PCR while only one exhibited symptom. One sample of the plum variety Jawhara and one sample of the Apricot variety Farlco also exhibited positive PCR results but their growth looked normal. The remaining treatments, growth development from almond scions grafted on Angelino and Red plums and Apricot Early Blush, were PCR negative and did not develop symptoms. Throughout the entire experiment the negative controls of each treatment showed no symptoms of disease and tested negative by PCR.

Table 3: Symptom development and detection of "*Ca*.P phoenicium" in grafted seedlings by PCR using specific primer pair Alm16sF/Alm23sR.

	Treatments	**14/07/15**		**31/08/15**		**21/09/15**	
		PCR *	Symptom**	PCR	Symptom	PCR	Symptom
1	**Almond**	2/4	S/NS	2/4	S/NS	3/4	S/NS/NS
2	**Plum (Jawhara)**	0	NS	1/2	NS	1/2	NS

3	**Plum (Angelino)**	0	NS	0	NS	0/3	NS
4	**Plum (Red)**	1/2	NS	0	NS	0/2	NS
5	**Apricot (Early blush)**	1/2	S	0	NS	0/2	NS
6	**Apricot (Farclo)**	0	NS	0	NS	1/3	NS

*PCR = Number of samples that tested positive for phytoplasma infection/ total number of samples tested.
**S= symptom appearance
**NS= no symptom appearance

c. Quantitation of "*Ca.* P. phoenicium" using qPCR

i. Generation of standard curves

Quantitation of "*Ca.* P. phoenicium" was performed using qPCR technique for the following treatments: Early blush apricot, Red plum and Farclo apricot against the positive control and negative controls. For quantitation of AlmWB phytoplasma in the newly developed scions, the standard curve for "*Ca.* P. phoenicium" was developed using the recombinant plasmid and the efficiency was 108.7% (Figure 18). In order to standardize the results obtained a standard curve of healthy almond was performed by diluting DNA from healthy almond, the curve had an efficiency of 91.5% (Figure 18).

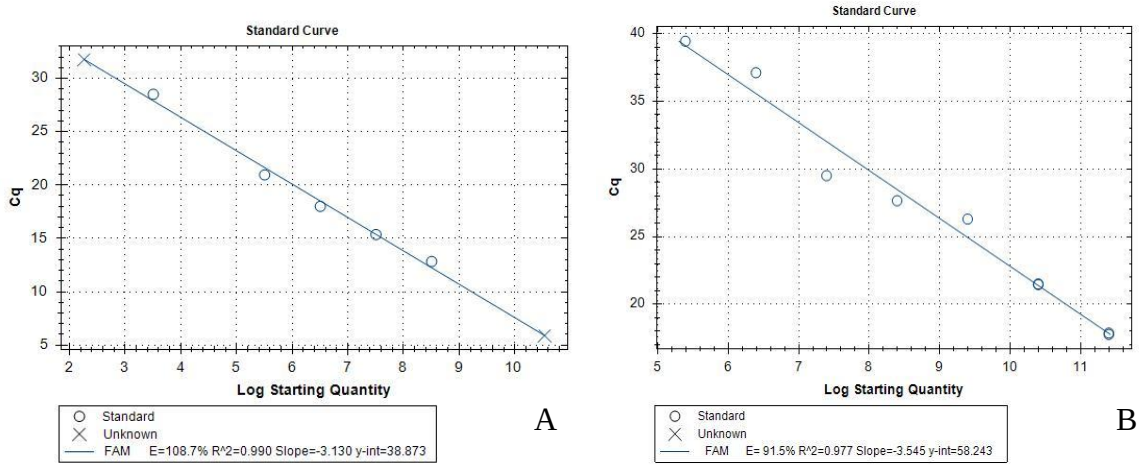


Figure 18: Standard curve for AlmWB circular plasmid (A); Total nucleic acid (TNA) extract of AlmWB-infected almond (B)

ii. *"Ca.* P. phoenicium" quantitation in different treatments

DNA extracted from the emerged almond scions during months July, August and September were subjected to two different qPCR runs; one targeting a "Ca. P. phoenicium" gene, while the second targeted the internal control gene. The qPCR tests were also performed on the negative controls of each treatment and the runs were replicated once. The Cq value of all the negative controls was higher than 30 cycles; as a result samples that had a mean Cq value equal to or higher than 30 were considered negative ie. Not infected by "*Ca*. P. phoenicium" (Tables 4). Apricot variety Angelino was considered negative due to Cq value higher than 30 cycles during the 3 months tested, the phytoplasma remained below detection level by the qPCR test. Therefore, this sample was not used in statistical analysis and copy number calculation.

Table 4 and Figure 18 were used to calculate the quantity of phytoplasma in terms of phytoplasma genomic units (GU/ng plant DNA). The quantity was calculated

for each sample and divided by 2 since phytoplasma is expected to have 2 rRNA operons.

Our data showed that the detection limit of the qPCR technique used was about 0.5 GU of phytoplasma/ ng of plant DNA. In the positive control the phytoplasma titer ranged between 83 and 604 phytoplasmas /ng plant DNA, the highest titer was detected in September. In both treatments that show recovery, almond grafted on Early Blush apricot and Red Plum, the occurrence of recovery from symptoms was also confirmed by qPCR. The phytoplasma titer was 44 and 3.1 phytoplasma GU/ ng of plant DNA in Early blush and Red plum, respectively in July when the symptoms were apparent, but this titer dropped to below detection level in August and September, when the symptoms disappeared. The other three treatments behaved somewhat differently. The phytoplasma was not detected in almond grafted on Angelino plum during the season. In Farclo apricot the phytoplasma titer was below detection limit in July and increased to 1.5 GU/ng plant DNA in September. However, in Jawhra plum the concentration varied between 11 and 42 GU/ng plant DNA in July and September, respectively. Noting that, no symptoms developed in the three latter treatments.

Table 4: Detection of "*Ca.* P. Phoenicium" by qPCR. Average Cq values of grafted and non-grafted (negative controls) stone fruit seedlings, during July, August and September for qPCR runs using primers and probes specific to the phytoplasma and to the plant DNA. Quantitation of "*Ca.* P. phoenicium" in different scion/rootstock treatments during July, August and September

	Treatments	Month	AlmWB DNA in plant DNA Cq (mean ± SD)	Quantity (GU)	Plant 18S rDNA Cq (mean ± SD)	Quantity (ng)	AlmWB DNA/ plant DNA (GU/ng)
1	Almond Negative	July August September	31.18 ± 1.22	1.42E+02	14.46 ± 0.43	1.23E+03	< 0.5
2	Plum Jawhara Negative	July August September	32.17 ± 2.59	6.93E+01	16.02 ± 0.06	8.09E+02	< 0.5
3	Red Plum Negative	July August September	30.93 ± 0.3	1.72E+02	13.72 ± 0.46	3.602E+03	< 0.5
4	Early blush Negative	July August September	30.90 ± 0.8	1.75E+02	13.23 ± 0.23	4.984E+03	< 0.5
5	Farclo Negative	July August September	32.8 ± 2.72	4.3E+01	14.75 ± 0.01	1.857E+03	< 0.5
6	Almond	July	22.53 ± 0.88	8.3248E+04	16.32 ± 0.43	6.69E+02	124
		August	23.42 ± 0.39	4.3254E+04	16.71± 0.11	5.20E+02	83.2
		September	18.19 ± 0.16	2.023E+06	13.23 ± 0.06	4.98E+03	406
7	Plum (Jawhara)	July	25.71 ± 0.21	7.8E+03	16.35 ± 0.99	6.6E+02	11.2
		August	27.83 ± 0.47	1.7E+03	12.86 ± 0.47	6.34E+03	< 0.5
		September	23.19 ± 0.48	5.1E+04	15.47 ± 0.42	1.2E+03	42.5
8	Plum red	July	28.47 ± 0.75	1.05E+03	17.35 ± 0.24	1.05E+03	3.1
		August	29.76 ± 0.06	4.1E+02	14.89 ± 0.60	1.7E+03	< 0.5
		September	31.51 ± 2.15	1.12E+02	16.14 ± 0.52	7.53E+02	< 0.5

9	Apricot (Early blush)	July	21.84 ± 0.23	1.4E+05	13.92 ± 0.68	3.2E+03	44
		August	31.50 ± 1.19	3.2E+03	14.14 ± 0.34	2.76E+03	< 0.5
		September	31.07 ± 1.05	8.6E+01	15.87 ± 0.50	8.97E+02	< 0.5
10	Apricot (Farclo)	July	30.74 ± 0.78	2.4E+02	14.80 ± 0.42	1.2E+04	< 0.5
		August	30.33 ± 0.18	1.75E+02	11.79 ± 0.59	2.5E+03	< 0.5
		September	30.97 ± 0.81	6.4E+02	13.57 ± 0.64	4.2E+02	1.5

*A Cq over 30, is considered qPCR negative, ie phytoplasma not present or below detection limit.

iii. Statistical analysis

Due to low sample size, mean values were not used for statistical analysis but each sample and its corresponding replicate were used.

The treatments used for statistical analysis were negative control, positive control, Apricot Early blush, Apricot Farclo and Red plum varieties. Based on statistical analysis of "*Ca.* P. phoenicium" concentration using One-Way ANOVA, treatments showed significant difference at 95% confidence level (Appendix III, A). Post Hoc multiple comparisons using Tukey method showed that treatment 2; positive control, was significantly different than all the other treatments while all the other 4 treatments were not significantly different than treatment 1; negative control (Appendix III, A).

Statistical analysis using One-Way ANOVA for "*Ca.* P. phoenicium" concentration in July, August and September of treatment apricot Early blush showed a significant difference at 95% confidence level (Appendix III, B). Post Hoc multiple

comparisons using Tukey method showed that the phytoplasma titer in July was significantly different than in August and September (Appendix III, B).

2. Long term field trial

Ten severely AlmWB-infected almond trees were grafted with scions of different plum and apricot varieties. PCR was conducted two and five months post grafting, in June and September 2013, respectively (Table 6). In June and September, PCR results, using semi-specific primer pairs AlmF2/AlmR2, proved that all almond trees used as rootstocks were infected; but the growth that developed from the scions of plum Fortune and apricot Farclo varieties gave negative results in PCR and did not show symptoms or mild symptoms except for the growth that developed from scions of Farclo Apricot. In fact, Farclo apricot was the only variety that showed clear witches' broom symptoms in June and was PCR positive. However, by September, most of the growth of that scion showed recovery from symptoms and looked normal except for a few leaves at the base of the shoot that remained small and chlorotic; PCR results from the healthy looking leaves (recovered) were negative, while the PCR results were positive for a small number of leaves that were symptomatic even though they originated from the same scion (Figure 19).

Most grafted trees died after a very dry summer, only 2 Farclo apricot trees survived because they received indirectly some irrigation water while irrigating neighboring ornamental or vegetable plants.

One year post grafting both Farclo apricot trees F2P2 and F2P4 showed no symptom of disease development. PCR analysis using AlmF2/R2 primers was positive

for Farclo F2P4 but negative for Farclo F2P2. Two years post grafting symptoms were not present and PCR results were similar to those of the previous year (Table 5) and (Figure 20).

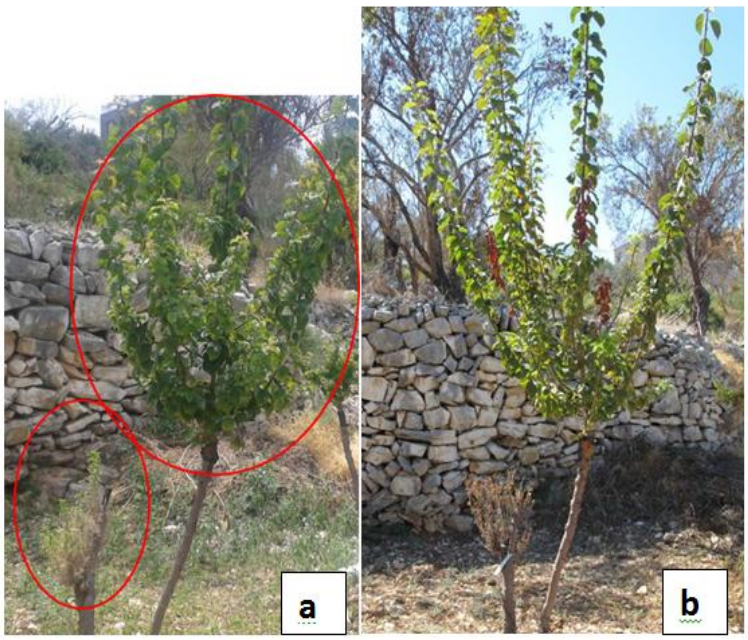


Figure 19: In field grafted AlmWB-infected almond rootstocks with plum Fortune scion. June 2013 symptom on rootstock and scion development (A). Same sample in September 2013 (B)

Figure 20: In field grafted AlmWB-infected almond rootstocks with Apricot Farclo scion. June 2013 symptom on rootstock and scion development(A). Same sample in September 2013(B)

Table 5: Symptom development on scions and PCR results using AlmF2/R2 primers 2 and 5 months post grafting and 1 and 2 years post grafting.

Tree Code	Scion variety	June 2013			September 2013		2014	2015
		PCR of scions	PCR of Rootstock	Symptom	PCR of scions	Symptoms	PCR of scions	PCR of scions
F1P2	Plum Abou Riha	-	+	Mild symptoms	-	Very mild / nutrient deficiency symptoms	Dead	Dead
F1P3	Plum Abou Riha	-	+	Mild symptoms	-	Scion dead	Dead	Dead
F1P4	Janarek plum	-	+	Mild symptoms	-	No symptoms	Dead	Dead
F1P5	Janarek plum	-	+	Mild symptoms	-	No symptoms	Dead	Dead
F2P4	Apricot Farclo	- (+)	+	Witches' broom	- +	Combination: no symptoms and symptoms	+ (NS*)	+ (NS*)
F2P2	Apricot Farclo	-(+)	+	Witches' broom	?	?	-	-

F3P2	Fortune plum	-	+	Mild symptoms	-	Mineral deficiency like symptoms	Dead	Dead
F3P1	Fortune plum	-	+	Mild symptoms	-	No symptoms	Dead	Dead
F1P1	Plum Santarosa	-	+	Mild symptoms	-	No symptoms	Dead	Dead
F3P4	Plum Santarosa	-	+	Mild symptoms	-	No symptoms	Dead	Dead

*NS: No symptom appearance

C. Epidemiology and strain identification of *"Ca.* P. phoenicium"

DNA from Almond samples infected with AlmWB phytoplasma and exhibiting disease symptoms collected from Feghal, Jeddayel, Rachana and Thoum areas in North Lebanon and subjected to nested PCR analysis using gep F1/R1 primers followed by gep F2/R2 primers amplified a 693 bp DNA region in infected samples but not in healthy samples (Figure 21).

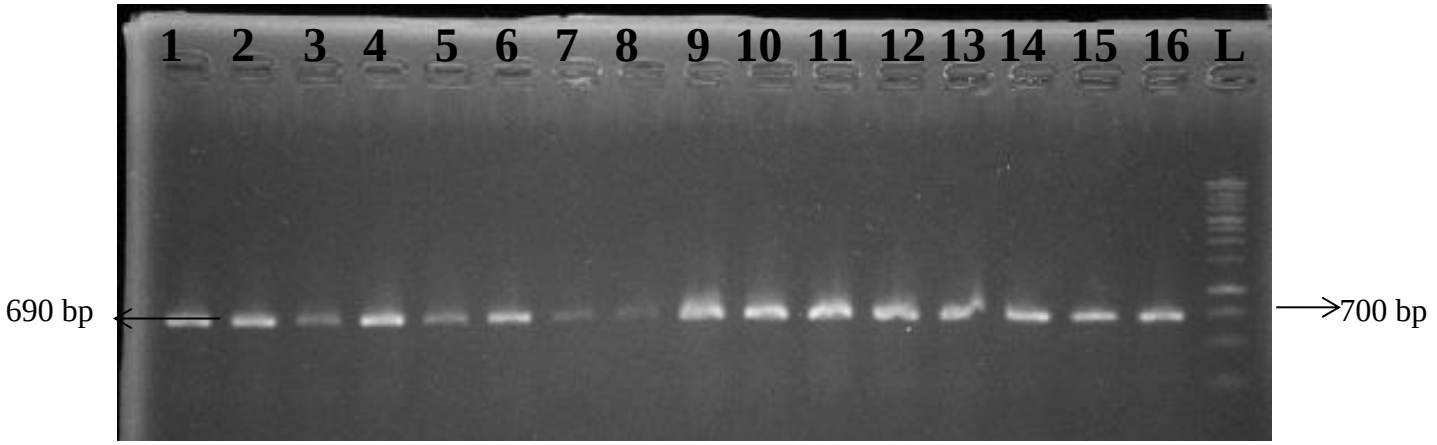


Figure 21: Agarose gel electrophoresis of nested PCR amplified fragments using gep F1/R1 and F2/R2 primer pairs. Samples from AlmWB-infeted almond trees collected from: 1-4: Feghal area; 5-8: Jeddayel area; 9-12: Rachana area; 13-16: Thoum area; L= 1Kb DNA bp Ladder

Two samples from each of the 4 regions were subjected to restriction digestion using Hpy18III restriction enzyme and run on 3% agarose gel. All samples showed one band at 693 bp level which is that of gep profile b where there is no restriction digestion and only one fragment is present (Figure 22).

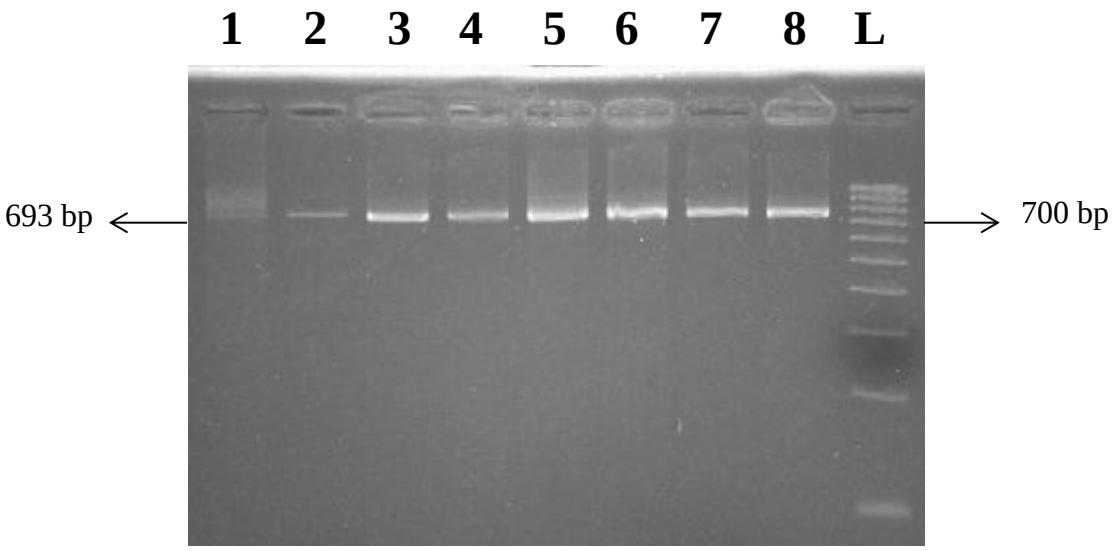


Figure 22: CAPS marker for AlmWB variability in the gep-gene: Electrophoresis in 3 % agrose gel showing the DNA pattern following digestion of nested PCR amplicons with Hpy18III restriction enzyme. 1-2: Feghal samples, 3-4: Jeddayel samples, 5-6: Rachana samples, 7-8: Thoum samples, L= 100 bp DNA ladder

CHAPTER V

DISCUSSION

In Lebanon, AlmWB phytoplasma has caused economically significant losses on stone fruit production especially that of almond. Infection of an almond tree with AlmWB phytoplasma can lead to gradual abnormal physiological growth, decrease in growth and vigor, complete yield loss within a year of infection and mortality after few years of infection (Abou-Jawdah, Y., Sobh, H., & Akkary, M. 2009). Diseases caused by phytoplasma are difficult to control by chemical measures. Therefore, preventive measures are targeted against the vector, eradication of infected trees, control of alternative hosts and replacement by tolerant or resistant crop varieties. To date, in Lebanon disease management focused on eradication of infected trees leading to loss of almond cultivation in several regions, mainly in North Lebanon (Abou-Jawdah et al., 2002). In Feghal region, some farmers removed infected trees and planted new almond seedlings; but four to five years after planting severe symptoms appeared on the newly planted seedlings. Therefore, an integrated approach should be followed for efficient management of the disease. The availability of efficient rapid and inexpensive detection method is required for early detection and eradication of infected trees as well as for detection of the phytoplasma in potential alternative hosts. The FAFS team has developed sensitive and specific detection methods based of PCR and qPCR. However, the PCR based techniques are relatively expensive compared to serological detection methods. Since phytoplasma can not be cultured in vitro, development of serological techniques has been hindered. Therefore, our research focused on,

a. development of serological techniques based on recombinant DNA technology
b. Development of efficient management technique that allows replanting almond trees in infected orchards.
c. Epidemiology/assessment of the diversity of AlmWB phytoplasma strains

A. Development of Serological Detection Methods for the Specific Detection of "*Ca.* P. phoenicium"

Phytoplasmas are phloem limited and present at relatively low concentration in the plant tissue therefore, very sensitive detection methods have to be developed. Relatively few serological methods were developed for phytoplasma. Therefore, phytoplasma detection during the last two decades has focused mainly on the use of PCR based techniques; in most cases nested PCR is required for detection of phytoplasma (Bertaccini & Duduk, 2010; Hogenhout *et al*., 2008; Abou-Jawdah et al., 2002). Since phytoplasma cannot be grown *in-vitro* to be used as antigen for antisera production, recombinant DNA technology may be used to produce the antigen. A team from AUB and University of Milano were able to sequence partially the genome of "*Ca.* P. phoenicium" the causal agent of AlmWB and to identify two potential membrane protein genes; AlmWB-1160 23.18 KDa and AlmWB-1850 29.09 KDa (Quaglino et al., 2015). Primers were designed in our laboratory and the two membrane protein genes were successfully amplified and cloned in the pGEM-T easy vector and then sub-cloned in two protein expression vectors; the pQE-32 vector which is suitable for expression in *E. coli* cells and the pIVEX 1.3 wg vector which is suitable for expression in either cell free system or *E. coli.* The second vector was included since some membrane protein

may be toxic to bacterial cells and in that case expression in cell free systems may constitute the alternative option. Sequencing of the cloned vectors proved that the inserts are effectively the AlmWB membrane protein genes and are positioned in frame for expression of the protein with a 6x histidine tag which allows efficient purification of the recombinant protein from other bacterial proteins. The experiments on protein expression are in progress, two *E. coli* strains will be used; M15 induced by IPTG and KRX induced by rhamnose, several protocols will be compared in order to identify the most efficient vector/host combination for recombinant protein expression.

Development of serological detection methods like tissue print immunoassay (TPIA) will help in surveys for early detection of the disease at fraction of the cost of using PCR based methods. TPIA does not require prior tissue extraction, are rapid and simple, do not require high level of expertise, the membranes can be transported to laboratories in distant regions and the results may be obtained within four hrs. However, the specificity of the produced antibodies should be verified, first against related phytoplasmas that attack stone fruits and then against most common bacterial pathogens or saprophytes on stone fruits. For example, the antibodies produced against Lime witches' broom phytoplasma gave also cross reaction with almond witches' broom "*Ca.* P. phoenicium" from Iran and sesame phyllody phytoplasma (Shahryari et al., 2013).

Few researchers used recombinant DNA technology to either express the membrane protein gene of phytoplasma or to produce serological detection methods. Due to the low expression level of ESFY phytoplasma immunodominant membrane protein gene protein expression has been performed under denaturing conditions using urea buffer (Mergenthaler, E., Viczian, O., Fodor, M., & Sule, S., 2001) whereas, antibody production from immunodominant membrane protein of Lime witches' broom

phytoplasma "*Ca.* P. aurantifolia" was performed under native lysis conditions (Shahryari et al., 2013).

B. Disease management: Evaluation of the potential of grafting on resistant rootstocks for management of almond witches' broom disease through Greenhouse and field trials.

One of the most effective preventive management measures against phytoplasma diseases is to plant resistant or tolerant cultivars. However, so far no resistant almond variety was identified. Almond cultivation in Lebanon experienced a big boom in the 1980's and early 1990s due to several factors; almond trees are drought tolerant; can be successfully grown on calcareous soil in rain fed areas where irrigation may not be available and almond fruits can be sold green or mature or saved dry for a long period (have a long shelf life) to be used for food or pastries. In rain fed areas with calcareous soils alternative crops with good economic returns are quite limited. Since AlmWB phytoplasma does not affect plums and apricots, this research addressed on one hand the possibility of grafting almond on apricot or plum rootstocks and on the other hand the possibility of reducing the economic loss to the farmer by grafting almond trees with apricot or plum as soon as the trees show signs of infection by AlmWB, i.e. before rapid decline of tree vigor. The preliminary experiments mimicked early infections by AlmWB, whereby, scions taken from AlmWB infected trees were grafted on apricot and plum rootstocks. The results proved very promising. Almond growth developing on rootstocks of Angelino and Jawhara plum and on Farclo apricot did not

develop symptoms five months after grafting, PCR results proved the presence of "*Ca.* P. phoenicium" in Jawhara and Farclo but not in Angelino. Data collection will continue for at least one year post grafting. Almond growth on the Red plum and Early blush apricot showed very interesting phenomenon: recovery. Symptoms were observed two months after grafting but the infected tissue recovered and looked like healthy. The recovery was also confirmed by qPCR analysis whereby the titer of the phytoplasma "*Ca.* P. phoenicium" was high two months after infection and dropped to low levels or below detection limits and the difference was statistically significant. Based on symptom monitoring and calculation of phytoplasma GU/ ng of plant DNA it was observed that symptom appearance is not only related to the quantity of phytoplasma found in the plant tissue, since this may also vary with the crop and rootstock scion combination. For example in Early Blush, a phytoplasma titer of 44 GU/ng DNA activates symptom appearance on the infected tree, while in the variety Jawhara this titer did not lead to appearance of symptoms.

The recovery of AlmWB-infected almond scions represents the first report of recovery from infections by "*Ca.* P. phoenicium". Noting that after over 15 years of follow up in the field, the AUB research team did not observe any recovery of infected almond trees.

Interestingly similar recovery results were observed in field trials where Farclo apricot scions were grafted on severely infected almond trees, the initial apricot growth showed severe symptoms but the new growth recovered within two-three months and most of the shoots were free from the symptoms. Understanding the mechanism of recovery may bring insights into successful curing of phytoplasma infection. Disappearance of symptoms from symptomatic trees has been observed in cases of

infection with AP phytoplasma, ESFY phytoplasma, pear decline phytoplasma and grapevine yellows phytoplasma, this change is known as natural recovery which can either be temporary or permanent and its causes are not fully understood (Carraro, L., Ermacora, P., Loi, N., & Osler, R. 2004). Along with the grafted Farclo apricot scions plum varieties were also grafted on severely AlmWB infected almond trees. No symptom development was observed and the trees exhibited good growth for one full year until struck by two consecutive dry years, most of the plants died except two Farclo apricot trees that received indirectly some water while irrigating other nearby plants. The recovered Farclo apricot, showed vigorous growth. PCR analysis in December 2014 gave positive result for "*Ca.* P. phoenicium" on one of the Farclo trees without any symptom appearance and the same result was obtained in June 2015.

Preliminary field and greenhouse results show a great potential for grafting to contribute to management of the disease and may give hope to replant almond in infested regions. However, disease resistance alone is not the major factor to consider the horticultural characteristics and yield potential in addition to resistance to drought, calcareous soils and adaptation to climatic and other edaphic conditions are important factors to take into consideration. Therefore about 100 plum rootstocks were distributed to farmers in Feghal region North of Lebanon where healthy almond scion from the variety Halwani will be grafted early spring 2016. Their performance including horticultural and disease resistance will be evaluated. These represent long term trials; their success will no doubt give hope to Lebanese farmers to plant almond seedlings in areas of infection.

C. Epidemiology and strain identification

While developing any resistant varieties for any pathogen, it is quite essential to test the developed variety against the most common or largest number of strains of the pathogen before releasing it in the market. Only little is known about the biodiversity of "*Ca*. P. phoenicium" strains in Lebanon. A recent study showed that "*Ca*. P. phoenicium" subgroup16SrIX-B; also designated as 16SrIX-D, is the most widespread in Lebanon (Quaglino et al., 2015). During the genome sequencing the Italian team identified a polymorphic gene. A technique based on nested PCR followed by RFLP was developed to differentiate between strains. All the samples collected from the North region (Feghal, Jeddayel, Thoum and Rachana); appear to belong to the same strain. Effectively, it is believed that "*Ca*. P. phoenicium" was introduced to Lebanon in a single event and then spread rapidly, therefore, it is expected that the biodiversity at this stage is still limited and may evolve with time depending on variability of vectors and potential other alternative hosts which may impart some selective pressures. At least two vectors belonging to two different families have been proven to transmit the disease, *Asymmetrasca decedens* family Cicadellidae (Abou-Jawdah et al., 2014) and *Cixius sp.* and *Tachycixius sp.* members of the Cixiidae family. Two plants were also found to be alternative hosts; *Anthemis sp.* and *Smilax aspera*, for "*Ca.* P. phoenicium" (Tedeschi, R et al., 2015).

This research in addition to the previous research conducted on the epidemiology of the AlmWB phytoplasma disease, will help in developing of a sound disease management approach.

CHAPTER VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Almond is considered an economically very important crop in Lebanon especially in rain fed areas with calcareous soil. Over two decades ago, AlmWB disease caused by the phytoplasma "*Ca.* P. phoenicium" has been introduced to Lebanon, spread rapidly and caused the loss of production in over 200,000 almond, peach and nectarine trees. This study firstly aimed at developing methods for facilitation of large scale surveys of "*Ca.* P. phoenicium" detection in Lebanon through developing rapid, sensitive, simple, reliable and relatively inexpensive serological detection techniques. Since the phytoplasma cannot be cultured in vitro, this study was conducted using recombinant DNA technology and accomplished through successful amplification and cloning of AlmWB *inmp* genes in two different types of expression vectors: pIVEX 1.3 wg vector normally used for *in-vitro* protein production and pQE-32 vector, used for bacterial cell protein expression systems such as in *E. coli*. In turn transformation for protein induction and expression was performed in two different *E. coli* strains of competent cells KRX and M15. For protein purification two different cell lysis protocols was compared, one being the native cell lysis technique also known as chemical cell lysis and the other being the denaturing cell lysis technique through using 6M Guanidine hydrochloride (GuHCl). Detection of the recombinant membrane proteins tagged with 6 x histidine was made possible through purification with Ni-NTA agarose beads and visualization on SDS-PAGE gel.

AlmWB-phytoplasma has caused significant loss on stone fruit production especially on almond trees. So far no resistant varieties have been identified and replacement of almond trees by other crops with equal economical returns to the farmers in many areas is very difficult. Therefore, development of an efficient approach is needed for the ability to reintroduce almond seedlings in infested orchards. Thus, the second aim of this research was to develop efficient and economically feasible management technique through grafting. This was achieved through two different trials, one in an almond orchard in Feghal area and the second in a greenhouse. In the field trial, severely infected almond trees were grafted with different plum and apricot cultivars and monitored for disease transmission and detection in newly developed plum or apricot shoots and for symptom appearance. Two and five months post grafting, growth from plum and apricot scions were found to be negative with PCR except Farclo apricot. Effectively Farlo apricot showed severe symptoms two months post- grafting but rapidly recovered and most of the new growth was symptomless. Phytoplasma was not detected by PCR in the symptomless part but was detected in few symptomatic leaves at the base of the shoots. One and two years post grafting samples were taken only from two apricot trees since all other grafted trees had died from two consecutive drought years, both trees showed no symptom of disease but one tree F2P4 was positive by PCR while the other; F2P2, was negative.

In the greenhouse trial, three plum cultivars; Jawhara, Red and Angelino, along with 2 apricot cultivars; Farclo and Early blush, were grafted with AlmWB-infected scions to mimic early infections. Almond rootstocks were also grafted as positive controls. Two months post grafting, apricot Early blush and Red plum treatments showed symptoms of AlmWB and "*Ca.* P. phoenicium" phytoplasma was detected by

PCR. Three to five months post-grafting, almond growth on the Red plum and Early blush apricot showed very interesting phenomenon: recovery. The symptoms observed two months after grafting disappeared, since the infected tissue recovered and looked like normal healthy growth. The recovery was also confirmed by qPCR analysis whereby the titer of the phtytoplasma "Ca. P. phoenicium" in Early Blush dropped from 44 (GU/ng plant DNA) in June to below detection limits in September, and the differences were statistically significant. This is the first report of recovery from infections by "Ca. P. phoenicium". It is worth mentioning that after over 15 years of follow up in the field, the AUB research team did not observe any recovery of infected almond trees. Understanding the mechanism of recovery may bring insights into successful curing of phytoplasma infection. Infected almond scions that were grafted on Angelino plum, developed normal growth and the phytoplasma was not detected in the new growth up to five months post-grafting. These observations will be followed up for at least one year post-grafting. These preliminary results are considered very promising and if confirmed may allow re-introduction of Almond production in the AlmWB infested regions. Therefore, a long term field trial was initiated whereby resistant/tolerant apricot and plum rootstocks were distributed to farmers in Feghal region, and will be grafted with healthy almond scions of the variety Halawani. Their performance, including horticultural characteristics and disease resistance will be monitored for few years.

In the course of this study the biodiversity of *"Ca.* P. phoenicium" was studied in 4 different areas in North of Lebanon; Feghal, Jeddayel, Thoum and Rachana, this technique was made possible through CAPS marker, whereby the amplicons obtained from a PCR amplification of a specific gene or DNA fragment is subjected to RFLP

using a restriction enzyme. In this case the gep gene was amplified and Hpy188III restriction enzyme was used. The RFLP results indicated that AlmWB-phytoplasma is of the same profile in all the studied regions. These results prove that this phytoplasma was introduced to Lebanon from one source of origin and suggests that it has been recently introduced. If no variability is observed within the phytoplasma populations in all infested regions in Lebanon, then the results from trials on disease resistance in one region may apply to the other region, in other terms, multiple research sites may not be needed.

In conclusion, two "*Ca.* P. phoenicium" integral membrane genes, AlmWB-1160 and AlmWB-1850, were successfully amplified by PCR and cloned in a cloning vector (pGEM-T easy vector) and then sub-cloned in two protein expression vectors, pIVEX 1.3 wg vector and pQE-32 vector. Transformation was successful for four recombinant plasmids or protein expression cassettes pIVEX 1.3 wg + AlmWB-1160 PIVEX 1.3 wg + AlmWB-1850, pQE-32 + AlmWB-1160 and pQE-32 + AlmWB-1850, in two types of competent cells M15 and KRX. Potentially promising results were obtained concerning protein expression however, these results need further confirmation.

Based on the results obtained from grafting trials whereby AlmWB-infected almond scions were grafted on plum or apricot rootstocks, three important observations were recorded: a- a combination did not show symptoms and the phytoplasma was not detected by PCRs like in Angelino plum; b- a combination that did not develop symptoms but the phytoplasma was detected by PCR like in the plum Jawhara and apricot Farclo; c- a combination that showed early symptoms but later on recovered like

Early Blush apricot and Red plum. These results should be confirmed in long term field trial and may lead the way for re-cultivation of almond in infested regions.

Based on the CAPS/RFLP pattern from 4 regions in North of Lebanon, it was established that "*Ca.* P. phoenicium" isolates in Lebanon have one genetic profile which may be due to recent introduction of the causal agent from the same source of origin.

Recommendations:

i. Optimization of protein expression and cell lysis protocols for protein expression at higher levels in order to get enough quantity of antigen to start antibody production for development of serological detection methods.
ii. Continue the full genome sequencing and identify other "*Ca.* P. phoenicium" *inmp* genes that can prove to be more antigenic for better protein expression and production.
iii. Follow up on the long term field trial for resistant cultivars through grafting technique for development of efficient long term disease management.
iv. Search for other resistant/tolerant rootstock cultivars and try to commercialize them for re-planting of almond seedlings in infested areas
v. Conduct epidemiological studies on AlmWB-infected samples from different regions in Lebanon
vi. Conduct further epidemiological studies concerning alternative hosts and methods and mechanisms of disease transmission by vectors between crops and between wild plants and crops

APPENDIX I

DNA SEQUENCES AND MAPS

A. Complete DNA sequence of *inmp* AlmWB-1160

5’ATGAATCGAAACGTATGGCCAAAAATAAAAAAATTTTTTAATGTTCTA
AAGAATTGTCTTTTTATCTTTTTATATGCTTTATTATTTTATTTGACTTTAA
TCCAAATTAGCAATTTTATTAATCCTACAAGAACAGTGGATTATTTATTT
TTTAATTTTTTTGAAGTTGCTAGCAGTAGTATGGAACCAGGTATAAAGAA
AAGTGATAAAGTGATTTTAAAGAGAATTCACGATAGAAAAACATTGCAA
CCAGGTGATATTATTTATTTCGAAACCAGTGATCCTTCATTGCAAAGTAT
CGGAATTAAACGTATTATTCATCGTGTTGTTAAAAATGATAAACAAAAC
GAAGAAATTACAACTCATGGTGATAATAATGAAAAAATAGCGCCTTTTG
AAAGACAAATACCTTATAAGGATGTAATAGCCGAACATTTTTATACTATT
CCTAACCAATATATCCAAAAATTGAATATTATCTTTTGGTTTGGGTTTATT
TATATGATCATTGAACTTTTTTATAAAATTATTTATGTTAAAAATGATCC
GAAAAATATTATTCTTTAA 3’

B. Complete DNA sequence of *inmp* AlmWB-1850

5’ATGACAGTTATGTTAAAATTAAATAAAGATAAACATTATTATTTCAAC
ATTTGGCTGACTAAATGGTTTTGGATGATTTTTTATACATTACTTTTATCT
TTAGGAGTTTATTTTTTTACTTTTGGATTTCAATTAGTGACTGGAGGGTTA
GACGGTTTAACTGTCTTAACCATAGAAATTTTACAAAATTGTGGTCTGCC

AAATGATTATATACCCCGAGTAGAATATTTATATGGATTTTACAATATTA

TTAGTTTAATAGCGGGATATAAGGTTTTTGGTAAAGATTTTTGTTATCAT

ACTGGTATTTTATGTATCATCTTATGTTTAAGTGTTTCTTTTTTAAGTTGG

CTTTTCGGCGACATTAGTATTGTAACCTGTTACCTCAGTCCTAATGATTAT

TTTAATTTAATTTTTGTTTCTATTGCAAGTGGTATCTTATTTGGTATTGCTT

TAGGAAATATTCGTAAATATAAATATACCACTGGTGGAATGGATATTTTT

CAAAAAATTTTAAAAGATATTTATGGTATAAATTTTATTTGGGTTGTTTT

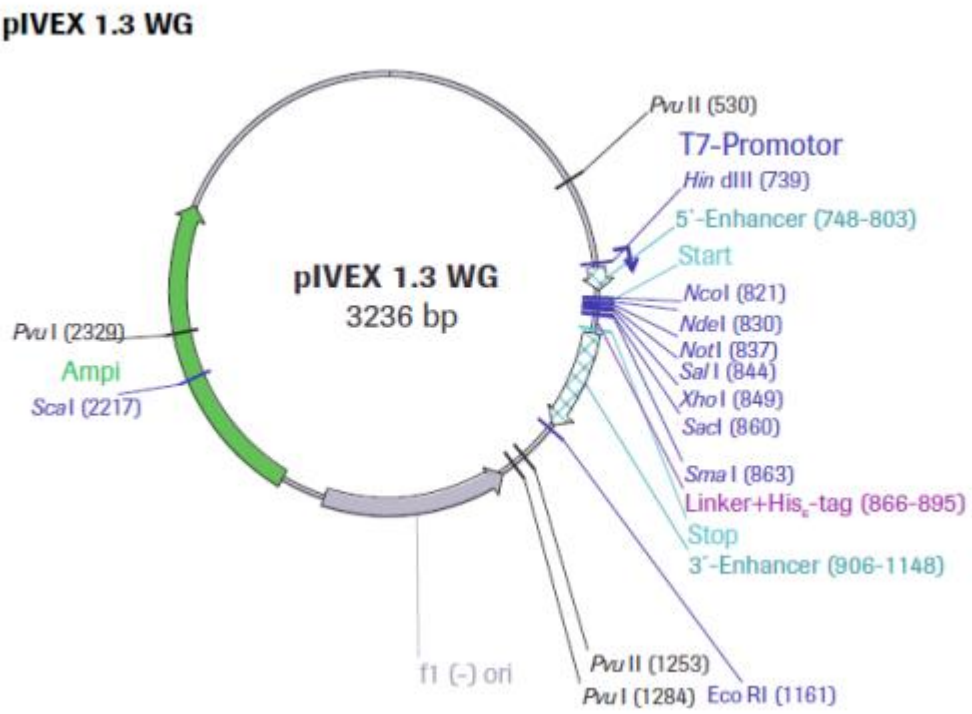
CATAACTGATGGAGTTTTAATATTGCTAACAGCAATCATTATTGCTACGA

AGAATCATGATTTAATGCAATTTATTATTAGATTTTTTTGCTCTTATTTAT

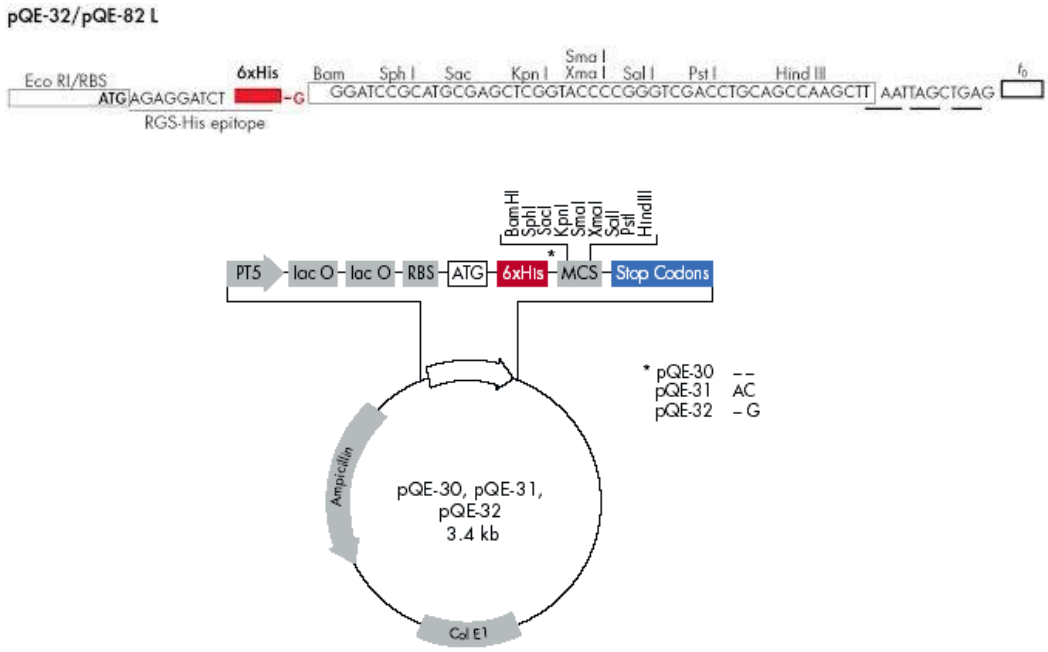
CTTCTTTTATTATGAGTTGTATTATCGAAAAAATCGCTCCTGAAATTCAA

AGTGTTAAATAA 3’

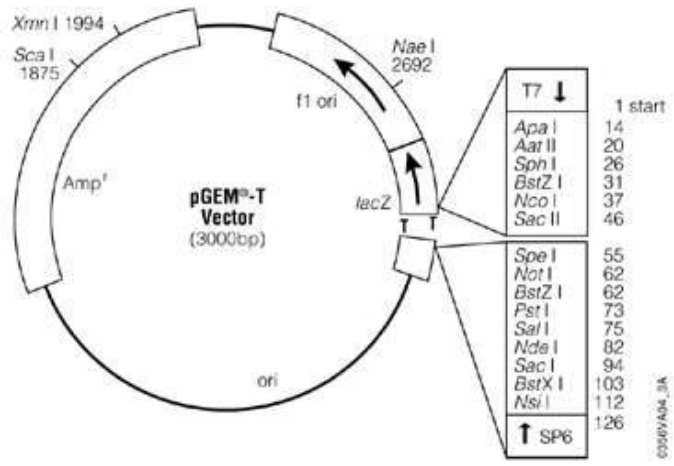
C. Vector map for pIVEX 1.3 wg expression vector



D. Vector map for pQE-32 expression vector



E. Vector map for pGEM-T easy cloning vector



APPENDIX II

REAGENTS, MEDIA AND BUFFERS

A. DNA extraction buffer

1. CTAB buffer pH= 8 (100 mL)

1.21 g Tris-Base

20 mM EDTA= 4 mL of 0.5 M EDTA

1 % Polyvinylpyrrolidone (PVP)

1.4 M 8.12 g NaCl

2 % Cetyltrimethylammonium bromide (CTAB)

B. Agarose gel electrophoresis

1. 6x loading dye

12.5 mg bromophenol blue

2 g sucrose

5 ml DEPC-treated water

2. 1% TAE agarose gel

1 g Agarose

100 mL 0.5x TAE buffer

3. 1x TAE Buffer (5 L)

100 mL 50x TAE buffer (Bio-Rad)

4900 mL distilled water

C. Culture media

1. LB medium (1 L)

10 g casein peptone

5 g yeast extract

5 g NaCl

For solid media add 7.5 g/L of agar

D. Chemicals

1. X-Gal solution

20 mg X-Gal

1 mL dimethylformamide

Store at -20 °C in dark

2. IPTG

2 g of IPTG

8 mL distilled water

Store at -20 °C

3. Lysozyme (1 mL)

20 mg Lysozyme

1 mL deionized water

Prepare fresh before use

4. Phenylmethanesulfonyl fluoride (0.1 M PMSF) (1 mL)

17.42 mg PMSF

1 mL anhydrous isopropanol

Store at -20 °C

5. Protease Inhibitor Cocktail II for bacterial extract (5 mL)

Reconstitute powder in 1 mL Dimethyl sulfoxide (DMSO)

Add 4 mL deionized water

Store at -20 °C

6. Deoxycholic acid (1 mL)

100 mg Deoxycholic acid

1 mL deionized water

Prepare fresh before use

7. Deoxyribonuclease I (DNAse I) (1 mL)

5 mg of DNAse

1 mL 0.15 M NaCl

Store at -20 °C

8. Magnesium Chloride ($MgCl_2$) (1 mL)

95.2 mg $MgCl_2$

1 mL deionized water

E. Protein expression and SDS- PAGE buffers

1. Lysis Buffer pH= 8 (1 L)

40 mM Na_2HPO_4 = 5.68 g

300 mM NaCl = 17.53 g

10 mM Imidazole = 0.68 g

Adjust pH using 0.5 M NaH_2PO_4

2. Wash Buffer pH= 8 (1L)

40 mM Na_2HPO_4 = 5.68 g

600 mM NaCl = 35.06 g

20 mM Imidazole = 1.36 g

Adjust pH using 0.5 M NaH_2PO_4

3. Elution Buffer

10 mL Wash Buffer

228 mM Imidazole = 0.155 g

4. 2x SDS gel loading Buffer

100 mM Tris-Cl

4 % (w/v) SDS

0.02 g bromophenol blue

20 % (v/v) glycerol

200 mM Dithiothreitol (DTT)

Store at -20 °C

5. 10% Sodium Dodecyl Sulfate (SDS) pH= 7.2 (1L)

100 g SDS

1 L Distilled water

Dissolve by heating at 68 °C and adjust pH with HCl

6. 5x running buffer pH= 8.3 (500 mL)

0.25 M Tris-Cl = 15.1 g

2.5 M Glycine electrophoresis grade = 94 g

50 mL 10 % SDS

Adjust pH with 0.5 M NaH_2PO_4

7. Coomassie Blue staining dye (500 mL)

225 mL methanol

225 mL distilled water

50 mL acetic acid

1.25 g Coomassie brilliant blue

8. De-staining buffer (500 mL)

225 mL methanol

225 mL distilled water

50 mL acetic acid

9. Lysis buffer A pH= 8 (1L)

6 M GuHCl = 573 g

100 mM NaH_2PO_4 = 13.8 g

10 mM Tris-Cl = 1.2 g

10. Wash Buffer C pH= 6.3 (1L)

8 M Urea = 480.5 g

100 mM NaH_2PO_4 = 13.8 g

10 mM Tris-Cl = 1.2 g

Adjust pH before use due to urea dissociation

11. Buffer E pH= 4.5 (1L)

8 M Urea = 480.5 g

100 mM NaH_2PO_4 = 13.8 g

10 mM Tris-Cl = 1.2 g

Adjust pH before use due to urea dissociation

F. SDS- PAGE gels

1. 1.5 M Tris-Cl pH= 8.8 (50 mL)

9.08 g Tris-Cl

50 mL distilled water

Adjust pH with HCl

2. 0.5 M Tris-Cl pH= 6.8 (50 mL)

3.03 g Tris-Cl

50 mL distilled water

Adjust pH with HCl

3. Ammonium persulfate (10% APS) (1 mL)

100 mg APS

1 mL distilled water

Stable at 4 °C for 1 week

4. Separating gel

3.2 mL of 30% bis-acrylamide

2 mL of 1.5 M Tris pH= 8.8

2.8 mL of Deionized water

100 µL of 10% SDS

140µL of 10% APS

20 µL TEMED

5. Stacking gel

750 µL of 30% bis-acrylamide

1,250 mL of 0.5 M Tris-Cl pH= 6.8

3,500 mL of deionized water

45 µL of 10% SDS

140 µL of 10% APS

20 µL TEMED

APPENDIX III

STATISTICAL ANALYSIS

A.

GUphytoplasma

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	194998.737	4	48749.684	9.959	.000
Within Groups	132163.246	27	4894.935		
Total	327161.984	31			

Tukey HSD						
(I) Treatment[Z]	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-207.26208*	37.78478	.000	-317.6194	-96.9048
	3.00	-19.36208	37.78478	.985	-129.7194	90.9952
	4.00	-14.83875	37.78478	.995	-125.1961	95.5186
	5.00	-1.35042	37.78478	1.000	-111.7077	109.0069
2.00	1.00	207.26208*	37.78478	.000	96.9048	317.6194
	3.00	187.90000*	40.39363	.001	69.9231	305.8769
	4.00	192.42333*	40.39363	.001	74.4464	310.4003
	5.00	205.91167*	40.39363	.000	87.9347	323.8886
3.00	1.00	19.36208	37.78478	.985	-90.9952	129.7194
	2.00	-187.90000*	40.39363	.001	-305.8769	-69.9231
	4.00	4.52333	40.39363	1.000	-113.4536	122.5003
	5.00	18.01167	40.39363	.991	-99.9653	135.9886
4.00	1.00	14.83875	37.78478	.995	-95.5186	125.1961
	2.00	-192.42333*	40.39363	.001	-310.4003	-74.4464
	3.00	-4.52333	40.39363	1.000	-122.5003	113.4536
	5.00	13.48833	40.39363	.997	-104.4886	131.4653

5.00	1.00	1.35042	37.78478	1.000	-109.0069	111.7077
	2.00	-205.91167*	40.39363	.000	-323.8886	-87.9347
	3.00	-18.01167	40.39363	.991	-135.9886	99.9653
	4.00	-13.48833	40.39363	.997	-131.4653	104.4886

[z] The following treatments were evaluated; 1: Negative control; 2: Positive control; 3: Plum Jawhara; 4: Apricot Early Blush; 5: Red plum

*. The mean difference is significant at the 0.05 level.

Tukey HSD

rootstock	N	Subset for alpha = 0.05	
		1	2
1.00	8	.0713	
5.00	6	1.4217	
4.00	6	14.9100	
3.00	6	19.4333	
2.00	6		207.3333
Sig.		.987	1.000

B.

ANOVA					
GUphytoplasma					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2626.724	2	1313.362	27.262	.012
Within Groups	144.529	3	48.176		
Total	2771.253	5			

Multiple Comparisons

Dependent Variable: GUphytoplasma

Tukey HSD

(I) Month	(J) Month	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	44.45500*	6.94091	.016	15.4509	73.4591
	3.00	44.31500*	6.94091	.016	15.3109	73.3191
2.00	1.00	-44.45500*	6.94091	.016	-73.4591	-15.4509
	3.00	-.14000	6.94091	1.000	-29.1441	28.8641
3.00	1.00	-44.31500*	6.94091	.016	-73.3191	-15.3109
	2.00	.14000	6.94091	1.000	-28.8641	29.1441

1: July; 2: August; 3:September

*. The mean difference is significant at the 0.05 level.

GUphytoplasma

Tukey HSD[a]

Month	N	Subset for alpha = 0.05	
		1	2
2.00	2	.0450	
3.00	2	.1850	
1.00	2		44.5000
Sig.		1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.000.

APPENDIX IV

OTHERS

Tree distribution for long term field trial 2

Farmer	Village	Phone number	GPS location	Kind and number of trees	Scions	Comments
Youssef wehbe	Feghal 205 m	03/548927	34°,12'16.1 N 35°,39'22.2 E	3 Almond 1 plum	Halawani scion for plum	3 Almonds grafted
Saab Wehbe	Feghal 196 m	03/313005	34°12'17.1 N 35°,39'22.6 E	4 Almond	Grafted already	
Michel Wehbe	Feghal 185 m	79/162466	34°,12'17.3 N 35°39'20.0 E	7 Almond 3 plum	Halawani scions for plum	7 plums gave away
Jean wehbe	Feghal 179 m	03/824255	34°,12'18.7 N 35°39'21.4 E	5 Almond 2 plum	Halawani scions for plum	
Youssef Tanous Wehbe	Feghal 181 m	03/347127	34°,12'18.7 N 35°39'22.2 E	5 Almond 2 plum	Halawani scions for plum	
Antoine Wehbe	Feghal	03/212005	34°,12'19.7 N 35°39'22.4 E	2 Almond 2 Plum	Halawani scions for plum	
Joseph Haddad	Shoushith	03/353905		2 Almond 4 Plum	Halawani scions for plum	4 plum gave away
Youssef Al Meer	Feghal 211 m	03/731379 09/794198	34°,12'19.3 N 35°39'37.1 E	6 Almond 12 Plum	Halawani scions for plum	
JeanPaul Jaber	Feghal 279 m	76/794400	34°,12'23.7 N	10 Plum	Halawani scions for	Not yet planted

			35°39’58.6 E		plum	7/2/2015
Youssef Said Khoury	Feghal 304 m	03/369843	34°,12’20.3 N 35°40’20.1 E	2 Almond 3 Plum	Halawani scions for plum	
Michel Said Khoury	Feghal	09/794707	34°,12’20.1 N 35°40’25.9 E	2 Almond 7-8 Plum	Halawani scions for plum	
Frozia Khoury	Feghal 332 m	09/794164	34°,12’20.1 N 35°40’25.9 E	2 Almond 1 Plum	Halawani scions for plum	
Noha Khairallah	Feghal 289 m	03/884860	34°,12’23.8 N 35°40’26.9 E	4 plum	Halawani scions for plum	
Tony Elias	Feghal 308 m	70/301488	34°,12’23.9 N 35°40’38.0 E	8 Plum	Halawani scions for plum	
Youssef Wadih Fghali	Feghal	03/772840	34°,12’30.1 N 35°40’39.5 E	2 Almond 4 Plum	Halawani scions for plum	A year to be ready for graft
Youssef Khalil	Feghal	09/794700	34°,12’25.8 N 35°39’02.7 E	1 Almond 5 Plum	Halawani scions for plum	Gave away 5 plums
Mounir Jaber	Feghal 377 m	03/270757	34°,12’18.4 N 35°40’31.6 E	2 Almonds 8 Plum	Halawani scions for plum	Not planted yet 7/2/2015
Moufid Khairallah	Feghal 145 m	76/616615	34°,12’22.0 N 35°39’11.6 E			
Youssef Khairallah	Fghal 145 m	03/198122	34°,12’22.0 N 35°39’11.6 E			

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