UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI SCIENZE AGRARIE E FORESTALI

Corso di Dottorato di Ricerca in

Protezione delle piante - XXVIII Ciclo

SURVEY OF BACTERIAL DISEASES ON STONE FRUITS IN LEBANON AND INVESTIGATION OF PHENOTYPIC AND GENETIC DIVERSITY OF THE ISOLATED *PSEUDOMONAS SYRINGAE* PATHOVARS

AGR/12

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27 May 2016

Summary

Stone fruits are of highly importance in Lebanon covering 17% of the total agricultural lands cultivated with permanent crops. These crops suffer from a diversity of diseases causing losses in production, including many of bacterial origin. Previously, two studies reported bacterial diseases of stone fruits in Lebanon but both of them were incomplete considering the number of samples collected and the identification protocols used at that time.

In order to accomplish this work and evaluate the incidence of bacterial diseases of stone fruits in Lebanon, we carried out a survey in 2013 when we collected 303 samples from all stone fruit growing areas and all commercial species. Results showed that bacterial canker is the main bacterial disease of stone fruits in this country where it appeared to be spread in all regions and on all cultivated species. In fact, preliminary identification of the isolated bacteria using physiological and biochemical tests allowed the identification of 102 *Pseudomonas syringae* pv. *syringae* (*Pss*), 30 *Pseudomonas syringae* pv. *morsprunorum* race1 (*Psm1*) and 3 *Pseudomonas syringae* isolates. None of the other common bacterial diseases of stone fruits including *Pseudomonas syringae* pv. *avii*, *Pseudomonas syringae* pv. *morsprunorum* race 2, *Pseudomonas syringae* pv. *persicae*, *Pseudomonas amygdali*, and *Xanthomonas arboricola* pv. *pruni* were found in the sampled orchards. Two gall symptoms suspected to be induced by *Agrobacterium tumefaciens* were observed on peach and plum, and the isolates obtained were conserved for further identification.

Pathogenicity of the collected isolates was assessed by inoculation on immature cherry fruits. With the exception of one *Pseudomonas syringae* isolate, all the others were able to produce disease symptoms. Interestingly, this technique clearly differentiate between isolates of the pathovar *syringae* that produced black necrotic lesions while isolates of the pathovar *morsprunorum* produced brown, water soaked superficial lesions.

Molecular tools were also used in this study to confirm results of classical identification techniques and to evaluate the genetic diversity within the Lebanese isolates of *Pseudomonas syringae*. In this context, we first conducted specific PCR for the detection of the gene coding for hopAP1 protein reported to be present on the pathovar *syringae*. Results showed that the majority of *Pss* isolates (94/102 isolates) possess this effector gene. Later on, BOX-PCR was used as a molecular fingerprinting technique to assess the genetic diversity of the collected isolates. UPGMA analysis of the fingerprint patterns divided the *Pseudomonas syringae* isolates into three major groups: A, B and C. *Pss* isolates showed a high genetic diversity producing 17 different patterns distributed according to their similarity level between the group C (87 *Pss* and 1 *Pseudomonas syringae*) and the group B (15 *Pss* and 2 *Pseudomonas*

syringae). *Psm* 1 isolates were very homogenous producing the same fingerprinting pattern forming together the group A.

In order to classify our isolates and to compare them with others from all over the world, 58 representatives of the collected *Pseudomonas syringae* isolates were analyzed by MLST. The selection of the isolates was based on all the techniques used before in addition to the host plant and the region of origin. MLST was performed by sequencing part of four housekeeping genes (*cts*, *gap* A, *rpo* D *and gyr* B) that were concatenated to produce a single sequence of 1859 bp. Concatenated sequences were used together with public sequences extracted from Genbank to construct a maximum likelihood phylogenetic tree. The topology of the phylogenetic tree obtained was similar to the one presented by Berge *et al*. (2014) with correct allocation of phylogroups and clades. MLST divided the Lebanese *Pseudomonas syringae* isolates in 2 phylogroups, named PG02 and PG03 according to Berge *et al*. (2014).

All isolates of the pathovar *morsprunorum* race 1 analyzed in this study were placed in the PG03 showing to be genetically closely related to each other and to *Psm* 1 strains from Genbank. Many other pathogens of woody and herbaceous plants were also enclosed in this phylogroup while the pathotype strain of the pathovar *morsprunorum* (M302280^{PT}=CFBP 2351) that is supposed to belong to the race 2 was allocated in the PG01b. In fact, many previous studies suggested the possibility that the 2 races of the pathovar *morsprunorum* can be separated into two species considering them as genetically distant and distinct pathogens adapted to the same hosts. The additional sequences of isolates of the race 1 we have added in this study strengthen this hypothesis, proofing the high genetic distance between the 2 races (>8.8 %). Moreover, we found that the PG03 can be divided into at least 2 clades following a threshold of genetic difference of 2.3% that was used for delineation of clades: PG03a (*Pseudomonas syringae*. pv. *lachrymans*, pv. *mori*, pv. *phaseolicola* race 6 and some unclassified *Pseudomonas syringae* strains) and PG03b (*Pseudomonas syringae*. pv. *morsprunorum* race 1 and pv. *miricae*).

Regarding the pathovar *syringae*, the Lebanese isolates were divided into 2 closely related clades within the PG02 (clades 2b and 2d). This phylogroup is considered to be the most ubiquitous group of *Pseudomonas syringae* found in all habitats analyzed to date. Nine *Pss* and one unclassified *Pseudomonas syringae* appear to belong to the clade 2b together with the type strain (CFBP 1392^{T}) and many other pathovars. To note here that isolates of this clade were isolated from all the stone fruit species surveyed, except apricot trees. The clade 2d includes the largest part of the Lebanese isolates with 37 *Pss* and one unclassified *Pseudomonas syringae*. This clade groups isolates from all stone fruit species and many *Pss* reference strains originated from different countries and hosts.

Results of the two typing techniques used, BOX-PCR and MLST, were analogous to each other. The groups A, B and C of BOX-PCR were equivalent to phylogroups 3, 2b and 2d of MLST, respectively. Interestingly, we found also that isolates that do not possess hopAP1 protein belong to the PG02b while all isolates of the PG02d possess this effector gene.

We also started a preliminary investigation to detect other effector genes in the genome of the collected *Pseudomonas syringae* isolates. Specific primers were designed for the detection of hopAE1 and hopI1 that appeared to be present in the majority of the tested isolates with the exception of one *Pss* isolate and one unclassified *Pseudomonas syringae* isolate. Later on, the amplified regions were sequenced for some isolates and the obtained sequences were used to construct a phylogenetic tree with maximum likelihood method. Congruence was found between the Hop gene and the MLST phylogenies in the case of both hopAE1 and hopI1. This indicates a similar phylogenetic resolution between the core genome (housekeeping genes) and those effector genes that needs to be investigated better in the future.

This study supports solid preliminary conclusions for any future studies dealing with bacterial diseases on stone fruits in Lebanon and *Pseudomonas syringae* in general. We conducted the first survey dedicated specifically to such kind of diseases and we characterized for the first time isolates of *Pseudomonas syringae* from Lebanon using different techniques. The data provided will help in investigating the epidemiology, ecology, population genetics, and molecular evolution of this multifunctional group of bacteria.

Thesis committee

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Acknowledgments

I still remember the first day when I arrived to Italy, it has been five years already. In my mind it was a complete mess. From one side, a lot of confusion, fear, sadness and worries for what would be waiting for me in my new life. Thinking about the beloved ones I left behind in my adorable country Lebanon. But from the other side, a great sense of joy for how much I am lucky to have the opportunity to come here and pursue my studies. Now, I am at the last step before the target that I would not have been able to achieve without the presence of many special people in my life.

First of all, I want to thanks the Mediterranean Agronomic Institute of Bari (CIHEAM-MAIB) that accepted me as a member and opened the door for a better future and a better life. My gratitude goes to the Director Dr. C. Lacirignola, the Deputy Director Dr. M. Raeli and every person working in this institution.

My sincere thanks and appreciations go to the coordinator of "Integrated Pest Management" department in CIHEAM-MAIB, Dr. Anna Maria D'Onghia for her continuous support throughout my studies.

A special thanks to my tutor Dr. Franco Valentini who I consider as a friend with his unique kindness. I also appreciate the help of Dr. Toufic El Beaino to accomplish part of my research study.

My deepest appreciation to my supervisor Prof. Leonardo Varvaro. I will always be thankful for your generous support and continuous interaction in the supervision of this work. Here also I would like to thank Tuscia University for the opportunity to do a PhD and all the great people working there especially Dr. Giorgio Balestra, Dr. Angelo Mazzaglia, Dr. Anna Maria Vettraino and my friend Dr. Alfredo Fabi for their help, each in a different manner.

I am particularly grateful to the enormous availability of Dr. Blanca Landa with whom I accomplished a big part of my thesis. I will always be thankful to you and your institution, the Institute for Sustainable Agriculture-CSIC (Córdoba/Spain).

It was also a pleasure for me to do the first part of my thesis in the laboratories of the Lebanese Agricultural Research Institute (LARI-Fanar) under the supervision of Dr. Claudine Sebaaly who I greatly admire.

My dearest friends in Lebanon, Georgio, Abdo and Michelle, thank you for being always present when I needed you even if thousands of kilometres apart.

My big family in Bari, those friends that encouraged me to continue forward, thank you. You know yourself one by one and I am sure that we will always be a family despite in which corner of this world each of us would eventually be. Among you I want to say a special thanks to my smart friend Bachir who helped me in my thesis and supported me in every detail of my daily life, Ramy my big brother who was always available since the first day I have got to know him and finally, Ali and Ali my housemates and my brothers from Iraq.

My friends in Viterbo, you made my life there very enjoyable. I arrived one day to Viterbo not knowing anyone and now I have many real friends. Francesco, Rocco, Matteo, Elisa, Valentina, Claudia, Giacomo, Silvio, Giulia, Anna, Diana, Davide and many others, you will always be in my heart.

I cannot forget to thank my dear friend Dr. Yousseph Rouphael who was the reason behind my coming to Italy. I will be always grateful to you.

Finally, I would like to thank every member of my family, especially my parents, my sweet sister, my brave brother and my dear aunt Marie-Therese, for their support and their prayers that helped me to continue forward and accomplish this title. I hope you are proud of me.

From all my heart... thank you all

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List of abbreviations

%	Percent
°C	Degree Celsius
BOX	BOX element of *Streptococcus pneumonia*
bp	Base pairs
cv.(s)	Cultivar(s)
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
ELISA	Enzyme-linked immunosorbent assay
ERIC	Enterobacterial Repetitive Intergenic Consensus
g	Gram
GATTa	Gelatin, Aesculin, Tyrosinase and Tartrate tests
h	Hour
ha	Hectare
KB	King's B medium
L	Litre
LARI	Lebanese Agricultural Research Institute
LB	Luria Bertani broth
LOPAT	Levan, Oxidase, Potato, Arginine and Tobacco tests
mg	Milligram
min	Minute
ml	Millilitre
MLST	Multilocus sequence typing
mM	Millimolar
MoA	Lebanese Ministry of Agriculture
NA	Nutrient agar
NaCl	Sodium Chloride

NAS	Nutrient agar sucrose
PCR	Polymerase Chain Reaction
pH	Potential Hydrogen
Psm1	*Pseudomonas syringae* pv. *morsprunorum* race 1
Pss	*Pseudomonas syringae* pv. *syringae*
REP	Repetitive Extragenic Palindromic sequence
Rep-PCR	Repetitive extragenic palindromic - PCR
rpm	Revolutions per minute
SDW	Sterile distilled water
sec.	Second
spp.	Species
TAE	Tris Acetate-EDTA
Taq	*Thermophilus aquaticus*
TTSS	Type three secretion system
UPGMA	Unweighted Pair Group Method with Arithmetic mean
UV	Ultra violet
Xap	*Xanthomonas arboricola* pv. *pruni*
YDC	Yeast extract-Dextrose-calcium Carbonate agar
YPGA	Yeast-Peptone-Glucose agar
µl	Microliter
µM	Micromol

Chapter 1: Literature review

1.1 Lebanon overview

Lebanon is a small country of the Middle East, bordered by Syria to the north and east, Palestine to the south and the Mediterranean Sea to the west with a cost line of 225 km. Its total surface is 10,452 km^2 divided into four distinct physiographic regions: the coastal plain, the western mountain range, the Bekaa valley and the eastern mountain range. Having a moderate Mediterranean climate, Lebanon receives a relatively large amount of rainfall but varying in space and time according to the geographical position of each area. In fact, along the coastal part, winters are cool and rainy while summers are hot and humid. Passing to the mountainous areas, temperatures usually drop below freezing during the winter with heavy snow cover that remains until early summer on the higher mountaintops. The Bekaa valley sits between the two mountain ranges and is known by its fertile soil with dry summer and semi-arid winter where the annual average of precipitations can vary from about 700 mm in the South to 250 mm in the North (FAO, 2007).

1.1.1 Main constraints facing the agricultural sector

In 2004, the agricultural sector was estimated to contribute by less than 10 % of the Lebanese economy with 73 % attributed to crops and 23 % to livestock production (FAO, 2007). In fact, this contribution has decreased a lot since the 1960's especially during the years of conflicts when the majority of the rural population was displaced.

In 2010, the total number of registered farmers was 169.512 showing a decrease of 2% comparing to 1998 with unequal distribution among regions. In marginal areas such as the south, the north and Baalbeck in Bekaa valley, the populations rely mainly on agricultural activities while in big cities and costal part, agriculture has a very low contribution in the economic cycle. A main problem of the remained farmers is the small surface of their lands. According to the Lebanese Ministry of Agriculture (MoA) (2010) 94% of farmers have less than 4 ha covering 49% of the total agriculture land, while those having more than 10 ha represent 2% of the total number of farmers although covering 33% of the arable lands.

Another difficulty for the agricultural sector is the low national budg*et al*located to the MoA that never exceeds 0.5 % of the national budget. This issue affects largely the development of this sector with only few investments in research and new technology implementation. This

sector suffer also from inefficient management of land and water use accompanied with poor breeding programs thus conventional techniques of production and cultivation of old cultivars are still widely present.

In the last few years, even with the increased function of the MoA, a lot of work still needs to be done for better agriculture management. Here comes the role of extension services by controlling the quality of the products and by supporting farmers especially regarding plant protection and implementation of new innovative techniques. Another factor to be taken into consideration is the absence of a clear market strategy and regional market agreements. This issue became worst in the last few years because of the conflicts in all surrounding countries which makes impossible the land transportation routes and no enough effort have been placed to find other ways. Here comes the problem of high levels of pesticide residues in the Lebanese fruits and vegetables which is a limiting factor for exportation of agricultural goods to many countries. Finally, an old-new problem is the competitive price that neighboring countries can offer compared to high agricultural production costs in Lebanon.

1.1.2 The occupation of the soil and distribution of agricultural lands

The total cultivated land surface is approximately 230,000 ha covering around 23% of the total surface of the country. Those lands resources are thus very limited, considering that Lebanon has a population around 4.6 million (MoA, 2010). Despite this issue, the topographical and landscape diversity in this country create diverse agro-ecosystems that enable a large variety of agricultural products ranging from tropical to temperate cultivation. Depending on the region, some crops are cultivated under rain fed conditions but nearly 49% of the lands are under irrigation. To mention here that only one third of the surface water available is used for irrigation (FAO, 2007).

As we can see in figure 1, fruit trees occupy 31% of the total surface of cultivated lands followed by olives, cereals and vegetables covering 23%, 20% and 17%, respectively. The remaining 9% of agricultural land is occupied by forages and industrial crops, mainly tobacco and sugar beet (Fig. 1). Accordingly, lands cultivated with permanent crops constitute 54% of the total agricultural lands of Lebanon where the most important crops are olives (43%), stone fruits (17%), pome fruits (11%), citrus (8%), grapes (8%), followed by banana, avocado, anona (cherimoya), pomegranate, kiwi and kaki (MoA, 2010).

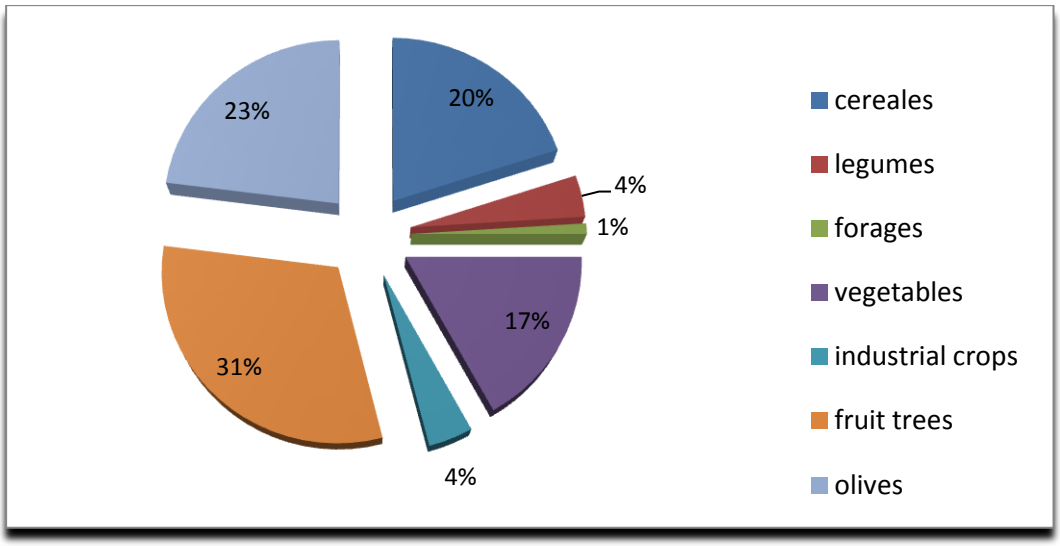


Figure 1: Importance of different crops cultivated in Lebanon (MoA, 2010)

The distribution of agriculture lands can vary from an area to the other ranking first Baalbek–Hermel region with 23% followed by the rest of the governorate of Bekaa with 19%. Aakar which belongs to North Lebanon comes third with 17 % and the rest of this governorate with 10 %. In the south, the two governorates South Lebanon and Nabtiyeh hold together 22 % of the total agricultural lands of the country and finally Mount Lebanon hold the least surface of agricultural lands with only 10 % that are about narrow terraces in the valleys of this mountainous region (Fig. 2).

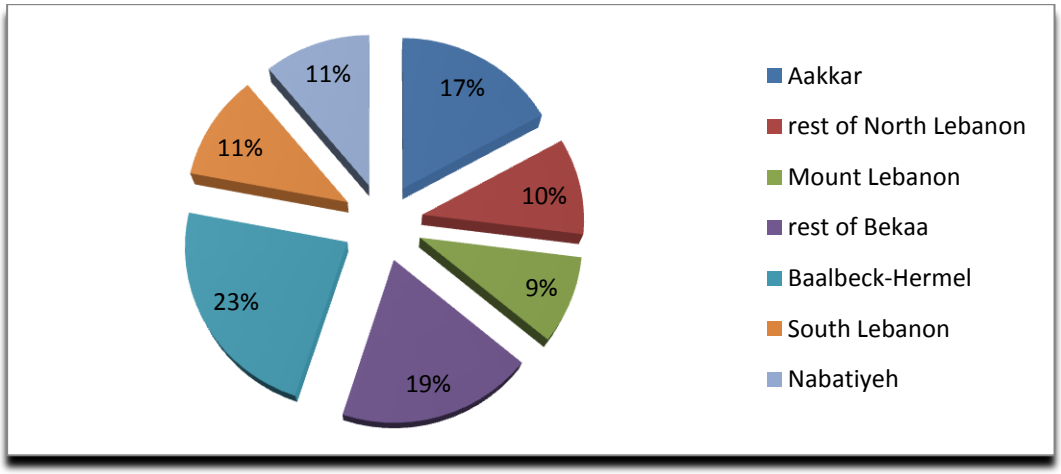


Figure 2: Distribution of agricultural lands according to Lebanese regions (MoA, 2010)

1.1.3 Stone fruits cultivation

The total surface cultivated with stone fruits in Lebanon is 21,715 ha equivalent to 17% of the total land covered with permanent crops. The main commercial species cultivated are cherry,

almond, apricot, peach, nectarine and plum. Their distribution among the Lebanese regions is presented in figure 3, showing that Bekaa valley holds 71% of the total lands cultivated by stone fruits where they are concentrated mainly in the northern part (Baalbek-El Hermel). Second ranks North Lebanon with 18% followed by Mount Lebanon 7% and finally South Lebanon and Nabatiye together with only 4% of the total stone fruits in Lebanon (MoA, 2010).

Regarding the importance of each of the present species, cherry comes first covering 6,172 ha in 2010, a percentage of 28% of the total stone fruit cultivation followed by almond with 5,427 ha (25 %). After those 2 species ranks apricot, peach and nectarine, and plum covering 21, 16 and 9% respectively of the total lands cultivated by stone fruits (MoA, 2010).

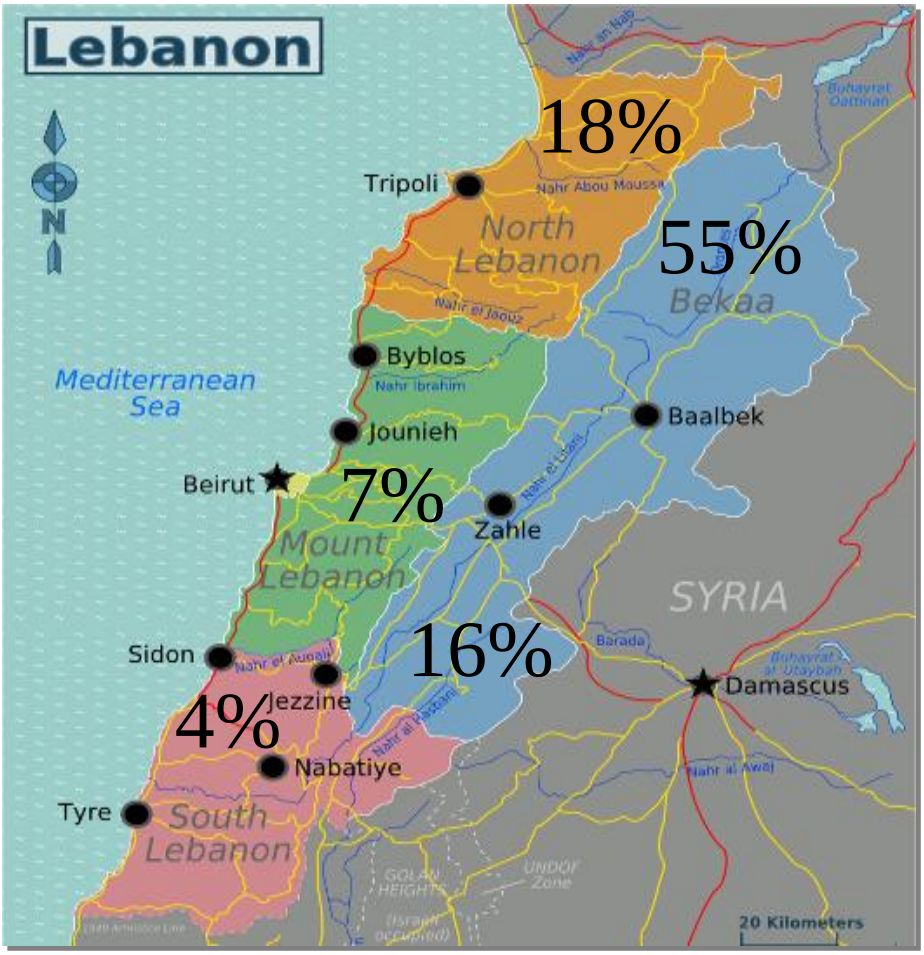


Figure 3: Distribution of stone fruits in Lebanon according to the governorates (MoA, 2010)

1.1.4 Main Diseases of stone fruits in Lebanon

Many fungal and bacterial diseases affect stone fruits in Lebanon as in other regions of the world where those species are present. Fungal diseases are considered of major importance

and are periodically treated with fungicides by farmers in order to reduce as much as possible economical losses. According to the survey conducted by the Lebanese ministry of agriculture in 2010 and to personal communications with farmers that we have made in 2013, the main fungal diseases of stone fruits in Lebanon are the following: leaf curl (*Taphrina deformans*), powdery mildew (*Sphaerotheca pannosa*), shot hole (*Wilsonomyces carpophilus*) and brown rot (*Monilia fructigena* and *Monilia laxa*). Those diseases are usually treated based on a calendar using in the majority of the cases wide spectrum preventive fungicides such Ziram, Tetraconazole and Cupper. Other diseases can also be found but their importance can vary in space and time that are treated when it is necessary (Phytophtora root and crown rot, Armillaria root rot, Verticilium wilt …).

Regarding bacterial diseases, bacterial canker caused by *Pseudomonas syringae* pv. *syringae* (*Pss*) and *Pseudomonas syringae* pv. *morsprunorum* (*Psm*) have been reported in Lebanon. Bacterial spot disease caused by the quarantine bacterium *Xanthomonas arboricola* pv. *pruni* (*Xap*) and, crown and root gall disease caused by *Agrobacterium tumefaciens,* were also reported previously (EPPO, 2012). In the literature, the only published report on the occurrence of bacterial diseases on stone fruits in Lebanon goes back to 1969's. This study was done by Saad and Nienhaus when they wrote a general report about plant diseases in Lebanon, including bacterial diseases of stone fruits. At that time they described that canker symptoms on almond trees that were observed in one location on the coast, were induced by *Xanthomonas pruni* (new name *Xap*). Also on peach, the same bacterium was isolated from Mount Lebanon and the symptoms were described as leaf-twig spots. Furthermore, *Pseudomonas morsprunorum* (new name *Psm*), causing cankers and gummosis, was isolated on cherry in Bekaa and on peach in Mont Lebanon. *Agrobacterium tumefaciens* was reported only on plum in Bekaa valley (Saad and Nienhaus, 1969).

Recently, the Lebanese Ministry of Agriculture conducted a survey dedicated to report different kind of diseases in stone fruit orchards. Regarding bacterial diseases, only few samples showing symptoms that may be of bacterial origin were collected, and it was reported that bacterial canker on cherry, almond and plum was caused by *Pss* while on one sample of peach it was caused by *Psm* race 1. None of the other bacterial diseases known to induce diseases on stone fruits, including *Xap* and *Agrobacterium tumefaciens,* were isolated during this survey (MoA, 2011, 2012).

1.2 Bacterial diseases of stone fruits with emphasis on bacterial canker

Many bacterial diseases affect stone fruit orchards in many regions of the world. In this thesis, we mainly discussed the most widely distributed diseases such as bacterial canker caused by different pathovars of *Pseudomonas syringae*, bacterial spot caused by *Xanthomonas arboricola* pv. *pruni* and, crown and root gall caused by *Agrobacterium tumefaciens*. Special emphasis was given to bacterial canker that is the main bacterial disease of stone fruits in Lebanon. Bacterial decline of peach caused by the quarantine bacterium *Pseudomonas syringae* pv. *persicae* is also of highly importance but its distribution is limited to few countries. Some other *Pseudomonas* spp. can also induce diseases on stone fruits but they are considered of less importance. The quarantine bacterium *Xylella fastidiosa* was also reported on peach, plum and almond from some countries but it will not be discussed in this thesis.

1.2.1 Bacterial canker of stone fruits

Pseudomonas spp. is a polyphagous bacterium causing diseases on both annual and perennial plants, including fruit trees, ornamentals and vegetables (Agrios, 2005). It is one of the most adaptive plant pathogenic bacteria able to produce a variety of symptoms such as leaf spot, leaf blight, leaf speck or bacterial canker of wide range of plant species all over the world (Vinatzer *et al*., 2006). It is known to live part of its life as epiphytic on plant surface than later on, under convenient conditions, it is able to infect the plant and reach the apoplast (intercellular space) as a pathogenic endophyte (Hirano and Upper, 2000).

Diseases of different fruit tree species caused by *Pseudomonas* spp. are of major concern in fruit producing areas worldwide. They are extremely difficult to control a reason that gives them the ability to cause significant economical losses. Those pathogens have a complicated genetic diversity and consistent methods of identification and discrimination between different pathovars and strains do not exist yet (Vicente *et al*., 2004; Donmez *et al*., 2010).

On stone fruits, 2 pathovars of *Pseudomonas syringae*, pathovar *syringae* and pathovar *morsprunorum*, cause a disease called 'bacterial canker of stone fruits'. Both causal agents are spread almost everywhere where stone fruits are cultivated. A third pathovar, pv. *persicae (Psp)*, classified as a quarantine bacterium in Europe and it is included on the European Plant Protection Organization A2 list, is also able to cause 'bacterial decline of peach' disease. This one still has a limited distribution in the world and it was reported so far in France, New Zealand and UK (EPPO, 2005, 2006). Other two species of less importance are *Pseudomonas*

amygdali the causal agent of 'hyperplastic bacterial canker of almond' and *Pseudomonas syringae* pv. *avii* newly reported to cause disease on wild cherry in France.

1.2.1.1 History and current geographical distribution

For a number of years, *Pseudomonas syringae* causing bacterial canker of stone fruits was known under different names according to the system of classification followed at the time and the wide range of hosts that this pathogen can infect. The name *Pseudomonas syringae* refers to Van Hall (1902) of the University of Amsterdam, when he proved the pathogenicity of this bacterium causing blight on lilac. At the same time in Poland, Brzezinski (1902) who was working on bacterial canker of stone fruits determined that gummosis and dieback of peach, plum, apricot and sweet cherry trees were of bacterial origin. In 1907, Aderhold and Ruhland described the pathogen causing death of sweet cherry trees in Germany as *Bacillus spongiosus*. Few years later, Griffin (1911) reported that *Pseudomonas cerasi* was the causal agent of gummosis and cankers on sweet cherries in USA (Bultreys and Kaluzna, 2010).

Pseudomonas morsprunorum was characterized for the first time in England by Wormald (1932) as the causal agent of bacterial canker of plum trees. Wormald noticed also that another bacterium, *Pseudomonas prunicola,* was frequently accompanied with bacterial canker of stone fruits and blossom blight of pear (Wormald, 1932, 1937). Later on, researches figure out that *B. spongiosus*, *Pseudomonas prunicola* and *Pseudomonas cerasi* are all the same bacterium and they had to be considered as *Pseudomonas syringae* other than *Pseudomonas morsprunorum* was retained as different species (Crosse and Garrett, 1963; Garrett *et al*., 1966). Few years later, *Pseudomonas morsprunorum* race 2 was described, differing from the one known before in some biochemical and pathological characteristics (Freigoun and Crosse, 1975).

Pseudomonas syringae pv. *persicae* was described for the first time in 1967 on nectarine and peach in France and almost simultaneously on nectarine, peach and Japanese plum in New Zealand (Young, 1988). It was also reported that the same pathogen was isolated once in the UK in 1966 from *Prunus cerasifera* (EPPO, 2005). Some unreliable data were also published about the presence of this bacterium in other countries but those reports cannot be taken into consideration (EPPO-PQR, 2015).

Pseudomonas syringae pv. *avii* isolated from wild cherries was described in 2003 by Menard and his colleagues. This finding explained the identity of many isolates obtained from cherry trees during subsequent years. Those were considered before as intermediate non typical forms, physiotypes, or ecotypes because they are different in some biochemical, physiological

and genetic properties from typical *Pseudomonas syringae* or *Pseudomonas morsprunorum* (race 1) (Dowler and Weaver, 1975; Sobiczewski, 1984; Bultreys and Gheysen, 2003; Renick *et al.*, 2008).

At the moment, the taxonomic position of *Pseudomonas syringae* and *Pseudomonas morsprunorum* has been changed to *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *morsprunorum*, respectively (Young *et al*., 1978; Young *et al.*, 1992). This classification of pathovars showed that *Psm* race 1 and *Psm* race 2 are clearly distinct organisms because they belong to the Genomospecies 2 and 3, respectively, whereas *Pss* belongs to the Genomospecies 1 (Gardan *et al.*, 1999; Ménard *et al.*, 2003).

Currently, bacterial canker of stone fruit occurs in all regions of stone fruit production in the world (Hattingh and Roos 1995; Agrios, 1997; Kennelly *et al.*, 2007). The two main pathovars causing bacterial canker disease, *Pss* and *Psm*, are well adapted to different climatic conditions and cause severe damages in many countries.

1.2.1.2 Damages and economical importance

Pseudomonas syringae can induce serious diseases on stone fruits causing often high economical losses (Scortichini *et al*., 2003; Vicente and Roberts, 2007; Renick *et al*., 2008; Gilbert *et al*., 2009; Kaluzna *et al*., 2010a). However, since the aggressiveness of the disease is not stable from season to season and varies between orchards and growing areas, only few quantitative data are available regarding this aspect. Damages can occur in nurseries, in wild cherry fields for wood production as well as in commercial orchards of stone fruits where reduction in fruit yield, quality and orchard life time can be dramatic (Vicente *et al*., 2004; Agrios, 2005; Janse, 2006; Kennelly *et al*., 2007). Losses can be the result of tree decline and death due to the development of cankers that girdle branches and main trunk or as direct reduction of the productivity due to cold induced by those bacteria leading to death of buds and flowers (Ogawa and English, 1991). In Germany, up to 30% of trees of plum orchards are uprooted every year because of bacterial canker, even with intensive copper sprays. Same losses were reported from Italy in one year old apricot orchards damaged by the same disease (Scortichini, 2006). In Turkey, almost 80% of apricot trees suffer from bacterial canker in Erzurum, Erzincan and Artvin (Kotan and Sahin, 2002) and 20% in Malatya (Donmez *et al*., 2010). Other countries such as Poland, Iran, France, USA, UK, Germany, New Zealand and Lithuania are also facing serious losses due to this disease (EPPO, 2005; Hinrichs-Berger, 2004; Vicente *et al*., 2004; Vicente and Roberts, 2007; Janse *et al.*, 2008; Karimi-Kurdistani and Harighi, 2008; Vasinauskiene *et al*., 2008; Kaluzna *et al*., 2010a).

1.2.1.3 The causal agents

Pseudomonas syringae is a gram negative bacterium belongs to the genus *Pseudomonas sensu stricto,* included in the γ subclass of the Proteobacteria (Kersters *et al*., 1996). It is an aerobic, motile, straight or slightly curved rod shape bacterium with one or several polar flagella (Holt *et al*., 1994; Palleroni, 2005). They also produce the pigmented iron chelating siderophores pyoverdins that are fluorescent under UV light. As many plant pathogenic bacteria they produce phytotoxins such as the two toxic lipodepsipeptides (TLP) syringomycins and syringopeptins produced by the pathovar *syringae* which play an important role in the virulence of the bacterium (Young *et al.*, 1992; Sorensen *et al.*, 1998; Bultreys and Gheysen, 1999; Gilbert *et al*., 2009).

Pseudomonas syringae includes saprophytic and pathogenic species harmful to human, mushrooms and one of the most important plant pathogenic *Pseudomonas* species. Some strains were also isolated from environmental habitats including those closely linked to the water cycle outside of agricultural contexts (Berge *et al*., 2014).

This complex group is known to be very heterogeneous, causing diseases to more than 180 plant species including fruit trees, vegetables, ornamentals, and other annual and perennial plants (Bradbury, 1986; Young *et al*., 1996). This diversity was the reason why this species was divided into at least 57 pathovars (Gardan *et al.*, 1997; Young, 2010) and nine Genomospecies (Gardan *et al.*, 1999).

The pathovar *syringae* is maybe the most heterogeneous pathovar having the ability to cause diseases to a large number of unrelated plant genera, including *Prunus* species (Bradbury 1986; Young 1991; Weingart and Völksch 1997; Little *et al.*, 1998; Vicente and Roberts 2007). Strains of *Pss* were isolated and identified from symptomatic plant tissues of woody and herbaceous hosts based on biochemical and physiological characteristics, and pathogenicity tests on different plant species (Little *et al*., 1998; Scortichini *et al*., 2003). In fact, because of the high diversity of strains of this pathovar, it is always recommended for an accurate identification to conduct pathogenicity tests on susceptible host plants since classical methods of identification are not enough (Little *et al*., 1998; Vicente and Roberts, 2007). Some studies reported that *Pss* strains isolated from a specific host are able to cause disease on a diversity of plant species (Scortichini *et al*., 2003; Vicente *et al*., 2004; Gilbert *et al*., 2009) while others are specific to the host of isolation such in the case of strains infecting grasses (Gross and De Vay, 1977) and beans (Cheng *et al*., 1989). Moreover, in a study conducted by Little *et al.,* (1998), results showed that *Pss* isolated from stone fruit formed a distinct cluster separate from most of strains isolated from other hosts. Also strains from

different zones showed a genetic diversity among each other's (Gonzalez *et al*., 2000) but it was not always the case that genetic diversity is related to host plant and/or region. According to Martín-Sanz *et al*. (2013), *Pss* strains isolated from peas were sometimes less virulent when artificially inoculated on the host of isolation itself than strains isolated from other plant species. They conclude that there is genetically and pathogenically distinct *Pss* strain groups from pea, a factor to be taken into consideration for the diagnostic and epidemiology of this pathogen and for disease resistance breeding.

This inconsistency makes the classification of this group of bacteria and the designation of pathovar a very complicated work. A standard protocol, either for identification or characterization must be followed in order to assess the host specificity and the virulence of different genetic and pathogenic groups of strains of the pathovar *syringae*.

The second pathovar, *Pseudomonas syringae* pv. *morsprunorum*, has a much narrower host range (Bradbury, 1986). It is more homogeneous than *Pss* but heterogeneity is known to occur within this pathovar since two genetically different races were described based on physiological and pathological characteristics (Freigoun and Crosse, 1975; Ménard *et al.*, 2003; Vicente *et al.*, 2004; Vicente and Roberts, 2007). *Psm* race 1 (Wormald, 1932) belongs to the Genomospecies 2 and it is pathogenic to cherry, plum and apricot, while *Psm* race 2 (Freigoun and Crosse, 1975) belongs to the Genomospecies 3 and it is pathogenic mainly to cherry (Bultreys and Kaluzna, 2010). Regarding isolates from cherry trees, it was noticed in Belgian orchards that *Psm* race 1 were more frequently isolated from sweet cherry and *Psm* race 2 mostly from sour cherry (Bultreys *et al.*, 2007; Gilbert *et al.*, 2009).

Pseudomonas syringae pv. *avii* is a newly described pathovar that infects wild cherry cultivated for wood production in France. It belongs to the Genomospecies 3 and strains of this pathovar show high genetic homogeneity among each other's (Ménard *et al*., 2003).

Furthermore, the pathovar *persicae* belongs also to the Genomospecies 3 and it is pathogenic to peach, nectarine and Japanase plum (Young, 1988; EPPO, 2005). This is the only *Pseudomonas syringae* pathovar classified as a quarantine bacterium A2 list by the European Plant Protection Organization (EPPO, 2005).

Here we have to mention also *Pseudomonas amygdali* the causal agent of hyperplastic bacterial canker of almond. This bacterium is of less importance and it was reported only from Afghanistan, Greece and Turkey (EPPO-PQR, 2015).

As in the case of the pathovar *syringae*, different phytotoxins are produced by the other pathovars, a characteristic used in identification either by direct detection of the secondary metabolite itself or by detection of the genes involved in its production or secretion.

Coronatine is produced by *Psm* race 1 strains and the siderophore yersiniabactin is produced by the pathovars *morsprunorum* race 2, *avii* and *persicae* (Bereswill *et al.*, 1994; Sorensen *et al.*, 1998; Bultreys and Gheysen, 1999; Bultreys *et al.*, 2006). Moreover, *Pseudomonas syringae* pv. *persicae* secretes several substances named persicomycins representing a new family among the phytobacterial toxins (Barzic and Guittet, 1996). All of them cause necrosis of peach tree tissues and they are involved in the die back disease of peach trees.

An important characteristic of some species of the genus *Pseudomonas*, including *Pseudomonas syringae*, is the ice nucleation active (INA). In fact, those bacteria are able to induce ice formation from water supercooled below 0°C (Lindow, 1983; Hirano and Upper, 2000). In *Pseudomonas syringae*, this ability is common for epiphytic strains not assigned to a pathovar and for the pv. *syringae*; but it was never reported for strains of the pathovar *morsprunorum* race 1 (Lindow, 1983; Mittelstädt and Rudolph, 1998). It is known that frost injury predisposes stone fruit to disease caused by *Pss* but it is not answered yet whether INA *Pss* could induce frost injury on fruit species (Sobiczewski and Jones, 1992; Bultreys and Kaluzna, 2010). However, according to Andrews *et al.*, (1986) flowers have a lower water super cooling temperature than stems which makes them vulnerable to freezing injury during spring frost with the presence of INA bacteria even under very mild and transient frost. This characteristic causes a direct loss in production and unfortunately the attempts to limit freeze injury in stone fruit and pear orchards by controlling INA *Pss* are not yet successful (Cody *et al.*, 1987; Mittelstädt and Rudolph, 1998).

1.2.1.4 Ecology and biology of the bacterium

Pseudomonas syringae pathovars are able to colonize leaf surfaces of host trees and weeds in the orchard, as epiphytic stage of the bacterium life. The epiphytism of plant pathogenic bacteria was first described on *Psm* by Crosse (1957, 1959). He found that the population of *Psm* race 1 is able to survive and multiplicate on sweet cherry leaves without causing symptoms. During hot dry summer, this population decreases considerably and reaches its lowest levels. Later on, an increase in bacterial population is observed during autumn when the temperature decrease and first rains start. In general, the bacteria responsible of bacterial canker have a facultative summer leaf spot stage, and an obligate spring, summer and autumn leaf epiphytic stage, with an overwintering stage within dormant buds and cankers (Crosse, 1955, 1956, 1957; Bultreys and Kaluzna, 2010; Scortichini, 2010). The natural drop of the leaves in autumn allows the pathogen transported by rain and wind or present already as epiphytic on plant surface to enter through leaf scars. Bacterial multiplication in cortical

tissues start from late autumn to early spring, but no important cankers develop before spring. Infected buds can remain symptomless or they can be killed by the pathogen before their opening in spring (Hatting *et al.*, 1989). At that time, the pathogen colonizes leaves, blossoms and young fruits which under favorable wet conditions show spot symptoms. Colonization of leaf stomata without symptoms formation (Roos and Hattingh, 1983) and systemic invasions from leaves through the veins to other tissues of the plant have also been reported (Roos and Hattingh, 1987; Sundin *et al*., 1989). However, when the weather becomes more and more dry during late spring and summer, the overwintered cankers of the previous year become dry and the bacterial population decrease dramatically (Bultreys and Kaluzna, 2010).

All events affecting the health of the tree or causing wounds can be used by the pathogens to increase the infection level. Consequently, pruning wounds, poor nutrition, plant parasitic nematodes, frost injury, hails injuries, favor the penetration and the spreading of the bacteria inside the tree tissues and among trees (Hinrichs-Berger, 2004; Bultreys and Kaluzna, 2010). For example, cultivar and rootstock sensitivity seems to be a main predisposing factor enhancing the virulence of *Pseudomonas syringae* to stone fruits (Scortichini, 2006). As well, low calcium content, sandy or very clayey can enhance the sensitivity of apricot and peach trees to *Pss* infection. Moreover, a period of freezing followed by thawing, promote the displacement of endophytic bacteria within the stem tissues (Weaver, 1978; Vigouroux, 1989). However, this appeared to favor the multiplication and longitudinal progression of *Pss* while *Psm* race 1 was more efficient in lateral infection of cortical tissues and it is not favored by frost (Sobiczewski and Jones, 1992; Bultreys and Gheysen, 1999; Gilbert *et al.*, 2010). It was also reported that *Pss* infection is more efficient through wounds while leaf scars are the main infection point of *Psm* race 1. Another difference between those 2 pathovars is that *Pss* population die out in cherry cankers earlier in dry summer than *Psm* race 1 (Garrett *et al*., 1966).

Knowing the behavior of the pathogen and the factors that can enhance or slow down the disease remain indispensable. This is the basic to determine the optimal method and time to apply control measures. To understand better this behavior there is need to have more knowledge about genetic characteristics of each pathovar and even strains within a specific pathovar that surely have a direct impact on epidemiology and pathogenicity of those bacteria.

1.2.1.5 Symptoms

Bacterial canker affects many parts of the tree and symptoms include blossom blast, spur dieback, leaf and fruit lesions, cankers associated with gummosis of woody tissue and overall tree decline (Hattingh and Roos, 1995; Renick *et al*., 2008; Bultreys and Kaluzna, 2010). The type and severity of the disease symptoms depends on many factors regarding cultivar, age of the infected tree, plant tissue invaded, strain of the pathogen and environmental factors (Gašić *et al.*, 2012). Anyhow, the most characteristic and destructive symptoms are cankers on trunk, limb and branches that are often located around spurs, pruning wounds, on twigs at the base of flowers, leaf buds and at branch junctions. At those points the pathogen enters and makes circular to elongated, brown, water-soaked lesions in the bark. Cankers may expand upward rapidly early in the spring accompanied with gum exudation with diebacks that appear on terminal shoots or twigs because of the girdling of the main trunk or branches. Infected buds may fail to grow during the next spring or if few leaves develop, they will wilt soon during first days of hot summer (Goto, 1992; Hattingh and Roos, 1995; Bultreys and Kaluzna, 2010; Gašić *et al.*, 2012).

When they are expressed, symptoms on the leaves are rounded to irregular lesions of different sizes, water-soaked, light brown color in early spring that turn darker with time surrounded by a yellow halo. Those spots become necrotic and rapidly drop out to produce a shot hole effect. Blossom blast is also common, showing brown, shriveled flowers often fall down before full opening. Immature fruit lesions are small, brown-black necrotic spots becoming sunken with dark center as the fruit matures. An important aspect is that leaves and fruits symptoms are not always formed depending on the susceptibility of cultivars and the amount of rainfall (Bultreys and Kaluzna, 2010; Kaluzna *et al*., 2012). Anyhow, when they are numerous on fruit surface they prevent the normal development of fruit epidermis, resulting in rusted areas or deformed fruits (Young, 1987).

Regarding bacterial decline caused by *P. s.* pv. *persicae* symptoms can be different on each of its hosts (EPPO, 2005). On Japanese plum, infection is mainly on nodes and a characteristic symptom is tip dieback with some occasional death of laterals and fruiting arms. On nectarine and peach, symptoms include shoot dieback, limb and root injury, tree death, leaf spots and fruit lesions. Dissection of the lesions show brown necrosis and water soaked areas developing along the vascular bundles (Young, 1987). They may extend more than one meter and girdle a big part of the tree with no distinct margin between healthy and necrotic tissue in lower parts of the tree. This is a distinctive aspect of bacterial decline from bacterial canker

(Hattingh and Roos, 1995). Moreover, in the case of bacterial decline the rootstock can also be infected, showing symptoms similar to those on woody shoots (EPPO, 2005).

In general, regarding host range and symptoms caused by *Pss*, *Psm*, *Psa* and *Psp*, it is difficult to distinguish between them in the field. This may have significant consequences because *Psp* is the only one considered as quarantine bacterium in many countries (EPPO, 2005; Gašić *et al.*, 2012).

1.2.1.6 Identification and characterization

Visual inspection of symptoms in the field would be very useful as first inspection to check the presence of diseases caused by *Pseudomonas* spp.. Cankers, necrotic twigs, leaf or fruit spots, gummosis or other symptoms should be taken into consideration. The problem is that those symptoms are not specific to diseases caused by this group of bacteria and they can be induced by other bacterial species or fungal pathogens and even by abiotic stress.

Accordingly, attention should be paid in order to accurately identify the causal agent of the disease to implement the most convenient control strategies. Traditionally, diagnostic and detection techniques used for plant pathogenic bacteria are based on microscopic observation, isolation on culture media, serological testing, bioassays and molecular assays. Isolation on culture media is time and labor consuming and confirmation of results may take several weeks (López *et al*., 2010). Anyway, it still indispensable in many cases to isolate the causal agent and to fulfill Koch postulates as it is mentioned in EPPO protocols.

Regarding phenotypic identification and characterization of *Pseudomonas syringae*, it is recommended to make isolation on King's B medium (King *et al*., 1954) since isolates of this group of bacteria are known to produce fluorescent pyoverdin observed under UV light. To be aware that some pathovars isolated from stone fruits are not fluorescent such as some strains of *Psm* and all strains of the pathovars *persicae* and *avii* (Bultreys and Kaluzna, 2010).

After this, LOPAT tests are generally used to discriminate between *Pseudomonas* spp. (Lelliot *et al*., 1966). Those 5 tests are able to divide species of this genus into different groups where *Pseudomonas syringae* are classified in the group Ia. Bacterial species of this group are able to produce levan and to induce hypersensitivity reaction on tobacco leaves but they are not able to produce cytochrome C oxydase, pectinase neither arginine dehydrolase. Continuing with classical identification tools, GATTa tests are used to differentiate between *Pseudomonas syringae* pathovars isolated from stone fruits (Latorre and Jones, 1979). Those 4 tests are very helpful with the exception of their incapability to clearly separate strains of the pathovar *morsprunorum* race 2 that show variable results (Gilbert *et al*., 2009).

Serological techniques are usually used as first screening for massive detection and the accuracy of data is related mainly to the quality of the antibodies. The main techniques used are indirect immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA) and recently lateral flow devices. In any case, serological techniques to detect *Pseudomonas* spp. are not commonly used because of the lack of specific polyclonal or monoclonal antibodies, so scientists do not advise their use for diagnosis or detection in the case of this group of bacteria (López *et al*., 2010).

In the last years, the use of molecular techniques has increased rapidly. Conventional Polymerase Chain Reaction (PCR), repetitive extragenic palindromic PCR (rep-PCR) and recently real-time PCR are more frequently used since they are highly sensitive techniques and relatively easy to perform. Many specific primers were developed to detect genes coding for the production of toxins or siderophores that are produced by *Pseudomonas* spp. such as *syr*B and *syr*D genes, involved in the synthesis and export of these lipodepsipeptides among *Pss* strains (Bultreys and Gheysen, 1999; Sorensen *et al*., 1998). A limit of this technique is that many toxins are not specific to one pathovar or even one species and results can be inaccurate (Bultreys and Gheysen, 1999). According to this fact, using PCR for the detection of secondary metabolites is not enough for the detection of all bacterial species and it is recommended to be combined with other techniques. Moreover, many specific primers were designed to detect different regions in the genome of *Pseudomonas syringae* pathovars such as *Psyr_1890* primer pair used for the detection of *Pss* (Vieira *et al*., 2007). It was reported that those primers are able to detect the presence of hopAP1 effector that is specific to the pathovar *syringae* enabling the discrimination of strains of this pathovars from others.

For molecular characterization of *Pseudomonas syringae* isolated from stone fruits, rep-PCR using REP, BOX and ERIC, may be the most used technique. It is able to discriminate between different pathovars and to illustrate the genetic diversity that exists among strains of each of them. The only difficulty is the case of strains of the pathovar *syringae* that according to rep-PCR showed to be very diverse while it is very easy to identify strains of the other pathovars from stone fruits using this technique (Ménard *et al*., 2003; Vicente and Roberts, 2007; Gilbert *et al*., 2009; Kaluzna *et al*., 2010b; EPPO, 2005). In fact, this technique divided *Pss* strains in many genetic groups according to pattern produced. In some studies, it was reported that it does not exist a clear relationship between host plant and bacterial genomic fingerprint (Schortichini *et al*., 2013) while according to other references it was reported the presence of host-pathogen relationships within *Pss* and possible specializations of clonal populations on different hosts (Little *et al*., 1998; Gilbert *et al*., 2009). Variability was also

observed in pathogenicity and virulence tests on a variety of plant species between strains from different host of isolation (Schortichini *et al*., 2013). For this reason, it was recommended that pathogenicity test on susceptible host should be always conducted in the case of *Pss* strains (Gašić *et al*., 2012, Latorre and Jones, 1979; Vicente *et al*., 2004).

Molecular techniques based on sequencing of specific genes are more frequently used in the last years. This is due to the decrease in the cost of sequencing comparing to the past, the assured results obtained and the possibility to compare results with a large data base. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses (Lane *et al*., 1985) and newly Multilocus Sequence Typing (MLST) were often used. This latter technique is based on housekeeping gene analysis revealing high discrimination among *Pseudomonas syringae* strains. Many studies were published during the latest years in which MLST was used to analyze the diversity and to classify strains of the complex *Pseudomonas syringae* group (Sarkar and Guttman, 2004; Hwang *et al*., 2005; Kaluzna *et al*., 2010b; Bull *et al*., 2011; Clarke *et al*., 2010). One of the most valuable studies was that of Berge *et al*. (2014) performing MLST on 216 *Pseudomonas syringae* isolates by partially sequencing four housekeeping genes (*cts*, *gap* A, *rpo* D, *gyr* B). They figure out that the *Pseudomonas syringae* complex is divided into 23 clades within 13 phylogroups. They also analyzed the phenotypic characteristics of strains that belong to each of these phylogroups and clades. The identified phylogroups have shown to be equivalent to the Genomospecies described by Gardan *et al*., (1999) based on DNA/DNA hybridization. In fact, Gardan *et al*. (1999) described 9 Genomospecies among the pathovars of *Pseudomonas syringae* where four of these represented the majority of species and pathovars of the complex. According to this classification, *Pseudomonas* spp. causing diseases on stone fruits were allocated in a way that the pathovar *syringae* belongs to the Gsp1 (=PG02 of Berge *et al*., 2014), the pathotype strain of the pathovar *morsprunorum*, the pathovar *avii* and the pathovar *persicae* belong to the Gsp 3 (PG01), and finally *Pseudomonas amygdali* belongs to the Gsp2 (PG03). To mention that it was reported that the pathotype strain of the pathovar *morsprunorum* is not representative of the pathovar (Young *et al*., 1996) and Gardan *et al*. (1999) showed that another strain of the pathovar *morsprunorum* (CFBP 2116) is a member of Genomospecies 2 (=PG03) and this strain is the proposed as neopathotype strain (Young, 2010).

1.2.1.7 Type three secretion system (TTSS)

Understanding the strategies used by microbial pathogens to infect and cause diseases in their host cells is taking more and more attention during the last years. It is known that many of

those pathogens transport effector proteins into the cytoplasm of the host cells in order to alter their metabolism, destabilize their immunity system and encourage their multiplication (Lindeberg *et al*., 2012).

Pseudomonas syringae is a model bacterium to study effector repertoires as dynamic systems. It possesses the type three secretion system (TTSS), as many groups of bacteria, which is the main factor that enables them to cause disease. This system secretes and/or translocates a group of effector proteins that alter host cellular processes and promote disease development (Jin *et al*., 2003). The TTSS is only expressed after the bacterium comes into direct contact with the host. After that, the TTSS pilus is produced forming a direct channel between the pathogen and its host. TTSS effector proteins are directly inject through this pilus into the cytoplasm of the host, where they target host proteins and modulate the host defense response. TTSS effectors are known to suppress the defense response by interfering with signal transduction, causing cytoskeletal changes, or by having direct cytotoxic effe*cts* (Collmer *et al*., 2002; Guttman *et al*., 2006).

Effectors are encoded by the hypersensitive reaction and pathogenicity genes *hrp*/*hrc* and in *Pseudomonas syringae* they are usually designated as Hop proteins (hrp outer protein) because they are able to move through the TTSS (Lindeberg, *et al*., 2005). Another set of effectors known as avirulence genes (*avr*), reduce the ability to cause disease if they get to be recognized by resistance proteins (R) of the plant. This reduction in pathogenicity is done by activation of the plant defense system (Lindeberg *et al*., 2005; Vinatzer *et al*., 2006).

According to this, the role of effectors can be divided into 2 functions. First of all, they suppress pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) which is evolved by common bacterial features such as flagellin, lipopolysaccharide (LPS), peptidoglycan and elongation factor Tu (Boller and Felix, 2009). Furthermore, the immunity system of plants is able to detect the effectors injected by the pathogen by resistance (R) proteins which results in localized programmed cell death known as the hypersensitive response (HR) (Lindeberg *et al*., 2012). As definition, hypersensitivity reaction (HR), is a phenomena that is produced when a high concentration of avirulent strain of bacteria is infiltrated in the apoplast of a non-host plant species or a resistant cultivar, that will cause a rapid programmed cell death of the infiltrated tissue (Lindgren *et al*., 1986; Greenberg and Yao, 2004).

Genomic sequence data of *Pseudomonas syringae* strains (Buell *et al*., 2003; Feil *et al*., 2005) in combination with *in vitro* and *in vivo* screening and bioinformatics analysis have revealed that strains of *Pseudomonas syringae* contain dozens of effectors that vary in size and

composition among strains (Guttman *et al*., 2002; Petnicki-Ocwieja *et al*., 2002; Greenberg and Vinatzer, 2003; Chang *et al*., 2005). According to the '*Pseudomonas syringae* genome resources' website (http://www.*Pseudomonas-syringae*.org), *Pseudomonas syringae* pv. *tomato* (DC3000), *Pseudomonas syringae* pv. *syringae* (B728a) and *Pseudomonas syringae* pv. *phaseolicola* (1448a) share only 13 effectors and the remaining effectors are either unique to one of these strains or only shared between two of them. It is believed that these differences in effector repertoires among strains are the main determinants of host range in *Pseudomonas syringae* (Alfano and Collmer, 2004; Baltrus *et al*., 2011).

Approximately 60 different TTSS gene families have been identified in gram-negative bacteria of both plants and animals, including *Pseudomonas syringae* (O'Brien *et al*., 2011). This number is supposed to increase with the increased number of genome sequences that have been done lately, allowing the identification of new members of these families as well as the identification of novel families.

1.2.2 Bacterial spot

Xanthomonas arboricola pv. *pruni* (*Xap*) is a plant pathogenic bacterium that causes bacterial spot disease on a wide range of commercial, ornamental and forest *Prunus* species (Ritchie, 1995). It is regulated as a quarantine pathogen in many countries as in the European Union phytosanitary legislation (Anonymous, 2000 and amendments) and the European Plant Protection Organization EPPO A2 List (Anonymous, 2003). This disease is rated as of little economic importance by the EPPO countries where it currently occurs in many of them but its behavior elsewhere in the world suggests that it would be likely to establish more widely in this area with no important threat to arid regions (EPPO, 2005). However the most severe epidemics have been reported on the Sino-Japanese plum group (*P. salicina* and *P. japonica*) and their hybrids, peach (*P. persicae)* and its hybrids, and nectarine (*P. persica* var. *nectarina*) (Ritchie, 1995; Stefani, 2010).

1.2.2.1 History and current geographical distribution

Since its first description on Japanese plum in the United States in 1903, *Xap* has been observed all over the world (Smith, 1903; Balestra and Varvaro, 1997; Battilani *et al*., 1999; Jami *et al*., 2005).

Smith (1903) named this bacterium *Pseudomonas pruni*, and described that symptoms appear on the foliage and green fruits with some notes about the growth of this organism on culture media. Two years later, Clinton identified the bacterial disease of peach leaves as that caused

by *Bactirium pruni* and he also published an excellent illustration of it. This name was used for many years till 1939, when Dowson calls it *Xanthomonas pruni*. Another change of the name was done by Dye in 1978, naming it: *Xanthomonas campestris* pv. *pruni*. The current name *Xanthomonas arboricola* pv. *pruni* was proposed by Vauterin and his colleagues in 1995 (Vauterin *et al*., 1995).

In Europe, bacterial spot has been identified first in northern Italy in 1934 (Petri, 1934). From the mid 70s to the late 80s, the bacterial spot of stone fruits has been severe and recurrent in many Italian regions. This was due to the introduction of a new Japanese plum (*P. salicina*) cultivar, named Calita, made in 1963 in the United States. This shift in plum production from European cultivars (*Prunus domestica*) to Japanese ones which are known to be moderate to highly susceptible (Topp *et al*., 1989; Bazzi *et al*., 1990) to bacterial spot disease was the cause of recurrent epidemics in Italy. This was accompanied with negative effe*cts* on the economic returns of this cultivation and by now it is considered as endemic (Battilani, 1999).

According to EPPO, 2012, bacterial spot is now widely distributed in all over the world where stone fruit are cultivated as showing figure 4.

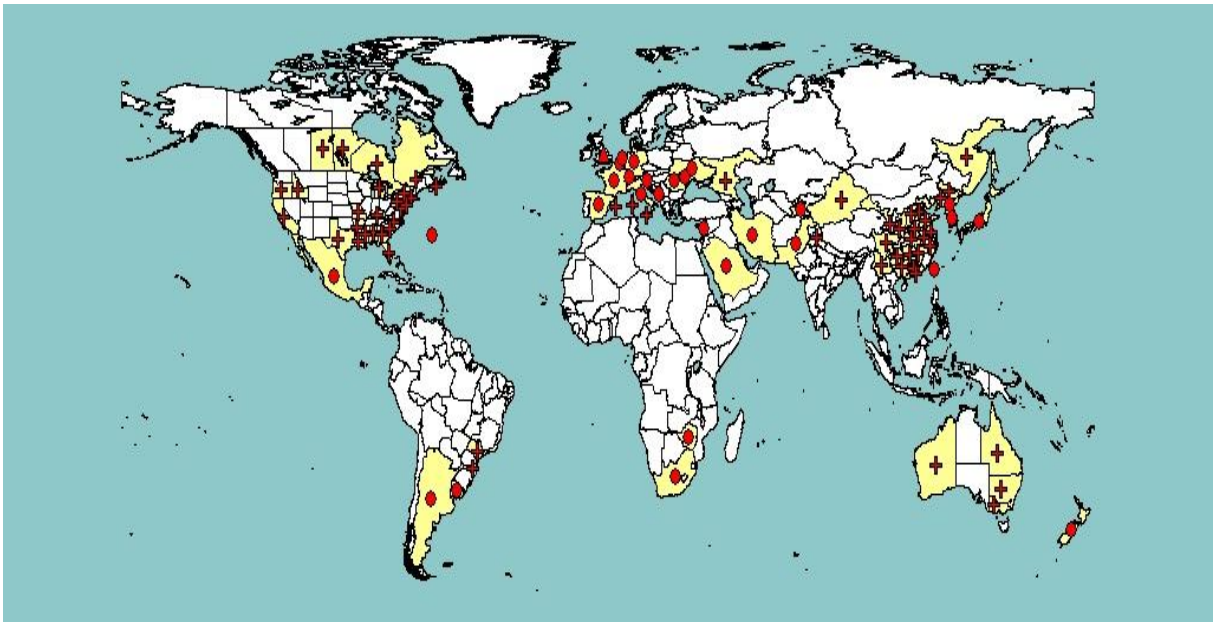


Figure 4: Distribution map of *Xap* (EPPO, 2015)

1.2.2.2 Economical importance

There is no a single opinion about the real economic impact of bacterial spot disease of stone fruit regarding productivity or tree losses. Petri in 1934 did not report significant damages or economic crop losses of plum, whereas Dunegan (1932) observed that injuries to peach fruits vary from 2% to 75% according to the environmental conditions and the management

strategies followed in each orchard. Greatest damage arises from developing a severe yellowing of leaves with shot holes that can be followed by severe defoliation leading to a reduction in yield, weakened trees and sometimes total death (Du Plessis, 1988; Crisosto *et al.*, 1995; Ritchie, 1995).

Moreover, According to Stefani (2010) epidemic conditions in a commercial plum orchard of northern Italy can easily affect 30% of the fruits, and result in crop losses estimated over 11,200 € per ha in the case of susceptible varieties. In fact, losses are related to many factors linked to environment, susceptibility of cultivars and cultural practices used in each area. This estimation was calculated by taking into consideration three major parameters: reduced quality and marketability of fruits, reduced orchard productivity and increased costs of nursery productions.

Another important fact is that *Xap* is considered a quarantine pest, the market standards regarding fruit quality, do not allow trading of affected fruits even if each fruit does not bear more than 1 or 2 superficial spots of very small diameter. Adding the extra labor to prune branches with cankers and the supplementary number of control sprays required to reduce the impact of this bacterium. Same for nurseries, regular field inspection of mother trees are required with the implementation of optimal hygienic conditions during the preparation of bud chips, rootstocks and scions, together with the need to implement the nurseries in pest free areas (Stefani, 2010).

1.2.2.3 The causal agent

The genus *Xanthomonas* spp. has been extensively revised with elevation of a number of pathovars to species level. As we mentioned before, the new name *Xap* proposed as part of the revision done by Vauterin and his colleagues in 1995.

Xap is a gram negative bacterium having a rod shape, belonging to the gamma proteobacteria group. It is motile by one flagellum, measuring 0.2–0.4x0.8–1.0µm, and strict aerobe with an optimum growth temperature ranging between 24 and 29°C (Ritchie, 1995; EPPO, 2005).

As isolates from different continents had shown a relatively low level of diversity, it has been suggested that the pathogen originated in the United States and has subsequently been disseminated to other regions (Boudon *et al*., 2005). This is due to international trade through contaminated material used for propagation (Goodman and Hattingh, 1986).

1.2.2.4 Ecology and biology of the bacterium

The rate of spreading of bacterial spot disease on stone fruits is related to two main factors: host susceptibility (Bazzi *et al.*, 1990; Simeone, 1991) and environmental factors that affect the epiphytic population survival, the penetration and the spread of the bacterium (Anderson, 1953; Foster and Petersen, 1954; Gasperini *et al.*, 1984; Du Plessis, 1986, 1987, 1990; Shepard and Zher, 1994). In fact, this disease is more common and most severe in areas where stone fruits are grown on light, sandy soils with a humid, warm environment during the growing season (Battilani *et al.*, 1999). The source of the primary inoculum can be leaf scars, buds, cankers, adding black tip in the case of peach (Zaccardelli *et al.*, 1998). As well, Xanthomonads generally can multiply and survive for several weeks on tissue surfaces of their hosts without showing any symptoms (Timmer *et al.*, 1987). A study conducted by Shepard and Zehr (1994) has shown that *Xap* can persist year round as epiphytic population on symptomless peach leaves, flowers, fruits, twigs and buds. It was also isolated from fallen leaves on the soil surface after 6 months of being artificially infected. Probably, this population plays also an important role as primary sources of inoculum (Zaccardelli *et al.*, 1998). During spring, the infections can occur any time after the leaves begin to unfold. From late bloom to a few weeks after petal fall, a temperature of 19-28°C with light, frequent rains accompanied by winds and dews are the optimal conducive factors for the disease development and spread. The bacteria in the plant tissues start to multiply and cause the epidermis to rupture, leading to lesions called spring canker. From those, the pathogen spread by windblown or splashing rain and comes in contact with healthy leaves, fruit and new twigs. Later on, the pathogen enters the tissues through natural opening stomata or lenticels where it multiplies and migrates systemically leading to symptoms development (Du Plessis, 1983; 1987). Secondary spread of the bacteria can occur from oozing leaf and fruit lesions during warm, wet weather. Those develop on green shoots causing summer cankers which will be healed after the formation of a new epidermic layer during early summer. Few months later, the host resistance mechanism decreases and rains started around autumn. At that time, infection of shoots constitutes the primary inoculum source for the following spring. As we can see, as long as the environmental conditions are favorable for the disease development, repeated infections occur throughout the growing season on all susceptible parts (Ritchie, 1995; Battilani *et al.*, 1999).

1.2.2.5 Symptoms

Symptoms on peach first appear on the lower part of the leaves as circular to irregular, small, water-soaked areas of green pale to yellow in color. With time, spots are visible on the upper surface of the leaves and become angular in shape taking a dark purple color that soon turns black or brown with a yellow halo. Mature spots become necrotic leading the center to drop resulting in a shot hole appearance symptom. Spots are more concentrated around the mid drip and the leaf tip, places where water from rain or dew accumulate (EPPO, 2005). Bacterial ooze may be excreted from the spots during wet weather but heavily infected leaves will turn completely yellow and fall down. Severe foliar infections and defoliation ultimately reduce yield and its quality, due to reduced photosynthetic competence and carbohydrate uptake (Crisosto *et al.*, 1995).

On plum, the shot hole effect is more pronounced than on other stone fruits, whereas chlorosis is minimal and less apparent than on peach leaves. On almond, apricot and cherry, leaf symptoms are similar to those on peach but rarely of importance. Symptoms of bacterial spot on leaves can sometimes be confused with injuries caused by fungi or copper preparations. However, copper lesions are larger and often round in shape (EPPO, 2005).

On twigs, two distinct types of damage appear on peach and nectarine as the result of *Xap* infection: "spring" and "summer" cankers. Spring cankers tend to be formed at nodes level, occurring on the upper part of the shoots of the previous year. They start after the bud brake as small, water-soaked, superficial blisters, then extend and may girdle the twig killing the part above the infection point keeping what is called 'black tip'. The epidermis of infected twigs ruptures and the bacteria is released in the surrounding environment. Later on, summer cankers appear after the leaf spots are evident. Those develop commonly between nodes as water soaked, dark-purplish spots surrounding lenticels. With time they dry out and become dark, sunken and circular to elliptical lesions with a water-soaked margin (Goodman and Hattingh, 1988; Du Plessis, 1988; Shepard, 1994). Cankers on plums and apricots are perennials and may survive for 2 or 3 years, resulting in deep cankered areas with discoloration of the inner bark. In this case, branches may be killed or broken because of fruits weight.

On fruits, bacterial spot first appears as small circular brown spots with water soaked margins becoming darker and pitted with time. On peach and nectarine, spots are usually of small size, grouped on the side of the sun and surrounded by a yellow halo. As the fruit enlarge, cracking enlarge and exudation of bacterial ooze is possible mainly after rain. Spots on plums and apricots are usually fewer but larger (Stefani, 2010). In general symptoms on fruits appear 3

to 5 weeks after petal fall and develop until the skin color changes. An important aspect is that fruits infected at an early age of the development are usually the most malformed while those infected later during the season are superficial, giving the fruit a mottled appearance (EPPO, 2005, 2006).

1.2.2.6 Diagnosis and detection

Since *Xap* is considered a quarantine pathogen in the EPPO region (Anonymous, 2003), effective measures must be applied for the detection of this bacterium in order to prevent its introduction and spread into new areas. An important challenge is to detect the pathogen during its epiphytic life stage or when the infection is still latent which may form the primary inoculum for dissemination (Dhanvantari, 1971; Goodman and Hattingh, 1986; Zaccardelli *et al*., 1998).

Isolation can be done from symptomatic leaves and fruits, cankered twigs and branches, and from asymptomatic sample detecting the epiphytic population. This operation is usually easy because of the high number of cultivable bacteria. However when environmental conditions do not favorite pathogen multiplication or heavy treatments with bactericides have been applied in the field, the number of cultivable cells should be expected to be very low in comparison with saprophytic bacteria (Pulawska *et al.*, 1997). The current EPPO standard protocol for the detection of *Xap* is based, on isolation using general agar media YDC (yeast extract-dextrose-calcium carbonate agar) or YPGA (yeast-peptone-glucose agar) where typical colonies are convex, smooth, mucoid, glistening with a creamy yellow color that turn yellow-orange with age. Suspected colonies are purified for physiological and biochemical tests such as gram reaction (-); presence of oxidase (-); glucose metabolism (oxidative); aesculin hydrolysis (+); gelatin liquefaction (+); protein digestion (+); starch hydrolysis (-); urease production (-); potato soft rot (slimy yellow growth); growth at 35°C in yeast broth (+); growth in 2% NaCl (+) and growth in 5% NaCl (-) (Fahy and Persley, 1983; Schaad, 1988).

From pure cultures, protein profiling SDS-PAGE and fatty acids methyl ester profile analysis (FAME), repetitive extragenic palindromic PCR (rep-PCR), specific PCR (Pothier *et al*., 2011a), real-time PCR (Ballard *et al*., 2011) and serological tests (IF) (Zaccardelli *et al*., 1995) can be used for the confirmation of results.

Biological tests are also used for the identification of the pathogen such as hypersensitivity reaction on tobacco leaves considering that a suspension of *Xap* produce typical symptoms

after 1 to 4 days (Klement *et al*., 1964). At the end, pathogenicity test should be done to full fill Koch postulates using susceptible plum or peach seedling (Anonymous, 2006a).

1.2.3 Crown and root gall disease

Agrobacteria are soil inhabitant microorganisms that include some plant pathogenic species able to induce crown and root gall disease. This disease is spread all over the world having a very large number of hosts. De Cleene and De Ley, 1976, reported that 1193 plant species belonging to more than 331 genera and 93 families can be infected by Agrobacteria. Not only dicotyledonous plant, some monocots are also susceptible to this pathogen such as some members of Liliales and Arales (Otten *et al*., 2008; DeCleene and De Ley, 1976).

Plant pathogenic *Agrobacterium* spp. are divided into four species according to the diseases they cause on different plant hosts: crown or root gall induced by *Agrobacterium tumefaciens* reported to be pathogen to 400 plant species (Bradbury, 1986), hairy root induced by *Agrobacterium rhizogenes* having a wide range of hosts belonging to 30 different genera (De Cleene and De Ley, 1986), cane gall induced by *Agrobacterium rubi* specific on Rubus plant and the new species *Agrobacterium vitis*, the causal agent of tumors on grape vine and few others plant species (Gelvin, 2003).

1.2.3.1 History and current geographical distribution

Crown gall was first described back to 1853 (Fabre and Dunal, 1853) as a neoplastic disease affecting various plant species. In 1897, Fridiano Cavara in Italy described a bacterium, termed *Bacillus ampelopsorae*, as the causal agent of crown gall of grape grapevine tumors, which was used for inoculation of plants of the same species yielding the formation of tumors (Cavara, 1897). Ten years later, Smith and Townsend (1907), reported and described *Bacterium tumefaciens* as the causal agent of crown gall disease. During the following years, the bacterium name was changed several times: *Pseudomonas tumefaciens* by Stevens in 1923 (Pulawska, 2010), *Phytomonas tumefaciens* (Bergey *et al*., 1923), *Polymonas tumefaciens* by Lieske in 1928 (Pulawska, 2010). In 1942, Conn created the genus *Agrobacterium* in which he classified some nonpathogenic species such as *Alcaligenes radiobacter*, *Phytomonas rhizogenes* (Riker *et al*., 1930) causing hairy roots, and *Polymonas tumefaciens* causing crown gall.

For several years, scientists were engaged in knowing the mechanisms of tumor formation by this bacterium when in 1970 it was confirmed that a part of the bacterial DNA called the T-DNA or transferred-DNA, is transferred to the plant genome (Chilton *et al.*, 1977). This

discovery represents a defining moment in *Agrobacterium* spp. research when scientists found in it a tool for plant transformation.

1.2.3.2 Economical importance

Crown gall can affect the growth of plants in various degrees. According to Smith *et al*. (1912), injury level can be related to factors such as the species infected, the parts attacked, and the size and the vigor of the individual.

This disease is considered the main bacterial disease of stone fruits in nurseries of the Mediterranean countries (Krimi *et al*., 2002) and one of the most important bacterial disease causing economic losses in nurseries producing fruit trees, roses and grapevines worldwide (Garrett, 1973; Kennedy and Alcorn, 1980; Sobiczewski *et al*., 1991). In addition to the direct impact of this disease on plants health, all nursery scions showing gall symptoms remain unmarketable and must be discarded. In Morocco, crown gall affects 15–20% of nursery plants and the infection reaches 80% in some regions. Similar results from Tunisia, where the infection rate reaches 30% (Boubaker, 1999) and 99% in some Algerian nurseries (Benjama *et al.,* 2002). On stone fruits, some authors reported no reliable impact of crown gall on the growth of cherry trees while according to others the disease causes stunting of peach trees and in some cases a total mortality is observed (Pulawska, 2010). Besides, Sobiczewski *et al*. (1991) reported that in water deficiency conditions, one-year-old shoots of crown galled cherries were 50% shorter and the crown diameter was 25% smaller than in healthy plants. In fact when tumors increase in size they inhibit the transportation of water and nutrients and may girdle roots and/or crown. This will lead to a reduction in plant growth and productivity with a stunted appearance (Flint, 2002). Moreover, when tumors breakdown they create wounds that are an entry point for other soil-borne pathogens and wood borer inse*cts* (Escobar and Dandekar, 2003).

1.2.3.3 The causal agent

The genus *Agrobacterium* spp. includes primarily saprophytic species that live in the soil microflora, occurring commonly in the rhizosphere (Escobar and Dandekar, 2003). Agrobacteria are gram negative, rods shape with perytrichous flagella. They are strictly aerobic, motile and do not produce any spores. They belong to the family Rhizobiaceae and have been included in the a-2 subclass of Proteobacteria on the basis of ribosomal characteristics (Kersters *et al.*, 1973; Woese, 1984). They produce hypertrophies on roots, crowns and stems of plants depending on the presence of a fragment called T-DNA present on

the Ti plasmid of the bacterium that is integrated in the genome of plant cells during the infection (Winans, 1992). The expression of those genes results in phytohormones synthesis leading to galls formation. It is important to mention that not all Agrobacteria are tumorogenic and some non tumorogenic strains were isolated from aerial and root galls (Moore, 1988).

The taxonomy of Agrobacteria was in the beginning on the basis of its pathogenicity to host plant. *A. tumefaciens* was considered to be the causal agent of crown gall whereas cane gall on raspberry was thought to be induced by *A.rubi* and *A.rhizogenes* classified as the causal agent of hairy roots symptoms and nonpathogenic strains were assigned to *A. radiobacter* (Allen and Holding, 1974). After that, there has been a disagreement over the classification and nomenclature of *Agrobacterium* and *Rhizobium* showing a lot of common characteristics (Young *et al.*, 2001; Farrand *et al.*, 2003). Keane *et al.* (1970) based on phenotypic and biochemical characteristics, suggested that the genus *Agrobacterium* spp. can be subdivided into two biovar, 1 and 2. Later on, a third biovar was described from the isolates of grapevine (Ophel and Kerr, 1990). Biovar 1 contains several strains of *A. radiobacter* and *A. tumefaciens*, biovar 2 contains many strains of *A.rhizogenes* and biovar 3 corresponds to *A. vitis* (Holmes, 1988; Ophel and Kerr, 1990). In 2003, Young and his colleagues suggested to incor*po*rate all members of the genus *Agrobacterium* into the genus *Rhizobium.* This suggestion was not accepted by Ferrand *et al.*, 2003, saying that the biovar 1 and biovar 3 of Agrobacteria are different from the genus *Rhizobium* and it is recommended to retain the genus *Agrobacterium*. They explained that the biovar 2 of Agrobacteria is more close to the genus *Rhizobium*, but they still differ in respect to their capacity to interact with plants.

1.2.3.4 Ecology and biology of the bacterium

Galls development is done through two steps after the infection by *Agrobacterium tumefaciens*: transformation and tumorigenesis (Escobar and Dandekar, 2003). Before this, the pathogen is able to detect in the soil some signal molecules, such as low molecular weight phenolic compounds (acetosyringone, hydroxy-acetosyringone) and sugar compounds released from the plant when it is wounded. Later on, the bacterium moves toward susceptible plant tissue by chemotaxis and enters using wounds colonizing the intercellular spaces (Zambryski, 1992). Directly after, the transformation of plant cells start by integration of a part of the Ti plasmid (T-DNA) into the plant genome. This transfer is controlled by virulence (*vir*) genes, located also on the Ti plasmid but not in the T-DNA region (Fig. 5). The integrated T-DNA genes will be expressed coding for auxin and cytokinin synthesis. A

secondary set of genes (6b and 5) are also integrated in this operation playing a role in modifying the effe*cts* of phytohormones in plant cells (Zhu *et al*., 2000). The expression of those 2 sets of genes will lead to an uncontrollable plant cell division and growth resulting in tumor formation just in few days after the infection.

Another set of T-DNA genes coding for opines synthesis are also present on the Ti plasmids. These derivatives of amino acids are actively produced by tumorigenic cells and they are used by *A. tumefaciens* as carbon and nitrogen sources (Bomhoff *et al*., 1976; Escobar and Dandekar, 2003; Valentine, 2003).

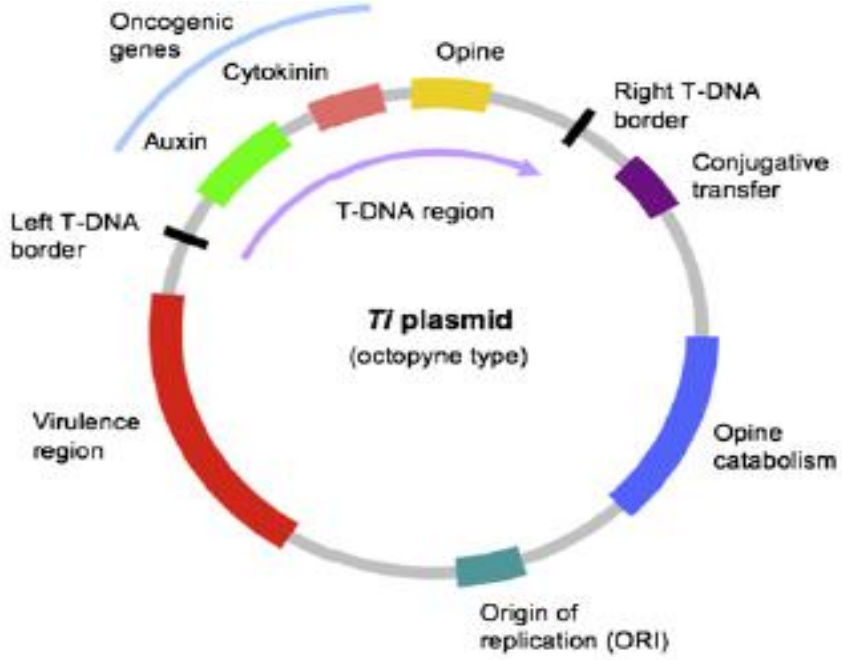


Figure 5: Ti plasmid of *Agrobacterium tumefaciens* (Pacurar *et al*., 2011)

As indicated before, Agrobacterium spp. are known to be a soil born disease, they are able to live as saprophytes in the soil by using nutrients present around. Up to now very little is known about survival of this tumorigenic bacterium in the soil, the environmental factors responsible for epidemic outbreaks and the structure of soil Agrobacteria populations (Peluso *et al.*, 2003). The difficulty exists in detecting pathogenic strains and the existence of a high level of non-pathogenic strains in soil were outbreaks occur. It happened that pathogenic strains of this bacterium have been isolated from pasture soil never cultivated (Schroth *et al.*, 1971) and from a contaminated soil left without any cultural practices for 6 years (Bouzar *et al.*, 1993). This gives us an idea about the long term survival of this pathogenic Agrobacteria in the soil. Moreover, using more modern technology of DNA sequencing, Pionnat *et al.*

(1999), concluded that pathogenic *A. tumefaciens* was transmitted on asymptomatic rootstocks of roses leading to buyer's fields contamination. In another hand, Moore and Cooksey (1981) concluded that fruit trees are generally infected from soil holding this bacterium or from ground water of galled orchards.

Seasonal fluctuation of the level of *Agrobacterium* spp. in the soil was also studied and it was demonstrated that during winter a low level of population in the soil was recorded while during the vegetative stage a high level is reached (Krimi *et al.*, 2002). Another important aspect is the possible systemic movement of this bacterium through the xylem tissue. This has been reported in some plant species where secondary tumors are produced on herbaceous and also on fruit trees species (Riker, 1923; Hill, 1928).

1.2.3.5 Symptoms

Gall symptom is considered to be specific for the infection caused by *Agrobacterium tumefaciens*. Galls start to appear soon after the infection occurs where most of them are located below the ground on the main roots or at the crown level. Young ones are usually soft and spongy, and they lack annual growth rings when are cut. They increase in size with age, become crooked and in some cases they grow to be visible above the ground. Those on the crown have more impact on the vigor of the tree while galls on the roots are generally smaller and appear to have little impact on most hosts (Epstein *et al.*, 2008). They are formed of unorganized tissues where transformed cells show a high level of differentiation. When galls become numerous, they may girdle part of big roots or trunk leading to a stunted plant with a decrease in productivity. Total death of the host plant can also occur (Flint, 2002).

1.2.3.6 Detection

Isolation of plant pathogenic Agrobacteria is usually done on selective media. Isolates suspected to belong to the genus *Agrobacterium* spp. are evaluated by physiological and biochemical tests, then a final pathogenicity test on a host plant. Aesculine utilization, urease production and 3-ketolactose production are considered primary tests for the preliminary discrimination of Agrobacteria (Moore *et al.*, 1988). Studies showed that 3-ketolactose test is highly reliable for the first separation between isolates of biovar 1 and 2 (Kersters *et al.*, 1973; Popoff *et al.*, 1981; Rid´e *et al.*, 2000). Other tests have been also used for biovar determination such as acid production from erythritol and melezitose, growth at 35 °C, growth in 5% NaCl broth, alkali production from mucic, malonic and tartaric acids, growth and pigmentation in ferric ammonium citrate broth and citrate utilization (Kerr and Panagopoulos,

1977; Janse, 2010). In a study conducted by Peluso *et al*. (2003), strains that passed 7 of the tests mentioned before are considered to belong to a given biovar and when the correspondence is lower, the strains were assigned to an intermediate biovar.

Pathogenicity test on susceptible host plant is also frequently used to evaluate the virulence of a specific isolate. This test is time consuming since symptoms may take more than 3 weeks to develop, depending on the host plant.

For this reason, other methods were developed for the detection of this pathogen. Serological tests (Bazzi *et al.*, 1987), DNA hybridization (Palleroni *et al.*, 1972) and PCR targeted to detect Ti plasmid and specifically the *vir* region in this plasmid (Ponsonnet *et al.*, 1994; Haas *et al.*, 1995) have been frequently used. Lately, an efficient nested PCR was developed targeting *pehA* which is a chromosomal gene and the *virA* gene which is on the Ti plasmid. Moreover, other techniques such as RAPD or PCR-RFLP of the 16S + ITS region showed to be able to differentiate almost all isolates of *Agrobacterium* despite the high level of diversity within this group of bacteria (Pulawska *et al.*, 2010).

1.3 Management of stone fruit bacterial diseases

Management of most fruit tree bacterial diseases is almost unattainable. This is due to the lack of effective chemical or biological control measures, lack of real host resistance, and the endophytic nature of the pathogens during some phases of the disease cycle (Kennelly *et al.*, 2007). Moreover, many bacterial species are able to survive for many years in the soil such as *A. tumefaciens* or as epiphytic on plant surface without producing any symptoms risking the dissemination of the pathogen from one place to another.

According to this, integrated strategies are needed to be implemented by combining cultural, biological and chemical control tools in order to reduce as much as possible the damage caused by bacterial diseases.

(i) **Production of certified plant material:** starting from the nurseries, efficient techniques for the detection of pathogenic bacteria must be used to produce disease free plant materials and to limit the dispersal of the pathogens. Here also, a reduction of infection level and of the epiphytic population can be achieved by protective sprays of Bordeaux mixture or copper oxychloride applied two or three times during leaf fall and subsequently at monthly intervals until bud development. Bud wood used for multiplication must be taken from disease free mother plants preferably grown in arid regions (Young, 1987). Moreover, tools

used during all the production process such as grafting, must be frequently disinfected (Rhouma *et al.,* 2005).

(ii) **Resistance**: so far, the use of disease resistant cultivars is one of the most viable options to control stone fruit bacterial disease since growers cannot support additional costs of production (Thomidis and Exadaktylou, 2008). Unfortunately, this target is not yet reached for many bacterial diseases. In the case of *Pseudomonas syringae,* the lack of accurate knowledge about the pathogenicity of different strains encountered in fruit orchards, limits the possibility to obtain precious resistant genotypes (Gilbert *et al.*, 2010; Abbasi *et al.*, 2011;). Same for crown gall, the high heterogeneity of the pathogen creates some limitation to find efficient resistant plant materials (Otten *et al.,* 2008). Regarding bacterial spot disease, resistant cultivars already exist and a breeding program is already established in North America trying to find satisfactory results (Anonymous (2006a).

(iii) **Cultural practices**: it was demonstrated that plants grafted higher than 0.5m above soil level are less susceptibility to *Pseudomonas syringae* pv. *syringae* than those grafted at the ground level (Day 1953; Duquesne *et al.*, 1974). Moreover, any factor that reduces root vigor increases the susceptibility of the tree and consequently increases the possibility of bacterial attack. Water logging, fluctuation of drought, hard pans; all are situations to be avoided (Taylor and Pohlen 1970; Duquesne *et al.*, 1974; Clothier *et al.*, 1978; Young 1987). It was also observed that bacterial canker was more dangerous in soils where pH is below 6 thus the maintenance of a pH range between 6 and 6.5 is recommended (Ritchie and Clayton, 1981; Weaver and Wehunt, 1975). Plant pathogenic nematodes have been also reported to accentuate the effect of bacterial canker, bacterial spot, and crown and root gall diseases (Nesmith and Dowler 1975). Pruning seems to be one of the most important practices that enhance bacterial infection on stone fruits. In fact, wounds resulted from this operation are an important entry point for bacteria and pruning tools are an efficient way for their dissemination. It was recommended that in orchards where bacterial diseases are reported, pruning should not be done during spring at bud swell since it is a stage of high risk of infection (Carroll *et al.*, 2010). According to the same study, the best time to prune sweet cherry in terms of limiting bacterial canker infection may be done shortly after harvest in late July. As well, a balanced fertilization of nitrogen, phosphorus, and potassium with some micronutrients, enhance the soil nutritional status which greatly reduce the severity of bacterial diseases (Renick *et al*., 2008). From the other side, the excesses of nutrients lead to the production of succulent growth that are susceptible to many bacterial diseases (English *et al.*, 1961; Melakeberhan *et al.*, 1993; Sayler and Kirkpatrick, 2003). Irrigation management is

also important to reduce the incidence of bacterial diseases. When this operation is done during late summer or autumn, it leads to the development of soft tissues vulnerable to bacterial attack especially when frost occurs. Adding that the use of overhead sprinklers irrigation has the same effect as rain in increasing the incidence and severity of bacterial diseases by carrying and spreading the pathogen (Kaluzna *et al*., 2012).

(iv) **Biological control**: a biocontrol system was discovered to be efficient in controlling *A. tumefaciens* after the isolation of a non-pathogenic strain of *Agrobacterium radiobacter* from diseased plants. It was observed the ability of this species to compete with pathogenic strains in mixed inoculations leading to a reduction of its population. Several non-pathogenic strains helped to reduce infection, but one strain in particular, *A. radiobacter* strain K84, had prevented the disease when added to wound sites with cells of *A. tumefaciens* (Farrand, 1990). For the other bacterial diseases of stone fruits, there are no yet effective biological control agents. Only few reports were published about the ability of some *Pseudomonas syringae* strains to moderately control pathogenic strains of the same species (Wilson *et al*., 2002).

(v) **Chemical control**: the only effective chemical registered to control bacterial diseases is copper. This product is frequently used to reduce the epiphytic population of *Xap*, *Pseudomonas* spp. and *Agrobacterium tumefaciens*. On stone fruits, copper has some limits because many of those species are susceptible to copper phytotoxicity during bud break in spring (Renick *et al.*, 2008). Anyhow, a reduction in bacterial canker occurrence of 67% was obtained in some studies when sprays are timed during leaf drop in autumn followed by subsequent treatments during winter (Olson and Jones, 1983; Wimalajeewa *et al.*, 1991). Unfortunately, efficient control using this product was not always achieved because many studies reported that copper applications were not enough in reducing the problem of bacterial canker. This may be due to copper tolerance in many *Pseudomonas syringae* strains or to an inappropriate timing of application (Sundin *et al.*, 1989; Scheck *et al.*, 1996).

Thesis objectives

The objectives of this thesis can be summarized in the following:

1- To determine the Sanitary status and incidence of stone fruit bacterial diseases in Lebanon.

2- To identify by means of physiological and biochemical tests, of *Pseudomnas syringae* pathovars causing bacterial canker in stone fruit Lebanese orchards and their distribution across geographical regions and host plants.

3- To characterize the identified *Pseudomonas syringae* isolates using different biological and molecular techniques.

4- To analyze the genetic diversity and profile the evolutionary relationship of the *Pseudomonas syringae* characterized isolates.

Chapter 2: Materials and Methods

2.1 Field survey and sampling

Specific survey to assess bacterial diseases of stone fruits in Lebanon was never carried out before. Only two reports treated this topic in the past, but they were incomplete and cannot be considered as basic for advanced studies in the future. In order to complement this work, survey and sampling from all stone fruit growing areas of Lebanon were carried out.

The survey started in April and ended in August 2013. During this period, almost all regions of Lebanon where stone fruits are cultivated were visited, with the exception of the North of Bekaa valley for security reasons. In the field, attention was given to find potential symptoms of bacterial origin such as cankers, leaf or fruit spots, gumming, dieback and galls. Samples were collected once every week, placed into polythene bags and conserved in a portable fridge before being transported to the laboratories of the "Lebanese Agricultural Research Institute" (LARI) where isolation and classical techniques of identification were done.

2.2 Isolation and purification

In the laboratory, samples were separated according to symptoms. Those showing disease symptoms that can be caused by *Pseudomonas* spp. or *Xanthomonas arboricola* pv. *pruni* (*Xap*), were first surface disinfected by 70% ethanol for 2-3 seconds followed by 3 rinses with sterile distilled water (SDW). The margin between necrotic and healthy areas was cut using sterile scalpel and forceps, and macerated in a sterile eppendorf tube containing 1 ml of SDW. An incubation period of 10 minutes at room temperature is necessary for the bacteria to be released in the water. Using a micropipette, 100 µl of the suspension were streaked on two different agar growth media: King's B (KB) medium (King *et al*., 1954) used mainly for *Pseudomonas* spp. containing the following ingredients (per liter): *Pseudomonas* Agar-F 35 g, Agar 3 g, Glycerol 10 ml; and Yeast Dextrose Carbonate agar (YDC) medium (Stolp and Starr, 1964) used mainly for *Xanthomonas* spp. isolation containing the following ingredients (per liter): yeast extract 10 g, dextrose or D-glucose 20 g, $CaCO_3$ 20 g, agar 15. For each sample, streaking was done consecutively on three plates using the same spreader without disinfection. This was done in order to obtain well separated colonies in the case of high bacterial concentration in the suspension (Schaad *et al*., 2001). Petri dishes were incubated at 27 ºC for 3 days and colonies growth was checked every day. From KB medium, colonies suspected to be *Pseudomonas* spp., having a white-creamy colonies and probably fluorescents

under UV light, were selected. Whereas suspected *Xap* were selected from YDC medium having typical convex, smooth, mucoid, creamy yellow colonies that turn yellow-orange with time. Purification of the isolates was done twice for each isolate on NA medium (containing per liter: Agar technical 15 g, Lab-Lemco Agar 8 g).

Same procedure of surface disinfection described before was used for gall disinfection suspected to be caused by *Agrobacterium tumefaciens*. Later on, each gall was cut into two parts and the inner water soaked areas were isolated using a scalpel and incubated for 10 min in SDW as described in the case before. The bacterial suspension were streaked on 1A medium, a semi-selective medium for *Agrobacterium tumefaciens* (containing per liter: L (-) arabitol 3.04g, NH_4NO_3 0.16g, KH_2PO_4 0.54g, K_2HPO_4 1.04g, $MgSO4.7H_2O$ 0.25g, Sodium taurocholate 0.29g, Crystal violet 2ml, Oxoid/Difco agar 15g, Actidione 1ml, $Na_2SeO_3.5H_2O$ 1ml). Typical colonies are mucoid having a purple to reddish color after 3 days of incubation. Purification was done on NA medium for two times to obtain pure colonies.

2.3 Conservation of the isolates

Isolates to be conserved were grown on NA medium for 48 hours. After checking the purity of the plates, 2 ml of 15% glycerol was placed on the agar and mixed with the colonies grown-up using a sterile pipette. The obtained suspension of each isolate was conserved in a cryovial tube at -80°C with a unique laboratory code.

2.4 Identification of the isolated bacteria

2.4.1 Physiological and biochemical tests

2.4.1.1 KOH test

KOH test was firstly performed for separation between gram positive and gram negative bacteria (Gregersen, 1978). Separation is based on the difference in cell wall structure and composition of those 2 groups. This test is easy and fast that substitutes the staining reaction test (Gram, 1884) which is more accurate but also more costly and time consuming.

To perform it a loop full of a fresh bacterial growth is mixed with a drop of 3% KOH placed on a glass slide. The formation of a thin strand of slime following the loop indicates that the bacterium is gram-negative while if a thread like is not formed indicate that the bacterium is gram positive. In the latter case, isolates were eliminated because all putative pathogenic bacteria of stone fruit are gram negative. *Pseudomonas syringae* pv. *syringae* (*Pss*) and

Rhodococcus fascians reference strains were used as gram negative and gram positive, respectively (Table 1).

2.4.1.2 Fluorescence on King's B medium

Putative *Pseudomonas* spp. isolates were first grown on KB medium for five days. The fluorescence on this medium under UV light is a characteristic known for a large number of *Pseudomonas* spp. which makes it easy to separate them from other bacteria that have similar morphological characteristics. In our study, we took into consideration that both fluorescent and non-fluorescent strains of *Pseudomonas* spp. can be found on stone fruits as it was reported in many previous studies (Menard *et al*., 2003; Vicente and Roberts, 2007). *Pseudomonas fluorescens* and *Agrobacterium tumefaciens* reference strains were used as positive and negative control respectively.

2.4.1.3 LOPAT tests

LOPAT tests are five tests able to differentiate between fluorescent plant pathogenic *Pseudomonas* spp. and divide them into 5 groups (Lelliott *et al*., 1966; González *et al*., 2003; Menard *et al*., 2003; EPPO, 2005; López *et al*., 2010). The group that gives positive results for levan production (L) and tobacco hypersensitivity (T), while negative results for oxidase reaction (O), potato soft rot (P) and production of arginine dehydrolase (A) belong to the LOPAT Ia group. This is the case of all suspected *Pseudomonas* spp. that are pathogenic to stone fruits. Suspected *Xap* isolates were also evaluated by LOPAT tests. Reference strains used are shown in (Table 1).

a) **L**evan test (Klement, 1990): bacteria were grown on 5 % sucrose nutrient agar (NAS) containing the following ingredients (per liter): Lab-Lemco broth 8 g, Agar tecnico 15 g, sucrose 50 g. After 3 days of incubation, white mucoid, dome-shaped colonies indicate a positive reaction due to the capacity of the bacterium to transform the sugar fructose into polyfructose (levan). *Pss* and *Pseudomonas syringae* pv. *papulans* were used as positive and negative control respectively.

b) **O**xidase test (Kovacs, 1956): a loop was used to pick a well-isolated colony from a fresh bacterial plate and rubbed onto a sterile filter paper soaked with a freshly prepared solution of tetramethyl 1-p phenylene-diammonium-dichloride. Oxidase positive reaction shows a blue-purple color on the paper within 10 sec indicating the presence of the enzyme cytochrome C oxydase. *P. fluorescens* was used as positive control and *Pss* as negative.

c) **P**ectolytic activity test (De Boer and Kelman, 2001): Potato tubers were cut into slices and disinfected using 70 % ethanol. Slices were putted in Petri dishes containing a sterilized filter paper moisten with SDW. Fresh bacterial colonies were placed in a hole made in the middle of the slices using a sterile scalpel. Petri dishes were incubated for 2 days at a temperature of 25 °C. Rotting of the potato slices indicate the presence of pectinase, considering the test as positive. *Pseudomonas viridiflava* was used as a positive control while *Pss* as negative.

d) **A**rginine dehydrolase test (Schaad *et al.,* 2001): A loop full of 2 days old bacterial culture was incubated in tubes of Thornley medium containing the following ingredients per liter: Peptone 1g, NaCl 5g, K2HPO4 0.3g, Agar 3g, Phenol red 1mg, arginine HCl 30g. An anaerobic condition was made by adding sterilized mineral oil on the top of each tube. Positive reaction is considered when the color of the media change from light red to pink after 4 days of incubation at 25 °C due to the secretion of the enzyme arginine dehydrolase. *P. fluorescens* was used as positive control and *Pss* as negative.

e) **T**obacco hypersensitivity reaction test (Klement *et al*., 1963): It is a specific plant test that gives an indication of the pathogenic nature of the tested bacterium. Practically all phytopathogenic bacteria that cause tissue necrosis in a susceptible host induce a hypersensitive reaction on tobacco leaves when inoculated with a highly concentrated bacterial suspension (approximately 10^8 cfu $ml^{-1}l$). To produce this reaction, a suspension of each isolate was infiltrated with a hypodermic syringe into the intercellular space of a healthy tobacco leaf (*Nicotiana tabacum* var. *avana* or var. *barley*). If the suspension contains a pathogenic bacterium, the injected tissue turns necrotic within 24 h, while in the case of non-pathogenic bacterium, some yellowing may appear after few days. *Pss* was used as positive control while SDW as negative.

2.4.1.4 GATTa tests

For the discrimination between *Pseudomonas syringae* pathovars, four tests known as GATTa tests were used (Latorre and Jones, 1979). Those tests were frequently used in many previous studies to differentiate *Pseudomonas syringae* isolated from stone fruits (Menard *et al*., 2003; Vicente *et al*., 2004; EPPO, 2005; Vicente *et al*., 2007; Gasic *et al*., 2012; Bultreys and Kaluzna, 2010). The list of reference strains used is shown in Table 1.

a) **G**elatin hydrolysis test (Frazier, 1926): The reduction of a gelatin culture medium to the liquid state occurs by the presence of the enzyme gelatinase produced by bacteria in a stab culture. The medium used contains per liter: gelatin 40 g and Lab-Lemco broth 8 g. The

liquefaction is controlled after 7 days of incubation at 27 °C. The positive reaction is seen when the medium remains liquid after a period of 30 min at 4 °C. *Pss* was used as positive control and *Psm* race 1 as negative.

b) **A**esculin hydrolysis (Sneath, 1956): A loop full of bacteria was inoculated in a tube containing aesculin medium composed of: Peptone 10 g, Aesculin 1 g, Sodium citrate ($NaH_2C_6H_5O_7$) 1 g, Ferric citrate 0.05 g and agar 15 g. After incubation for 2-5 days, the development of a dark brown color indicates the presence of β-glycosidase and the test is considered as positive. *Pss* was used as positive control and *Psm* race 1 as negative.

c) **T**yrosinase activity (Lelliott *et al*., 1966): On a specific agar plate (containing in 1L: Glycerol 5 ml, Casein hydrolyzate (Oxoid) 10 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.25 g, L-tyrosine 1 g and agar 15 g), bacteria were streaked and incubated for 2-5 days. A reddish-brown diffusible pigment indicates a positive tyrosinase activity. *P. savastanoi* pv. *savastanoi* was used as positive control and *Pss* as negative.

d) L (+) **T**artrate utilization (Ayers *et al.*, 1919): On a specific agar plate (containing in 1L: $MgSO_4.7H_2O$ 0.2 g, NaCl 5 g, $NH_4H_2PO_4$ 1 g, K_2HPO_4 1 g, Bromothymol blue 10 ml (1.5 % in ethanol), agar 15 g and sodium tartrate 2g), bacteria were streaked and incubated for 2-5 days**.** A production of a blue diffusible pigment indicates a positive test. *P. fluorescens* was used as positive control and *Pss* as negative.

Table 1: Reference strains of *Agrobacterium* spp., *Erwinia* spp., *Pseudomonas* spp. and *Rhodococcus* spp. used in this study.

Strain codes	**Strain names**	**Host**	**Isolation date**	**Source**
CFBP 42 ^{T}	*A. tumefaciens* biovar 1	*Lycopersicon esculentum*	1935	-
CFBP 5770	*Agrobacterium sp.* biovar 1	*Prunus persica*	1967	Australia
CFBP 3205 ^{T}	*P. amygdali*	*Prunus amygdalus*	1967	Greece
DPP 334	*P. fluorescens*	-	-	Italy
CFBP5062 ^{PT}	*P. savastanoi* pv. *Fraxini*	*Fraxinus excelsior*	1978	Netherlands
CFBP 1670 ^{T}	*P. savastanoi* pv. *savastanoi*	*Olea europea*	-	Yugoslavia
CFBP 6013	*P. savastanoi* pv. *savastanoi*	*Olea europea*	1984	Syria

CFBP 6574	*P. syringae*	*Prunus persicae*	1992	France
CFBP 2351 ^{T}	*P. syringae* pv. *morsprunorum*	*Prunus domestica*	1983	USA
CFBP 3801	*P. syringae* pv. *morsprunorum* race 1	*Prunus sp.*	1996	UK
CFBP 3800	*P. syringae* pv. *morsprunorum race 2*	*Prunus cerasus*	2003	UK
CFBP1573 ^{PT} *	*P. syringae* pv. *persicae*	*Prunus persica*	1974	France
CFBP 1580	*P. syringae* pv. *syringae*	*Citrus lemon*	1968	Corse
CFBP 5426	*P. syringae* pv. *syringae*	*Capsicum annuum*	1996	Macedonia
CFBP 5472	*P. syringae* pv. *syringae*	*Malus domestica*	1988	Canada
CFBP 1773	*P. syringae* pv. *syringae*	*Cotoneaster sp.*	1975	France
CFBP 1779	*P. syringae* pv. *syringae*	*Citrus sinensis*	1962	Greece
CFBP 5880	*P. syringae* pv. *syringae*	*Pyrus communis*	-	-
CFBP 1392 ^{T} = CFBP 4364	*P. syringae* pv. *syringae*	*Syringa vulgaris*	1950	UK
CFBP 2105 ^{PT}	*P. syringae* pv. *pisi*	*Pisum sativum*	1978	New Zealand
CFBP 1754 ^{T}	*P. syringae* pv. *papulans*	*Malus sylvestris*	1973	Canada
DPP 321	*P. viridiflava*	-	-	Italy
NCPPB 2551	*R. fascians*	*Lathyrus odoratus*	1958	UK

CFBP: Collection Francaise de Bacteries Phytopathogenes, France. **NCPPB**: National Collection of Plant Pathogenic Bacteria, UK. **DPP:** Collection of the University of Tuscia, Viterbo-Italy. * DNA extracted courtesy of Joel Pothier (Agroscope, Switzerland).

2.5 Pathogenicity test on immature cherry fruits

Pseudomonas syringae is a very heterogeneous group of bacteria and strains of this group can show differences in virulence when artificially inoculated in their host plant (Scortichini *et al.* 2003; Vicente *et al.* 2004; Gilbert *et al.* 2009). Moreover, pathogenic *Pseudomonas syringae* isolates cannot be distinguished from non-pathogenic ones on the basis of physiological, biochemical or serological tests (Vicente *et al*., 2004). Hypersensitive reaction of tobacco

leaves is a reliable indication of the pathogenic nature of the tested bacterium but it is not a substitute for pathogenicity test on susceptible host plants (Gašić *et al*., 2012, Latorre and Jones, 1979). Therefore, inoculation of isolates of the *Pseudomonas syringae* group should be always performed on the host of isolation to be sure of the pathogenic ability of each isolate.

In our case, the inoculation of woody tissue of stone fruit plants is time and money consuming. For this reason, pathogenicity test was done by inoculating immature cherry fruits that according to many previous studies showed to be a suitable technique giving consistent results (Gilbert *et al*., 2010; Ivanović *et al*., 2012; Kałużna and Sobiczewski, 2009).

Fresh bacterial cultures grown overnight at 25°C on KB medium were used for the preparation of the inocula. Bacterial colonies were suspended in SDW and the concentration was adjusted using a spectrophotometer in order to obtain a cell density of $10^7 – 10^8$CFU/ml (OD_{600}=0.050). Cherry fruitlets cv. Ferrovia were surface sterilized by dipping for 2 min in 2 % sodium hypochlorite and then rinsed three times with SDW (Klement, 1990; Ivanović *et al*., 2012). Using a sterile needle, one puncture was made on each fruit and a 20 µl drop of the bacterial suspension was placed on each wound. Ten replicates were made for each isolate and the negative control fruitlets were inoculated with SDW. Inoculated fruitlets were placed in Petri dishes and to provide ample humidity for disease development, a wet paper towel was included in every dish. The dishes were sealed with parafilm and incubated at room temperature (≈22°C). Results were evaluated 7 days after the inoculation by measuring the diameter of the lesion according to the following rating system: 0 = no symptoms, 1 = lesion diameter between 0.1 and 1 mm, 2 = lesion diameter between 1.1 and 2 mm, 3 = lesion diameter between 2.1 and 3 mm, 4 = lesion diameter between 3.1 and 4 mm, 5= lesion diameter over 4 mm (Xu and Gross, 1988). Subsequently, one-way ANOVA was performed and the mean values were separated using Tukey's test at $P \leq 0.05$ using 'Statistix 10'.

2.6 Molecular characterization

From this point and forward, our study was limited to the molecular characterization of the *P. syringae* isolates we have collected. Different techniques were used in order to determine the diversity within this group of bacteria present in stone fruit orchards in Lebanon. At the same time, techniques used are powerful to differentiate between pathovars of *Pseudomonas syringae*, confirming or denying the results obtained before.

2.6.1 DNA extraction

Isolates were grown for overnight in Luria Bertani broth LB (containing per liter: Tryptone 10g, yeast extract 5g and NaCl 10g) at 28 ºC with continuous shaking. The next day, the total DNA was extracted from 1ml of the liquid culture using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer instruction. The initial concentration of the total genomic DNA was checked using a spectrophotometer and then adjusted to a final concentration of 50 ng.µl^{-1}.

2.6.2 Detection of *Psyr*_1890 gene specific to *Pss*

In order to confirm the identity of the isolates classified as *Pss* by physiological and biochemical tests, specific primers to detect the type III effector HopAP1 were used. It was reported that this effector exist only on the pathovar *syringae* enabling an easy detection of this group of bacteria (Vieira *et al*., 2007).

The PCR reactions were carried out according to the protocol of Vieira *et al.* (2007) with some modifications. Primers were obtained from 'Eurofins Genomics' (Table 2) and the 25 µl of reaction mix contained 1X reaction buffer (GoTaq Flexi Buffer, Promega, USA), 2 mM $MgCl_2$, primer at 50 pmol, 0.2 mM of each dNTP, 1 U GoTaq and 50 ng of template DNA. SDW was added to reach the final volume.

PCR amplification was performed in a Thermo Cycler (BIO-RAD, PCR system C1000^{TM}) under the following conditions: an initial denaturation cycle at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 sec, annealing at 63°C for 30 sec, extension cycle at 72°C for 30 sec, a single final extension cycle at 72°C for 10 min, and final soak at 4°C.

PCR products were separated by electrophoresis on 1% TAE agarose gel containing GelRed™ Nucleic Acid Gel Stain (from BIOTIUM), run at 5 V cm^{-1} for 1 h. The external wells of the gel were loaded with GeneRuler 1 kp DNA Ladder (Thermo Fisher Scientific). Bands were visualized under UV camera and a digital image of the gel was made.

2.6.3 BOX-PCR

In our study, BOX primer (Table 2) targeting the DNA sequences of the BOXA subunit of the BOX element of *Streptococcus pneumonia* was used (Versalovic *et al.*, 1994). This technique was used frequently in many previous studies to discriminate between *Pseudomonas syringae* pathovars isolated from stone fruits and to analyze their diversity (Gilbert *et al*., 2009; Ménard *et al.*, 2003; Vicente and Roberts, 2007; Scortichini *et al*., 2003; Renick *et al*., 2008;

Bultreys and M. Kaluzna, 2010; Gašić *et al*., 2012; Ivanović *et al*., 2012). All *Pseudomonas syringae* isolates we have collected during the survey were subjected to BOX-PCR together with many reference strains from international bacteria collections. Those latter were used for comparison during the analysis of results and they are listed in Table 1.

2.6.3.1 PCR conditions and data analysis

BOX-PCR was carried out according to the protocol of Louws *et al*. (1994) with some modifications. Primers were obtained from 'Eurofins Genomics' (Table 2) and the 25 µl reaction mix contained 1X reaction buffer (GoTaq Flexi Buffer, Promega, USA), 3 mM $MgCl_2$, primer at 50 pmol, 1.25 mM of each deoxynucleoside triphosphate, 1.5 U GoTaq and 50 ng of template DNA. SDW was added to reach the final volume.

PCR amplification was performed in a Thermo Cycler (BIO-RAD, PCR system C1000^{TM}) under the following conditions: an initial denaturation cycle at 95°C for 7 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 90 sec, extension cycle at 65°C for 8 min, a single final extension cycle at 65°C for 16 min, and final soak at 4°C.

PCR products were separated by electrophoresis in 1.5% TAE agarose gels containing GelRed™ Nucleic Acid Gel Stain (BIOTIUM), and run for 8 h at 60 V (1.8 V.cm^{-1}). The external wells of the gel were loaded with 5 µl of 'GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific). Patterns were visualized under UV camera and a digital image of the gel was made. Bands shorter than 500-bp and longer than 3000bp in size were eliminated from the analysis because they were not clear for all patterns. In order to be sure of our results, PCR with same conditions was repeated two times for all the isolates. For each pattern, every amplification band was treated as a unit character and scored as absence (0) or present (1). A dendrogram with distance matrix was constructed according to the equation proposed by Nei and Li (1979) and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm using TFPGA software (version 1.3; M. Miller, Northern Arizona University, Flagstaff).

2.6.4 MultiLocus Sequence Typing (MLST)

2.6.4.1 Isolates selection, PCR condition and sequencing of the amplified gene fragments

A total of 58 representative isolates divided between 46 *Pss*, 9 *Psm*1 and 3 unclassified *Pseudomonas syringae* isolates were chosen from the collection to be analyzed by MLST. The selection of those candidates was made on the basis of their diversity in BOX-PCR, host

of isolation, region of origin, virulence on cherry fruitlets and the presence/absence of the type III effector hopAP1 (Tables 4 and 7).

MLST analysis was performed by sequencing four housekeeping genes: *cts* (also known as *glt*A) encoding citrate synthase, *gap*A encoding glyceraldehyde-3-phosphate dehydrogenase A, *rpo*D encoding RNA polymerase sigma factor 70 and *gyr*B encoding DNA gyrase B. These four genes were chosen from the seven primers used in the original paper about MLST of *Pseudomonas syringae* (Sarkar and Guttman, 2004) and later were used frequently in many similar studies (Hwang *et al*., 2005; Yan *et al*., 2008; Ferrante and Scortichini, 2010; Berge *et al*., 2014). In fact, they play a key role in carbohydrate metabolism, they consistently provide robust data, and their combined level of polymorphism is sufficient to reliably resolve evolutionary relationships (Hwang *et al*., 2005). For the amplification of a fragment of those 4 genes, primers of Sarkar and Guttman (2004) were used (Table 2). PCR reactions were carried out in a total volume of 25 µL containing 50 ng of DNA, 1.25 U of Fire *Taq* DNA polymerase (Novazym Polska), 1× reaction buffer, dNTPs at 0.1 mM each, 2 mM of $MgCl_2$ and 50 pmol of forward and reverse primers. PCR amplification was performed in a BIO-RAD thermocycler (PCR system C1000^{TM}) under the following conditions: one initial cycle at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 sec, annealing under a uniform temperature for all primers at 60°C for 30 sec, extension at 72°C for 1 min and a final extension step at 72°C for 7 min. PCR products were separated by electrophoresis using 1% TAE agarose gels containing GelRed™ Nucleic Acid Gel Stain (BIOTIUM) and specific bands were visualized and photographed under UV light. PCR products were purified and sequencing was done at STABVIDA sequencing facilities (Monte da Caparica, Portugal).

2.6.4.2 Data analysis

For MLST analysis, we followed the schema used by Berge *et al.* (2014) since a large amount of sequence data already exists which permit the comparison of our results with many others all over the world. This schema combines the Morris and the Hwang schemas of the Plant Associated and Environmental Microbes Database (PAMDB, http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl). According to Berge *et al*. (2014), 13 phylogroups and their associated clades (23 clades) form the *Pseudomonas syringae* complex. This classification takes into consideration representative strains of all collections described to date and provides a comprehensive analysis of the phenotypic differences in these phylogroups and clades relative to traits that have commonly been used to identify *Pseudomonas syringae*. In order to classify our isolates, we selected a range of strains from PAMDB and NCBI representing all

phylogroups and clades which practically represent all the 9 Genomospecies classified by Gardan *et al*. (1999) based on DNA/DNA hybridization.

For each locus, sequences were clipped to standard start and finish position, and then aligned using MEGA6 software. The sequence fragments of the 4 genes were concatenated to produce a single sequence of 1859 bp for all isolates. The concatenated sequences were used to construct a phylogenetic tree with maximum likelihood method in MEGA6 using Kimura 2-parameter model (Kimura, 1980) to infer the evolutionary relationships among *Pseudomonas syringae* strains. Confidence levels of the branching points were determined using 1,000 bootstrap replicates. The Lebanese *Pss* strains with identical concatenated sequences were represented by a single isolate in the tree.

Genetic distances among all the analyzed isolates and strains were determined with the Kimura 2-parameter model using MEGA 6 software.

In order to evaluate the capability of each of the four genes sequenced in this study to allocate correctly *Pseudomonas syringae* strains to MLST phylogroups, four phylogenetic trees were also constructed for each locus separately. Similarly to MLST, maximum likelihood method in MEGA6 software with 1,000 bootstrap replicates was used. All strains were included in this analysis in addition to the isolate C16, excluded in MLST, due to its lack of the *gap* A gene sequence.

2.6.5 Preliminary trials on the type three secretion system effectors present in the Lebanese *Pseudomonas syringae* isolates

2.6.5.1 Primers design and PCR amplification conditions:

Primers were designed using Primer-Blast in NCBI using the type three secretion system (TTSS) sequences of *Ps*. pv. *syringae* available in Genbank database and in the *Pseudomonas syringae* Genome Resources (http://www.pseudomonas-syringae.org). Two sets of primers were designed for each of the following effectors: hopX1, hopI1, hopAE1 and *avr* RPM1, and one set of primers for hopA1 and *avr* B3 (Table 2).

PCR was first conducted on only few isolates in order to check the effectiveness of the designed primers. The amplified products were sequenced and the obtained sequences were blasted in NCBI against the non-redundant nt database. According to the results, we chose 2 pairs of primer sets (AE1A and I1A in Table 2), one for the detection of hopI1 and one for the detection of hopAE1 that appeared to be specific and sensitive by amplifying the required sequences.

The 58 *P. syringae* isolates that we studied previously using MLST were all tested by PCR for the detection of hopI1 and hopAE1 effectors. The PCR reactions were carried out in a total volume of 25 µL containing 1 µL of 50 ng DNA, 1.25 U of Fire *Taq* DNA polymerase (Novazym Polska), 1× Buffer, dNTPs at 0.1 mM each, 2 mM of MgCl2 and 50 pmol of forward and reverse primers. PCR amplification was performed in a BIO-RAD thermocycler (PCR system C1000TM) under the following conditions: one initial cycle at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 sec, annealing under a uniform temperature of 54°C for 30 sec that appeared to be appropriate for both primers, extension at 72°C for 1 min and a final extension step at 72°C for 7 min. PCR products were separated by electrophoresis using 1% TAE agarose gels containing GelRed™ Nucleic Acid Gel Stain (from BIOTIUM) and specific bands were visualized and photographed under UV light. A total of 20 and 21 representative isolates, corresponding to hopAE1 and hopI1 respectively, were sequenced at STABVIDA sequencing facilities (Monte da Caparica, Portugal).

The obtained sequences were quality checkd to keep high quality base calls at both ends. The corresponding translated amino acid sequences were multiple aligned with the available public sequences from PPI website and Genbank. Those were used to construct phylogenetic trees of each of the effectors using maximum likelihood method in MEGA6 software according to Kimura 2-parameter model (Kimura, 1980). Confidence levels of the branching points were determined using 1,000 bootstrap replicates.

Table 2: Primers used in this study.

Primers names		***Tm*** **(°C)**	**Length (bp)**	**Sequence (5′→3~′)**	**Reference**
X1A	F	60	20	TCACAGCGGTCCATCAGAAC	This study
	R	59.7	20	CGGTTGAAAGCGTGAACGAA	
X1B	F	59.8	20	AAAAGGCCGTGCAATCATCG	This study
	R	60.9	20	GCTTTGGCAGCGGTTGAAAG	
AE1A	F	59.7	19	ACTTGCCAGCGAGGATCAG	This study
	R	59.8	20	GTTGTTCCTGCGTGAGATGC	
AE1B	F	60.9	19	GGCGATTCCGGATCTGCTG	This study
	R	60.1	20	CCTCGCTCAATACCAGCGAA	
I1A	F	59.9	20	GCCATATCGCTGGCAAAGTG	This study
	R	57.9	19	CGCAATTCTGGATCGGACA	
I1B	F	56	19	GCAGGTCAACAGCTCATTG	This study
	R	57	19	CGGACAAAATCCTGAACGC	

A1A	F	60.2	20	AGCCCAGGTACTCAACGAGA	This study
	R	60.8	20	AACGTGTGTTCTCGCCTGTC	
AvrB3A	F	57	20	ATGGGTTGCGTATCGTCAAA	This study
	R	59.9	22	TCCACCAATGTATCTCTGCGAG	
AvrRPM1 A	F	59.9	20	AAAGCCATGAGGAACCCAGG	This study
	R	60.3	20	CATCGGGGTCAGGGAAGTTG	
AvrRPM1 B	F	60.3	20	CGAAAGCCATGAGGAACCCA	This study
	R	60.5	20	TCATCGGGGTCAGGGAAGTT	
gyr B	F	63	24	MGGCGGYAAGTTCGATGACAAYTC	Sarkar and Guttman (2004)
	R	63	25	TRATBKCAGTCARACCTTCRCGSGC	
rpo D	Fpc	63	25	AAGGCGARATCGAAATCGCCAAGCG	Sarkar and Guttman (2004)
	Fs	53	19	AAGCGTATCGAAGAAGGCATYCGTG	
	Rpsc	63	25	GGAACWKGCGCAGGAAGTCGGCACG	
gap A	F	62	16	CGCCATYCGCAACCCG	Sarkar and Guttman (2004)
	R	62	19	CCCAYTCGTTGTCGTACCA	
cts	Fp	56	23	AGTTGATCATCGAGGGCGCWGCC	Sarkar and Guttman (2004)
	Rp	56	22	TGATCGGTTTGATCTCGCACGG	
	Fs	53	22	CCCGTCGAGCTGCCAATWCTGA	
	Rs	50	24	ATCTCGCACGGSGTRTTGAACATC	
*Psyr*_1890	F		25	TGTCAATGGGCAACCTGACCCAAGC	Vieira *et al*. (2007)
*Psyr*_1890	R		20	TGGCGTTGAGCGATCTGAGC	
BOX A1R			22	CTACGGCAAGGCGACGCTGACG	Versalovic *et al*., 1994

F: forward primer; R: reverse primer; p: PCR primer; s: sequencing primer; Tm: melting temperature

Chapter 3: Results

3.1 Orchards survey

The survey conducted is considered the first in Lebanon dedicated specifically for bacterial diseases of stone fruits. We were able to cover all the Lebanese territory with the exception of a small region in the north of Bekaa valley. In fact, 70 villages cultivating stone fruits were visited and a total of 303 samples of cherry, peach, apricot, nectarine, plum and almond were collected from 200 orchards (Table 3). The majority of those orchards showed symptoms that can be of bacterial origin. Large cankers, leaf spot, gumming, bud necrosis, dieback and totally perished trees were always found (Fig. 6). Furthermore, symptoms were more pronounced on high mountains than in other regions. In fact, the situation on mountainous regions was dramatic, especially on cherry trees that have an average lifespan of only 5 to 6 years. It was clear for the farmers that the disease is of bacterial origin but a scientific study was necessary to confirm this hypothesis and identify the causal agents. Strangely, galls were observed only twice during this survey; one in Bekaa region on a plum tree and the other one in Mont Lebanon region on a peach tree (Fig. 7).

Table 3: Regions of origin and plant species of the collected samples during the survey conducted on stone fruits in Lebanon in 2013.

Governorate	**Province**	**Peach (P)**	**Plum (PL)**	**Nectarine (N)**	**Apricot (A)**	**Almond (AL)**	**Cherry (C)**	**Total**
Mont Lebanon (ML)	Jbeil	5	7	3	8	9	3	**35**
	Keserwan	6	5	2	3	2	0	**18**
	Chouf	22	11	0	4	1	4	**42**
	Aley	8	5	3	2	0	0	**18**
	Maten	9	3	0	2	2	11	**27**
South & Nabatiyeh (SH&NB)	South	1	0	3	1	1	0	**6**
	Nabatiyeh	3	2	4	1	3	1	**14**
North Lebanon (NL)	Bchareh	2	0	2	3	1	4	**12**
	Batroun	0	0	0	1	0	0	**1**
	Doniyeh	8	7	4	7	0	0	**26**
	Koura	1	1	0	0	1	0	**3**

Bekaa (BK)	Baalbeck	3	2	0	4	3	9	**21**
	West Bekaa / Rachaya	5	6	1	5	2	3	**22**
	Zahle	4	9	10	8	5	22	**58**
	Total	**77**	**58**	**32**	**49**	**30**	**57**	**303**

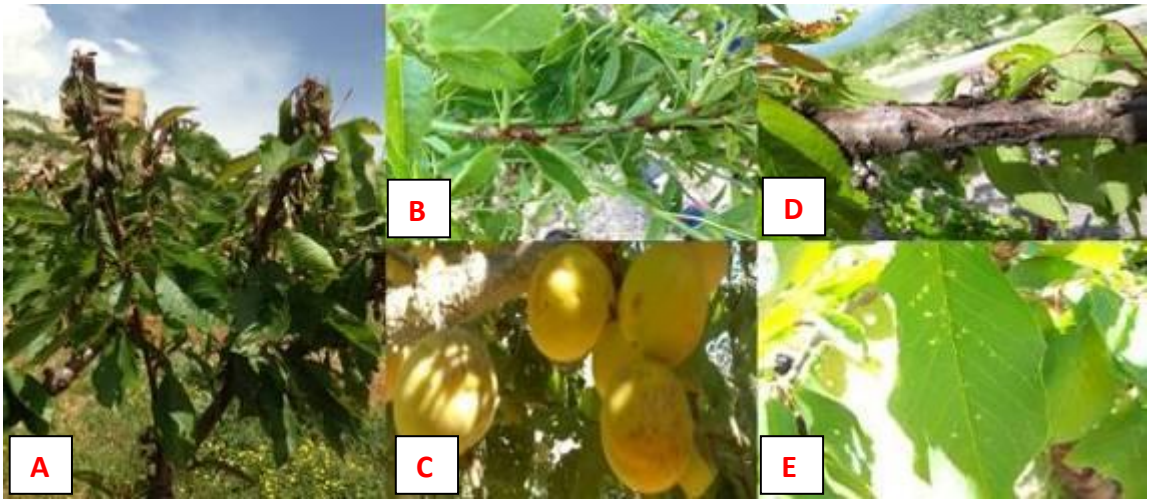


Figure 6: Symptoms of bacterial canker disease of stone fruits observed in the field during the survey.

A: dieback symptoms on cherry; B: cankers and gumming on almond twigs; C: spots on apricot fruits; D: cankers on cherry branches; E: leaf spots on cherry.

Figure 7: Crown gall symptoms observed in the field during the survey.

A: gall on plum; B: gall on peach

3.2 Strain isolation and identification

3.2.1 Physiological and biochemical tests

A total number of 326 isolates were selected from all the isolation we have made according to their morphological characteristics on each media. Most of those were putative *Pseudomonas* spp. selected from KB medium and few were suspected to be *Xap* that were selected from YDC medium. Only 2 isolates suspected to be *Agrobacterium tumefaciens* obtained from a gall were selected from 1A medium and conserved at -80 °C at the laboratory of LARI-Fanar in order to be used in future studies.

Isolates were subjected to many physiological and biochemical tests and after each test many were eliminated according to the obtained results.

a) KOH test: all the gram positive bacteria were eliminated at this step and 263 gram negative isolates of our interest were conserved (Fig. 8A).
b) Fluorescence on KB medium: suspected *Pseudomonas* spp. isolates were grown on KB medium and after observation under UV light we found that 132 of them were fluorescent. Isolates having morphological characteristics similar to *Pseudomonas* spp. but not fluorescent on KB medium were also conserved, taking into consideration the possibility that many of them may not be fluorescent.
c) LOPAT tests: results showed that 135 isolates suspected to be *Pseudomonas* spp. belonged to LOPAT Ia group. They were able to produce levan on NAS medium and to induce hypersensitivity reaction on tobacco leaves but they didn't produce cytochrome C oxydase neither pectinase and arginine dehydrolase. Accordingly, those isolates are considered to be *P. syringae* (Lelliott *et al*., 1966). From another side, none of the yellow colonies suspected to be *Xap* induced hypersensitivity reaction on tobacco leaves which means that they are not the expected bacterium (Fig. 8B, 9 and 10).

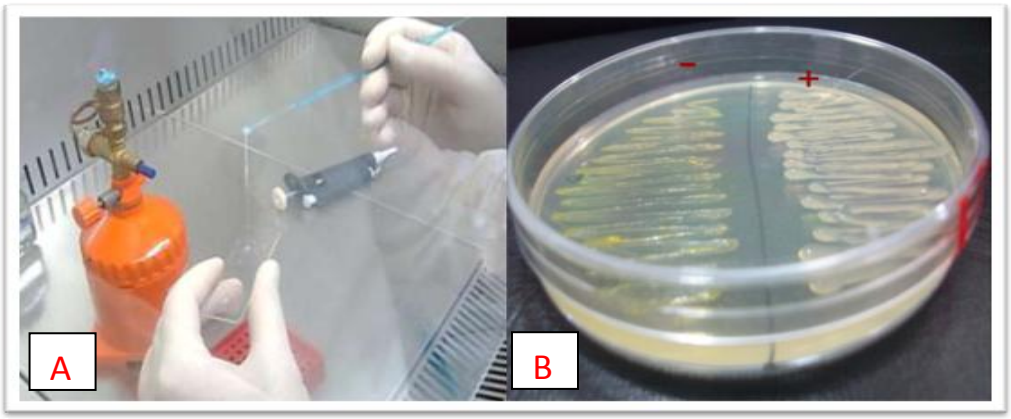


Figure 8: A: KOH test (Gram negative bacteria); B Levan production on NAS medium.

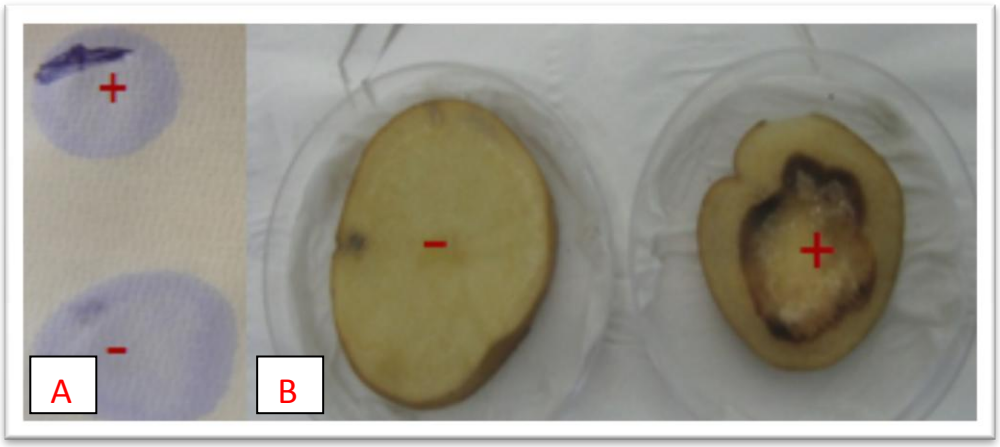


Figure 9: A: Oxidase production test; B: Potato rotting test.

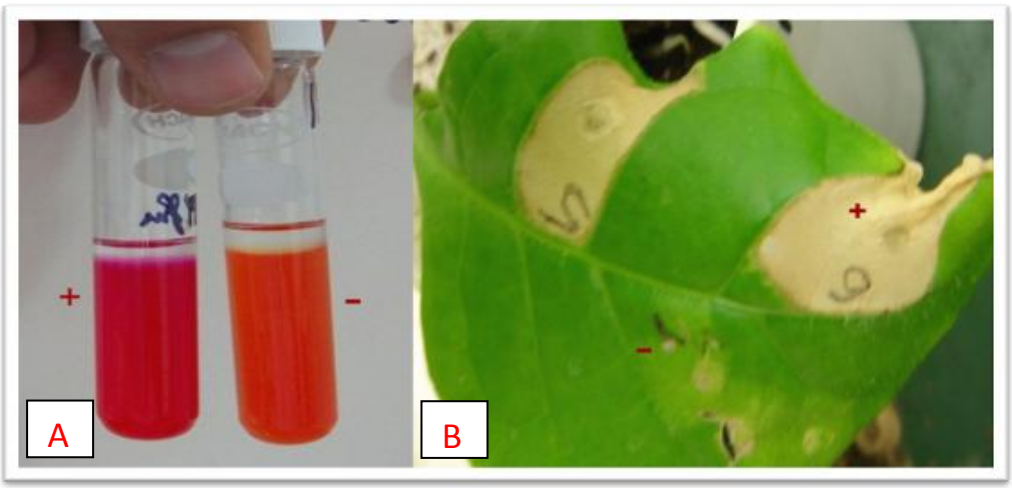
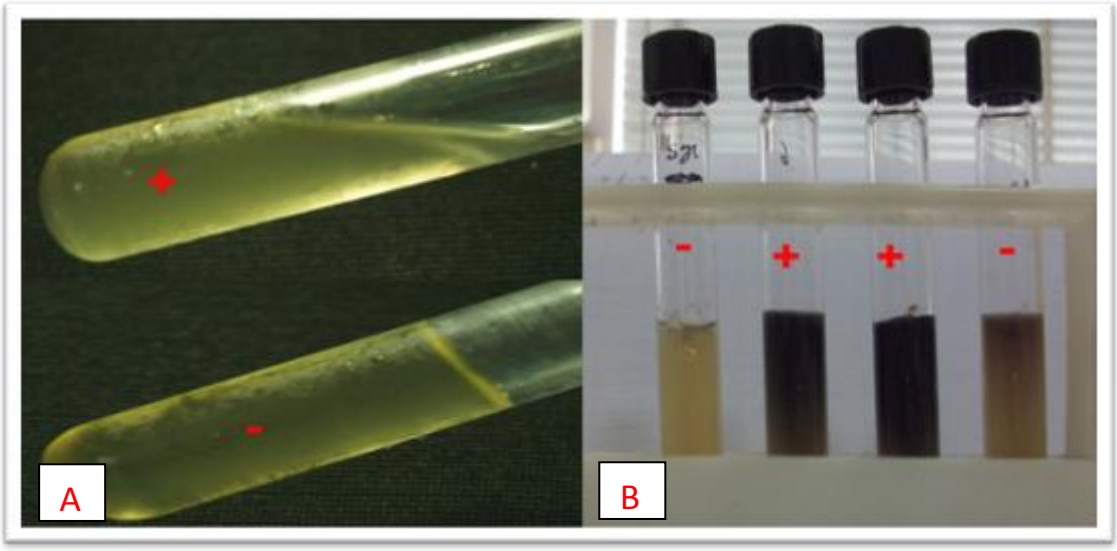


Figure 10: A: Arginine dehydrolase test; B: Tobacco hypersensitivity test.

d) GATTa tests: Results obtained for the 135 *Pseudomonas syringae* isolates divided them into 3 groups. The biggest group contains 102 fluorescent isolates positive for gelatin liquefaction and aesculin hydrolysis, but negative for tyrosinase activity and tartrate utilization (G+A+T−Ta−). Isolates of this group were considered to be *Pseudomonas syringae* pv. *syringae* (*Pss*) as many previous studies have reported (Latorre and Jones, 1979; Hinrichs-Berger, 2004; Vicente *et al*., 2004). Those were obtained from all stone fruit species sampled during our survey and from all Lebanese regions covered. The second group includes 30 fluorescent isolates that were negative for gelatin liquefaction and aesculin hydrolysis, but positive for tyrosinase activity and tartrate utilization (G-A-T+Ta+). Isolates of this group were considered as *Psm* 1 and they were obtained from apricot, plum, cherry and nectarine but never from almond and peach in this study. This keep us in doubt specially in the case of almond because according to Kaluzna *et al*. (2012) the pathovar *morsprunorum* is able to cause disease on peach and almond, while according to Lopez *et al.* (2010), *Pss* and *Pa* are the only described phytopathogenic *Pseudomonads* reported on almond trees. Regarding the case of peach, this issue seems to be due only to chance since *Psm* 1 was isolated previously in Lebanon from a peach tree (MoA, 2011-2012). Finally, the third group contains only 3 isolates that were not assigned to a specific pathovar at this level of our study. The 3 of them were not fluorescent on KB medium with diverse GATTa results. AL12 showed G+A-T-Ta- and the other two, C16 and C53, showed G+A+T−Ta−. Results of physiological and biochemical tests are shown in Table 4 (Fig. 11).



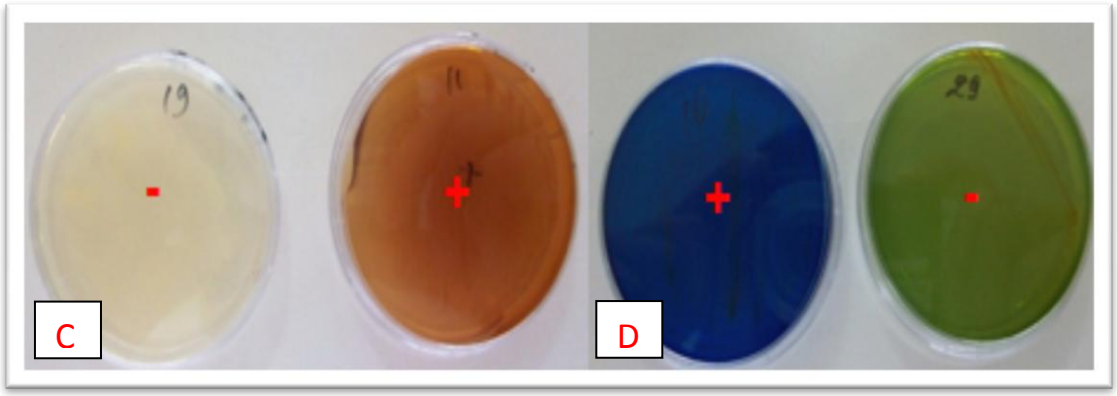


Figure 11: A: Gelatine hydrolysis test; B: Aesculin hydrolysis test; C: Tyrosinase activity; D: L(+) Tartrate test.

Table 4: Original source, host plant and biochemical characteristics of the Lebanese *Pseudomonas syringae* isolates.

Strain names	Number of isolates	Host	Governorate [a]	KB	LOPAT					GATTa				Hop AP1	*Ps* pathovars [b]
					L	O	P	A	T	G	A	T	Ta		
A3,A43,A47, A48,A51,A54	6	Apricot	ML	+	+	-	-	-	+	+	+	-	-	+	*Pss*
A22,A24,A25	3	Apricot	NL	+	+	-	-	-	+	+	+	-	-	+	*Pss*
A21,A29,A33, A37,A57,A58, A61	7	Apricot	BK	+	+	-	-	-	+	+	+	-	-	+	*Pss*
AL1,AL2,AL10, AL11,AL3,AL4, AL5,AL7,AL8	9	Almond	ML	+	+	-	-	-	+	+	+	-	-	+	*Pss*
AL13	1	Almond	SL&N	+	+	-	-	-	+	+	+	-	-	+	*Pss*
AL16,AL17	2	Almond	NL	+	+	-	-	-	+	+	+	-	-	+	*Pss*
AL18	1	Almond	BK	+	+	-	-	-	+	+	+	-	-	+	*Pss*
C1,C11,C13, C15,C18,C19, C21,C3,C4,C6,C 7,C8,C9,C42, C45	15	Cherry	ML	+	+	-	-	-	+	+	+	-	-	+	*Pss*

C23,C24,C25, C28,C29,C46, C48,C49,C50, C51,C54	11	Cherry	BK	+	+	-	-	-	+	+	+	-	-	+	*Pss*
C40,C41,C47	3	Cherry	BK	+	+	-	-	-	+	+	+	-	-	-	*Pss*
C32,C34,C37	3	Cherry	NL	+	+	-	-	-	+	+	+	-	-	+	*Pss*
														+	
N13	1	Nectarine	BK	+	+	-	-	-	+	+	+	-	-	+	*Pss*
N2,N3	2	Nectarine	ML	+	+	-	-	-	+	+	+	-	-	-	*Pss*
N5,N6,N7	3	Nectarine	NL	+	+	-	-	-	+	+	+	-	-	+	*Pss*
P1,P3,P5,P6*, P7,P9,P11,P14, P29,P33,P35, P42,P43,P44, P49	15	Peach	ML	+	+	-	-	-	+	+	+	-	-	+	*Pss*
P18	1	Peach	SL&N	+	+	-	-	-	+	+	+	-	-	-	*Pss*
P23,P24	2	Peach	NL	+	+	-	-	-	+	+	+	-	-	+	*Pss*
P40	1	Peach	ML	+	+	-	-	-	+	+	+	-	-	-	*Pss*
P50	1	Peach	BK	+	+	-	-	-	+	+	+	-	-	+	*Pss*
PL3,PL15,PL17, PL4, PL26,Pl29, PL35,PL39,PL7, PL12	10	Plum	ML	+	+	-	-	-	+	+	+	-	-	+	*Pss*
PL2	1	Plum	ML	+	+	-	-	-	+	+	+	-	-	-	*Pss*
PL24,PL25, PL41,PL42	4	Plum	BK	+	+	-	-	-	+	+	+	-	-	+	*Pss*
A10,A11,A12, A13,A14,A15 A16, A41, A50	9	Apricot	ML	+	+	-	-	-	+	-	-	+	+	-	*Psm1*
A5,A23,A62, A65	4	Apricot	NL	+	+	-	-	-	+	-	-	+	+	-	*Psm1*
C12,C31	2	Cherry	ML	+	+	-	-	-	+	-	-	+	+	-	*Psm1*
C30	1	Cherry	BK	+	+	-	-	-	+	-	-	+	+	-	*Psm1*
N12	1	Nectarine	ML	+	+	-	-	-	+	-	-	+	+	-	*Psm1*
PL1*,PL5,PL13,	12	Plum	ML	+	+	-	-	-	+	-	-	+	+	-	*Psm1*

PL10,PL11, PL37,PL40,PL8, PL9,PL27,PL28, PL36															
PL44	1	Plum	NL	+	+	-	-	-	+	-	-	+	+	-	*Psm1*
Al12	1	Almond	ML	-	+	-	-	-	+	+	-	-	-	-	*Ps*
C53	1	Cherry	BK	-	+	-	-	-	+	+	+	-	-	+	*Ps*
C16	1	Cherry	ML	-	+	-	-	-	+	+	+	-	-	-	*Ps*
Total	135														

[a]: Codes in Brackets represent the Lebanese Governorates (BK: Bekaa; NL: North Lebanon; ML: Mount Lebanon; SL & N: South Lebanon and Nabatiyeh); [b]: identification based on biochemical tests and the abbreviations mean the following: *Pss* = *Pseudomonas syringae* pv. *Syringae*; *Psm* 1= *Pseudomonas syringae* pv. *morsprunorum* race 1 and *Ps* = *Pseudomonas syringae*; KB: fluorescence on King'B medium. LOPAT (L: levan production; O: oxidase production; P: pectinolitic activity; A: arginine dihydrolase production; and T: tobacco hypersensibility); GATTa (G: gelatin hydrolysis; A: aesculin hydrolysis; T: tyrosinase activity; and Ta: utilization of tartrate).

3.3 Infection by stone fruit species and governorates

The incidence of bacterial canker disease was different between stone fruit species. Cherry, apricot, plum and almond showed a high percentage of infection with 63, 59, 48 and 47 % infected samples, respectively. Peach and nectarine showed to be less vulnerable to bacterial canker with 26 and 22% of infected samples, respectively (Fig. 12). The causal agent of this disease was different among stone fruit species with predominance of *Pss* compared to *Psm* 1. In fact, *Pss* was the only bacterium isolated from peach and almond during this survey while both *Pss* and *Psm* 1 were isolated from plum, apricot, cherry and nectarine. *Psm* 1 incidence on plum and apricot was high since it was isolated from 22 and 26 % of the collected samples, respectively. In the case of cherry and nectarine, *Psm* 1 was isolated from only few samples and *Pss* was the main causal agent of bacterial canker (Fig. 13).

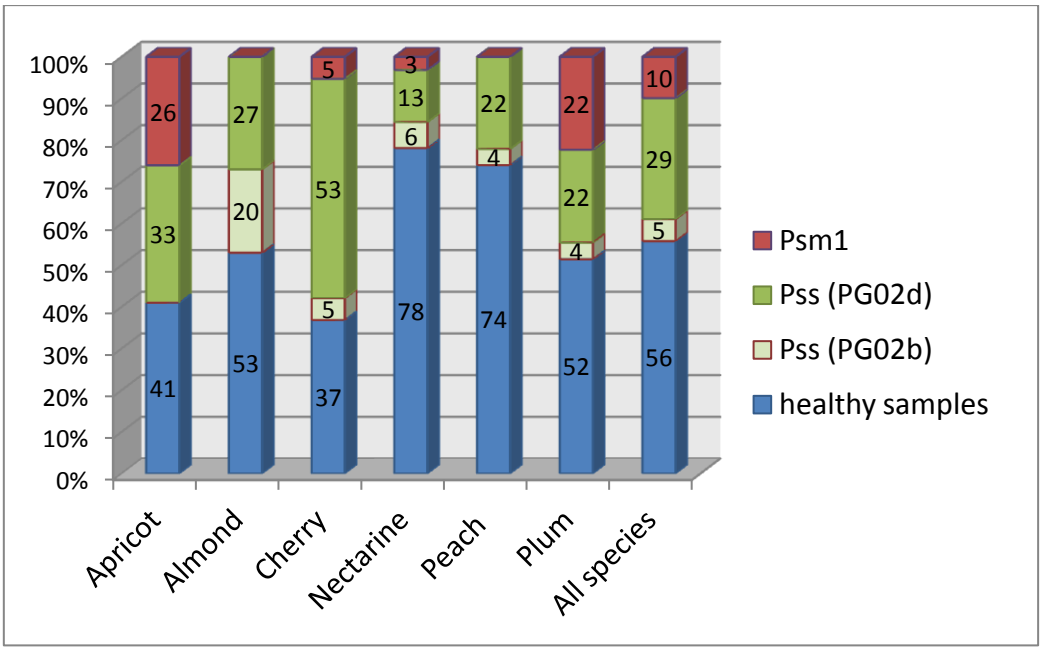


Figure 12: Incidence of *Pseudomonas syringae* pathovars and genotypes according to stone fruit species.

Pss = *Pseudomonas syringae* pv. *Syringae*; *Psm* 1= *Pseudomonas syringae* pv. *morsprunorum* race 1 and *Ps* = *Pseudomonas syringae*. PG02d: MLST phylogroup 2d, PG02b: MLST phylogroup 2b.

According to results, we found also some differences in the percentage of infected samples from one region to the other. Regardless the inequality of the number of samples collected from each governorate, the highest incidence of bacterial canker was in Mount Lebanon where 60% of the samples suffered from this disease. The infection was mainly induced by *Pss* that was isolated from 47 % of the samples while *Psm* 1 was isolated from only 17 % of them. In North Lebanon, we were able to isolate *Pss* and *Psm* 1 from 31 and 12 % of the samples, respectively, and in Bekaa valley 30 % of the samples were infected by *Pss* whereas *Psm* 1 was isolated only from one sample. Finally in South Lebanon and Nabatiyeh governorates, only 10 % of the samples suffered from bacterial canker and the unique bacterium isolated was *Pss* (Fig.13).

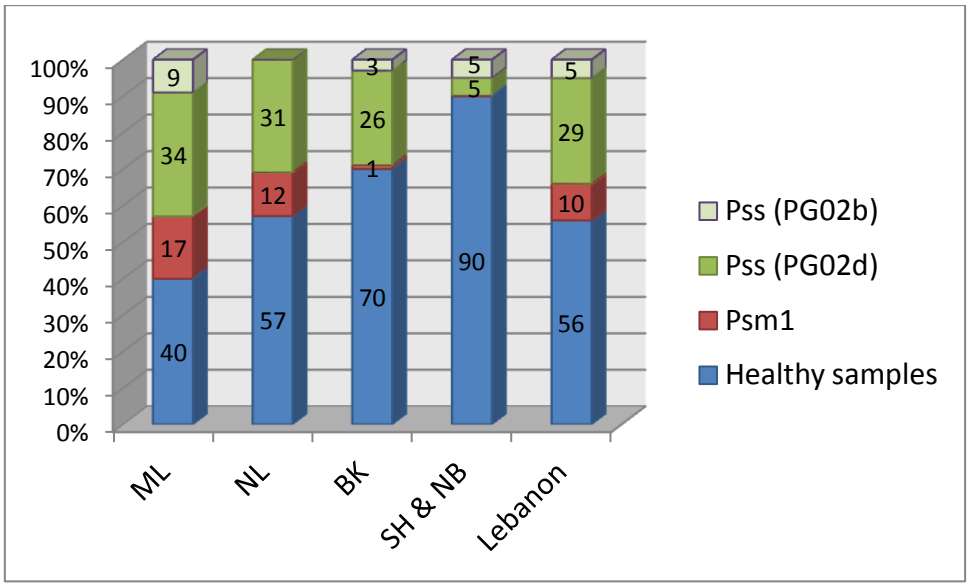


Figure 13: Incidence of *Pseudomonas syringae* pathovars and genotypes according to Lebanese governorates.

BK: Bekaa; NL: North Lebanon; ML: Mount Lebanon; SL & N: South Lebanon and Nabatiyeh. *Pss* = *Pseudomonas syringae* pv. *Syringae*; *Psm* 1= *Pseudomonas syringae* pv. *morsprunorum* race 1 and *Ps* = *Pseudomonas syringae*. PG02d: MLST phylogroup 2d, PG02b: MLST phylogroup 2b.

3.4 Pathogenicity test on immature cherry fruits

Isolates evaluated in this test were already classified previously as *Pseudomonas syringae* according to biochemical tests. All of them are considered to be pathogenic because they induced hypersensitivity reaction when bacterial suspension was infiltrated in tobacco leaves. Evaluating the pathogenicity on the host of isolation is considered to be essential to fulfill Koch postulate and it was reported that HR cannot substitute it to confirm the pathogenicity of an isolate on a specific host plant (Gašić *et al*., 2012).

The results showed that all the isolates tested were pathogenic on cherry fruits, with the exception of one *Pseudomonas syringae* isolate (C16) that was obtained from a symptomatic cherry plant. Similarly to the results obtained by Kałużna and Sobiczewski (2009), difference of symptoms between the 2 pathovars was clear. In fact, black-brown sunken necroses symptoms were obtained when fruits were inoculated with *Pss* while brownish water soaked superficial lesions symptoms when inoculated with *Psm* 1 (Fig. 14).

Figure 14: Symptoms obtained on cherry fruitlets *cv.* 'Ferrovia' inoculated with *Pseudomonas syringae.*

A: fruitlets inoculated with *Pss* isolates showing black sunken necroses; B: fruitlets inoculated with *Psm* 1 isolates showing brownish water soaked superficial lesions.

The rating data from the experiment analyzed using one-factor analysis of variance (ANOVA) in 'Statistix 10' software showed that there is significant difference of virulence between the isolates. Tuckey's test ordered the Lebanese *Pss* isolates into 9 groups according to the severity of symptoms. Those groups were not well separated and they overlap each other's (Table 5). In any case, 88 of the 102 tested isolates had a mean of grade higher than 3 and were considered as highly virulent. The remained 2 *Pseudomonas syringae* isolates showed symptoms similar to those of *Pss* and one of them was highly virulent (C53) while the other was less virulent (AL12). Another important observation is that the isolates from apricot were the most uniform and they all had means of grade >3.8 considering them as highly pathogenic.

Also for the case of *Psm* 1, all the isolates were pathogenic to immature cherry fruits and Tuckey's test showed a significant difference between the virulence of the tested isolates. To mention here that 23 from the 29 (one isolate was lost) isolates tested didn't show significant difference of virulence between each other's and the presence of bacterial exudates after 4-5 days of inoculation was very frequent in the case of this pathovar.

Table 5: Pathogenicity test performed on immature cherry fruits *cv.* Ferrovia with the Lebanese *Pseudomonas syringae* isolates

Isolate names	Species [a]	Host	Means of necrosis diameter (mm) [b]	Number of isolates
A29	*Pss*	Apricot	**4.4**[a]	1
AL16	*Pss*	Almond	**4.4**[a]	1
C8, C25, C45, C51	*Pss*	Cherry	**4.4**[a]	2
P14	*Pss*	Peach	**4.4**[a]	1
PL12, PL24, PL42	*Pss*	Plum	**4.4**[a]	3
A3, A21, A24, A33, A37, A43, A51, A58, A61	*Pss*	Apricot	**4.2**[ab]	9
AL1, AL4, , AL17	*Pss*	Almond	**4.2**[ab]	3
C1, C4, C7, C11, C24, C29, C34, C41, C42, C46, C49, C53, C54	*Pss and Ps* [c]	Cherry	**4.2**[ab]	13
N13	*Pss*	Nectarine	**4.2**[ab]	1
P1, P9, P18, P24	*Pss*	Peach	**4.2**[ab]	4
PL2, PL3, PL17	*Pss*	Plum	**4.2**[ab]	3
A22, A25, A47, A54, A57	*Pss*	Apricot	**4**[a-c]	5
C9, C15, C21, C23, C37, C48	*Pss*	Cherry	**4**[a-c]	6
N2, N3, N5, N7	*Pss*	Nectarine	**4**[a-c]	4
P23, , P29, P35, P50	*Pss*	Peach	**4**[a-c]	4
PL4, PL25, PL26, PL39, PL41	*Pss*	Plum	**4**[a-c]	5
A48	*Pss*	Apricot	**3,8**[a-d]	1
AL13	*Pss*	Almond	**3,8**[a-d]	1
C28	*Pss*	Cherry	**3,8**[a-d]	1
P7	*Pss*	Peach	**3,8**[a-d]	1
PL7, PL15, PL29	*Pss*	Plum	**3,8**[a-d]	3
C32	*Pss*	Cherry	**3,6**[a-d]	1
P3, P33	*Pss*	Peach	**3,6**[a-d]	2
N6	*Pss*	Nectarine	**3,4**[b-d]	1
AL3, AL11	*Pss*	Almond	**3,2**[c-e]	2
C13, C18, C40, C50	*Pss*	Cherry	**3,2**[c-e]	4
AL2, AL5, AL7	*Pss*	Almond	**3**[d-f]	3
C6	*Pss*	Cherry	**3**[d-f]	1
P44	*Pss*	Peach	**3**[d-f]	1
AL10	*Pss*	Almond	**2,4**[e-g]	1

C3	*Pss*	Cherry	**2,4**$^{\mathbf{e-g}}$	1
CFBP1754 ^{PT}	*Ps* pv. *papulans*	*Malus sylvestris*	**2,4**$^{\mathbf{e-g}}$	1
P11, P43	*Pss*	Peach	**2,4**$^{\mathbf{e-g}}$	2
PL38	*Pss*	Plum	**2,4**$^{\mathbf{e-g}}$	1
AL12	*Ps*	Almond	**2,2**$^{\mathbf{f-h}}$	1
P42, P49	*Pss*	Peach	**2,2**$^{\mathbf{f-h}}$	2
PL35	*Pss*	Plum	**2,2**$^{\mathbf{f-h}}$	1
AL8	*Pss*	Almond	**2**$^{\mathbf{gh}}$	1
C47	*Pss*	Cherry	**2**$^{\mathbf{gh}}$	1
AL18	*Pss*	Almond	**1,8**$^{\mathbf{gh}}$	1
P5	*Pss*	Peach	**1,8**$^{\mathbf{gh}}$	1
C19	*Pss*	Cherry	**1,6**$^{\mathbf{gh}}$	1
P40	*Pss*	Peach	**1,6**$^{\mathbf{gh}}$	1
CFBP1392 ^{T}	*Pss*	*Syringa vulgaris*	**1,4**$^{\mathbf{h}}$	1
C16	*Ps*	Cherry	**0**$^{\mathbf{i}}$	1
CFBP2105	*Ps* pv. *pisi*	*Pisum sativum*	**0**$^{\mathbf{i}}$	1
A14	*Psm* 1	Apricot	**3.2**$^{\mathbf{a}}$	1
N12	*Psm* 1	Nectarine	**3.2**$^{\mathbf{a}}$	1
PL8, PL9, PL10, PL11, PL28, PL36	*Psm* 1	Plum	**3.2**$^{\mathbf{a}}$	6
A10, A11, A23, A41	*Psm* 1	Apricot	**2.8**$^{\mathbf{ab}}$	4
PL40, PL44	*Psm* 1	Plum	**2.8**$^{\mathbf{ab}}$	2
A12, A15, A65	*Psm* 1	Apricot	**2.6**$^{\mathbf{a-c}}$	3
PL5, PL27	*Psm* 1	Plum	**2.6**$^{\mathbf{a-c}}$	2
PL13, PL37	*Psm* 1	Plum	**2.4**$^{\mathbf{a-d}}$	2
A13, A62	*Psm* 1	Apricot	**2.2**$^{\mathbf{a-d}}$	2
A5	*Psm* 1	Apricot	**2**$^{\mathbf{b-f}}$	1
A50	*Psm* 1	Apricot	**1.8**$^{\mathbf{c-f}}$	1
A16	*Psm* 1	Apricot	**1.6**$^{\mathbf{d-f}}$	1
C12	*Psm* 1	Cherry	**1.6**$^{\mathbf{d-f}}$	1
C30	*Psm* 1	Cherry	**1.4**$^{\mathbf{ef}}$	1
C31	*Psm* 1	Cherry	**1.2**$^{\mathbf{f}}$	1

[a]: The abbreviations means the bacterial species/pathovars names (*Pss*: *Pseudomonas syringae* pv. *syringae*; *Psm* 1: *Pseudomonas syringae* pv. *morsprunorum*; *Ps*: *Pseudomonas syringae*); [b]: Mean values are based on ten replications. [c] : Isolate C53. Numbers in column followed by the same letter are not significantly different using the Tukey's mean separation test at P £ 0.05. Lesion diameter was recorded after 7 days according to the following rating system: 0= no symptoms; 1 = necroses between 0.1 and 1 mm in diameter, 2 = necroses between 1.1 and 2 mm in diameter, 3 = necroses between 2.1 and 3 mm in diameter, 4 = necroses between 3.1 and 4 mm in diameter, 5= necroses over 4 mm in diameter (Xu and Gross, 1988). Results of the two pathovar were compared separately from each other.

3.5 HopAP1 detection:

From the total 102 *Pss* isolates, we were able to detect HopAP1 from 94 of them while the other 8 gave negative reaction. Moreover, 1 *Pseudomonas syringae* isolate (C53) was positive in this specific PCR test and the remaining 2 *Pseudomonas syringae* (AL12 and C16) were negative. All putative *Psm* 1 were also tested for the presence of this effector gene but they all gave negative response. According to Vieira *et al*. (2007), this effector is specific to the pathovar *syringae* and does not exist in other pathovars of the *Pseudomonas syringae* complex group. In fact, the study of Vieira *et al*. (2007) was based on the unique strain of *Pss* completely sequenced at that time (B728a) that was isolated from snap bean leaflet in Wisconsin-USA (Feil *et al*., 2005). A representative photo of the gels we obtained is shown in figure 15 and the results in table 4.

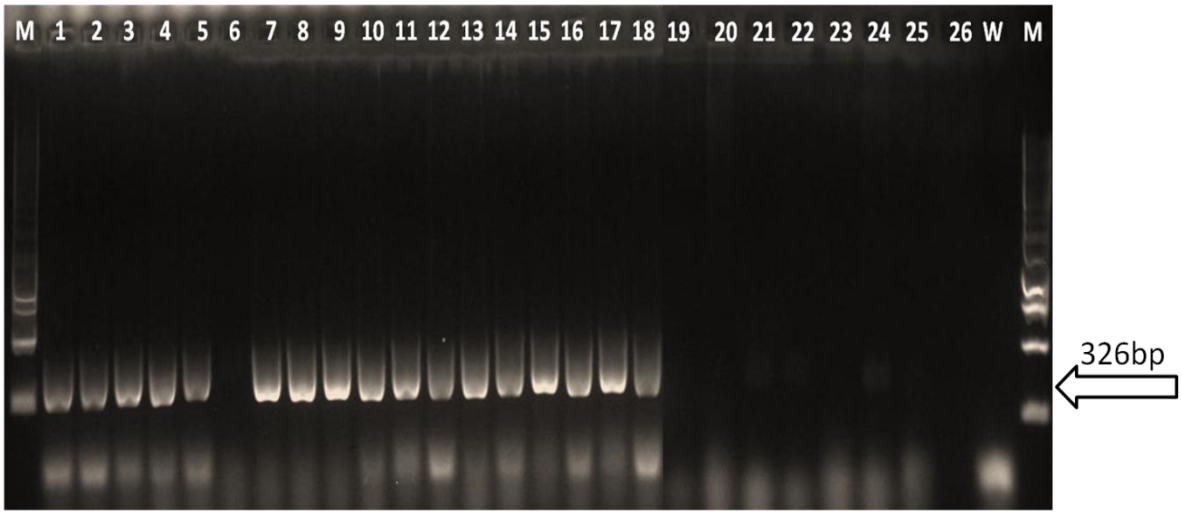


Figure 15: Representative gel for the detection of the effector hopAP1.

M: Molecular marker, Lane 1: *Pss* strain CFBP 1773, Lanes 2 till 18: Lebanese *Pss* isolates; Lanes 19 till 25: Lebanese *Psm* 1 isolates; Lane 26: *P. savastanoi* pv. *savastanoi* type strain (CFBP 1670) W: SDW; M: molecular marker (GeneRuler 1 kp DNA Ladder, Thermo Fisher Scientific).

3.6 BOX-PCR

The fingerprint patterns of the Lebanese isolates generated by BOX-PCR were complex and 30 reproducible bands were obtained ranging in size between 500 and 3000 bp. Results revealed that *P. syringae* isolated from stone fruit orchards in Lebanon show a high level of heterogeneity producing 19 different patterns. On the basis of similarity level, the patterns obtained could be grouped into three major clusters: A, B and C, as it is specified in the dendrogram in the figure 17. A photo of a gel representing some of the patterns we obtained are shown in the figure 16, and the pattern composition and grouping in table 6.

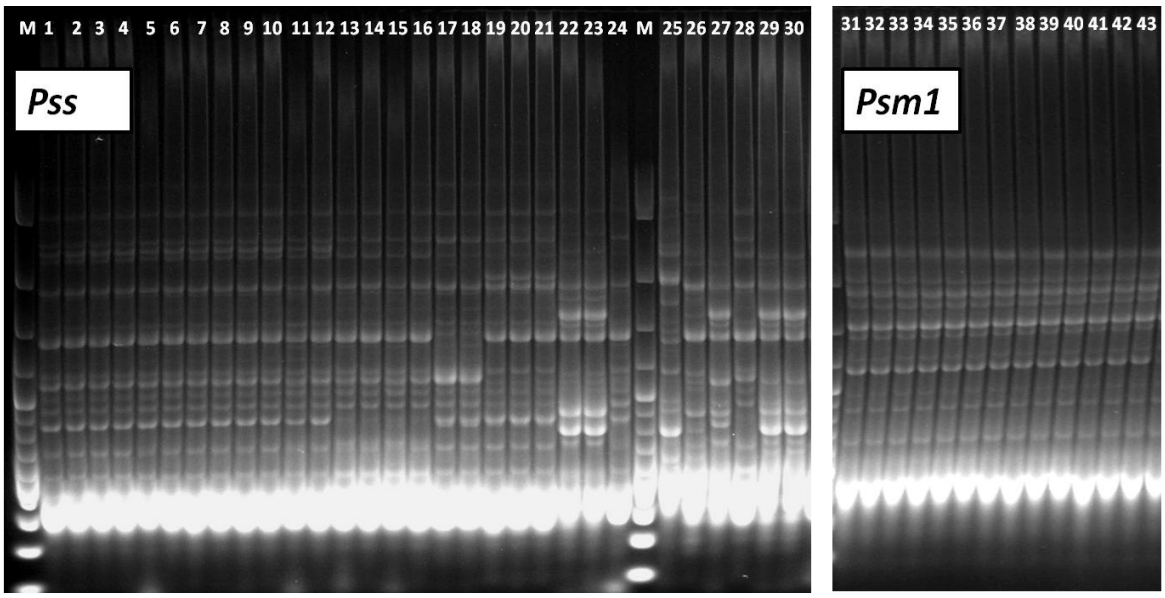


Figure 16: BOX fingerprints of the Lebanese *Pseudomonas syringae* isolates showing variability among strains of the pathovar *syringae* and homogeneity among strains of the pathovar *morsprunorum* race 1.

M: GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific); Lanes 1 to 30: Lebanese *Pss* isolates; Lanes 31 till 43: Lebanese *Psm* 1 isolates.

BOX-PCR supported phenotypic identification since this technique was able to discriminate between *Pseudomonas syringae* pathovars isolated from stone fruits. In fact, group A includes all the Lebanese *Psm* 1 isolates (30 isolates), having all exactly the same pattern (pattern 19). *Psm* 1 strain CFBP 3801 from the French collection of plant pathogenic bacteria produced also exactly the same pattern.

Group B enclose 15 isolates identified previously as *Pss* and 2 *Pseudomonas syringae* isolates. They are distributed between the patterns 5, 6, 7, 9, 10, 11 and 12 (Table 6). This group can be divided into 2 subgroups according to the similarity level. Subgroup B1 enclose

one *Pss* isolate (C47) having the pattern 12 and 1 *Pseudomonas syringae* isolate (C16) having the pattern 9. The 2 Lebanese isolates of this subgroup were both isolated from cherry trees and the isolate C16 was not fluorescent on KB medium. Subgroup B2, enclose 14 *Pss* isolates producing different patterns and one *Pseudomonas syringae* isolate (AL12) that was not fluorescent on KB medium producing the pattern 7. Four *Pss* reference strains isolated from different plant species and different countries belong also to this subgroup. Those strains included the type strain CFBP 4364^{T} that was isolated from *Syringa vulgaris* in the UK, CFBP 1779 isolated from *Citrus sinensis* in Greece, CFBP 1580 isolated from *Citrus lemon* in France and CFBP 5426 isolated from *Capsicum annuum* in Macedonia. Isolates of this subgroup share around 71 % of similarity between each other's and 42 % with the subgroup B1.

Finally, Group C include the majority of the Lebanese *Pss* isolates (87 isolates) producing the following patterns 1, 2, 3, 4, 8, 13, 14, 15, 16, 17 and 18. Fifty five of them had exactly the same pattern (1) while the patterns 8, 13, 14, 15, 16, 17 and 18 were produced by a unique isolate each (Table 6). One *Pseudomonas syringae* isolate (C53) that was not fluorescent on KB medium but having the same GATTa results as *Pss* isolates belong also to this group producing the pattern 2 exactly as many others *Pss* isolates. Moreover, many *Pss* reference strains belong to this group such as 3 French strains isolated from pear (CFBP 5880), cotton easter (CFBP 1773) and *Prunus persica* (CFBP 6574). Lebanese isolates of this group shared around 58% of similarity level with each other's and the 2 groups of *Pss* share around 40 % of similarity between each other's and 27 % with the group of *Psm* 1 (group A).

The distribution of *Pss* isolates between different patterns or groups was not related to the host plant from which they were isolated. The isolates from apricot, almond, cherry, nectarine, peach and plum were able to produce at least 4 different patterns each. The pattern 1 was the most dominant with 55 isolates, followed by the patterns 2, 4 and 5 (11, 13 and 8 isolates, respectively). Moreover, *Pss* isolates from all stone fruit species, with the exception of apricot, were distributed between the 2 groups (B and C). To keep in mind that the number of samples from each stone fruit species and the number of isolates obtained from each was not equal to enable the comparison.

Here we have to mention that the 2 *Psm* strains were analyzed in this study (CFBP 3800 and CFBP 2351^{PT}) that produced exactly the same pattern and they form a separate group in the dendrogram. This group share around 47% of similarity with the group of *Psm* 1 and 27 % of similarity with the 2 groups of *Pss*.

Table 6: Number of strains of *Pseudomonas syringae* generating 1 of 18 BOX genomic fingerprinting patterns.

	Number of strains according to BOX patterns																		
Groups	**C**										**B2**					**B1**		**A**	
Patterns / **Host**	1	2	3	4	8	13	14	15	16	17	5	6	7	10	11	9	12	19	**Total**
Apricot	9	3	2	2														13	**29**
Almond	3			4	1						5		1						**14**
Cherry	20	8		2										1	1	1	1	3	**37**
Nectarine	4											2						1	**7**
Peach	8		1	3		1	1	1	1	1	2	1							**20**
Plum	11			2							1	1						13	**28**
Total	**55**	**11**	**3**	**13**	**1**	**1**	**1**	**1**	**1**	**1**	**8**	**4**	**1**	**1**	**1**	**1**	**1**	**30**	**135**

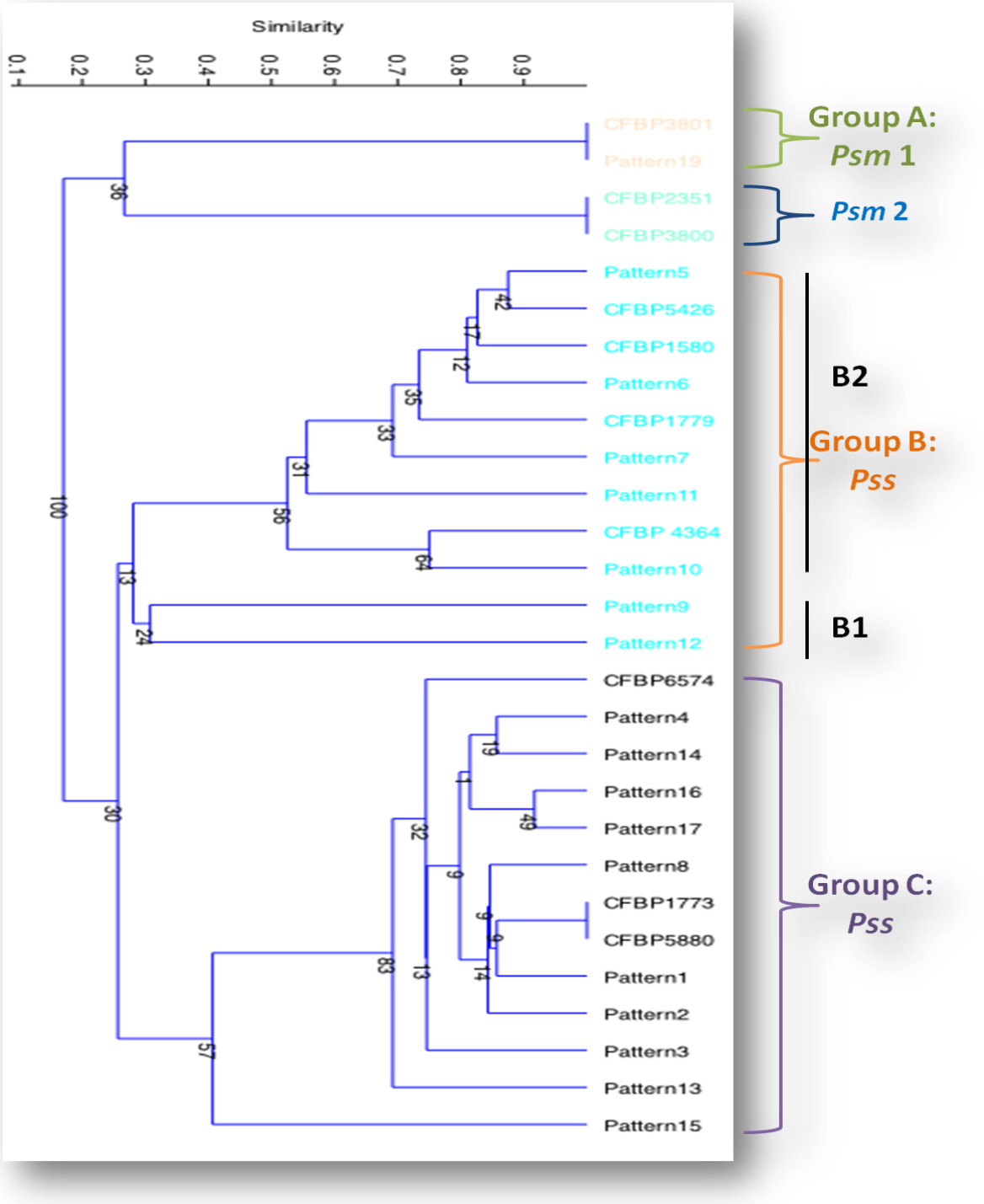


Figure 17: Dendrogram of genetic similarity of BOX fingerprint patterns generated by 135 *Pseudomonas syringae* isolates from stone fruit orchards in Lebanon.

Patterns of the Lebanese isolates are presented by 'Pattern' followed by the code number of each of them while patterns generated by reference strains are presented directly by strain names (Table 1). The similarity is the result of data set of BOX primer using Jaccard's coefficient and UPGMA algorithm using TFPGA software (version 1.3; M. Miller, Northern Arizona University, Flagstaff). The scale on the top indicates the degree of genetic relatedness between strains.

3.7 Multilocus Sequence Typing

We succeed to amplify the four loci *cts*, *gapA*, *gyrB*, and *rpoD* for 57 of the 58 representative strains of our collection that we chose to be studied by Multilocus Sequence Typing (MLST). The only exception was the strain C16, for which we were not able to amplify the g*ap* A gene fragment even if we tried many PCR reaction mixtures and amplification conditions. This isolate was excluded from the MLST analysis. In any case, all the obtained amplicons were sequenced and part of them is already deposited in PAMDB.

The sequences obtained were aligned and cut at the same site according to the schema followed by Berge *et al*. (2014). Aligned sequences for the 4 gene fragments of strains representative of all Genomospecies (Gardan *et al*., 1999) and Phylogroups (Berge *et al.*, 2014) were downloaded from NCBI and PAMDB (Annex 1). Sequences were concatenated and used to construct the phylogeny with maximum likelihood method (Fig. 18 and 19). The topology of the phylogenetic tree we obtained was similar to the one presented by Berge *et al*. (2014), confirming the reproducibility of this technique and the validity of the model used. In fact, phylogroups and clades are clearly separated and supported by high bootstrap values (Fig. 18 and 19).

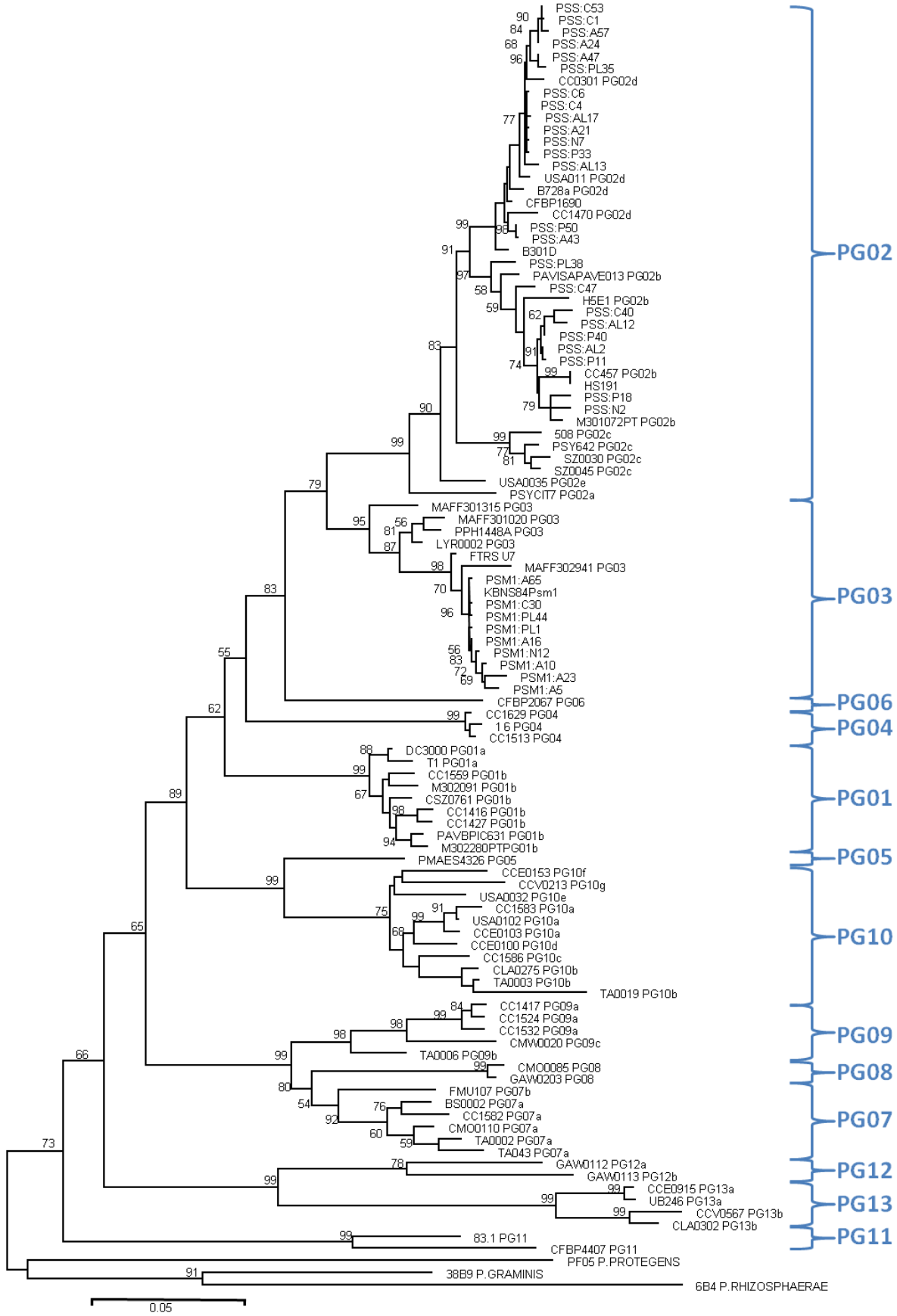


Figure 18: Phylogenetic tree constructed on concatenated sequences (*cts*, *gyr* B, *gap* A and *rpo* D) of 70 *P. syringae* strains and 58 Lebanese *Pseudomonas syringae* isolates

Bootstrap values are showed at each node; Names of the strains are indicated at tree branches and the tree was rooted on *P. protegens;* Phylogroups are specified according to the classification of Berge *et al.* (2014); The Lebanese isolates are written starting by the species (*Pss* or *Psm* 1) followed by the lab code of each isolate. Lebanese isolates having the same concatenated sequence are represented by only one of them as the following: *Pss* strain A47 represents also the strains PL15, C19, AL18 and P43; *Pss* strain C4 represents the strains A3, C23, P1, PL26, PL24, C45, A37, A51, AL1, C3, A25, C34, C50, N6, P23, P5 and PL41; *Pss* strain P50 represents also the strain A61; *Pss* strain AL2 represents also the strains PL7 and AL8; *Pss* strain C53 represents also the strain C46.

MLST of the 4 housekeeping genes divided the Lebanese *Pseudomonas syringae* isolates into 2 phylogroups (PG2 and PG3 of Berge *et al*., 2014). All the pathovar *morsprunorum* race 1 isolates analyzed in this study, appear to belong to the phylogroup 3 and showed to be genetically closely related to each other. *Psm* strain MAFF301436 (=FTRS_U7) isolated from *Prunus mume* in Japan and KBNS84 isolated from sweet cherry in Serbia belong also to this phylogroup and showed to be closely related to the Lebanese *Psm* 1 isolates. Moreover, many pathogens of woody plants are enclosed in this phylogroup such as *Pseudomonas savastanoi* pv. *savastanoi* (*e.g.* NCPPB3335), *Pseudomonas syringae* pv. *aesculi* (*e.g.* NCPPB 3681) and pv. *mori* (MAFF301020) but also many pathogens of herbaceous plants (*Pseudomonas syringae* pv. *phaseolicola* Pph1448A and pv. *lachrymans* MAFF301315). In addition, some isolates from rain such as LYR0002 and CMO0010 isolated in France, fall also in the PG03. To mention here that the *Psm* pathotype strain (MAFF302280^{PT}=CFBP2351) belong to the Phylogroup 01b.

Regarding the pathovar *syringae*, the Lebanese strains were divided into 2 closely related clades in the phylogroup 2 (clades 2b and 2d). This group is considered to be the most ubiquitous phylogroup of *Pseudomonas syringae* found in all habitats analyzed to date (Berge *et al*., 2014). The subgroup 2b enclose many *Pseudomonas syringae* pathovars including the *Pss* type strain (CFBP 1392^{T}), *Pseudomonas syringae* pv. *aptata* (CFBP1906), *Pseudomonas syringae* pv. *atrofaciens* (CFBP2256), *Pseudomonas syringae* pv. *japonica* Pathotype strain (MAFF 301072^{PT}), *Pseudomonas syringae* pv. *pisi* (H5E1) and many other strains isolated from diverse environmental substrates (*e.g.* CC457). From all the Lebanese isolates evaluated by MLST, 10 of them belong to the clade 2b and those were isolated from all stone fruit species surveyed, excluding apricot trees (Table 7).

The subgroup 2d contains the largest part of the Lebanese *Pss* isolates evaluated in this study. In fact, 37 of them were grouped in this clade and those were isolated from all stone fruit species we surveyed (Table 7). *Pss* strains B728a and B301D isolated from *Phaseolus*

vulgaris and *Pyrus communis* belong also to this subgroup, together with *Pseudomonas syringae* pv. *aceris* Pathotype strain (MAFF 302273^{PT}). In addition, many strains from environmental substrates such as snow (CC1475) and stream water (USA011) isolated from France and USA, respectively, belong to this subgroup (Annex 1).

Table 7: List of the Lebanese *Pseudomonas syringae* isolates evaluated by MLST

Isolates	Host	Governorate	BOX-PCR group	hopAP1	MLST-PG [a]	**V** [b]
A21	Apricot	BK	C	+	2d	***
A24	Apricot	NL	C	+	2d	***
A25	Apricot	NL	C	+	2d	***
A3	Apricot	ML	C	+	2d	***
A37	Apricot	BK	C	+	2d	***
A43	Apricot	ML	C	+	2d	***
A47	Apricot	ML	C	+	2d	***
A51	Apricot	ML	C	+	2d	***
A57	Apricot	BK	C	+	2d	***
A61	Apricot	BK	C	+	2d	***
AL1	Almond	ML	C	+	2d	***
AL13	Almond	SL & N	C	+	2d	***
AL17	Almond	NL	C	+	2d	***
AL18	Almond	BK	C	+	2d	*
AL2	Almond	ML	**B**	+	**2b**	**
AL8	Almond	ML	**B**	+	**2b**	*
C1	Cherry	ML	C	+	2d	***
C19	Cherry	ML	C	+	2d	*
C23	Cherry	BK	C	+	2d	***
C3	Cherry	ML	C	+	2d	**
C34	Cherry	NL	C	+	2d	***
C4	Cherry	ML	C	+	2d	***
C40	Cherry	BK	**B**	**-**	**2b**	***
C45	Cherry	ML	C	+	2d	***
C46	Cherry	BK	C	+	2d	***
C47	Cherry	BK	**B**	**-**	**2b**	*
C50	Cherry	BK	C	+	2d	***

C6	Cherry	ML	C	+	2d	**
N2	Nectarine	ML	**B**	**-**	**2b**	***
N6	Nectarine	NL	C	+	2d	***
N7	Nectarine	NL	C	+	2d	***
P1	Peach	ML	C	+	2d	***
P11	Peach	ML	**B**	+	**2b**	**
P18	Peach	SL & N	**B**	**-**	**2b**	***
P23	Peach	NL	C	+	2d	***
P33	Peach	ML	C	+	2d	***
P40	Peach	ML	**B**	**-**	**2b**	*
P43	Peach	ML	C	+	2d	**
P5	Peach	ML	C	+	2d	*
P50	Peach	BK	C	+	2d	***
PL15	Plum	ML	C	+	2d	***
PL24	Plum	BK	C	+	2d	***
PL26	Plum	ML	C	+	2d	***
PL35	Plum	ML	C	+	2d	**
PL41	Plum	BK	C	+	2d	***
PL7	Plum	ML	**B**	+	**2b**	***
AL12	Almond	ML	**B**	**-**	**2b**	**
C53	Cherry	BK	C	+	2d	***
C16	Cherry	MT	B	-	- [c]	X [d]
A10	Apricot	ML	A	-	3	**
A16	Apricot	ML	A	-	3	*
A23	Apricot	NL	A	-	3	**
A5	Apricot	NL	A	-	3	*
A65	Apricot	NL	A	-	3	**
C30	Cherry	BK	A	-	3	***
N12	Nectarine	ML	A	-	3	*
PL44	Plum	NL	A	-	3	**
PL1	Plum	ML	A	-	3	L [e]

[a]: MLST Phylogroups according to Berge *et al.* (2014); [b] : Virulence level. *: low virulent (mean of rating ≤ 2 in pathogenicity test on cherry fruitlets); **: average level of virulence (mean of rating between 2.1 and 3 in pathogenicity test on cherry fruitlets); ***: highly virulent (mean of rating > 3 in pathogenicity test on cherry fruitlets). More information about the pathogenicity test are presented in Table 5; [c]: Isolate not included in

MLST analysis because the gap1 fragment sequence is missing; [d]: Isolate did not induce disease on cherry fruitlets; [e]: Lost isolate. BK: Bekaa; NL: North Lebanon; ML: Mount Lebanon; SL & N: South Lebanon and Nabatiyeh.

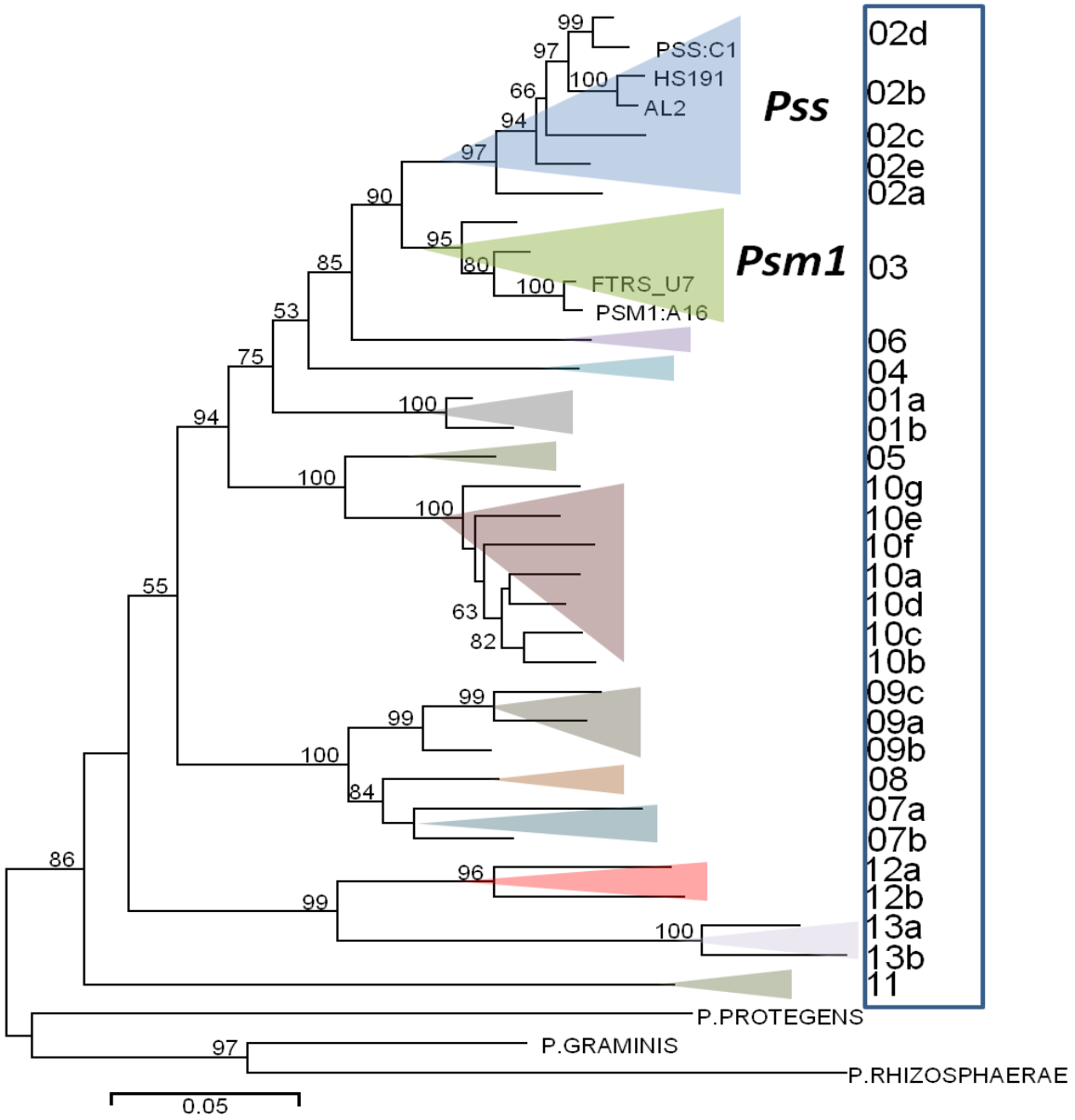


Figure 19: Contracted phylogenetic tree constructed on concatenated sequences (*cts*, *gyr* B, *gap* A and *rpo* D).

Phylogroups and clades of Berge *et al.* (2014) are specified and they are represented by a single candidate. Similarly, the Lebanese isolates are represented by a single candidate in the PG02b, PG02d and PG03. Bootstrap values are showed at each node.

The four phylogenetic trees constructed using partial sequences of each of the four housekeeping genes used in this study were also consistent supported by high bootstrap

values (Fig. 20, 21, 22 and 23). The delimitation of phylogroups by each of the four genes was similar of the one obtained by MLST with some exceptions. Anyhow, the topology of the phylogenetic tree constructed by *cts* partial sequences was the closest to the one of MLST. This gene gives an acceptable accuracy regarding the delimitation of phylogroups and clades with the best information about the evolutionary relationship between *Pseudomonas syringae* strains compared to the other three genes (Fig. 21). The position of phylogroups by the *gyr* B gene was the most deviated from the one of MLST eventhought that strains of the same phylogroup were placed together (Fig. 23). Interestingly, this gene divided the PG03 into two well separated groups. It allocate strains of the pathovar *morsprunorum* race 1 with the pathovar *miricae* in a group alone and strains of the pathovars *phaseolicola*, *mori*, *lachrymans* and *Pseudomonas syringae* strains from environmental substrates in another group.

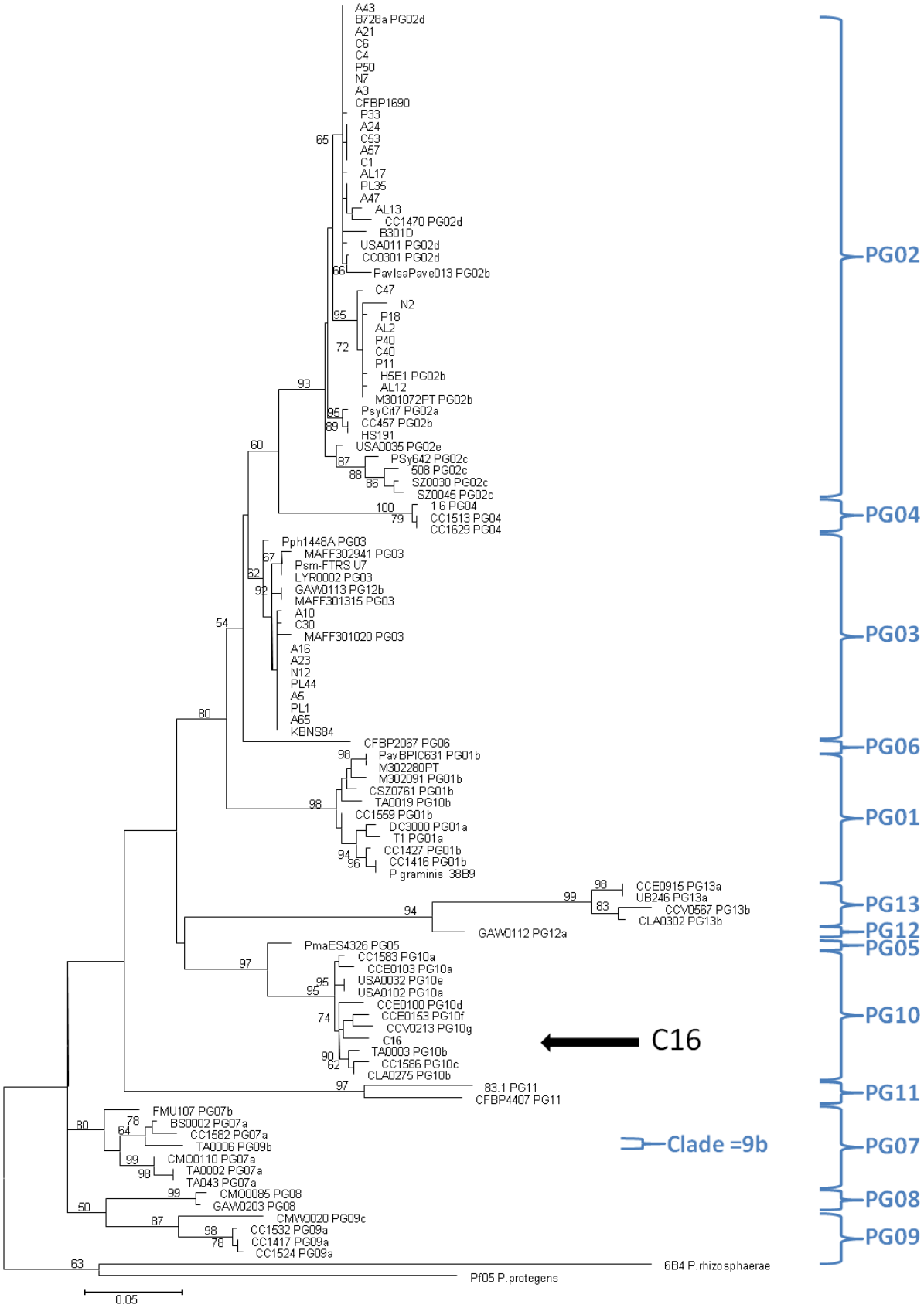


Figure 20: Phylogenetic trees constructed based on *rpo* D partial sequences of *P. syringae* strains.

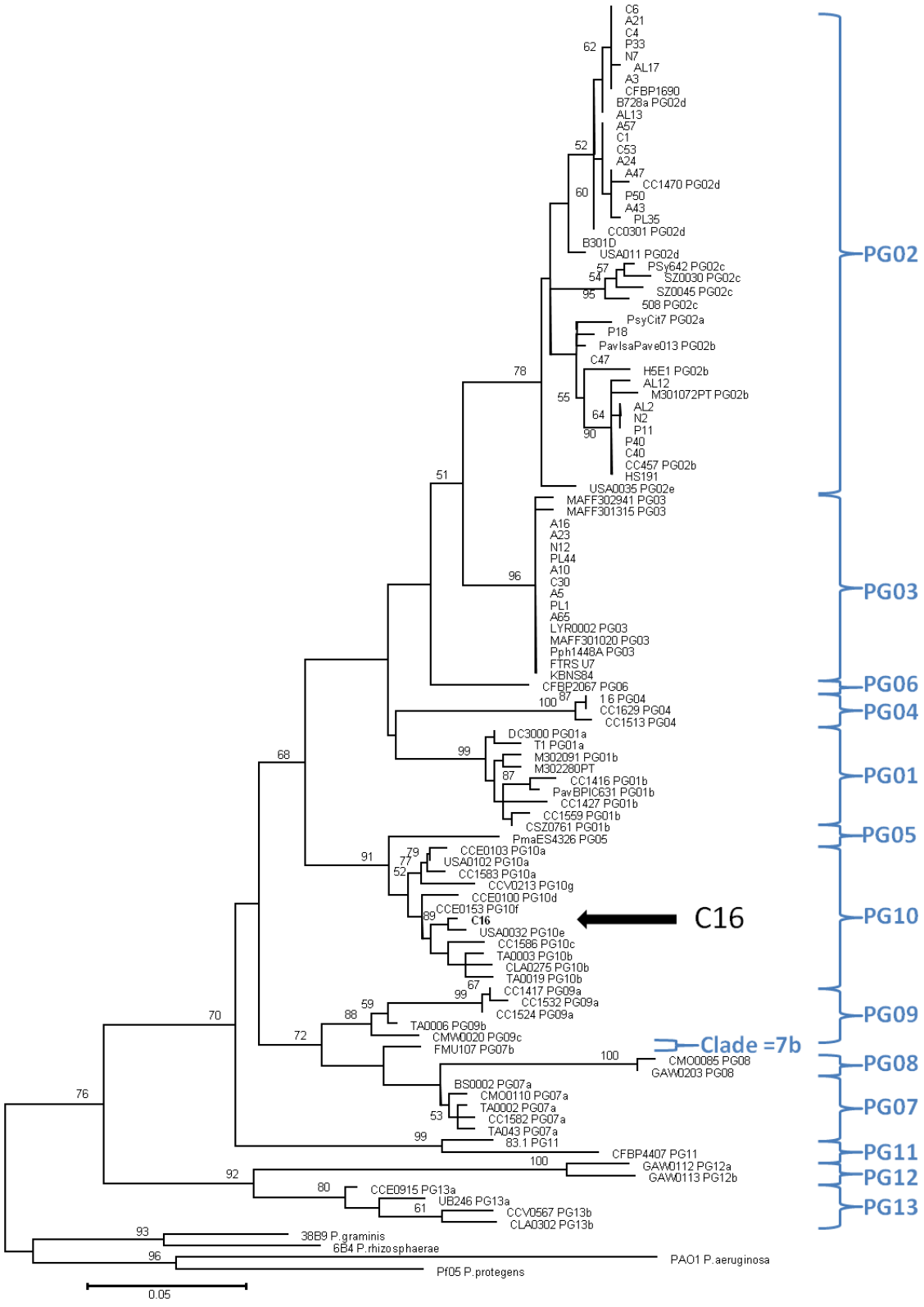


Figure 21: Phylogenetic trees constructed based on *cts* partial sequences of *P. syringae* strains**.**

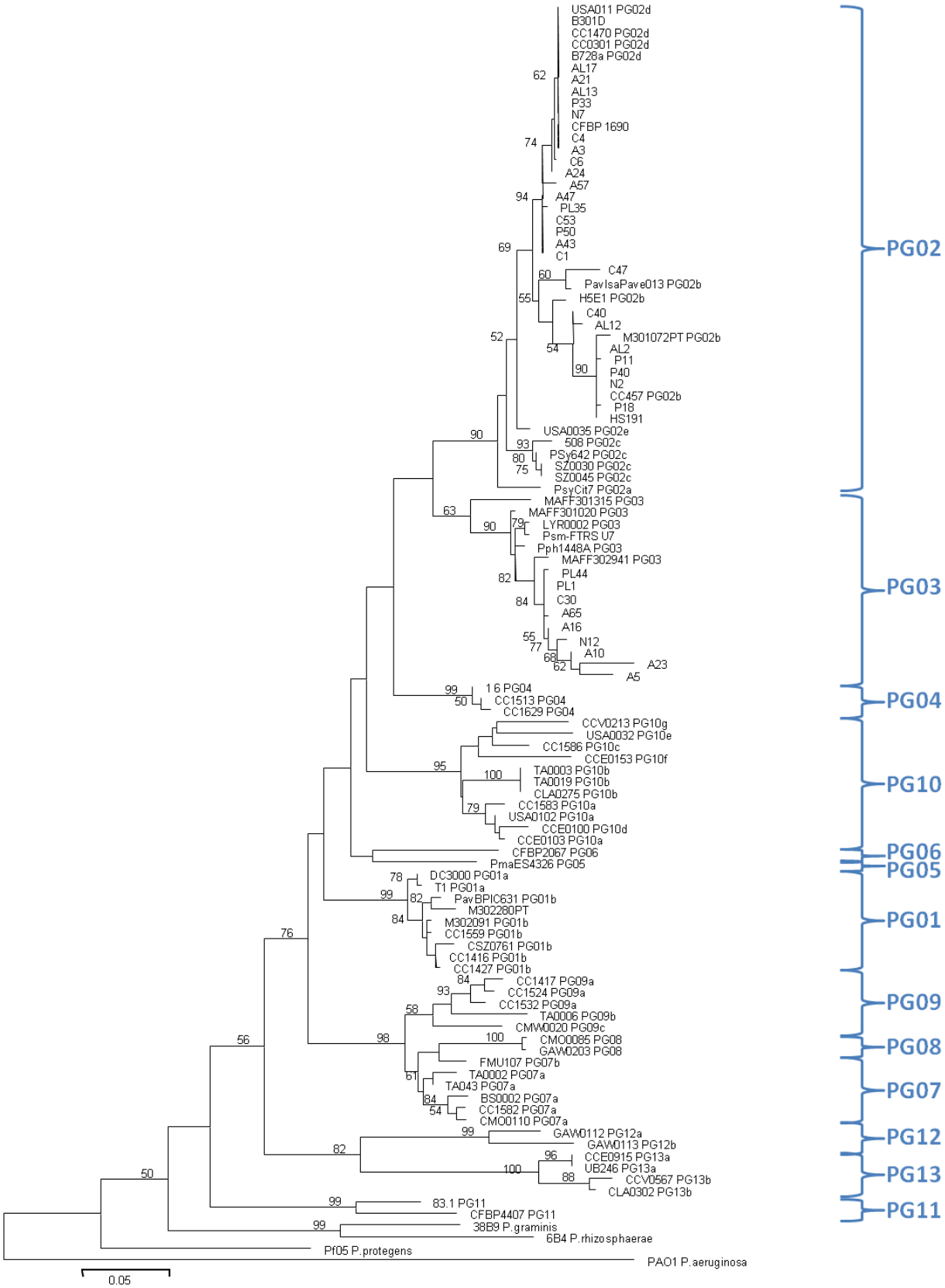


Figure 22: Phylogenetic trees constructed based on *gap* A partial sequences of *P. syringae* strains**.**

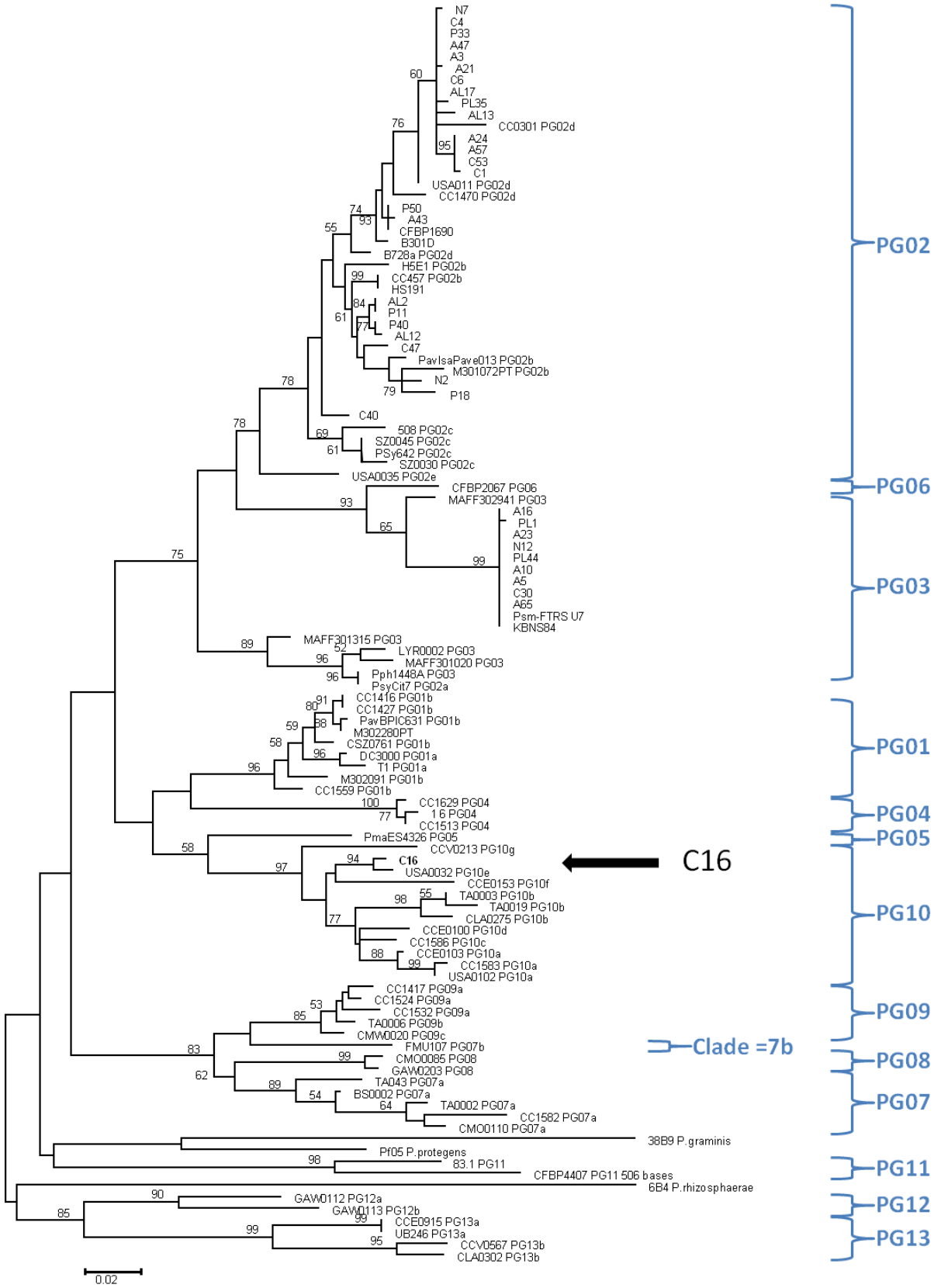


Figure 23: Phylogenetic trees constructed based on *gyr* B partial sequences of *P. syringae* strains.

Lebanese isolates used here are those included in MLST phylogeny adding the isolate C16 (C16 is missed in the phylogeny of gap A). Bootstrap values >50 are showed at each node. Name of the strains are indicated at tree branches and the tree were rooted on *P. protegens.* Phylogroups are specified according to the classification of Berge *et al*. (2014). The Lebanese isolates are named by their lab code (Table 4) and the list of strains used is presented in Annex 1.

3.8 Preliminary detection of some type three secretion system effectors:

PCR results showed that hopAE1 and hopI1 are present in all the tested isolates that belong to the pathovar *morsprunorum* race 1. Also in the case of isolates of the pathovar *syringae*, both effectors were detected in the majority of the tested isolates with a unique exception that hopAE1 was not amplified for the isolate C40. Solitary, the primers used did not amplify any of the 2 effectors for the *Pseudomonas syringae* isolate C16.

Phylogenetic trees constructed using amino acids sequences of each of the 2 effectors are presented in the figures 24 and 25. As we can see, the tree obtained with both effectors divided the Lebanese isolates into 2 groups, supported by high bootstrap values. Interestingly, those groups were equivalent to the phylogroups that we obtained previously by MLST analysis. Those 3 groups are equivalent to the phylogroups described by Berge *et al*. (2014) and therefore to the Genomospecies of Gardan *et al*. (1999). The first group encloses all strains of the pathovar *syringae* together with pathovars that belong to the PG02 (Gsp 1) such as the pathovars *aptata, lapsa, pisi, japonica, atrofaciens, coryli, solidagae* and *aceris,* the second group encloses pathovars that belong to the PG03 (Gsp 2) such as the Lebanese *Psm* 1 isolate and the pathovars *savastanoi, phaseolicola, aesculi, glycinea, mori,* and *tabaci,* and the third group encloses pathovars that belong to the PG01 (Gsp 3) such as the pathovars *tomato, maculicola* and *actinidiae*.

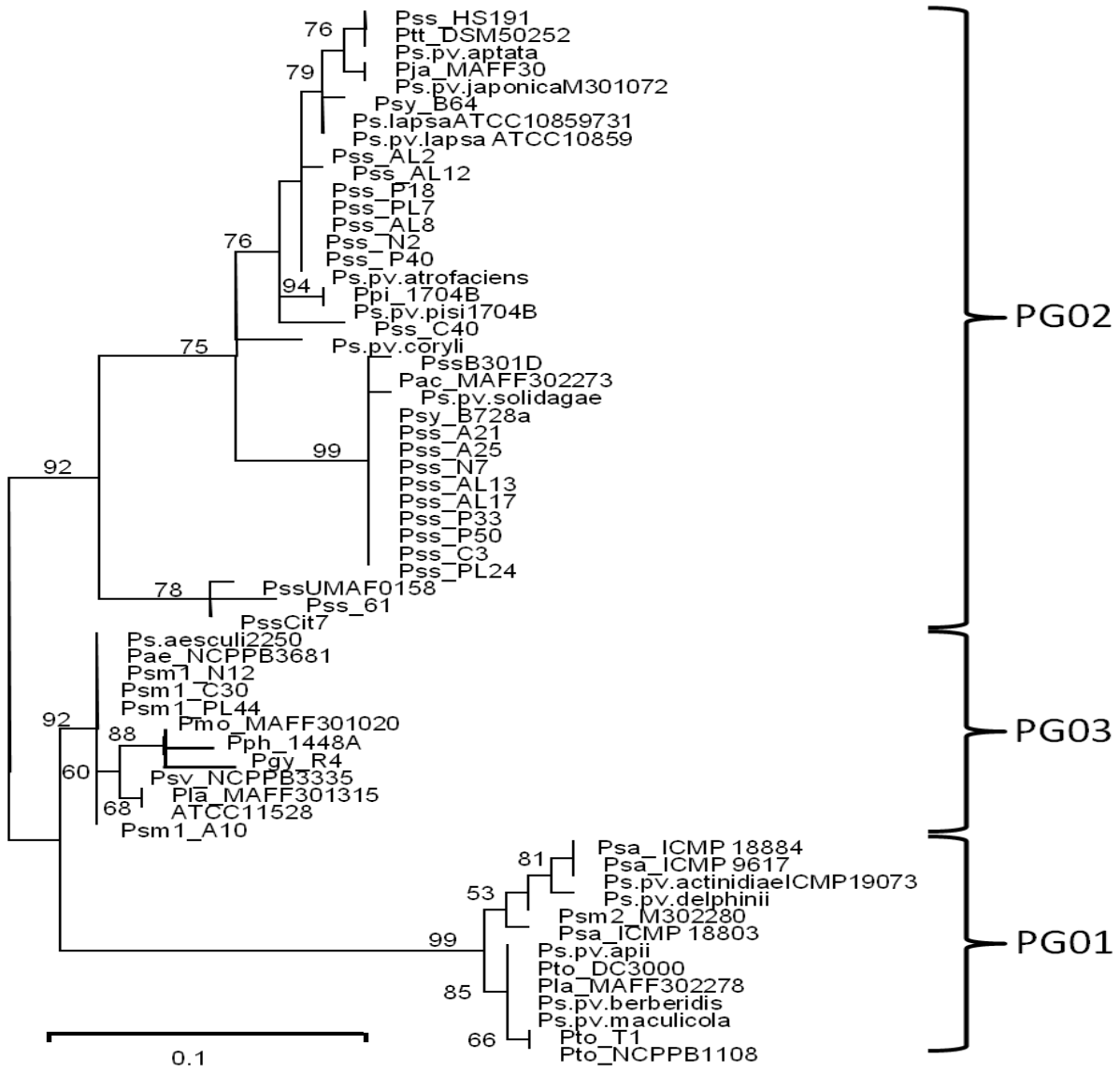


Figure 24: Maximum likelihood phylogenetic tree constructed using amino acid sequence of HopI1 effector.

Numbers above the nodes are bootstrap scores. Only bootstrap scores >50 are presented. On the right of the tree are shown the MLST phylogroups to which strains in the tree belong too. The Lebanese isolates are named by their lab code (Table 4) and the list of strains used is presented in annex 2.

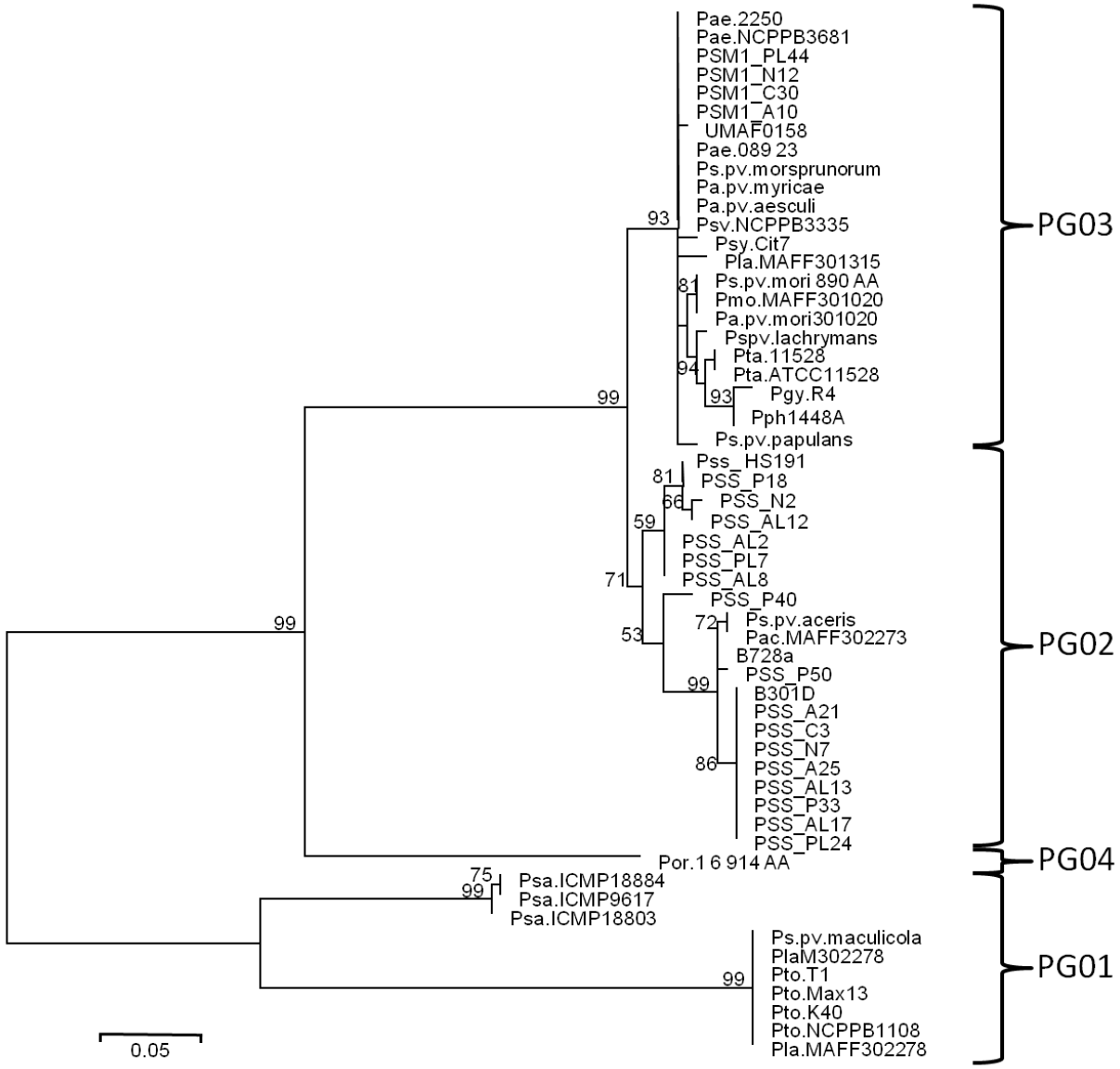


Figure 25: Maximum likelihood phylogenetic tree constructed using amino acid sequence of HopAE1 effector.

Numbers above the nodes are bootstrap scores. Only bootstrap scores >50 are presented. On the right of the tree are shown the MLST phylogroups to which strains in the tree belong to. The Lebanese isolates are named by their lab code (Table 4) and the list of strains used is presented in annex 3.

Chapter 4: Discussion

Many farmers and agronomists reported lately different disease symptoms on stone fruits in many Lebanese regions. These diseases of unknown aetiology were described to cause cankers on trunk and branches, dieback, leaf and fruit spots, excessive gumming, and in some cases total perishing of the trees. Those symptoms are not specific for any known stone fruit diseases and can be caused by a diversity of biotic and even abiotic factors. The Lebanese Ministry of Agriculture (MoA) through the Lebanese Agricultural Research Institute (LARI), conducted in 2011-2012 a survey that reported different stone fruit diseases in Lebanon. This survey did not pay enough attention on bacterial diseases that may exist on these crops and reported the presence of *Pseudomonas syringae* pv. *syringae* (*Pss*) on cherry, almond and plum, and *Psm* race 1 on peach. None of the other bacterial species that may cause diseases on these crops, including *Pseudomonas syringae* pv. *avii*, *Pseudomonas syringae* pv. *morsprunorum* race 2, *Pseudomonas syringae* pv. *persicae*, *Pseudomonas amygdali*, *Agrobacterium tumefaciens* and *Xanthomonas arboricola* pv. *pruni* (*Xap*), were isolated during this survey (MoA, 2011, 2012). Going back to 1969, Saad and Nienhaus published one of the first works about plant diseases in Lebanon including bacterial diseases of stone fruits. They reported that cankers and shot holes were common on almond trees and the causal agent was *Xanthomonas pruni* (new name *Xanthomonas arboricola* pv. *pruni*). According to them, the same bacterium was isolated from a mountainous region on peach and the symptoms were described as leaf-twig spots. Furthermore, they reported that *Pseudomonas morsprunorum* (new name *Pseudomonas syringae* pv. *morsprunorum*) was the causal agent of cankers and gummosis on cherry and peach that was isolated in Bekaa and in Mount Lebanon. *Agrobacterium tumefaciens*, the causal agent of root and crown gall, was also observed at that time on plum in Bekaa (Saad and Nienhaus, 1969). The two studies we mentioned before cannot be considered sufficient to describe the real situation regarding bacterial diseases of stone fruits in Lebanon. Thus, the number of samples in the survey conducted by the Ministry of Agriculture (MoA, 2011, 2012) was very small and the identification of the isolates was based only on physiological and biochemical tests without any confirmation of results by any other techniques. In the case of the report of Saad and Nienhaus, (1969), no information was given about the methodology followed for bacterial isolation and identification so the results remain unreliable.

According to this, there was a need to investigate the symptoms that are observed frequently in many regions of Lebanon and to verify if the disease is caused by a bacterial agent. Our

objective was also to characterize the obtained isolates using different techniques in order to determine the genetic diversity among the strains present in Lebanon. Moreover, it was important to make sure if the bacterium identified in 1969 as *Xanthomonas pruni* was correctly identified at that time and rule out the possibility of being the quarantine bacterium *Xanthomonas arboricola* pv. *pruni*.

As this study is the first scientific research conducted in Lebanon specifically adressing bacterial diseases of stone fruits, it will be considered as a preliminary analysis for more advanced studies in the future. Additionally, isolates collected and deeply characterized using different techniques can be used in any further research related to the management of bacterial diseases such as evaluating the resistance of stone fruit varieties to the local strains of those plant pathogenic bacteria.

Also at the international level, this work will help to understand better the complex group of *Pseudomonas syringae* by putting in accessibility strains and sequences of *Pseudomonas syringae* from Lebanon in international bacterial collections and Genbank.

4.1 Current status of bacterial diseases in stone fruits orchards in Lebanon

This study demonstrated that bacterial canker is the main bacterial disease of stone fruits in Lebanon. It is widespread and present on all the six commercial stone fruit species that were surveyed. The severity of symptoms seems to be related to climatic conditions in each region. Even though Lebanon is a small country, climatic conditions can vary in just few kilometers. In fact, symptoms were more pronounced in the governorate of Mount Lebanon especially in orchards present on an altitude higher than 1200 m. This is probably due to the harsh climate during winter in regions higher than this altitude taking into consideration the frequent snow storms, hail and frost periods that may occur until late spring. In many villages such as Bchareh, Sannine, Tarchich and others situated on high altitudes, farmers were forced to shift their cultivation from stone fruits to pome fruits such as apples and pears. Those regions that used to be important producers of cherry are losing their high quality production and their precious local varieties. Unfortunately, no enough effort was given to investigate the bacterial species causing bacterial canker in stone fruit orchards and subsequently make decisions that at least can limit or mitigate the problem. Farmers with their own expertise, were focusing on treating fungal diseases without paying attention to bacterial ones which need different managing approaches.

According to our survey, we found that the incidence of bacterial canker is considerably high in stone fruit orchards in Lebanon with some differences between species. Peach and nectarine appeared to be less vulnerable to bacterial canker and in the field they showed much less pronounced symptoms when compared to cherry, almond, plum and apricot. This fact may due to the tolerance of those two species to this disease or at least the tolerance of the varieties that are cultivated in Lebanon to the present strains of *Pseudomonas syringae*.

It was also clear that the predominant *Pseudomonas syringae* pathovar causing bacterial canker in Lebanon is the pathovar *syringae*. This pathovar was isolated from almost 34 % of the samples and it is present on all stone fruit species. *Psm* 1 showed to have a similar incidence as *Pss* on apricot and plum but it was never isolated from peach and almond. Our results disagree with those of the Lebanese Ministry of Agriculture (MoA, 2011-2012) which reported the isolation of *Psm* 1 from one sample of peach. According to Kaluzna *et al*. (2012), *Psm* 1 can infect peach and almond. Regarding almond, Lopez *et al.* (2010) wrote that *Pss* and *P. amygdali* are the only described phytopathogenic Pseudomonads on this tree species. In a minireview wrote by Bultreys and Kaluzna (2010) about bacterial canker of stone fruits, they indicated that *Psm* 1 is mainly pathogenic to cherry, plum and apricot; the latter agrees with our results. To consider that in the bibliographic research we have done, *Psm* 1 was never reported previously on nectarine even if artificially this pathovar is able to cause disease on immature nectarine fruits (Gasic *et al*., 2012).

The incidence of bacterial canker was also different among regions. In some governorates, such as Mount Lebanon and North Lebanon, this disease was widely spread while in others, Bekaa and the south of Lebanon, its presence was very restricted. In fact, Mount Lebanon and North Lebanon have similar geographic and climatic conditions since both belong to the western mountain range of Lebanon and stone fruits are cultivated and distributed almost in the same way. Also in Bekaa, the majority of the infected samples was obtained from the mountainous part of this governorate (*e.g.* Quaa el Rim and Hezzerta) while the healthy samples were mainly from the plan part. In the south of Lebanon (South Lebanon and Nabatiyeh governorates), the incidence of bacterial canker was the lowest and only 2 samples were infected. It is obvious that *Pseudomonas syringae* is much more harmful in mountainous regions where climatic conditions help the bacterium survival and the development of the disease while it is almost absent in softer climatic conditions such as in the plan of Bekaa and the south of Lebanon. This fact is well known since winter and spring frost, adding hail storms can dramatically favor the penetration and/or the spreading of *Pseudomonas syringae* pathovars within and among orchards by inducing wounds along the branches and the trunk

(Hinrichs-Berger, 2004; Bultreys and Kaluzna, 2010; Janse, 2010). We found also that *Pss* was spread in all Lebanese regions while *Psm* 1 seems to be present in restricted zones. In fact, *Psm* 1 was isolated only from samples collected from mountainous regions such as Mount Lebanon, North Lebanon and the mountainious part of Bekaa. This pathovar was never isolated from the plan of Bekaa or the south of Lebanon. It seems that *Psm* 1 is more adapted to climatic conditions that favored its survival and development while the pathovar *syringae* is much more widespread under diverse climatic conditions. This may due to the specificity of *Psm* 1 to stone fruits whereas *Pss*, that has a wide range of hosts and is probably able to shift to another host species when survival conditions are not suitable in stone fruit orchards.

Regarding other bacterial species pathogenic to stone fruits, our study supports the results obtained by the Lebanese Ministry of Agriculture in 2011-2012, confirming that only *Pss* and *Psm* 1 are present on stone fruits in Lebanon. Both studies disagree with the report of Saad and Nienhaus (1969) since we did not isolate the quarantine bacterium *Xap*. Probably the identification of *Xap* at that time was not supported by molecular tools for accurate pathogen identification and characterization. Gall symptoms suspected to be caused by *Agrobacterium tumefaciens* were also observed during this study but this disease appeared to be of minor importance on stone fruits.

4.2 Molecular characterization of *Pseudomonas syringae* isolated from stone fruits in Lebanon

Pseudomonas spp. is one of the most complex genus of gram-negative bacteria comprising 144 species (Gomila *et al*., 2015) where *Pseudomonas syringae* alone is divided into at least 60 pathovars (Young *et al*., 2010). Strains of this species are able to cause disease on more than one hundred host plants and they were also isolated from diverse environmental substrates (Berge *et al.*, 2014).

Despite that *Pseudomonas syringae* is one of the most studied species of bacteria, it still an import challenge to assign correctly strains of this group to the correct pathovar. Many papers have dealt with different methods of genetic characterization and clustering of strains of this group of plant pathogenic bacteria by using different molecular techniques such as DNA/DNA hybridization (Gardan *et al*., 1999), rep-PCR and MLST.

Regarding pathovars of *Pseudomonas syringae* causing bacterial canker of stone fruits, rep-PCR can be considered as one of the most used molecular tool to identify and analyze the diversity that exist between strains of this species. According to many previous studies, this technique using BOX, REP or ERIC primers is able to identify strains of the 2 races of the pathovar *morsprunorum* since isolates of both of them are known to be very homogeneous. Differently, strains of the pathovar *syringae* are known to be very heterogeneous and this technique alone is not able to give a clear-cut response about the identity of such isolates. However, it is still very useful as an additional tool for identification and an efficient method for analyzing the genetic diversity of such complex group of bacteria (Ménard *et al.,* 2003; Vicente and Roberts, 2007; Renick *et al*., 2008; Gilbert *et al*., 2009; Bultreys and Kaluzna, 2010; Gašić *et al*., 2012; Ivanović *et al*., 2012).

Nowadays, more advanced molecular techniques based on sequencing of specific locus in the genome of bacteria are more frequently used. Among these, Multilocus sequence typing (MLST) is one of the most preferred to identify and classify *Pseudomonas syringae* strains and to unravel population genetics and molecular evolution (Sarkar and Guttman, 2004; Berge *et al*., 2014; Kaluzna *et al*., 2010; Hall *et al*., 2015; Słomnicka *et al*., 2015). A precious advantage of this technique is that it gives us the possibility to analyze the phylogenetic relationship among a large set of strains much better than any previous genotyping technique such as DNA/DNA hybridization, AFLP, or rep-PCR. Sequences of new strains can be compared to others coming from all over the world and isolated from different hosts or substrates. Moreover, MLST can accurately allocate new pathogens directly to *Pseudomonas syringae sensu lato* Genomospecies and describe diversity within each pathovar (Bull *et al.,* 2011).

The work conducted by Berge *et al*. (2014) performing MLST on 216 isolates through sequencing fragments of four housekeeping genes (*cts*, *gap* A, *rpo* D, *gyr* B), demonstrated that the *Pseudomonas syringae* complex is divided into 23 clades within 13 phylogroups. The robustness of the MLST-phylogroups was confirmed by core genome phylogeny on 29 strains representative of 9 of the 13 phylogroups. This classification can be considered till now as the most suitable one to rely on considering the number of strains studied and the standards used to select them. They also analyzed the phenotypic variation within these phylogroups and clades relative to traits that have commonly been used to identify *Pseudomonas syringae*. Moreover, it is believed that the 4 loci they sequenced provide robust and accurate data, and their combined level of polymorphism is sufficient to reliably resolve evolutionary

relationships (Hwang *et al*., 2005). Those four genes were also used frequently in many similar studies (Hwang *et al*., 2005; Yan *et al*., 2008; Ferrante and Scortichini, 2010; Berge *et al*., 2014) which offer a huge amount of data for comparison.

In our study, molecular characterization was indispensable to figure out the genetic diversity of *Pseudomonas syringae* causing bacterial canker in Lebanon. As first screening, we started by conducting rep-PCR using BOX primer. This technique is considered to be easy and low cost that enables the discrimination between pathovars of *Pseudomonas syringae* and analyzes their genetic diversity. According to results we obtained, representative isolates were subsequently selected and analyzed using MLST. This molecular technique is much more reliable and accurate with better outcome, taking into account that it is much expensive and additional expertise is required.

After analyzing the results of BOX-PCR, we found that they were consistent with phenotypic identification of pathovars confirming the identity of the collected isolates. In fact, this technique was able to clearly separate between the two pathovars that cause bacterial canker in the Lebanese stone fruit orchards. All isolates that were identified phenotypically as *Psm* 1 produced a unique pattern and they were placed in the group A together with *Psm* 1 strain CFBP 3801. Isolates we have considered previously as *Pss*, were divided in two groups: B and C. Those latter were very heterogeneous producing 17 different patterns distributed between the two groups. The 3 isolates that were not assigned previously to any pathovar (isolates C16, C53 and AL12), fall also in the 2 groups of *Pss*. The isolate AL12 (pattern 7) appears to belong to the group B and the isolate C53 (pattern 2) belongs to the group C. The only doubt we still have is regarding the isolate C16 that produced the pattern 9 and form a small cluster within the group B (subgroup B1) together with a *Pss* isolate (C47). To mention that the isolate C16 did not induces any disease symptoms when inoculated on cherry fruitlets, while the isolate C47 was weakly pathogenic.

In MLST, representative isolates having different BOX patterns and isolated from different regions in Lebanon and different host species were chosen. They were selected also according to their virulence to cherry fruitlets and the presence of hopAP1 effector gene in their genome that was evaluated previously. The main objective here was to have an accurate idea about the population genetics and the molecular evolution of the Lebanese isolates. Accordingly, we wanted to classify them using the most suitable scheme available for to date and compare them with other strains from other countries and different host species. We found that MLST

analysis performed according to Morris MLST schema of the Plant Associated and Environmental Microbes Database in combination with *gap* A and *gyr* B of the Hwang schema is indeed a powerful tool for accurate strain identification and clustering of *Pseudomonas syringae*. Following this schema, that was adopted by Berge *et al*. (2014); our isolates were divided into 3 main groups. All *Psm* 1 isolates clustered in one group and they all belonged to the phylogroup 3 (PG03), and *Pss* isolates were divided between the clades 2b and 2d of the phylogroup 2 (PG02). The two phylogroups, PG02 and PG03, are equivalent to genomospecies 1 and genomospecies 2 respectively (Gsp1 and Gsp2) of Gardan *et al*. (1999) based on DNA/DNA hybridization. In any case, in both techniques that we used the grouping of our strains was always equivalent. The PG02d is equivalent to the group C in BOX-PCR, PG02b is equivalent to the group B and the PG03 is equivalent to the group A. This finding supports the credibility of both techniques used and the protocols we followed. Here we have to note that we assumed that the 3 groups of BOX-PCR fit well the 3 phylogroups of MLST as the representative isolates evaluated in the latter technique did.

4.2.1 Pathovar *morsprunorum*

Rep-PCR and MLST analysis put in evidence the homogeneity of isolates of the pathovar *morsprunorum*. As we mentioned before they all produced in BOX-PCR a unique pattern and in MLST analysis they formed a separated group with a variation in only few nucleotides between sequences among strains. This homogeneity was not related to the host species from which each strain was isolated neither to the place of isolation. Here we are not talking only about isolates from Lebanon, but identical pattern was produced also by an isolate from *Prunus* sp. in UK (CFBP 3801) that was included in our BOX-PCR analysis. In fact, the homogeneity of this pathovar is well known and similar results are reported in maybe all previous similar studies (Ménard *et al*., 2003; Vicente and Roberts, 2007; Gilbert *et al*., 2009; Kaluzna *et al*., 2010b). This characteristic is valid also for phenotypic traits since all *Psm* 1 isolates were fluorescent on KB medium with same results in LOPAT and GATTa tests. Symptoms produced by each isolate on cherry fruitlets were also similar with some small differences of virulence of few isolates.

In MLST analysis, the Lebanese *Psm* 1 isolates were closely similar to *P. syringae* pv. *miricae* (MAFF302941) on *Myrica rubra* and *Psm* 1 strain FTRS_U7 (=MAFF301436) on *Prunus mume* both isolated in Japan. The pathotype strain of the pathovar *morsprunorum* (M302280^{PT}=CFBP 2351) was placed in the PG01b of Berge *et al.* (2014) and not in the

PG03. However, strains of the Clade 1b and those of the PG03 have many common characteristics including the possession of genes for degradation of aromatic compounds and the incapacity to degrade aesculin that was frequent in both of them (Berge *et al.*, 2014). Anyhow, it was reported previously that this pathotype strain is not representative of the pathovar *morsprunorum* (Young *et al*., 1996; Bull *et al*., 2011) and according to Gardan *et al*. (1999) it is a member of Genomospecies 3. In fact, Gardan *et al*. (1999) included in their study a second strain of *Psm* (CFBP 2116) that appears to belong to the Genomospecies 2 and not 3 as the pathotype strain. Same results were obtained by Clarke *et al*. (2010) and Sawada *et al*. (1999) when strains of the pv. *morsprunorum* were always distributed over two groups regardless the molecular technique used. In the latter study, the pathotype strain (MP1= M302280^{PT}) was placed in one group with pv. *maculicola* (MA1 and MA2), pv. *lachrymans* (LA1=MAFF 302278^{PT}), pv. *syringae* (SY7=T–1), pv. *actinidiae* (AC30) and pv. *theae* (TH2, TH3), and 2 other *morsprunorum* strains (MP2, MP3) in a different group with the pv. *myricae* (MY1), pv. *eriobotryae* (ER1), pv. *tabaci* (TB1), pv. *lachrymans* (LA2=MAFF 301315), pv. *castaneae* (CA1), pv. *phaseolicola* (PA1, PU4), pv. *glycinea* (GL1), pv. *mori* (MR1, MR2, MR6) and pv. *broussonetiae* (BR1). This classification is similar to the one we obtained since strains of *Pseudomonas syringae* pv. *mori*, pv. *phaseolicola*, pv. *tabaci*, pv. *glycinea*, pv. *broussonetiae* and pv. *myricae* were placed in the PG03 with the Lebanese *Psm* 1 isolates while the *Psm* pathotype strain was allocated in the PG01 together with strains of the pathovar *actinidiae* and *maculicula*.

In any case, according to biochemical and physiological tests (Garrett *et al*., 1966; Burkowicz and Rudolph, 1994; Luz, 1997 Vicente *et al*., 2004), rep-PCR (Ménard *et al*., 2003; Vicente and Roberts, 2007) and MLST (Sarkar and Guttman, 2004; Hwang *et al*., 2005; Kaluzna *et al*., 2010b) analysis, *Psm* is subdivided into two races, race 1 (Wormald, 1932) and race 2 (Freigoun and Crosse, 1975). Strains within each of the two races appear to be quite homogeneous and are thus reliably identified and distinguished. Previously, many studies suggested the possibility that the 2 races can be divided into separate species considering that they are genetically distant enough to be distinct pathogens although adapted to same hosts (Bultreys and Kaluzna, 2010; Scortichini *et al*., 2013, Marcelletti and Scortichini, 2014). Confirming this hypothesis is not yet accomplished because of the lack of genomic data about strains belonging to the race 1 adding that in the majority of the previous studies the race of *Psm* strains was not specified which creates some confusions.

In our study, we used the *Psm* strain FTRS_U7 from PAMDB that was demonstrated to be *Psm* race 1 by Young *et al.* (2012) and another one from NCBI isolated from sweet cherry in Serbia (KBNS84). Those 2 strains and the Lebanese *Psm* 1 strains fall in the PG03 of Berge *et al*. (2014). We confirmed previously that the Lebanese *Psm* isolates belong to the race 1 according to biochemical tests and this was supported by BOX-PCR analysis. Also according to our results from BOX-PCR analysis, the pathotype strain M302280^{PT} (= CFBP 2351) had an identical pattern as *Psm* 2 reference strain CFBP 3800. This proves that the pathotype strain M302280^{PT} is *Psm* 2 and CFBP 2116 used by Gardan *et al*. (1999) is *Psm* 1 as it was suggested by Marcelletti and Scortichini (2014).

Our results clearly confirmed the identity of strains of *Psm* used frequently in previous similar studies and the race they belong to, including the pathotype strain. We also put in evidence the genetic distance between the 2 races of *Psm* that was always higher than 8.8 % between the concatenated sequences used. By adding more sequences of *Psm* 1, we give strong support for many previous opinions that suggested that the 2 races of *Psm* should be divided into 2 distinct species

Here we have to add that the phenotypic characteristics of strains of the PG03 as given by Berge *et al*. (2014) fit well to those we evaluated for the Lebanese *Psm* 1 isolates. One characteristic was exceptional that was the utilization of tartrate, where all *Psm* 1 isolates were able to use it as a sole carbon source while according to Berge *et al*. (2014) none of the strains in this group can do this.

4.2.2 Pathovar *syringae*

Contrary to the results described for *Psm* 1, both BOX-PCR and MLST analysis put in evidence the high genetic diversity between the Lebanese *Pss* isolates. Such results were always obtained in many previous studies, not only when isolates are originated from stone fruits but also from different host species and countries. According to a study conducted by Martín-Sanz *et al*. (2013), rep-PCR grouped *Pss* isolated from peas into two groups together with strains from other hosts. In the same study, isolates from non-legume species formed a third group and the authors found that two of the three groups were more virulent to peas independently of their host of isolation. They concluded that genetically and pathogenically different *Pss* groups are able to cause disease on peas. In another study, *Pss* strains isolated from sweet and wild cherries were divided into 3 groups in the combined dendrogram of REP, ERIC and BOX-PCR fingerprints (Vicente and Roberts, 2007). The distribution of the isolates

between groups was not related to host plant neither place nor year of isolation. Moreover, within the same group some isolates were highly virulent while others were not pathogenic at all on Lilac. They concluded that rep-PCR cannot replace pathogenicity test on susceptible host for the identification of *Pss* isolates.

In our study, the analysis showed that the diversity within the pathovar *syringae* was not related to the host plant or the place of isolation. When reference strains were included in the analysis, we found that isolates of the PG02d (or C in BOX-PCR) and isolates of the PG02b (or B in BOX-PCR) were obtained from different plant species and different countries. Among Lebanese regions, isolates of the two groups were obtained from different governorates, with the exception of North Lebanon from where only *Pss* of the PG02d were isolated.

When analyzing *Pss* according to stone fruit species from where they were isolated, we found that isolates of the PG02d (or C in BOX-PCR) are obtained from all species while isolates of the PG02b (or B in BOX-PCR) were obtained from cherry, plum, peach, nectarine and almond but never from apricot. In fact, isolates from apricot were all placed in a tight cluster in the group C of the dendrogram of genetic similarity of BOX fingerprint patterns. This means that they are genetically closely related and probably a host-pathogen specific relationship that should be investigated better in the future exists. To take into consideration that the number of *Pss* isolated from apricot was only 16 so it would be better to collect more samples from this species and to try a pathogenicity test by inoculating isolates of the group B on apricot plant material. This will clarify the hypothesis that we can ask here regarding the inability of strains of this group to induce bacterial canker on apricot.

According to all the characterization analysis we performed, only molecular typing tools were able to separate between the 2 genotypes of *Pss*. In fact, they did not show any differences between each other according to phenotypic characteristics. Only some variation of virulence of some isolates was observed on cherry fruitlets but still 86% of the Lebanese *Pss* were highly virulent having a mean score of lesion diameter >3 on the used scale. Few isolates were weakly virulent and only the isolate C16 was not pathogenic. This isolate was clustered separately in the subgroup B1 of BOX-PCR and in MLST we were not able to amplify its *gap* A gene so it was excluded from the MLST analysis. In any case, C16 was classified in the PG10 with single gene phylogeny using *cts* sequences. This classification was unusual since

isolates of the PG10 have been reported exclusively from environmental reservoirs outside of agricultural areas (Berge *et al*., 2014) while the isolate C16 isolate was isolated from cherry.

Statistical analysis showed that the variability of virulence was related to phylogroups. Isolates of the PG02d were significantly more virulent than isolates of the PG02b. Interestingly it appeared also that all isolates of the PG02d possess the hopAP1 effector while only some isolates of the PG02b possess it. Supporting this finding, our MLST analysis allocated the *Pss* strain B301D in the PG02d and the *Pss* strain HS191 in the PG02b that according to Ravindran *et al*. (2015) the first one possesses hopAP1 gene while the second do not. This finding disagrees with the suggestion of Vieira *et al.* (2007) reporting that the primers they designed to amplify this effector can be used for a specific detection of isolates of the pathovar *syringae*. Anyhow, it is well known the role of effectors in the pathogenicity of *Pseudomonas syringae* and their role in host specificity of this group of bacteria (Guttman *et al.*, 2002; Greenberg and Vinatzer, 2003; Vinatzer *et al*., 2006). The short analysis conducted in this work seems to be interesting but it is not enough to make any conclusion about the role of hopAP1 alone and more investigations about other effectors most be conducted.

Sawada *et al*. (1999) when analyzing the phylogeny of *Pseudomonas syringae* pathovars, suggested that the pathovar *syringae* is divided into 2 groups. They added that this separation may due to an incorrect or incomplete pathovar definition or strain identification. Supporting this, many previous researches proposed that this pathovar is very heterogeneous and it regroups many pathovars and even species that may be synonyms such as the pathovars: pv. *aceris*, pv. *aptata*, pv. *atrofaciens*, pv. *dysoxyli*, pv. *japonica*, pv. *lapsa*, pv. *panici*, pv. *papulans*, and pv. *pisi* (Gardan *et al*. 1991, 1994; Young 1991; Young *et al*. 1992). In fact, Sawada *et al.* (1999) studied 4 of those nine pathovars (pv. *aceris*, pv. *aptata*, pv. *japonica* and pv. *pisi*) and results confirm the close relation between them and the pathovar *syringae*. They were clustered together with all strains of the pv. *syringae* with the exception of one strain isolated from *Citrus iyo* that was placed in another group. During the same year, Gardan *et al*. (1999) indicate the presence of 9 Genomospecies among the pathovars of *Pseudomonas syringae*. The technique used was DNA-DNA hybridization and ribotyping, differently from the one used by Sawada *et al*. (1999) based on sequencing of four index genes (*gyr* B, *rpo* D, *hrp* L, and *hrp* S). The pathovar *syringae* was classified in the Gsp1 *sensu stricto* of Gardan *et al.* (1999) with the same nine pathovars mentioned before, without being divided into different groups. Exactly the same results were obtained by Bull *et al*.

(2011) using MLST and by Parkinson *et al.* (2011) using *rpo*D phylogeny of species type strains and pathovar type strains. Two additional pathovars were added to this Genomospecies by Bull *et al*. (2011): pv. *coryli* and pv. *solidagae*.

According to the classification of Berge *et al.* (2014) that we followed, all strains of the pv. *syringae* that we studied were placed in the phylogroup PG02. This phylogroup is equivalent to Gsp 1 of Garden *et al*. (1999) and it was divided into 5 clades, 2a, 2b, 2c, 2d and 2e. Three of them (2a, 2b and 2c) where previously described by Clarke *et al*. (2010) using MLST based on the 4 loci of Hwang *et al*. (2005). Anyhow, strains of PG02 are very diverse and they are found in all habitats analyzed to date such as plants, stream water, snow, ground water, rain, litter (Berge *et al*., 2014). In agreement with previous studies, many pathovars belong to this group (pv. *syringae*, pv. *aceris*, pv. *aptata*, pv. *atrofaciens*, pv. *dysoxyli*, pv. *japonica*, pv. *lapsa*, pv. *panici*, pv. *papulans*, pv. *coryli*, pv. *solidagae*. and pv. *pisi*), in addition to many strains that were not isolated from plants thus not assigned to a pathovar. The subgroup 2d that enclose most of the Lebanese *Pss* isolates, enclose also 2 important strains of the pv. *syringae*, B728a isolated from *Phaseolus vulgaris* in USA and B301D isolated from *Pyrus communis* in UK, that were completely sequenced (Feil *et al*., 2005 and Ravindran *et al*., 2015, respectively). Whereas the clade (2b) contain the rest of Lebanese isolates and enclose the type strain of the pathovar *syringae* CFBP 1392^{T} isolated from *Syringae vulgaris* in UK and HS191 isolated from a diseased monocotyledon plant (*Panicum miliaceum*) in Australia.

Here also, the results of phenotypic traits of strains representing the genetic diversity in the *Pseudomonas syringae* complex discussed by Berge *et al*. (2014) are in agreement with the results of phenotypic traits that we evaluated in our study. The only exceptions were 2 of our isolates (AL12 and C53) that were not fluorescent on KB medium contradicting what was published by Berge *et al*. (2014) that all strains of the PG02 are fluorescent on KB medium.

Even though the complexity of the pathovar *syringae* and the high genetic and phenotypic diversity of strains of this group of bacteria, this study demonstrate that *Pss* causing bacterial canker of stone fruits in Lebanon followed 2 different genetic evolutions. It is clear that the two genotypes described in this study are widely spread in different regions of the world and many of them were isolated from environmental substrates not related to agriculture. More investigations must be done to find any possible relationship between the genotypes and their behavior in a specific ecosystem.

4.2.3 Allocation of strains using single gene phylogeny and division of the PG03 into 2 clades

Nowadays, MLST offers many advantages over other molecular typing techniques which make it one of the most used to identify and classify *Pseudomonas syringae* strains. Many previous studies performed this molecular tool by sequencing different housekeeping genes or different locus of those genes (Sarkar and Guttman, 2004; Bull *et al*., 2010; Hwang *et al*., 2005; Berge *et al*., 2014). All results obtained can be considered accurate and the classification of *Pseudomonas syringae* strains was almost similar. Even if currently the cost of sequencing is much lower than any time, it is still an important factor to be taken into consideration when it is about a large number of isolates. For this reason, we tried to figure out the capability of each of the genes we used in MLST to identify and allocate correctly a set of *Pseudomonas syringae* strains when used alone in phylogeny analysis.

Accordingly, it appeared that the partial sequence of *cts* gene was able to allocate all the isolates almost equally to MLST. The only exception was the separation between the 2 clades, 'a' and 'b', of the PG07. This finding supports the suggestion of Berge *et al.* (2014) that the citrate synthase (*cts*) housekeeping gene can accurately predict the phylogenetic affiliation for more than 97% of the tested strains.

The other genes were also able to allocate the majority of the strains in the correct phylogroup with some exceptions. The main problem was the allocation into the correct clade inside each phylogroup rather than within phylogroups. In a previous study, *rpo* D was shown to be useful in grouping pathovars of *Pseudomonas syringae* into phylogroups equivalent to genomospecies classification of Gardan *et al*. (1999) (Parkinson *et al*., 2011). According to Bull *et al*., (2011), this approach is valuable but represents the phylogeny of a single gene which may be different from the phylogeny of the organisms.

The classification of *gyr* B was interesting showing the highest divergence from the one of MLST especially in the case of the PG03 that was divided into 2 distantly separated groups. In fact, strains of the pathovars *morsprunorum* race 1 and *miricae* were placed together in one group, while strains of the pathovars *phaseolicola*, *mori*, *lachrymans* and *Pseudomonas syringae* strains from environmental substrates in another group. In order to investigate better this division, we analyzed the matrix of genetic distance obtained from the MLST analysis. We found that if we follow a threshold of genetic difference of 2.3% used by Berge *et al*. (2014) for delimitation of clades, the PG03 can be divided into at least 2 clades. In fact, the

mean genetic distance between strains of those 2 clades was equal to 3.1%. We suggest a Clade named ‘b’ grouping the pv. *morsprunorum* race 1 and pv. *miricae* and a clade named ‘a’ that groups the remaining pathovars of the PG03. To mention here that only 14 isolates of the PG03 were used in the study of Berge *et al*., (2014) consequently they were not enough to divide this phylogroup into many clades. However, by including *Psm* 1 isolates from Lebanon and others from the Genbank, it becomes clear that this phylogroup should be divided into 2 clades. From this discussion we can understand how much it is critical the choice of genes used in MLST for an accurate affiliation of *Pseudomonas syringae* strains to phylogroups. Only one gene such as *gyr* B, is able to greatly change the classification and even the delimitation of clades. We suggest here, that even if *cts* gene showed to be the best for an easy identification and classification of new *Pseudomonas syringae* strains, results will not be always accurate when only one gene is used for new strains allocation.

4.2.4 Type three secretion system preliminary analysis

P. syringae is composed of pathovars and races differing in host range among crop species and cultivars, respectively (O’Brien *et al*., 2011). Nowadays, it is known that the type three secretion system (TTSS) is essential for pathogenicity and it is involved in defining host range between strains of the complex groups of the bacteria *Pseudomonas syringae* (Alfano and Collmer, 2004; Baltrus *et al*., 2011). Three strains that belong to different phylogroups and are considered to be the most studied ones were completely sequenced with the aim to identify the molecular basis of pathogenesis across them. Those strains are *P. syringae* pv. *tomato* DC3000 (*Pto* DC3000), *P. syringae* pv. *syringae* B728a (*Psy* B728a) and *P. syringae* pv. *phaseolicola* 1448A (*Pph* 1448A), which respectively belong to the phylogroups 1, 2 and 3, (Sarkar, Guttman, 2004; Hwang *et al*., 2005; Clarke *et al*., 2010, Berge *et al*., 2014). However it still remains unclear the genetic and the evolutionary basis of those three closely related pathogens, that differ dramatically in host range. In the last few years, the use of next-generation genome sequencing of many strains has further enhanced our understanding of the dynamic nature of the *P. syringae* genome and it become clear the high variability of the type III effectors even among strains of the same pathovar. In order to better understand how host shifts and specialization occur and to understand how those pathogens are able to adapt to a specific host, additional sequencing of very closely related strains that differ in their host ranges and of divergent strains with similar host ranges will need to be performed (O’Brien *et al*., 2011).

In this part of our study, our objective was to detect some effector genes that may be present in the genome of our isolates and then to sequence some of those genes. The sequences that we obtained will help in future studies to design much more accurate primers. In fact, the ones we designed were based only on few sequences available in public databases which keep some doubt about their ability to amplify the requested region in strains that may show some genetic diversity. The more sequences available, better primers can be designed to detect not only closely related strains but even diverse ones. It was also interesting to find any link between the presence and the diversity of those effectors from one side, and the phylogroups obtained by MLST from the other side.

As we mentioned before, results of this preliminary investigation showed that the 2 effectors, hopAE1 and hopI1, were present in almost all the tested *Pseudomonas syringae* isolates from stone fruits in Lebanon. The only exception was the *Pss* isolate C40 for which no PCR amplification product could be obtained when testing for the presence of hopAE1 and the *Pseudomonas syringae* isolate C16 that gave negative results for both effectors. To mention here that both isolates, C16 and C40, do not even possess the hopAP1 effector that was evaluated previously. According to Vinatzer *et al*. (2006), the 2 effectors hopI1 and hopAE1 are present in the genome of *P. syringae* pv. *syringae* strain B728a but a recent study aimed to explore host specificity in *Pseudomonas syringae* showed that hopAE1 is missed in some isolates of *Pss* from stone fruits (Hulin *et al.,* 2015). In the same study, it was reported also that both hopI1 and hopAE1 are present in strains of the pathovar *morsprunorum* race 1. Regarding the effector hopAP1 detected previously, it was reported by Vieira *et al.* (2007) that this effector is specific to the pathovar *syringae* but it appeared later that it is not present in all strains of the pathovar *syringae* (Ravindran *et al*., 2015).

The phylogeny of both effectors was completely equivalent to the phylogeny of our previous MLST analysis constructed using partial sequences of housekeeping genes. Isolates of each of the PG01, PG02 and PG03, including our isolates, were grouped together. This finding gives us an idea about the relationship between the core genome of *Pseudomonas syringae* strains and at least the two effector genes evaluated here. Regarding those 3 phylogroups, previous studies reported that strains of PG01 have the greatest number of TTSS genes among all phylogroups followed by the PG03 (Baltrus *et al*., 2011; O'Brien *et al*., 2012). Differently, strains of the PG02 that enclose the pathovar *syrinage*, can be considered to carry the fewest number of TTSS genes among all phylogroups (Baltrus *et al*., 2011), just after the PG10 (Young, 2010). To mention here that the isolate C16 that do not possess any of the effector

genes we evaluated, belong to the PG10 according to *cts* phylogeny and was not pathogenic to cherry fruitlets.

Further investigation will be conducted in the future to figure out the presence of other effector genes that may play a role in the host range and the virulence of strain that belong to a specific phylogroup. Moreover, a deep study must be done in order to determine the level of amino acid diversity of those effectors within and between subgroups and elucidate the role of this diversity in *Pseudomonas*-plant interaction.

Conclusions and future perspectives

Upon evaluating the state of the art of bacterial diseases affecting stone fruits in Lebanon, we conducted an extended survey to tackle the critical and most important occurring pathogenic bacteria unveiling their biochemical, biological and molecular identity.

Our survey demonstrated that bacterial canker is the main and widely spread bacterial disease of stone fruits in Lebanon. The highest disease incidence was observed on cherry and apricot followed by plum, almond, peach and nectarine, respectively. As causal agents of bacterial canker we identified two pathovars of *Pseudomonas syringae*, pathovar *syringae* and pathovar *morsprunorum* race 1. The pathovar *syringae* was isolated from all the commercial stone fruit species (peach, almond, nectarine, plum, cherry and apricot) and all Lebanese sampled regions, while the pathovar *morsprunorum* race 1 was present on nectarine, plum, cherry and apricot only in mountainous areas of Lebanon (Mount Lebanon, North Lebanon and the mountainous part of Bekaa). None of the other bacterial species pathogenic to stone fruits were isolated in this study unless gall symptoms suspected to be induced by *Agrobacterium tumefaciens* that were observed twice.

Molecular characterization using both MLST and BOX-PCR showed that isolates of the pathovar *morsprunorum* race 1 were genetically homogenous while high genetic diversity was observed among isolates of the pathovar *syringae* which were divided into two genetic groups. It is important to note that isolates of the phylogroup 02b (PG02b) were never isolated from apricot trees whereas those of the phylogroup 02d (PG02d) were detected from all stone fruit species. PG02b and PG02d are standard phylogroups named following an international code for *Pseudomonas syringae* classification. Differences in virulence was observed among *Pseudomonas syringae* isolates when inoculated on cherry fruitlets and *Pseudomonas syringae* pv. *syringae* isolates of the PG02d showed to be more virulent than those of the PG02b.

Single gene phylogeny using partial sequence of *cts* gene allocated all isolates used in our analysis similarly to MLST. This gene is potentially useful for rapid and regular identification of an unknown isolate especially in routine disease monitoring programs. Moreover, the genetic distance between PG01 and PG03, representing strains of the pathovar *morsprunorum* race 2 and race 1 respectively, revealed that the two races are distant and both distinct pathogens can be adapted to the same hosts. This finding supports previous researches that suggest dividing them into 2 different species. Furthermore, having enough isolates in PG03

enabled us to divide them into two clades according to the distance threshold of clades delimitation, separating isolates of different pathovars in this phylogroup.

The data we are offering in this study and bacterial isolates we have collected during the conducted survey will be useful in any subsequent studies related to diagnosis and epidemiology of *Pseudomonas syringae* in stone fruit orchards in Lebanon and worldwide. The acquired knowledge will be used in the future for the implementation of the most convenient control strategies of bacterial diseases on these tree species.

The awareness about the bacterial species present in the field and the understanding of the structure and the diversity of strains within each species are crucial since those aspects are directly related to the disease symptom expression. More investigation should be conducted to evaluate the behavior and the pathogenic characteristics of the two genotypes of the pathovar *syringae* identified in this study and if possible to determine a pathogenicity fingerprint even at race level. Such kinds of information are crucial to assess the availability of resistant varieties or to implement breeding for disease resistance programs). Pathogenicity trials on plant species other than stone fruits should be also studied in order to figure out the host range of the local strains being aware of the risk that the polyphagous bacterium *Pseudomonas syringae* pv. *syringae* supposes since it may be able to shift to other crops.

The lack of efficient tools to control bacterial canker increases the challenges to identify novel alternatives. Plant-pathogen interaction is taking more and more attention during the last decade to understand better the mechanism used by the pathogen to infect and the reaction of the plant to resist. Accordingly, preliminary investigations on the type three secretion system known to be the main factor used by *Pseudomonas syringae* to induce disease, have already started in this research study. According to our preliminary results the same phylogenetic resolution, in term of species identification, was obtained using MLST and the sequenced effectors when they are present.

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Annexes

Annex 1: Information about strains used in MLST analysis

Strain name	**Year of isolation**	**Identification according to authors**	**Reference**	**Origin country, region)**	**Substrate**	**MLST Phylogroup**	**Accession to sequences used in MLST**
233	2009	*P. syringae* pv. *syringae*	Berge *et al*. 2014	Italy (Ravenna)	*Beta vulgaris*	PG 02d	PAMDB*
601	1966	*P. syringae* pv. *aptata*	NIAS Genbank	Japan (Kagawa)	*Beta vulgaris*	PG 02b	PAMDB*
0893_23	1969	P. syringae pv. aesculi	Green *et al*. 2010	India	*Aesculus hippocastanum*	PG 03	PAMDB*
1_6	1991	P. syringae pv. Oryzae	Takeuchi *et al*. 1992	Japan (Hokkaidou)	*Oryza sativa*	PG 04	PAMDB*
38B9	2010	*P. graminis*	Vaïtilingom *et al* 2012	France	Water phase of Cloud	*P. graminis*	PAMDB*
6B4	2004	*P. rhizosphaerae*	Amato *et al*. 2007	France	Water phase of Cloud	*P. rhizosphaerae*	PAMDB*
83.1	NA**	*P. cichorii*	Cottyn *et al*. 2011	USA (Arizona)	NA	PG 11	PAMDB*
AI0003	2007	*P. syringae*	Morris *et al*. 2010	New Zealand	Stream water	PG 02d	PAMDB*
AI0027	2007	*P. syringae*	Morris *et al*. 2010	New Zealand	Stream water	PG 02b	PAMDB*
ATCC11528	1905	*P. syringae* pv. *tabaci*	ATCC collection	USA	*Nicotiana benthamiana*	PG 03	PAMDB*
B728a	1987	*P. syringae* pv. *syringae*	Loper & Lindow 1987	USA (Wisconsin)	*Phaseolus vulgaris*	PG 02d	PAMDB*
BS0002	2008	*P. viridiflava*	Bartoli *et al*. 2014	Italy (Brescia)	*Actinidia deliciosa*	PG 07a	PAMDB*
CAW0019	2011	*P. syringae*	Berge *et al*. 2014	France (Montfavet, 84)	Groundwater	PG 02d	PAMDB*
CC0125	1997	*P. Syringae*	Morris *et al*. 2000	France (Castelnau, 46)	*Cucumis melo* leaf	PG 02b	PAMDB*
CC0206	1999	*P. syringae*	Morris *et al*. 2010	France (Bollène, 84)	*Cucumis melo*	PG 02d	PAMDB*
CC0301	2001	*P. syringae*	Morris *et al*. 2010	France (Quercy, 46)	*Cucumis melo*	PG 02d	PAMDB*
CC0301	2001	*P. syringae*	Morris *et al*. 2010	France (Quercy, 46)	*Cucumis melo*	PG 02d	PAMDB*
CC0393	2001	*P. syringae*	Morris *et al*. 2010	France (Quercy, 46)	Irrigation reservoir water	PG 02d	PAMDB*
CC0654	2004	*P. syringae*	Morris *et al*.	France	*Primula*	PG 02d	PAMDB*

			2008	(Mézel, 04)	*officinalis*		
CC0658	2004	*P. syringae*	Morris *et al*. 2008	France (Mézel, 04)	*Primula grandiflora*	PG 02d	PAMDB*
CC1416	2004	*P. syringae*	Morris *et al*. 2007	USA (Gallatin Cy, MT)	Epilithic biofilm	PG 01b	PAMDB*
CC1417	2004	*P. syringae*	Morris *et al*. 2007	USA (Gallatin Cy, MT)	Epilithic biofilm	PG 09a	PAMDB*
CC1427	2004	*P. syringae*	Morris *et al*. 2007	USA (Park Cy, MT)	Epilithic biofilm	PG 01b	PAMDB*
CC1470	2005	*P. syringae*	Morris *et al*. 2008	France (Ardèche, 07)	Stream water	PG 02d	PAMDB*
CC1470	2005	*P. syringae*	Morris *et al*. 2008	France (Ardèche, 07)	Stream water	PG 02d	PAMDB*
CC1475	2005	*P. syringae*	Morris *et al*. 2008	France (Villard-de-Lans)	Snow	PG 02d	PAMDB*
CC1484	2006	*P. syringae*	Morris *et al*. 2008	France (La Clusaz, 74)	Snow	PG 02b	PAMDB*
CC1513	2006	*P. syringae*	Morris *et al*. 2008	France (Col Vieux, 05)	*Hutchinsia alpina*	PG 04	PAMDB*
CC1524	2006	*P. syringae*	Morris *et al*. 2010	France (Col de Vars, 05)	Stream water	PG 09a	PAMDB*
CC1532	2006	*P. syringae*	Morris *et al*. 2010	France (Bès lake, 48)	Lake water	PG 09a	PAMDB*
CC1559	2006	*P. syringae*	Morris *et al*. 2008	France (Col Vieux, 05)	Alpine meadow	PG 01b	PAMDB*
CC1582	2006	*P. syringae*	Demba Diallo *et al*. 2012	France (Col Vieux, 05)	Epilithic biofilm	PG 07a	PAMDB*
CC1583	2006	*P. syringae*	Morris *et al*. 2008	France (Col Vieux, 05)	Epilithic biofilm	PG 10a	PAMDB*
CC1586	2006	*P. syringae*	Morris *et al*. 2008	USA (Hyalite Lake MT)	Lake water	PG 10c	PAMDB*
CC1629	2007	*P. syringae*	Morris *et al*. 2010	USA (Bozeman, Montana)	*Avena sativa*	PG 04	PAMDB*
CC1671	2011	*P. syringae*	Berge *et al*. 2014	France	*Actinidia deliciosa*	PG 02d	PAMDB*
CC457	2003	*P. syringae*	Morris *et al*. 2010	France (Plan de Robion)	*Cucumis melo*	PG 02b	PAMDB*
CC457	2003	*P. syringae*	Morris *et al*. 2010	France (Plan de Robion)	*Cucumis melo*	PG 02b	PAMDB*
CCE0100	2009	*P. syringae*	Berge *et al*. 2014	France (Ceillac, 05)	Alpine meadow	PG 10d	PAMDB*
CCE0103	2009	*P. syringae*	Berge *et al*.	France	Alpine	PG 10a	PAMDB*

			2014	(Ceillac, 05)	meadow		
CCE0153	2009	*P. syringae*	Berge *et al*. 2014	France (Ceillac, 05)	Stream water	PG 10f	PAMDB*
CCE0633	2010	*P. syringae*	Berge *et al*. 2014	France (Ceillac, 05)	Litter	PG 02b	PAMDB*
CCE0915	2010	*P. syringae*	Berge *et al*. 2014	France (Ceillac, 05)	Litter	PG 13a	PAMDB*
CCV0213	2009	*P. syringae*	Berge *et al*. 2014	France (Vars, 05)	Stream water	PG 10g	PAMDB*
CCV0567	2010	*P. syringae*	Berge *et al*. 2014	France (Vars, 05)	Litter	PG 13b	PAMDB*
CFBP1392	1950	Type strain of *P. syringae*	Gardan *et al*. 1999	United Kingdom	*Syringa vulgaris*	PG 02b	PAMDB*
CFBP1906	1979	*P. syringae* pv. *aptata*	CIRM CFBP collection	France	*Beta vulgaris*	PG 02b	PAMDB*
CFBP2067	1972	*P. syringae* pv. *helianthi* Pathotype strain	Gardan *et al*. 1999	Mexico	*Helianthus annuus*	PG 06	PAMDB*
CFBP2256	1983	*P. syringae* pv. *atrofaciens*	CIRM CFBP collection	Greece	*Triticum aestivum*	PG 02b	PAMDB*
CFBP4407	1984	*P. cichorii*	CIRM CFBP collection	France (Lot-et-Garonne)	*Lactuca sativa*	PG 11	PAMDB*
CLA0275	2010	*P. syringae*	Monteil *et al*. 2013a	France (Lautaret, 05)	Litter	PG 10b	PAMDB*
CLA0302	2010	*P. syringae*	Monteil *et al*. 2013a	France (Lautaret, 05)	Litter	PG 13b	PAMDB*
CMO0010	2009	*P. syringae*	Berge *et al*. 2014	France (Montfavet, 84)	Rain	PG 03	PAMDB*
CMO0043	2009	*P. syringae*	Berge *et al*. 2014	France (Montfavet, 84)	Rain	PG 02b	PAMDB*
CMO0085	2010	*P. syringae*	Bartoli *et al*. 2014	France (Montfavet, 84)	Rain	PG 08	PAMDB*
CMO0110	2010	*P. syringae*	Bartoli *et al*. 2014	France (Montfavet, 84)	Rain	PG 07a	PAMDB*
CMW0020	2011	*P. syringae*	Berge *et al*. 2014	France	River water	PG 09c	PAMDB*
CSZ0761	2010	*P. syringae*	Bartoli *et al*. submitted	France (Sauze, 06)	Alpine meadow	PG 01b	PAMDB*
DC3000	1960	*P. syringae* pv. *tomato*	Cuppels 1986	United Kingdom	*Solanum lycopersicum*	PG 01a	PAMDB*
Fmu-107	1986	*P. viridiflava*	Morris *et al*. 1992	China	*Brassica rapa*	PG 07b	PAMDB*
GAW0112	2011	*P. syringae*	Berge *et al*.	France	Irrigation canal	PG 12a	PAMDB*

			2014	(Gadagane, 84)	water		
GAW0113	2011	*P. syringae*	Berge *et al*. 2014	France (Gadagane, 84)	Irrigation canal water	PG 12b	PAMDB*
GAW0203	2011	*P. syringae*	Bartoli *et al*. 2014	France (Gadagane, 84)	Irrigation canal water	PG 08	PAMDB*
H5E1	1993	*P. syringae* pv. *pisi*	NIAS Genbank	Japan (Wakayama)	*Pisum sativum*	PG 02b	PAMDB*
H5E1	1993	*P. syringae* pv. *pisi*	NIAS Genbank	Japan (Wakayama)	*Pisum sativum*	PG 02b	PAMDB*
JT7	2007	P. *syringae* pv. *syringae*	Berge *et al*. 2014	Italy (Viterbo)	*Actinidia deliciosa*	PG 02b	PAMDB*
KOZ8101	1980	P. *syringae* pv. *broussonetiae*	Takahashi *et al*. 1996	Japan (TOTTORI)	*Morus papyrifera*	PG 03	PAMDB*
LYR0002	2011	*P. syringae*	Monteil *et al*. In press	France (Montfavet)	Rain	PG 03	PAMDB*
LYR0002	2011	*P. syringae*	Monteil *et al*. In press	France (Montfavet)	Rain	PG 03	PAMDB*
M301072PT	1951	*P. syringae* pv. *japonica* Pathotype strain	Mukoo, H., 1955	Japan (Tochigi)	*Hordeum vulgare*	PG 02b	PAMDB*
M301072PT	1951	*P. syringae* pv. *japonica* Pathotype strain	Mukoo, H., 1955	Japan (Tochigi)	*Hordeum vulgare*	PG 02b	PAMDB*
M302091	1984	*P. syringae* pv. *actinidae*	Takikawa *et al*. 1989	Japan	*Actinidia deliciosa*	PG 01b	PAMDB*
M302280PT	NA	*P. syringae* pv. m*orsprunorum*(CFBP 2351)	Sawada *et al*. 1999	USA	*Prunus domestica*	PG 01b	PAMDB*
MAFF301020	1966	*P. syringae* pv. *mori* (JD04, PmoM301020)	Sawada *et al*. 1999	Japan (Nagano)	*Morus alba*	PG 03	PAMDB*
MAFF301020	1966	*P. syringae* pv. *mori* (JD04, PmoM301020)	Sawada *et al*. 1999	Japan (Nagano)	*Morus alba*	PG 03	PAMDB*
MAFF301315	1975	*P. syringae* pv. *lachrymans*	Ohuchi *et al*. 1980	Japan	*Cucumis sativus*	PG 03	PAMDB*
MAFF301315	1975	*P. syringae* pv. *lachrymans*	Ohuchi *et al*. 1980	Japan	*Cucumis sativus*	PG 03	PAMDB*
MAFF301765	1982	*P. syringae pv. glycinae*	Moriwaki *et al*. 1996	Japan	*Glycine max* Merrill	PG 03	PAMDB*
MAFF302273PT	1939	*P. syringae* pv. *aceris* Pathotype strain	Sawada *et al*. 1999	USA	*Acer sp*	PG 02d	PAMDB*
MAFF302941	1989	*P. syringae* pv. *miricae*	NIAS Genbank	Japan (Tokushima)	*Myrica rubra*	PG 03	PAMDB*
MAFF302941	1989	*P. syringae* pv.	NIAS	Japan	*Myrica rubra*	PG 03	PAMDB*

		miricae	Genbank	(Tokushima)			
NCPPB3335	1984	*P. savastanoi pv. savastanoi*	Pérez-Martínez *et al*. 2007	France	*Olea europaea*	PG 03	PAMDB*
P6	2007	*P. syringae* pv. syringae	Berge *et al*. 2014	Portugal	*Actinidia deliciosa*	PG 02b	PAMDB*
PavBPIC631	1976	*P. avellanea* Type strain	Janse *et al*. 1996	Greece (Drama)	*Corylus avellana*	PG 01b	PAMDB*
PavISPave013	1992	*P. syringae* pv. *avellanea*	Scortichini & Tropiano 1994	Italy (central)	*Corylus avellana*	PG 02b	PAMDB*
PavISPave013	1992	*P. syringae* pv. *avellanea*	Scortichini & Tropiano 1994	Italy (central)	*Corylus avellana*	PG 02b	PAMDB*
Pf-5	1979	*P. protegens*	Howell & Stipanovic 1979	USA	Root surface of cotton	*P. protegens*	PAMDB*
PmaES4326	1965	*P. cannabina* pv. *asilensis*	Davis *et al*. 1991	NA	*Raphanus sativus*	PG 05	PAMDB*
Pph1448A-PT	1985	*P. syringae* pv. p*haseolicola race 6 pathotype*	Teverson 1991; Taylor *et al*. 1996	Ethiopia	*Phaseolus vulgaris*	PG 03	PAMDB*
PseHC_1	1997	*P. syringae* pv. *sesami*	NIAS Genbank	Japan (Ibaraki)	*Sesamum indicum*	PG 03	PAMDB*
PSy642	2007	*P. syringae* pv. *syringae*	Clarke *et al*. 2010	USA (Virginia)	Unidentified plant	PG 02c	PAMDB*
PsyCit7	2008	*P. syringae* pv. s*yringae*	Hirano & Upper 1990	USA (Visalia, California)	Plant in Tech campus	PG 02a	PAMDB*
Pta6606	1967	*P. syringae* pv. *tabaci*	Ono 1976	Japan	*Nicotiana tabacum*	PG 03	PAMDB*
R4_A29-2	1977	*P. syringae* pv. *glycinea*	Fett & Sequiera 1981	USA (Wisconsin)	*Glycine max*	PG 03	PAMDB*
R6_1704B	1986	*P. syringae* pv. *pisi race 6*	CIRM CFBP collection	France	*Pisum sativum*	PG 02b	PAMDB*
SZ0030	2007	*P. syringae*	Morris *et al*. 2010	France (Sauze, 06)	Stream water	PG 02c	PAMDB*
SZ0045	2007	*P. syringae*	Morris *et al*. 2010	France (Sauze, 06)	Stream water	PG 02c	PAMDB*
T1	1986	*P. syringae* pv. *tomato*	Cai *et al*. 2011	Canada	*Solanum lycopersicum*	PG 01a	PAMDB*
TA0002	2007	*P. syringae*	Morris *et al*. 2010	France (Tarn, 48)	Stream water	PG 07a	PAMDB*
TA0003	2007	*P. syringae*	Morris *et al*. 2010	France (Tarn, 48)	Stream water	PG 10b	PAMDB*
TA0006	2007	*P. syringae*	Morris *et al*. 2010	France (Tarn, 48)	Stream water	PG 09b	PAMDB*
TA0019	2007	*P. syringae*	Morris *et al*. 2010	France (Tarn, 48)	Stream water	PG 10b	PAMDB*
TA043	2007	*P. syringae*	Morris *et al*. 2010	France (Tarn, 48)	*Primula* sp	PG 07a	PAMDB*

UB246	2006	*P. syringae*	Morris *et al*. 2010	France (Ubaye, 05)	Stream water	PG 13a	PAMDB*
USA0032	2007	*P. syringae*	Morris *et al*. 2010	USA (Cascade creek)	Stream water	PG 10e	PAMDB*
USA0035	2007	*P. syringae*	Morris *et al*. 2010	USA (Cascade creek)	Stream water	PG 02e	PAMDB*
USA0102	2007	*P. syringae*	Morris *et al*. 2010	USA (Pilgrim creek)	Stream water	PG 10a	PAMDB*
USA011	2007	*P. syringae*	Morris *et al*. 2010	USA (Pine creek)	Stream water	PG 02d	PAMDB*
USA011	2007	*P. syringae*	Morris *et al*. 2010	USA (Pine creek)	Stream water	PG 02d	PAMDB*
FTRS_U7=MAFF301436		*P. syringae* pv. *morsprunorum*	Jung and Lee 2010	JAPAN (SHIGA)	*Prunus mume*	PG 03	PAMDB*
B301D		*P. syringae* pv. *syringae*	Ravindran *et al*., 2015	United Kingdom	*Pyrus communis*	PG 02d	CP005969.1
CFBP1690		*P. syringae* pv. *tagetis*	Cunty *et al*. 2013	USA	*Phaseolus vulgaris*	PG 02d	KF937691.1; KF937594.1; KF937497.1; KF937400.1
HS191		*P. syringae* pv. *syringae*	Ravindran *et al*., 2015	Australia	millet	PG 02b	CP006256.1
KBNS84		*Ps. yringae* pv. *morsprunorum*	Balaz and Popovic 2015	Serbia	Sweet cherry	PG03	KR051367.1; KR051339.1; KR051311.1; KR051283.1

*: http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl

**: No Data

Annex 2: Strains' information used in hopI1 phylogeny

Hop names	**Strain names**	**Pathovar abbreviations**	**Pathovars**	**Reference**	**Source or NCBI accession number**
hopI1	T1	*Pto*	*tomato*	Almeida *et al*. 2008	PPI*
hopI1	NCPPB 1108	*Pto*	*tomato*	Vinatzer (unpublished)	PPI*
hopI1	NCPPB 3681	*Pae*	*aesculi*	Green *et al*. 2010	PPI*
hopI1	2250	*Pae*	*aesculi*	Green *et al*. 2010	PPI*
hopI1	B728A	*Psy*	*syringae*	Greenberg and Vinatzer 2003	PPI*

hopI1	B64	*Psy*	*syringae*	Dudnik and Dudler 2013	PPI*
hopI1	NCPPB 3335	*Psv*	*savastanoi*	Rodríguez-Palenzuela *et al*. 2010	PPI*
hopI1	MAFF302278	*Pla*	*lachrymans*	Baltrus *et al*. 2011	PPI*
hopI1	MAFF 302273	*Pac*	*aceris*	Baltrus *et al*. 2011	PPI*
hopI1	ICMP 18884	*Pan Psa-V*	*actinidae*	McCann *et al*. 2013	PPI*
hopI1	ICMP 9617	*Pan Psa-J PT*	*actinidae*	McCann *et al*. 2013	PPI*
hopI1	ICMP 18803	*Pan LV*	*actinidae*	McCann *et al*. 2013	PPI*
hopI1	089_23	*Pae*	*aesculi*	Baltrus *et al*. 2011	PPI*
hopI1	R4	*Pgy*	*glycinea*	Baltrus *et al*. 2011	PPI*
hopI1	MAFF 30	*Pja*	*japonica*	Baltrus *et al*. 2011	PPI*
hopI1	MAFF301315	*Pla*	*lachrymans*	Baltrus *et al*. 2011	PPI*
hopI1	ES4326	*Pma*	*maculicola*	Baltrus *et al*. 2011	PPI*
hopI1	MAFF301020	*Pmo*	*mori*	Baltrus *et al*. 2011	PPI*
hopI1	1704B	*Ppi*	*pisi*	Baltrus *et al*. 2011	PPI*
hopI1	ATCC11528	*Pta*	*tabaci*	Baltrus *et al*. 2011	PPI*
hopI1	DSM50252	*Ptt*	*aptata*	Baltrus *et al*. 2011	PPI*
hopI1	1448A (race 6)	*Pph*	*phaseolicola*	Joardar,*et al*. 2005	PPI*
hopI1	Pss strain61	-	*syringae*	Jelenska *et al*. 2007	DQ401067.1
hopI1	PssCit7	-	*syringae*	Jelenska *et al*. 2007	DQ401068.1
hopI1	PssHS191	-	*syringae*	Ravindran *et al*., 2015	CP006256.1
hopI1	PssB301D	-	*syringae*	Ravindran *et al*., 2015	CP005969.1
hopI1	PssUMAF0158	-	-	Martinez-Garcia *et al*. 2013	CP005970.1
hopI1	Ps.lapsa(ATCC10859)	-	*lapsa*	Kong *et al*. 2015	CP013183.1
hopI1	Ps.pv.*solidagae*	-	*solidagae*	Thakur *et al*. 2015	KPY56707.1
hopI1	Ps.pv.*atrofaciens*	-	*atrofaciens*	Thakur *et al*. 2015	KPW11380.1
hopI1	Ps.pv.*coryli*	-	*coryli*	Thakur *et al*. 2015	KPW98445.1
hopI1	Ps.pv.*aptata*	-	*aptata*	Thakur *et al*. 2015	KPY99334.1
hopI1	Ps.pv.*japonica* (M301072)	-	*japonica*	Baltrus *et al*. 2011	EGH27852.1
hopI1	Pa.pv.*morsprunorum* (M302280)	-	*morsprunoru m*	Baltrus *et al*. 2011	EGH14784.1
hopI1	Ps.pv.*berberidis*	-	*berberidis*	Thakur *et al*. 2015	KPW50099.1
hopI1	Ps.pv.*delphinii*	-	*delphinii*	Thakur *et al*. 2015	KPX20976.1
hopI1	Ps.pv. *maculicola*	-	*maculicola*	Thakur *et al*. 2015	KPX76415.1
hopI1	Ps.pv.*apii*	-	*apii*	Thakur *et al*. 2015	KPW27661.1
hopI1	Ps.pv.*actinidiae* (ICMP19073)	-	*actinidiae*	McCann *et al*. 2013	EPM45399.1

*: http://www.pseudomonas-syringae.org/

Annex 3: Strains' information used in hopAE1 phylogeny

Hop names	Strain names	Pathovar abbreviations	Pathovars	Reference	Source or NCBI accession number
hopAE1	B728A	*Psy*	*syringae*	Greenberg and Vinatzer. 2003	PPI*
hopAE1	T1	*Pto*	*tomato*	Almeida *et al*. 2008.	PPI*
hopAE1	Max13	*Pto*	*tomato*	Vinatzer, B.(unpublished)	PPI*
hopAE1	K40	*Pto*	*tomato*	Vinatzer, B.(unpublished)	PPI*
hopAE1	NCPPB 1108	*Pto*	*tomato*	Vinatzer, B.(unpublished)	PPI*
hopAE1	11528	*Pta*	*tabaci*	Studholme *et al*. 2009	PPI*
hopAE1	2250	*Pae*	*aesculi*	Green *et al*. 2010	PPI*
hopAE1	NCPPB 3681	*Pae*	*aesculi*	Green *et al*. 2010	PPI*
hopAE1	NCPPB 3335	*Psv*	*savastanoi*	Rodríguez-Palenzuela *et al*. 2010	PPI*
hopAE1	Cit7	*Psy*	*none*	Baltrus *et al*. 2011	PPI*
hopAE1	MAFF302278	*Pla*	*lachrymans*	Baltrus *et al*. 2011	PPI*
hopAE1	MAFF 302273	*Pac*	*aceris*	Baltrus *et al*. 2011	PPI*
hopAE1	089_23	*Pae*	*aesculi*	Baltrus *et al*. 2011	PPI*
hopAE1	R4	*Pgy*	*glycinea*	Baltrus *et al*. 2011	PPI*
hopAE1	MAFF301315	*Pla*	*lachrymans*	Baltrus *et al*. 2011	PPI*
hopAE1	MAFF301020	*Pmo*	*mori*	Baltrus *et al*. 2011	PPI*
hopAE1	1_6	*Por*	*oryzae*	Baltrus *et al*. 2011	PPI*
hopAE1	ICMP 18884	*Pan Psa-V*	*actinidae*	McCann *et al* (2013)	PPI*
hopAE1	ICMP 9617	*Pan Psa-J PT*	*actinidae*	McCann *et al* (2013)	PPI*
hopAE1	ICMP 18803	*Pan LV*	*actinidae*	McCann *et al* (2013)	PPI*
hopAE1	ATCC11528	*Pta*	*tabaci*	Baltrus *et al*. 2011	PPI*
hopAE1	1448A (race 6)	*Pph*	*phaseolicola*	Joardar,*et al*. 2005	PPI*
hopAE1	Pa.pv.*aesculi*	-	*aesculi*	Thakur *et al*. 2015	KPW10499.1
hopAE1	Pa.pv.*myricae*	-	*myricae*	Thakur *et al*. 2015	KPX98913.1
hopAE1	Ps.pv.*mori*	-	*mori*	Thakur *et al*. 2015	EGH21419.1
hopAE1	Pspv.*lachrymans*	-	*lachrymans*	Thakur *et al*. 2015	KPX57714.1
hopAE1	Ps.pv.*morsprunorum*	-	*morsprunorum*	O'Brien *et al*. 2015	KPC43215.1
hopAE1	Ps.pv.*maculicola*	-	*maculicola*	O'Brien *et al*. 2015	KPB81218.1
hopAE1	Ps.pv.*papulans*	-	*papulans*	Thakur *et al*. 2015	KPY35564.1
hopAE1	Ps.pv.*aceris*	-	*aceris*	Thakur *et al*. 2015	KPW06338.1
hopAE1	B301D	-	*syringae*	Ravindran *et al*., 2015	CP005969.1
hopAE1	HS191	-	*syringae*	Ravindran *et al*., 2015	CP006256.1
hopAE1	UMAF0158	-	-	Martinez-Garcia *et al*. 2013	ALE00493.1

*: http://www.pseudomonas-syringae.org/

Annex 4: Media used in this study

NA	Quantity for 1L of SDW (g)
Nutrient agar	28
NAS	Quantity for 1L of SDW (g)
Nutrient agar	28
Sucrose	50
KB	Quantity for 1L of SDW (g)
Protease peptone 3	20
K_2HPO_4	1.5
$MgSO_4.7H_2O$	1.5
Glycerol	10 ml
Agar	16
1A	Quantity for 1L of SDW (g)
L (-) arabitol	3.04
NH_4NO_3	0.16
KH_2PO_4	0.54
K_2HPO_4	1.04
$MgSO_4.7H_2O$	0.25
Sodium taurocholate	0.29
Crystal violet	2 ml
Agar	15
Actidione	1 ml
$Na_2SeO_3.5H_2O$	1 ml
YDC	Quantity for 1L of SDW (g)
Glucose	20
Yeast extract	10
Calcium carbonate	20
Agar	18

LB medium	Quantity for 1L of SDW (g)
Tryptone	10
Yeast extract	5
NaCl	10
Arginine	Quantity for 1L of SDW (g)
Peptone	1
NaCl	5
K_2HPO_4	0.3
Agar	3
Phenol red	1 mg
Arginine HCl	30
Gelatine	Quantity for 1L of SDW (g)
Lab-Lemco broth	8
Gelatin	40
Aesculine	Quantity for 1L of SDW (g)
Peptone	10
Aesculin	1
Sodium citrate ($NaH_2C_6H_5O_7$)	1
Ferric citrate	0.05
agar	15
Tyrosinase	Quantity for 1L of SDW (g)
Glycerol	5ml
Casein hydrolyzate (Oxoid)	10
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.25
L-tyrosine	1
Agar	15

Tartrate	Quantity for 1L of SDW (g)
$MgSO_4.7H_2O$	0.02
NaCl	5
$NH_4H_2PO_4$	1
K_2HPO_4	1
Bromothymol blue (1.5 % in ethanol),	10ml
Agar	15
sodium tartrate	2

Annex 5: Buffers and solutions used in this study

TAE buffer (50X)	Quantity for 1L of SDW
Tris	242g
Na_2EDTA	100ml (0.5 M at pH 8)
Glacial acetic acid	57.1 ml
Oxidase	
N,N,N'N'-Tetramethyl-*1p*-phenylene-diammonium-dichloride solution	1%