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DEVELOPMENT OF NEW TOOLS FOR ON-SITE DETECTION OF *SPIROPLASMA CITRI* THE CAUSAL AGENT OF CITRUS STUBBORN DISEASE

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DEVELOPMENT OF NEW TOOLS FOR ON-SITE DETECTION OF *SPIROPLASMA CITRI* THE CAUSAL AGENT OF CITRUS STUBBORN DISEASE

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To my parents

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Abstract

This research concerns the development of new tools for the on-site detection of *Spiroplasma citri* the causal agent of citrus Stubborn Disease (CSD). Firstly, a Direct Tissue Blot ImmunoAssay (DTBIA), which consist on the transfer of protein antigens from a freshly cut tissue surface to nitrocellulose membrane, that are reacting with enzyme-labelled antigen-specific antibodies, was developed to allow the mass detection of the causal agent of the CSD. The developed protocol was validated through a field survey on two citrus varietal collections located in the main Algerian citrus growing areas. This investigation consented to detect two *S. citri* infected trees among the tested samples. The obtained results and the efficiency of the test were confirmed by molecular assays. The molecular characterization of the newly detected Algerian *S. citri* isolate, evidenced 99% nucleotide homology of the spiralin gene with the Iranian fasa I, isolated from a leafhopper vector. Interestingly, the Algerian isolate also reacted positively with the primer pairs, targeting the *TraG* gene, which is essential for insect transmission and predicts a natural diffusion of the pathogen in the case of the presence of insect vectors. Following these findings, a monitoring of the putative insect vectors of *S. citri*, was carried out in the previously surveyed area, in order to evaluate their presence. Fortunately, among the captured and identified insects, none of them was reported as possible vector of this pathogen.

Furthermore, the development and application of the innovative Loop Mediated Isothermal Amplification detection techniques to *S. citri,* was also performed. In this context, a rapid method of crude plant extract preparation from infected trees was developed for on-site testing. The developed LAMP assay targeting the spiralin gene showed high specificity to *S. citri* and detected DNA at 70 fg/µl, with no crude plant extract inhibition. This isothermal assay was validated in open field using a portable BioRanger device. The LAMP assay detection diagnostic reliability of the on-site procedure was shown to be 100%, efficient, considering that all the tested samples, originated from different cultivars, showed similar results with the qPCR assay detection.

Finally, the genetic diversity among the Mediterranean and American *S. citri* isolates was investigated. In this framework, spiralin gene sequences of eleven isolates were obtained and analyzed. The comparison of the translated amino acid, confirmed the presence of conserved region in all the tested isolates.

Keywords: DTBIA, Loop-Mediated Isothermal Amplification, pathogen, crude plant extract, Molecular characterization, Algeria

Riassunto

Questa ricerca ha riguardato lo sviluppo di nuovi strumenti per la diagnosi *in-situ* di *Spiroplasma citri*, l'agente causale della malattia dello "Stubborn" degli agrumi (*Citrus Stubborn Disease*, CSD). In primo luogo, è stato messo a punto il metodo di diagnosi massale *Direct Tissue Blot ImmunoAssay* (DTBIA), che consiste nel trasferimento di antigeni proteici da una sezione di tessuto appena tagliata su una membrana di nitrocellulosa, e successiva aggiunta di anticorpi marcati con enzima specifici per *S. citri.* Il protocollo sviluppato è stato applicato per la validazione nel monitoraggio in campo su due collezioni varietali di agrumi site in importanti aree agrumicole Algerine. L'indagine ha consentito di rilevare la presenza di due piante infette da *S. citri* tra i campioni saggiati. I risultati ottenuti e l'efficienza del test sono stati confermati dai risultati dei saggi molecolari. La caratterizzazione molecolare del gene spiralina dei due isolati di *S. citri* Algerini ha evidenziato un'omologia di sequenza nucleotidica del 99% con il corrispondente gene dell'isolato Iraniano fasa I, ottenuto da cicalina vettrice del patogeno. È interessante notare che l'isolato Algerino è stato identificato anche dalle coppie di primer che rilevano il gene *TraG*, che è essenziale per la trasmissione, suggerendo quindi la sua potenziale diffusibilità in natura in presenza di insetti vettori. A seguito dei risultati ottenuti, è stato effettuato uno studio sui potenziali insetti vettori di *S. citri* presenti nella zona monitorata. Fortunatamente, tra gli insetti catturati ed identificati, nessuno era segnalato come potenziale vettore di questo patogeno.

Inoltre, è stata messa a punto una procedura diagnostica con il metodo LAMP per lo *S. citri.* A questo scopo è stato sviluppato un metodo rapido di preparazione degli estratti vegetali grezzi per il saggio *in-situ*. Il saggio LAMP sviluppato per il gene spiralina ha mostrato un'elevata specificità per *S. citri* e ha rilevato DNA a concentrazioni di 70 fg/µl, senza alcuna inibizione da parte dell'estratto grezzo della pianta. Questo test isotermico è stato validato in pieno campo utilizzando un dispositivo BioRanger portatile. I campioni testati sono stati prelevati da cultivar diverse e le analisi qPCR del saggio LAMP *in-situ* hanno dato risultati simili a quelli ottenuti con la LAMP tradizionale, rivelandosi pertanto totalmente affidabili.

Infine, è stata studiata la variabilità genetica tra gli isolati di *S. citri* mediterranei e quelli americani. A questo scopo sono state analizzate sequenze del gene spiralina di undici isolati. Dal confronto delle sequenze amminoacidiche è stato confermato che il gene spiralina contiene una zona conservata che non varia nei diversi isolati.

Parole chiavi: DTBIA, Loop-Mediated Isothermal Amplification, patogeno, estratto grezzo, Caratterizzazione molecolare, Algeria.

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Chapter I. General introduction

Citrus

The genus Citrus belongs to the Rutaceae family, sub-family Aurantoideae. The various species of this genus are all believed to be native to the subtropical and tropical regions of Asia and the Malay Archipelago, and to have spread from there to other sections of the world and most of them have been cultivated from remote ages (Reuther, 1989; Davis and Albrigo, 1994).

Four groups of citrus are commercially cultivated worldwide as scions or rootstock. Because of its adaptability to different climatic conditions and its wide range of cultivars, sweet orange *(Citrus sinensis)*, which originated from the northeastern region of India and central China, is considered to be the most economically important group of citrus (Davis and Albrigo, 1994; Timmer, 1999).

The world citrus production and consumption have grown strongly since the mid - 1980s. The production of the different citrus species has expanded rapidly, and even faster growth has been realized for processed citrus products like juices. Citrus fruit are cultivated in the Northern and Southern Hemisphere, accounting for around 70% of total citrus production, in over 140 countries on five continents. The worldwide citrus estimated production for 2014 was almost 140 million tons (FAO STAT, 2015).

As any other commercial crop, Citrus can be affected by several pest problems. The diseases control caused by fungi, bacteria, viruses or nematodes, is one of the most expensive elements in the citrus production, representing around 5% of the total direct costs of production. (Garnsey and Lee, 1988). Among these pests, Huanglongbing (HLB), also known as citrus greening, is currently the most devastating disease of citrus worldwide. The disease is associated with the phloem-limited bacterium *Candidatus* Liberibacter asiaticus, *Candidatus* Liberibacter africanus and *Candidatus* Liberibacter americanus (Duan *et al.*, 2009). In addition, *Citrus Tristeza Virus* (CTV) represents also a worldwide problem whose management usually involves vector control, cross-protection, and the use of disease free buds and rootstock, among other practices. On the other hand, Stubborn, which is caused by *Spiroplasma citri*, showed a recent re-emergence in California, after a relatively quiescent period. This disease constitutes a significant threat to California citrus industry and for other countries (Turkey, Israel, Syria, Morroco and Egypt).

Stubborn disease

The citrus disease caused by *S. citri* was initially called “stubborn” in California, while in Israel it was called “little leaf” because of its symptoms (Markham *et al.*, 1974; Calavan and

Oldfield, 1979). Later, the name of Stubborn became universal and started to be used by the scientific community. Symptoms of stubborn are relatively nonspecific and can be confused with those of other biotic citrus diseases such as citrus tristeza or other pathogens (Mello *et al.*, 2008). The mottling in leaves is similar to that seen with abiotic conditions caused by iron, zinc and manganese deficiency (Timmer, 1999). Although the stubborn disease is rarely lethal, the affected trees show varying degrees of stunting and have a condensed or bunchy type and upright position of growth with shortened stem internodes and numerous axillary buds. The trees usually develop unseasonal growth flushes and blossoms, which results in various ages and sizes of fruit on the tree (Calavan, 1979; Yokomi *et al.*, 2008; Nejat *et al.*, 2011). Leaves usually are smaller than normal, mottled, cupped shaped with rounded tip and leathery appearance. Leaves show variable chlorotic patterns that may resemble nutritional deficiency (Bové, 1995). Fruits of infected trees usually look lopsided with a curved columella, the albedo may become blue (mainly in grapefruit and tangelos). Fruits often do not color at the stem end (acropetal) and the seeds produced are often aborted, moreover, the small fruits drop is also common. Finally, the infected fruits may have an insipid or bitter flavor (Bové and Garnier, 2000; Yokomi *et al.*, 2008).

When the citrus are grafted with *S. citri* infected plant material and kept in greenhouses under temperatures of 35 °C/27 °C (day/night), they have a latent period of around two months. The occurrence of mildly and severely symptomatic CSD trees under field conditions is affected by temperatures, and can also be associated with bacterial titer within the plant and/or strain virulence (Calavan and Oldfield, 1979; Calavan and Bové, 2012). Little is known about the mechanisms of *S. citri* pathogenicity and plant symptom expression. Mutagenesis using random insertions of the transposon Tn4001 demonstrated that the fructose operon is somehow related to *S. citri* virulence and to the length of the latent period (Gaurivaud *et al*., 2000). Fructose and glucose play distinct roles in spiroplasmal pathogenicity; the fructose utilization by the spiroplasmas deprives the companion cells of fructose, thereby impairing sucrose loading in the sieve elements. Alternatively, preferential use of fructose increase invertase activity, leading to glucose accumulation in source organs (mature leaves), sugar depletion in sink organs (young leaves, roots), inhibition of photosynthesis, physiological disorders and stunting (Bové and Garnier, 2003; Renaudin, 2006).

Among all citrus species, *C. sinensis, C. paradisi, C. reticulata x C. paradisi* and *C. reticulata* are considered the most susceptible to the disease in the field (Calavan and Oldfield, 1979). In addition to citrus, *S. citri* can also infects other commercial crops, firstly horseradish

(*Armoracia rusticana*) (Lee *et al*., 2006) and more recently, it has also been isolated from the carrot (*Daucus carota*) (Mello *et al.*, 2009; Cebrián *et al.*, 2010; Swisher *et al.*, 2016) and from celery in Spain (Alfaro-Fernández *et al.*, 2015). In total, at least 38 plant species comprising 12 botanical families are considered to be naturally infected by *S. citri.* Possible migratory routes of the leafhopper vector *Circulifer tenellus* (Fletcher *et al.*, 1996) probably introduced *S. citri* into the Northern states of United States, causing a disease in Illinois and Maryland horseradish called "brittle root", which is characterized by stunting and chlorosis (Fletcher *et al*., 1981; Fletcher, 1983). The first discovered plant infected with *S. citri* in California (Granett *et al.*, 1976), in Arizona (Allen and Donndelinger, 1981) and Morocco (Bové *et al*., 1978) were not belonging to citrus family (*Rutaceae*) but periwinkle of Madagascar (*Catharanthus roseus*). In California, *S. citri* was isolated from weeds like plantin (*Plantago sp*.), ornamental plants such as marigold (*Tagetes erecta*), aster (*Callistephus chinensis*), cultivated plants such as lettuce (*Lactuca sp*.), radish (*Raphanus sativum*), watermelon (*Citrullus lanatus*) and some fruit trees, cherry trees, peach and pear trees (Calavan and Bové, 1989). Additionally, *S. citri* could be transmitted experimentally by grafting or insect vector to about 80 different plant species.

Spiroplasma citri

S. citri belongs to the genus *Spiroplasma* and taxonomically included in the kingdom of *Bacteria*, Phylum *Tenericutes*, Class *Mollicutes*, Order *Entomoplasmatales*, Family *Spiroplasmataceae* and Genus *Spiroplasma* (Figure 1) (Gasparich, 2010). *Mollicutes* represent the smallest and simplest free-living and self-replicating cells (Yus *et al.*, 2009). They are a unique class of organisms: in the Gram test, they are negatively stained but they are phylogenetically related to gram-positive eubacteria with low G+C content (Weisburg *et al.*, 1989; Bové, 1993). The *Mollicutes* species are characterized by a complete lack of cell walls and external or internal flagella (Fraser *et al.*, 1995); they have smaller genomes than most bacteria, including those with whom they share a common ancestor, this led to the general idea that this class of bacteria has evolved by reductive evolution involving significant gene losses. One landmark in *Mollicutes* evolution is the loss of the ability to synthesize a cell wall; these characteristics, explains the ability of these organisms to adopt different cell morphologies (Andersson and Kurland, 1998; Sirand-Pugnet *et al.*, 2007). Plants harbor two types of Mollicutes: the phytoplasmas and the spiroplasmas; both are endogenous, wall-less bacteria and localized exclusively in the sieve tubes of affected plants (Bové and Garnier, 2003). In the early 1970s, the spiroplasmas were described for the first time and were demonstrated to be the causal agents of several plant diseases (Davis, 1973).

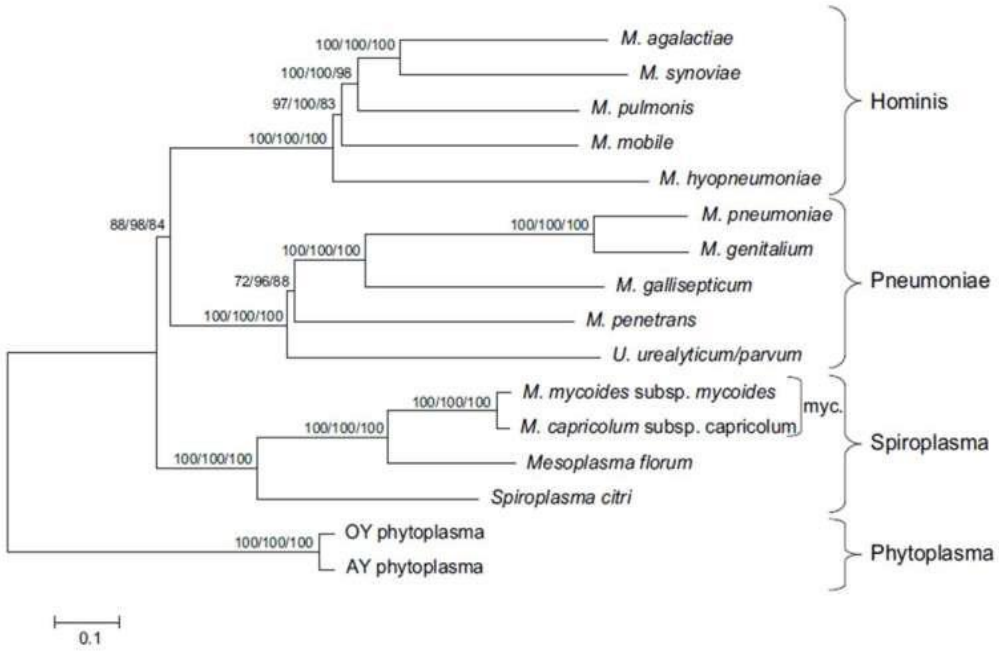

Figure 1. Phylogenetic tree based on the alignment of 91 shared proteins of 16 *Mollicute* (Sirand-Pugnet *et al.* 2007)

The spiroplasma was the first cultured mollicute of plant origin. The first spiroplasma seen to be helical in plant sap, was the corn stunt agent, but it was cultured only in 1975, andcharacterized in 1986 as *Spiroplasma kunkelii* (Chen and Liao, 1975; Whitcomb *et al*., 1986). The second sieve-tube restricted spiroplasma was *Spiroplasma phoeniceum*, cultured in 1983 from naturally infected periwinkle plants in Syria. *Spiroplasma citri* is probably the most studied and the best understood phytopathogenic mollicute today (Saillard *et al.*, 1987; Garnier *et al.*, 2001; Bové and Garnier, 2003). After being obtained in pure culture, *S. citri* became the best characterized phytopathogenic mollicute at the moment. The first observations with a dark-field microscope reveal that *S. citri* has an helical morphology and demonstrates a rotary motility despite the absence of demonstrable organelles usually associated with motile, helical microorganisms (Cole *et al.*, 1973). *S. citri* owes motility to two types of movements, the first was rapid rotatory motions, which could reverse, leading to minimal back and forth progress, and the second was a slow undulation and bending of filaments sometimes with a slight rotatory component, but not leading to change of position of the filament (Davis and Worley, 1973; Daniels *et al.*, 1980). This rotational motion associated with its helical morphology allows the spiroplasma to move in viscous medium. On solid medium, this motility has repercussions on the colonies appearance that are diffuse and surrounded by satellites. More recently, some researchers have highlighted the involvement of the *S. citri* cytoskeleton in its motility. This cytoskeleton is composed of fibril protein (Williamson *et al.*, 1991) and the actin-like protein MreB. This cytoskeleton would act as a linear motor capable of flexing thus allowing the cell movement (Trachtenberg, 2004). Complementary work showed that Spiroplasma swimming is driven by the propagation of a pair of kinks along the cell body and that these kinks are generated by a processive change in body helicity (Shaevitz *et al.*, 2005). The growth of *S. citri* is carried out by elongation of an elementary helix with one blunt and one tapered end that develops into an elementary helix with two blunt ends. Growth will start at one end, during the same time; parental helices, which are ready to divide, grow around the constriction to yield two elementary helices with one blunt end and one labeled, tapered end (Garnier *et al.*, 1984). Today, 92% of the genome sequence of *S. citri* GII3 is known (Carle *et al.*, 2010). This genome consists of a 1820 kbp chromosome with 26% G + C bases and seven plasmids (pSciA and pSci1 to 6) which do not have known homologs (Saillard *et al.*, 2008). The size of these plasmids varies from 7.8 kbp for pSciA to 35.3 kbp for pSci6 and their numbers of copies by spiroplasms were estimated between 10 and 14. The plasmidic DNA would thus represent nearly 1.6 Mpb or 47% of the total DNA of *S. citri* GII3. The pSci1-6 have a percentage in G + C (25.6 to 29%) close to that of the chromosome (26 %) while the pSciA at a lower percentage

of 21.3%. Interestingly, none of the coding sequences (CDS) carried by the pSci show homology with a protein of replication (Saillard *et al.*, 2008). Recent work has nevertheless been able to restrict the origin of replication of *S. citri* pSci plasmids to a region containing a single CDS, named pE, and its downstream sequence having binding motifs to the initiator protein DnaA (Breton *et al.*, 2008). In addition, three viral replicative forms *SpV1*, *SpV2* and SpV3 have been identified in the chromosome of *S. citri* (Renaudin *et al.*, 1990; Renaudin and Bové, 1994). SpV2 and SpV3 are two types of tailed bacteriophage, whereas the *SpV1* virus is filamentous with 8.3 kb circular single-stranded DNA. One of the characteristics of this *SpV1* virus is the large number of DNA copies, distributed throughout the chromosome (Carle *et al*., 2010).

***Spiroplasma citri* dispersal**

S. citri is graft-transmissible through infected budwood but is neither seed nor mechanically transmissible. It can be also transmitted via the parasitic plant dodder *Cuscuta compestris* or *C. subinclusa* (Lee *et al.*, 2001; Rangel *et al.*, 2005).

Insect transmission of *S. citri* occurs by leafhoppers which belong to the Order Hemiptera: suborder Homoptera: family Cicacellidae: subfamily Deltocephalinae. *Scaphytopius nitridus* (DeLong) was the first reported insect vector, transmitting *S. citri* from citrus to periwinkle and citrus under greenhouse conditions (Kaloostian *et al.*, 1975; Oldfield *et al.*, 1977a). Later, *Circulifer tenellus* (the beet leafhopper), collected from California orchards, was shown to harbor and to transmit *S. citri* to citrus and periwinkle (Oldfield *et al.*, 1976) and to the weed London rocket (Oldfield *et al.*, 1977b). Under experimental conditions only, *Macrosteles fascifrons* transmited the spiroplasma from aster (*Callistephus chinensis*) to aster, and from horseradish to the Brassicaceous weed yellow rocket (*Barbarea vulgaris*), aster, and turnip (O'Hayer *et al.*, 1983). *Circulifer haematoceps* (synonym: *Neoaliturus haematoceps*) is a key vector in the Mediterranean region including Turkey, Morocco, Syria and France (Corsica) (Fos *et al.*, 1986). In southern Turkey five other leafhoppers were found to carry *S. citri* in their bodies, but only one of them, *Circulifer opacipennis* (Lethierry), was effective in transmission to *Catharanthus roseus* L. (Kersting and Ŝengonca, 1992).

During the transmission cycle, spiroplasmas ingested by leafhopper vectors cross two physical barriers, the gut epithelium (from the lumen to the hemocoel) and then the salivary gland-associated membranes (Figure 2) (to reach the salivary duct). The ingested spiroplasmas infect gut epithelial cells where they multiply to high titers (10^6–10^7/CFU/insect), cross the intestine midgut lining and subsequently invade other organs, including the salivary glands,

from which they are injected into the phloem via salivary secretions during insect feeding (Fletcher *et al.*, 1998; Kwon *et al.*, 1999; Bové *et al.*, 2003; Labroussaa *et al.*, 2011). The crossing of these different barriers involves protein interactions between the spiroplasma cells and insect host cells.

Genetic information and determinants in *S. citri* transmission

S. citri has one of the largest genomes among Mollicutes, with a size around 1.8 Mbp. It is characterized by a high adenosine-thymidine content and utilization of UGA to encode tryptophan instead of being a stop codon as in other organisms. Beyond the circular chromosome, *S. citri* also contains plasmids and virus genomes that contribute to genetic information (Ye *et al.*, 1997; Melcher and Fletcher, 1999). Changes in the DNA sequence can occur because of nucleotide acquisition and loss during DNA replication or repair, deletion, rearrangement or duplication of DNA fragments, integration of extra chromosomal DNA into the spiroplasma chromosome, or DNA exchange between spiroplasma cells. Frequent rearrangements in spiroplasma chromosome partly due to integrated sequences of spiroplasma viruses lead to a remarkable genome instability. The viral DNA sequences usually integrate into the chromosome in one or more copies, either intact or fragmented (Barroso *et al.*, 1990; Melcher and Fletcher, 1999).

Additionally, the genes of several membrane-associated proteins identified in the genomes of spiroplasma species are similar to those of highly variable proteins in mycoplasma species (Yu *et al.*, 2000; Berg *et al.*, 2001; Comer *et al.*, 2007). Membrane-associated proteins were shown to interact with insect microfilaments by colocalization of spiroplasmas with the actin filaments of the leafhopper salivary glands (Sc76, Spiralin, P89, SARP1). A correlation was established between the presence of plasmids pSci and the transmissibility of this bacterium by its insect vector (Berho *et al.*, 2006; Killiny *et al.*, 2006). It appeared that transformants possessing pSci6 are transmitted but with less efficiency than that of the transmissible strain GII3. These results indicate that unlike other pSci plasmids, plasmid pSci6 carries determinants that are critical for insect transmission. The first of these determinants are the ScARPs (*S. citri* adhesion related proteins) proteins (Figure 3). These membrane proteins have strong homologies with the protein ARP1 (adhesion related proteins) of *S. citri* BR3 which is involved in *in vitro* adhesion to insect cells (Yu *et al.*, 2000). In the *S. citri* GII3 strain, eight genes encode eight ScARPs that are carried by plasmids pSci 1 to 5 (Berho *et al.*, 2006).

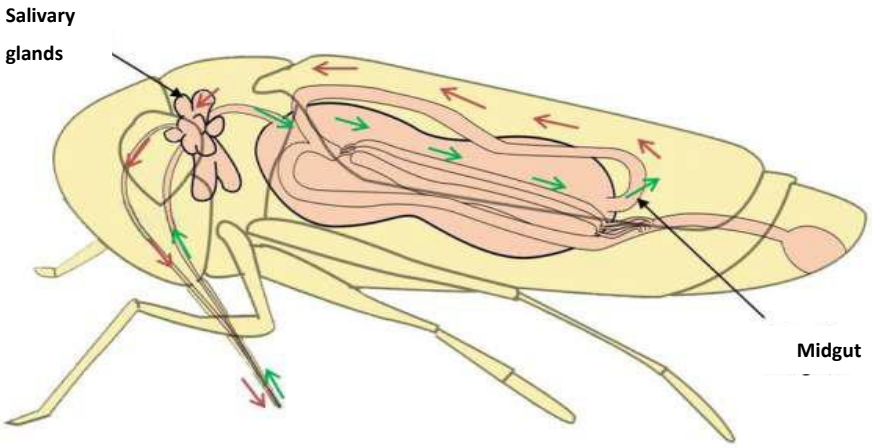

Figure 2. Schematic representation of *S. citri* cycle inside the leafhopper vector

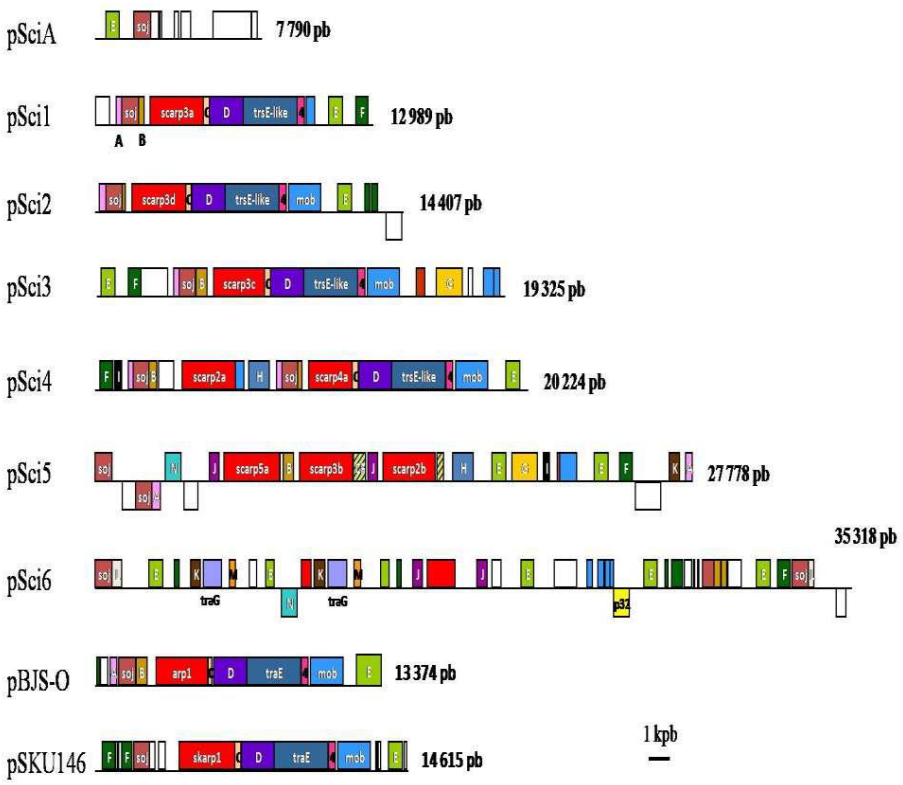

Figure 3. The plasmid of *Spiroplasma citri* annotated pSciA and pSci1-6, pBJS-O and pSKU146. Conserved CDS are represented in colored boxes. Circular plasmids are represented by linear maps to facilitate comparisons (Saillard *et al.,2008*)

The P32 protein is a determinant carried by the plasmid pSci6 and could intervene in the transmission of spiroplasma. This hydrophilic protein of 238 amino acids does not have characteristic domains and has no homology with known proteins. The introduction of the p32 gene in strain 44 seems to modify the localization of spiroplasms at the salivary gland. On the other hand, it does not allow restoring the transmissibility (Killiny *et al.*, 2006). More recently, the involvement of CDS present on the pSci6 has been described. This coding region, annotated *TraG*, is required for the transmission of *S. citri* by the insect vector *C. haematoceps* (Breton *et al.*, 2010). This gene encodes a protein that has homologies with the C-terminal portion of ATPases of the *TraG* / VirD4 family involved in type IV secretion systems. This cytosolic protein, could allow the pathogen to cross the salivary gland barrier or survive inside the insect cells. In *S. citri*, the proteins involved in adhesion are still few and their roles are poorly understood.

Even though Sc76 protein as well as ScARPs proteins that are contributing to transmission, assuming this role of adhesins, till now, only the spiralin was implicated in an interaction with insect proteins (Killiny *et al.*, 2005). Originally, spiralin is known to form a protective "coat" on the spiroplasma surface (Castano *et al.*, 2002). However, this lipoprotein was shown to be be a lectin-like protein that can interact with two glycoproteins of the insect vector *C. haematoceps* (Killiny *et al.*, 2005). Therefore, it seems to be an ideal candidate to have this role in *S. citri* adhesion to insect cells, which is an essential step in crossing the intestinal and salivary glands.

Diagnostic methods

It is crucial to accurately detect and identify pathogens to initiate preventive disease control measures. *S. citri* can be detected by graft inoculation of indicator plants, the Madame vinous sweet orange seedling is recommended as a superior indicator for it. Inoculations for stubborn are most successful when done by side grafting, rather than by bud-grafting practiced with most graft transmissible pathogens of citrus (Rangel *et al*., 2005; Nejat *et al*., 2011). Development of symptoms requires warm or greenhouse temperatures kept at 32-38°C maximum during the day and not below 27°C at night. Definitive symptoms can be predictable eight to 12 weeks after inoculation (Roistacher, 1991).

Furthermore, The enzyme-linked Immunosorbent assay (ELISA) with polyclonal and monoclonal antibodies has been used to detect, compare Spiroplasma isolates for taxonomic purposes and for identifying spiroplasmas directly in plant or insect material (Archer *et al*., 1982; Nejat *et al*., 2011).

Moreover, it was shown by Archer and Best (1980) that ELISA may also be used quantitatively to estimate cell numbers in Spiroplasma cultures. Saillard *et al.* (1987) developed an ELISA protocol but obtained inconsistent results when using citrus leaves as samples; the detection of *S. citri* in leafhoppers by ELISA is more useful for screening the vectors, but the choice of the grinding buffers is essential to avoid positive ELISA reactions with uninfected material. However, in some cases the positive ELISA reaction need to be combined with culturing for a reliable *S. citri* detection when using citrus showing typical and atypical CSD symptoms (Bové *et al*., 1988).

Continuous advances in DNA-based detection methods have provided fast, sensitive and reliable detection and quantification of pathogens, when compared to culture-based identification methods . Most of these techniques rely on polymerase chain reaction (PCR) involving the use of primers developed from sequences of housekeeping genes such as spiralin, 16s ribosomal RNA and adhesion genes (Lee *et al.*, 2006; Saponari *et al.*, 2008; Yokomi *et al.*, 2008; Carle *et al.*, 2010; Yokomi and Sisterson, 2011). Real time PCR assays have been extensively applied to plant pathology. (q) PCR has been a standard molecular method for detection of *S. citri* and improved diagnosis was demonstrated by targeting the high copy number Prophage gene (*SpV1-ORF1*) (Wang *et al.*, 2015). Moreover, the potential of digital droplet ddPCR assay was demonstrated for absolute quantification of *S. citri*. The ddPCR targeting a multi-copy gene such as *SpV1 ORF1* significantly improved the *S. citri* detection in culture and field samples. The increased sensitivity can have additional value for early detection of the pathogen (Maheshwari *et al., 20*17).

Isothermal amplification detection method

For the stubborn disease, no isothermal amplification detection methods have been developed previously. These techniques enabled to overcome the use of PCR thermocyclers for a possible on-site testing. As the name suggests, isothermal amplification of DNA (or RNA) occurs at a constant temperature, which confers to some of these methods the potential to be used in the field using portable instruments (Chang *et al.*, 2012). Loop-mediated isothermal amplification (LAMP) is a very simple, cost-effective and sensitive technique for the detection of specific DNA sequences, first described by Notomi *et al*. (2000). The addition of reverse transcriptase makes it possible to amplify DNA from RNA sequences (RTLAMP) (Curtis *et al.*, 2008).

LAMP does not require initial template denaturation (Nagamine *et al.*, 2001) and employs a DNA polymerase with strand-displacement activity, along with two forward and backward

inner primers (FIP, BIP) and outer primers (F3, B3) which recognize six separate regions within a target DNA (Figure 4) (Tomita *et al.*, 2008). The loop primers (LF, LB) are additional primers designed to anneal at the loop structure in LAMP amplicons, can accelerate and enhance the sensitivity of the LAMP reaction (Nagamine *et al.*, 2002) . The LAMP assay is highly specific as the amplification reaction occurs only when the primers correctly recognize all six regions, within a target DNA. The procedure is rapid and is able to amplify from a single copy to 10^9in one hour at constant temperature; LAMP does not need a sophisticated heating block system that requires a high temperature (90–100 °C), as the amplification take place at a constant temperature of 63 °C for 60 min (Notomi *et al.*, 2000). It is consistently insensitive to extraneous nucleic acids or interference from sample or media components that are problematic for other detection methods (Kaneko *et al.*, 2007; Niessen, 2015).

Frequently used detection methods include turbidity, agarose gel electrophoresis, calorimetric detection using naked eyes, and detection using UV light. Real-time monitoring based on turbidity using real-time turbidity meter (e.g. Loopamp Real time Turbidimeter LA-200, LA-320, LA-500) is commonly used for LAMP product detection. LAMP assay was monitored for the white precipitation caused by the presence of magnesium pyrophosphate, $Mg_2P_2O_7$(a by-product of LAMP reaction) at optical density 650nm every six seconds. However, visualization of LAMP DNA product by gel electrophoresis required the opening of the reaction tubes, therefore increase the risk of carry-over contamination. An alternative to gel electrophoresis, intercalating dyes were used for direct detection of the DNA products, for example, calcein, SYBR green I and hydroxynapthol blue (HNB) dye. Calcein dye, a metal ion binding fluorophore, which was added before isothermal incubation will form an insoluble salt complex, manganese-pyrophosphate (manganese ion, $Mn^{2+}$from calcein whereas pyrophosphate is the by-product of LAMP reaction), thus making the reaction fluorescent. Subsequently, the fluorescence is further intensified when free calcein binds to magnesium ions ($Mg^{2+}$from LAMP reaction mixture) and could be easily be observed under UV light (365nm) (Sahoo *et al.*, 2016). In addition, monitoring the isothermal LAMP reaction in real-time has been made available using a LAMP master mix that contains an engineered GspSSD LF DNA Polymerase with strand displacement and reverse transcriptase activities and a double-strand DNA binding dye (OptiGeneLimited). Moreover, a fluorescence resonance energy transfer (FRET)-based probes technology was described by Kubota *et al.* (2011), namely, assimilating probes. These probes are designed in a particular architecture, where a quenching strand is displaced from a partially complementary fluorescent strand, during the process of DNA synthesis. Assimilating probes

have been used for sequence specific detection of the LAMP amplicon, but also for quantification and multiplexing purposes (Tanner and Evans, 2014; Kubota and Jenkins, 2015).

LAMP could be one of the diagnostic method alternative in relation to its rapidity, sensitivity and specificity. The most significant advantage of LAMP would be its rapidity where LAMP allows immediate diagnosis. Unlike any other nucleic acid amplification technique for example PCR, LAMP could be performed in just 30 min rather than at least 90 min for PCR. In addition, a LAMP amplification proceeds rapidly since the initial heat denaturation for the DNA template is not required. It works as a fast and simple diagnostic tool for the quick detection and identification of microbiological infections. Besides being simple to operate and easily adapted to any field circumstances and environments, LAMP has all the features required for real-time assays, especially in high sensitivity. LAMP is also a beneficial and valuable tool for use in developing countries. Thanks to its ease of performance without needing any advanced equipment or experts to operate.

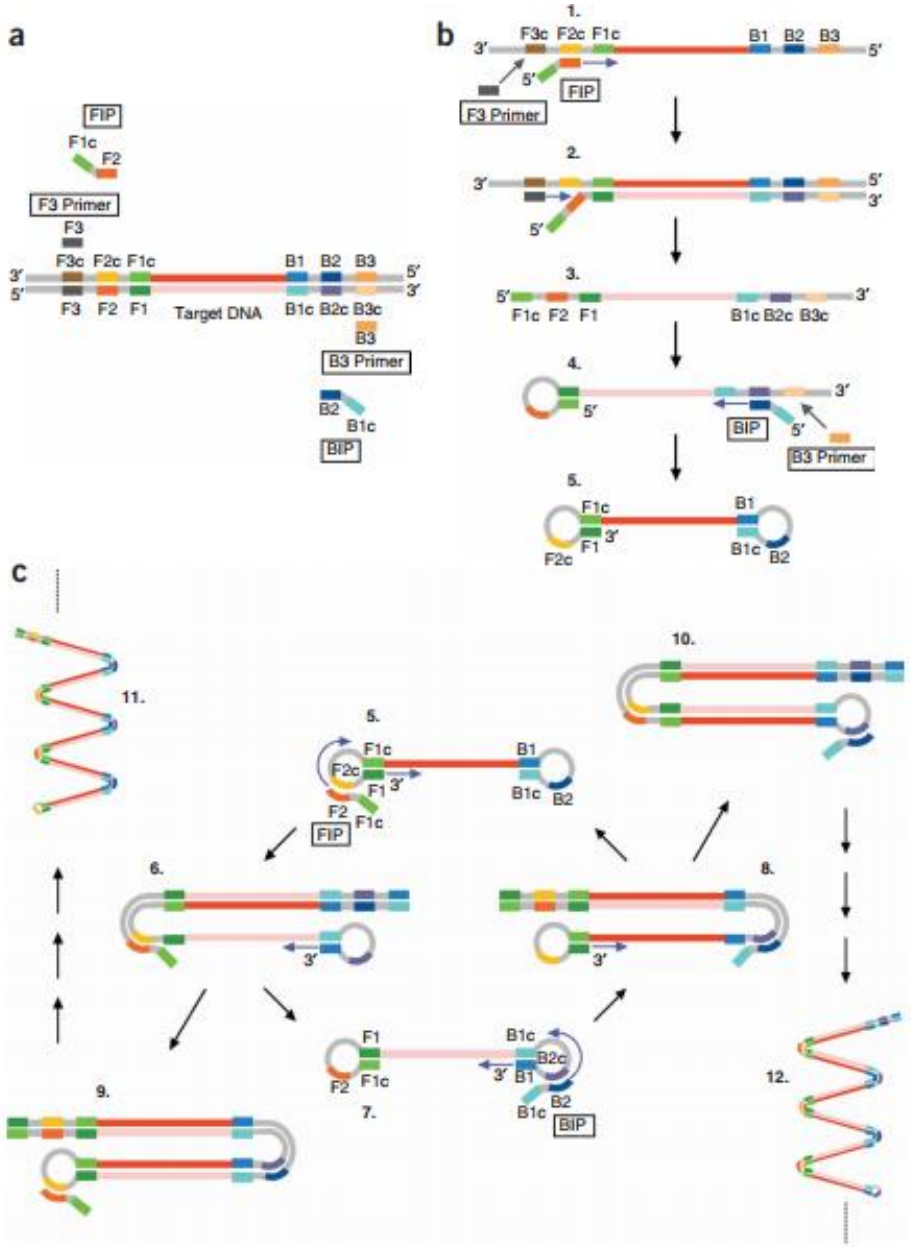

Figure 4. Principle of loop-mediated isothermal amplification (LAMP) method.

(a) LAMP outer primers (F3 and B3) and inner primers (FIP and BIP) targeting six distinct regions within a target DNA; **(b)** Starting structure producing step: FIP primer anneals to the targeted sequence and initiates DNA synthesis. F3 primer carries out the strand -displacement DNA synthesis and produces a single stranded DNA that forms a DNA loop structure at 5' end. This structure works as a template for B3 and BIP primers that anneal to the 3' end. A double stem-loop structure is formed;**(c)** Cycling amplification step: FIP and BIP primers bind to the loops and synthesize new DNA strands. The extension of each primer opens the loop end and elongated structures are continuously produced (Tomita *et al. 2008*).

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Thesis objectives

The aim of this research project was the development of new tools for the potentially suitable on-site detection of *Spiroplasma citri* the causal agent of Citrus Stubborn Disease (CSD).

Specific goals of this research were:

1. Setting up of Direct Tissue Blot ImmunoAssay (DTBIA) protocol for large-scale detection of *Spiroplasma citri* and molecular analysis of the identified *S. citri* isolates in Algerian citrus groves (Chapter II);
2. Development and application of Loop Mediated Isothermal Amplification (LAMP) assay for the detection of *S. citri* (Chapter III);
3. Evaluate the genetic diversity among isolates of *S. citri* from different locations, Mediterranean and American (Chapter IV).

Chapter II

Setting up of Direct Tissue Blot ImmunoAssay (DTBIA) protocol for large scale detection of *Spiroplasma citri* and molecular analysis of the identified *S. citri* isolates in Algerian citrus groves

The experiments described in this chapter were mostly carried out in the CIHEAM-Bari (Italy). While, the field investigation was performed in the ITAFV (Institut Technique de Arboriculture Fruitière et de la Vigne), Algeria.

Introduction

Citrus stubborn disease (CSD), is an important vascular disease in several hot and arid citrus growing regions, it was first noticed around 1915 in stunted trees of Navel orange in California (Calavan and Oldfield, 1979). The disease is caused by a phloem limited bacterium, *Spiroplasma citri*, which was the first mollicute of plant origin to be obtained in culture (Saglio *et al.*, 1973a).

The pathogenic spiroplasmas are transmitted from plant to plant by phloem-feeding leafhoppers in a propagative manner. *Circulifer tenellus* (Baker) was identified as the major vector of *S. citri* in California (Oldfield *et al.*, 1977), whereas in the Mediterranean area, *C. haematoceps* is regarded as the main vector of this pathogen (Bové *et al.*, 1988).

The CSD epidemiology is influenced by several factors, associated to the strain of the causal agent, its plant hosts and the ability of the vectors to feed on different plant species (Saglio *et al.*, 1973b; Mello *et al.*, 2008).

Field diagnosis of CSD is difficult and often inaccurate. Routine detection of *S. citri* is mainly carried out by culturing in cell-free liquid medium and examination of the organism for helical morphology and motility by dark field microscopy; however, Polymerase chain reaction (PCR) detection of *S. citri* is routinely used, with primers targeting different genes. Meanwhile, qPCR can be more sequence specific and faster than PCR because no post amplification processing is required. However, both PCR and qPCR requires skilled technicians and PCR reaction optimization is critical. Relatively to this aspect and considering that DTBIA was already successfully applied in the mass detection of citrus disease agents, such *Citrus tristeza virus*, *Citrus psorosis virus* (Garnsey *et al.*, 1993; Cambra *et al.*, 2000; D'Onghia *et al.*, 2001), *Xylella fastidiosa* from citrus affected by variegated chlorosis (Garnier *et al.*, 1993) and other infectious agents (Lin *et al.*, 1990), and does not require equipped laboratory and minimal technical expertise to analyses plant samples directly in the field. One of the objective of this study is to set up and validate the DTBIA for the detection of *S. citri* from plant material, and successively to the molecular characterization of the identified new strains of this pathogen. Finally, in order to investigate on the possible diffusion of the pathogen, a monitoring of the putative insect vectors of *S. citri* was also scheduled in the surveyed area.

Materials and Methods

I.1.1 Setting up of Direct Tissue Blot ImmunoAssay (DTBIA) protocol

In order to overcome limitations of some available *S. citri* detection techniques for large-scale detection, DTBIA assay, a process of transferring protein antigens from a freshly cut plant tissue to nitrocellulose membrane and then detected with enzyme-labelled antigen-specific antibodies, has been developed. The protocol developed was based on similar approach applied recently for the mass detection of *Xylella fastidiosa* in olive trees (Djelouah *et al.*, 2014).

To assess the efficiency of the developed technique in citrus plants, several parameters were compared; including the type of the explant printed (petiole, stem, ovaries, fruit peduncle), the type of blotted nitrocellulose membrane (0.20 and 0.45 µm pore size); the solutions for the saturation of protein-binding sites (bovine serum albumin (BSA), fat milk, gelatin and polyvinyl alcohol (PVA). All these components were also coupled with an evaluation of the different time incubations (1 hour for BSA, gelatin and fat milk, from 2 to 10 min for PVA) and concentration of the solution used (2% BSA, 2 % gelatin, 1% fat milk, 1 mg/ml PVA).

To this aim, two types of Protran nitrocellulose membranes (Sigma-Aldrich) with high affinity for binding proteins were cut to an appropriate size (7 cm X 7 cm) and delimited with a grid of suitable size (1cm X 1cm). Different types of explants belonging to *S. citri* infected and healthy citrus plants were blotted on the membranes. Following these steps, the membranes were placed in the blocking solution using different incubation times and concentrations of BSA, fat milk, gelatin, PVA (Sigma Aldrich). After saturation of protein-binding sites with the cited solutions on a shaker (orbital shaker) and washing the blotted membranes with PBS containing 0.05% Tween 20, they were exposed to *S. citri* alkaline phosphatase-conjugated polyclonal antibodies (Sediag, France) at different dilutions of conjugate buffer (from 1:50 to 1:200). Membranes were then stained by immersion in a solution including one tablet of SigmaFastTM BCIP-NBT dissolved in 10 ml of distilled water, and then incubated at room temperature until a purple-violet color appeared in the positive controls. The reaction was then stopped by washing membranes with tap water. After drying at room temperature, the membranes were observed under a low power magnification lens (×10 or ×20).

I.1.2 Sampling

The developed protocol was validated through a survey performed in the Mitidja region, belonging to the main Algerian citrus growing area, where symptoms associated to CSD i.e. stunted trees, short leaf internodes, leaf mottling, unseasonal blossoming and lopsided fruits have been already observed, but without any investigations on the identification of the causal agent. During the summer season 2014, 112 samples were collected from the same area. Among

these samples, 57 were belonging to the varietal collections located at Boufarik, whereas the remaining 55 collected samples were belonging to the Tessala el Merdja, varietal collection. Both orchards were managed by the *Institut Technique d'Arboriculture Fruitière et de la Vigne* (I TAFV), where a fairly large bank of local and imported citrus germplasm are located.

The type of sampling focused on visual inspections and the trees showing putative symptoms associated to the CSD were primarily sampled. The collected samples consisted of leaves, fruit collumella, petiole, stem, and fruit peduncle. All sample type were collected from single tree, The samples were printed onto the nitrocellulose membranes (Fig. 1) and the performance of the DTBIA assay was assessed. All the 112 samples were tested again by PCR, using SC1 primer pairs targeting the spiralin gene, the most abundant membrane proteins in *S. citri* (Foissac *et al.*, 1996).

I.1.3 DNA extraction and PCR

Spiroplasma genomic DNA was extracted from 600-800 mg of fresh tissues (Columella/leaves) following CTAB/Chloroform-Isoamyl Alcohol DNA Extraction Protocol described by (Doyle, 1990). *S. citri* detection and characterization were performed by following the protocols developed by Foissac *et al.* (1996), Yokomi *et al.* (2008) and Breton *et al.*(2010) and using SC1, P89 and *TraG* primer pairs targeting Spiralin gene, putative adhesion gene and insect transmission gene, respectively (Table 1). Each PCR mixture included 1.5µl of DNA template and 23.5µl of reaction mix, which included 0.25µl DreamTaq polymerase (Promega), 2.5µl DreamTaq Buffer10x, 0.5 µl of 10mM dNTPs and 0.5µl of 10µM forward and reverse primers. The reactions were performed on Bio-Rad™ C1000 thermal cycler. The cycling parameters consisted of 3min of denaturation at 95°C, followed by 40 cycles of 30-45 s at 95°C, 30-60 s at 50-60°C, 30s at 72°C. Each run included two replications each for the unknown sample, the *S. citri* negative control, the positive control consisting of *S. citri* infected tissue extract and the non-template control (NTC).

I.1.4 Cloning and sequence analysis

PCR amplicons were inserted in the cloning pUC18 vector (Agilent Technologies StrataClone PCR Cloning Kit) that was used to transform *Escherichia coli* DH5αn cells. Inserts from four different clones were sequenced in both directions to eliminate any sequence ambiguity (GATC Biotech, Germany).

Table 1. List of the samples collected from both fields

Specie	N° samples	Orchard
Citrus jambhiri	2	Boufarik station
Citrus lumia	2	
Citrus maxima	4	
Citrus medica	10	
Citrus paradisi	10	
Citrus deliciosa	8	
Fortunella margarita	2	
Citrus unshiu 'Owari'	2	
Citrus reticulata	4	
Citrus sinensis	13	
Citrus sinensis	55	Tessala el Merdja station

Table 2. List of PCR primers used for amplifying different genes of *S. citri*

Gene	Primers	Primer sequence	Length (bp)	Acces N°	Ref
Spiralin	SC1-f	ATTTTCAATTTGATGTTTATCAAGACAAC	336	AF012877.1	Foissac *et al.*, 1996
	SC1-r	CAAAATCACTTGCTCCTGCATTTGG			
P89	P89-f	TTGACTCAACAAACGGGATAA	707	AJ969072.1	Yokomi *et al.*, 2008
	P89-r	CGGCGTTTGTTAATTTTTGGTA			
TraG	*TraG*-f	TCTACCATTCGTGGAGAACAA	561	AJ969074	Breton *et al.*, 2010
	TraG-r	*AGTTGCTAATTCTTCGGCACTC*			

- **Ligation**

Two µl of each PCR product were mixed with 3 µl of cloning buffer and 1 µl of vectors and the mix was incubated at room temperature for 15 min.

- **Transformation of competent cells**

Fifty microliters of bacterial suspension were added to the mix and immerged in ice for 20 min. After a heat shock at 42°C for 45 sec, the mix was replaced in ice. The samples were added to 800 µl of LB (Lysogeny Broth) without ampicillin and shacked at 37°C for 90 min. after, the samples were centrifuged at 5 000 rpm for 3 min, and the pellet (100 µl) was plated using sterile glass rod onto solid LB plate previously prepared adding 35 µl of Xβ galactosidose. Plates were incubated overnight at 37°C.

- **Extraction of DNA**

From each plate, single white colonies, likely containing the recombinant plasmids, were collected and diluted in bacterial tubes containing 3 ml of LB with ampicillin and incubated overnight at 37°C with agitation. An aliquot (1.5 ml) from each tube was transferred to another eppendorf tube and centrifuged at 13 000 rpm for 30 sec. The supernatant was discarded and the pellet re-suspended in the remaining 1.5 ml in the bacterial tube. After a new centrifugation at 13 000 rpm for 30 sec, the supernatant was discarded. The isolation of the plasmid DNA was done using Quick Lyse Qiagen Mini prep kit. Thereby, 400 µl of lysis buffer and the mix were homogenized on vortex. This mixture was transferred onto collection tubes with filters and centrifuged at 13 000 rpm for 1 min; then 400 µl of washing buffer (QLW buffer) and the mix were added and again centrifuged at 13 000 rpm for 1 min. The liquid was discarded keeping the covering filters, and again centrifuged to eliminate the remaining ethanol. Then, the covering filters containing the DNA were transferred on new eppendorf tubes where 50 µl of elution buffer (QLE buffer) were added and centrifuged at 13 000 rpm for 1 min. Covering filters were then discarded and the DNA kept into the eppendorf tubes.

- **Enzymatic digestion**

Three microliters of DNA were mixed with 1 µl of restriction enzyme buffer H (10X), 0.2 µl of EcoRI and 5.8 µl of sterile water. The mix was incubated at 37°C for 1h until used for electrophoresis analyses.

I.1.5 Data Analysis

The obtained sequences were compared using the GenBank database and basic local alignment search tools (Blast) for nucleotide analyses. Multiple sequence alignments and Phylogenetic relationships were generated using the software Mega 6.06 (Tamura *et al*., 2013).

I.1.6 Capture and Identification of putative Insect vectors

From April 2016 to September 2016, leafhoppers insects were collected monthly from six citrus groves located in the Mitidja province of Algeria where the *S. citri* infected trees were identified. A circular sweep net (38-cm diameter) was used to manually collect insects from 6 selected locations from the Algerian citrus groves where the *S. citri* infected trees were present. In each location, samples were collected from a citrus tree and ground vegetation under the tree canopy. Captured insects were collected on site using an aspirator, placed in vials containing 70% ethanol and transported to the laboratory for identification. The classification and nomenclature of captured leafhoppers were based on keys to accomplish their identification. Species dominance was characterized according to the categories described by Tischler (1949): eudominant, more than 10% of the total number captured; dominant, between 5 and 10%; subdominant, between 2 and 5%; rare, between 1 and 2%; sub-rare, less than 1 %.

Results

I.1.7 Development of Direct Tissue Blot ImmunoAssay (DTBIA) protocol

The nitrocellulose membrane 0.45µm pore sizes comparing to the 0.20µm showed higher performance in terms of blot colour reaction. The localization and distribution of the stain within 2-3 min after the substrate addition, was particularly intense and homogeneous in blots from the shoots of the *S. citri* infected samples and less clear-cut or sometimes absent in those from petioles peduncles; while, the blots from negative controls remained virtually unstained (Figure 5). Similarly, concerning the protein-binding sites, the use of PVA solution, coupled with a gentle stirring (150 rpm) on a shaker for 2 min, gave better results than other used blocking solutions. Furthermore, the best results were obtained with the membranes exposed for 2h to 1/50 dilution of specific alkaline phosphatase-conjugated polyclonal antibodies (Sediag France).

Interestingly, comparative tests of the field application of DTBIA with laboratory PCR assays did not show any relevant differences when the 112 field samples were tested for validation. In fact, two out of the 112 assayed field-grown plants showed stained areas evidencing their positivity and unstained blots for the negative samples;

The preliminary trials on the use of DTBIA, clearly indicate that DTBIA is a reliable technique for the mass detection of *S. citri* infecting citrus trees in the field.

I.1.8 Molecular analysis of the identified *S. citri* isolates in Algerian citrus groves

Two out of the 112 assayed field-grown plants produced amplicons of the expected sizes (336 bp), from the sample G and 34, using the primers targeting the Spiralin gene (Figure 6). The *S. citri* infected trees were a common orange belonging to the ITAFV Boufarik varietal collection and a Navelate orange collected from Tessala el Merdja varietal collection. Moreover, it is important to highlight that the P89 primer pairs were also efficient to identify the previously infected *S. citri* trees; in fact, the amplification with these primers enabled the appearance of three amplicons of the expected size (707 bp), associated to the two previously detected *S. citri* Algerian isolates G, 34 and the positive control (Fig 7). The obtained partial spiralin gene nucleotide sequence (336bp) from the *S. citri* infected tree revealed high percentage of homology (99%) with Fasa I that represents an Iranian *Spiroplasma citri* (GenBank Accession No.FJ755921.1), 95% identity with Palmyre strain that represent a Syrian strain (GenBank Accession N° U13997.1) and 94 % identity with the Corsican strain from France (GenBank Accession N° U13995.1). The obtained sequence of the partial Spiralin gene

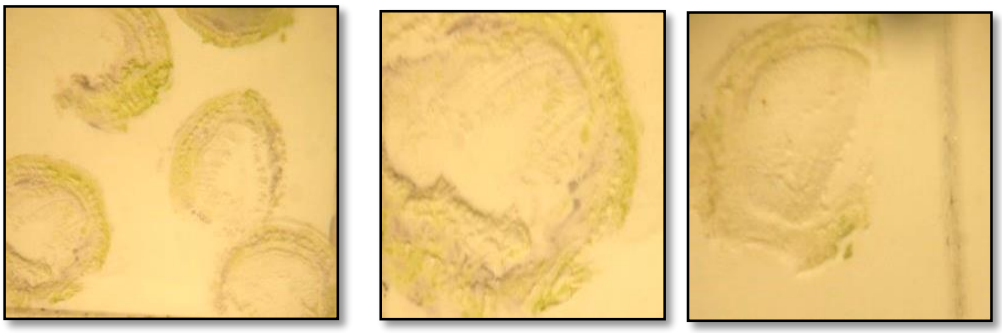

Figure 5. Purple coloration observed on stem sections of *S. citri* infected citrus samples, negative control (right)

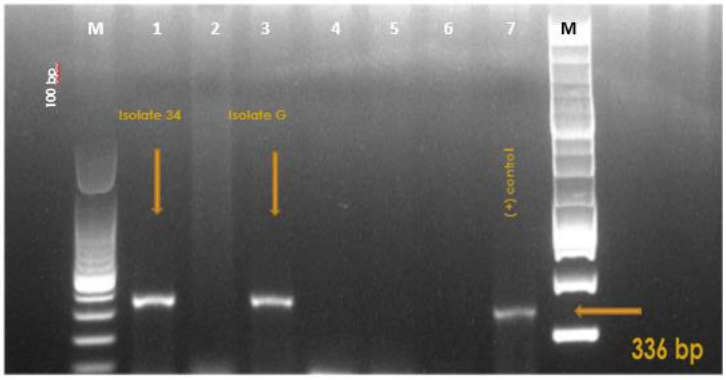

Figure 6. Polymerase chain reaction amplicons produced using spiralin-f/r primers with *S.citri* DNA extracted from plant tissue from the field plants. Lane 1-5 Algerian samples, lane 6 deionized water control, lane 7 positive control., (M) DNA 1-kb-plus ladder**;**

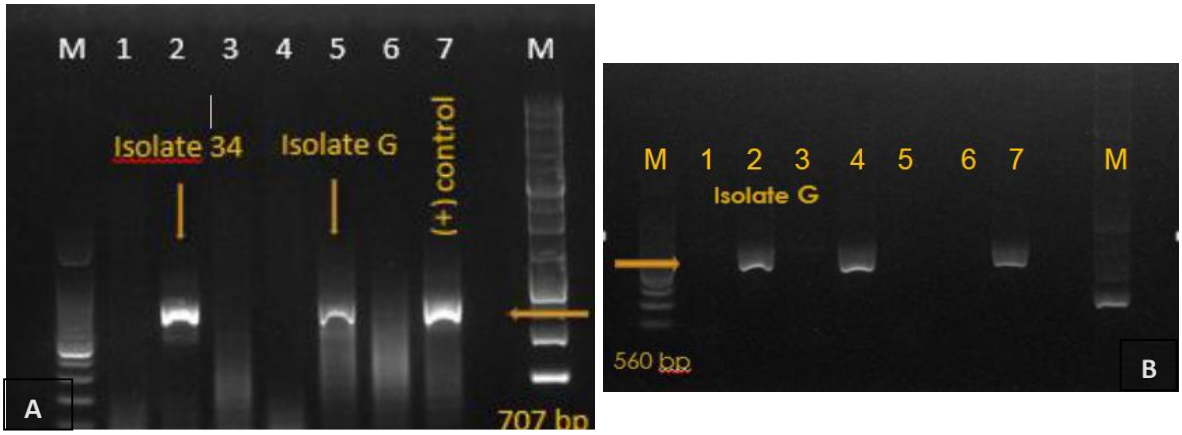

Figure 7. Polymerase chain reaction amplicons produced using P89-f/r (A), *TraG*-f/r (B) primers with *S. citri* DNA extracted from plant tissue from the field plants. Lane 1-5 Algerian samples, lane 6 deionized water control, lane 7 positive control., (M) DNA 1-kb-plus ladder

of the Algerian isolate G was registered on NCBI (GenBank Accession N°LN713947.1) and the designed phylogenetic tree included the Algerian (Accession LN713947.1) and Iranian strains (Accession FJ755921.1) in the same cluster (Figure 8).

The nucleotide sequences were translated into amino acid sequences in Mega. The amino acid sequences translated from the amplified nucleotide sequences represented 100-101 amino acids in length. The aligned amino acids were highly conserved among the 16 strains. Spiralins of the Algerian isolate contained 100 amino acids same as the spiralins of strains fasa I (Iran), R8A2 (Morocco), corse (Corsica), shiraz (Iran), Palmyre (Syria); whereas the other 10 strains contained 101 amino acids. The Algerian strain compared with the other Spiralin strains mentioned has a unique glycine at position 45; which is existing only for this isolate. As expected by phylogenetic and gene sequence homology analyses, the Algerian isolate possesses a spiralin which is mostly similar to the Iranian strain Fasa I but differs somehow from the other spiralins. Indeed, 11 amino acids changes were found occurring for the Algerian strain with some amino acid replacements. Most of these changes were localized within the first amino acids; in which Asparagine replaces Histidine at position 13, Serine replaces Leucine at position 31, Asparagine replaces Lysine at position 43, Glycine replaces Aspartic acid at position 45, Leucine replaces Isoleucine at position 64, Isoleucine replaces Asparagine at position 65, Glutamine replaces lysine at position 71, Isoleucine replaces Threonine at position 74, Glutamine replaces Asparagine at position 76, Methionine replaces lysine at position 77, Methionine replaces Leucine at position 98.

Moreover, the P89 partial gene nucleotide sequence (707bp) was also obtained (Accession N°LN908965) and revealed a 96% of nucleotide homology with the Spanish strain (273-14) of *S. citri* isolated from carrots; the designed phylogenetic tree evidenced a genetic diversity between the Algerian *S. citri* strain and the other available sequences retrieved from the database (Figure 9).

Relatively to the PCR assays targeting the *TraG* gene, the 560 bp fragments that were amplified were associated to the two previously detected *S. citri* Algerian isolates confirming the presence of the pSci6 plasmid in the genome (Figure 7B), the relative nucleotide sequence (Accession N°LN908966) was analyzed using BLAST and revealed 97% nucleotide homology with *Spiroplasma citri* plasmid pSci6 from the Moroccan strain GII3.

I.1.9 Investigations on the presence of the potential insect vector in the area

Overall, 380 adults of Auchenorrhyncha were captured. Twenty-five species were identified, belonging to two infra orders (Cicadomorpha and Fulgoromorpha) and four different families

(i.e., Cixiidae (one specie), Delphacidae (three species) Aphrophoridae (one specie), Cicadellidae (18 species)) (Table 3). The Tischler's abundance classification in classes of abundance revealed two specie as eudominant, i.e. *Neophilaenus campestris* (62%), *Psammotettix gr. alienus* (Dhlb.) (Hemiptera: Cicadellidae) (21 %) and three as subdominant, i.e. *Toya propinqua* (Fieb.) (3.16%), *Anaceratagallia laevis* Rib (Hemiptera: Cicadellidae). (2.11%), *Exitianus taeniaticeps* (Kbm.) (Hemiptera: Cicadellidae) (3.95%); and one as rare *Zyginidia sp*. (Hemiptera: Cicadellidae) (1.05 %) (Hemiptera: Cicadellidae) while the remainder of the captured species were classified as sub-rare with less than 1% abundance (Table 2). *Neophilaenus campestris* was clearly the most captured species in the citrus groves with 62% (Figure 10).

Table 3. Classification and total number of captured species during the survey, N: number of individuals captured, P (%): Percentage of individuals of each specie

Infraorder	Superfamily	Family	Specie	N	P%
Fulgomorpha	Fulgoroidae	Cixiidae	*Reptalus melanochaetus*	1	0,26
		Delphacidae	*Laodelphax striatella (Fall.)*	1	0,26
			Toya propinqua (Fieb.)	12	3,16
			Sogatella vibix (Haupt)	1	0,26
			Delphacidae		
	Cercopoidea	Aphrophoridae	*Neophilaenus campestris (Fall.)*	236	62
Cicadomorpha	Cicadoidea	Cicadellidae	*Anaceratagallia laevis Rib.*	8	2,11
			Anaceratagallia sp.	3	0,79
			Hecalus sp.	1	0,26
			Zyginidia sp.	4	1,05
			Aconurella prolixa (Leth.)	2	0,52
			Exitianus taeniaticeps (Kbm.)	15	3,95
			Allygus provincialis (Ferr.)	1	0,26
			Allygus theryi (Horv.)	2	0,52
			Allygus sp.	1	0,26
			Euscelidius sp.	3	0,79
			Euscelis alsius	1	0,26
			Oxytettigella viridinervis (Kbm.)	1	0,26
			Psammotettix gr. alienus (Dhlb.)	82	21
			Paralimnini sp	1	0,26
			Zygina sp.	1	0,26
			Balclutha rosea cf. (Scott)	1	0,26
			Goniagnathus brevis (H.-S.)	1	0,26
			Macrosteles sp.	1	0,26
			Deltocephalinae		
			Total	380	

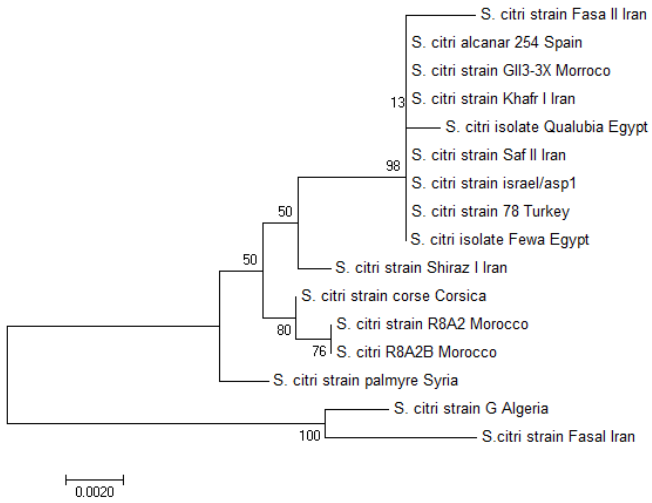

Figure 8. Phylogenetic tree based on Spiralin nucleotide sequences from different *Spiroplasma citri* strains. The neighbor-joining method using MEGA 6.06 was utilized, Bootstrapping consensus values are shown on trees.

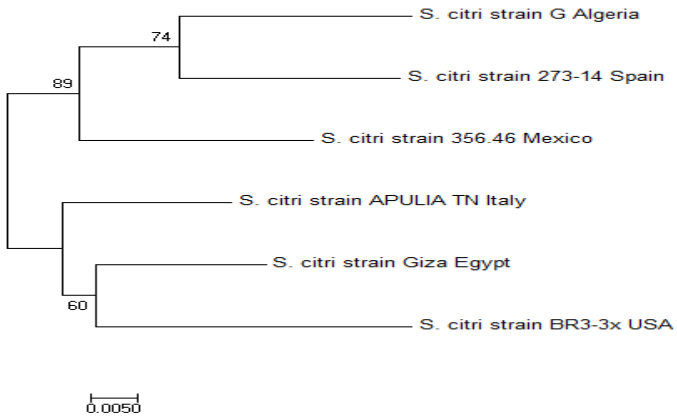

Figure 9. Phylogenetic tree based on P89 nucleotide sequence analysis of different *Spiroplasma citri* strains. The neighbor-joining method using MEGA 6.06 was utilized, Bootstrapping consensus values are shown on trees.

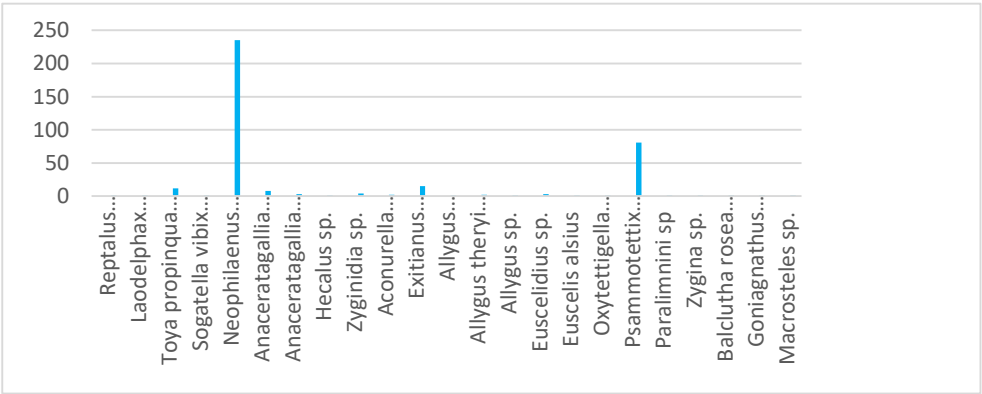

Figure 10. Total number of individuals captured for each species during the survey**.**

Discussion

The citrus industry is facing major threats from severe invasive diseases caused by bacterial pathogens such as HLB and citrus variegated chlorosis, as well as re-emerging endemic bacterial diseases such as CSD (Bruening *et al.*, 2010). These diseases are especially difficult to manage because the causal pathogens are transmitted by prolific insect vectors and reside in the plant vascular systems. Because, there is no cure for these diseases once trees are infected, early detection becomes extremely important for disease management. Moreover, detection of these pathogens is further complicated due to the uneven distribution or low titers in infected trees coupled with large seasonal variation (Bové, 2006; Polek, 2007; Yokomi *et al.*, 2011).

Results obtained from this study indicate that the developed DTBIA protocol can be a reliable technique for the detection of *S. citri* in citrus orchards and as an alternative to PCR. Interestingly, this technique does not require a sophisticated equipment or highly skilled operators; thus, reducing time processing and costs compared to other detection techniques. In addition, the printing of samples can be done directly in the field, avoiding the movement of infected plant material and reducing the risk of spreading the bacterium to new un-infested areas. However, the suitability of the developed protocol for its validation and a large-scale field deployment needs more investigations on a large number of field samples from several additional citrus species, at different tree ages and development stages, and also co-infected with other bacterial and viral pathogens of citrus, at different times of the year and in different geographic locations.

Beside the efficiency of this DTBIA assay to detect *S. citri* in citrus orchards, it is important to highlight that recently the qPCR targeting high-copy-number genes such as *SpV1-ORF1* significantly improved *S. citri* detection in culture and field samples. The increased sensitivity has additional value for early detection of the disease agent (Wang *et al.*, 2015) but nucleic-acid-based assays require complex sample preparations, which make them costly and time consuming, especially when multiple samples have to be collected and tested from one tree to compensate for erratic pathogen distribution and titer. Whereas, according to Shi *et al. (2014)* the new developed assay ScCCPP1-based direct tissue print detection, which is similar to the developed DTBIA assay, can overcome uneven distribution or low titers of *S. citri* in the infected plants, thereby reducing false negative diagnosis. This challenge was clearly demonstrated where qPCR results were inconsistent with imprint data in detecting CSD-infected field trees (Shi *et al.*, 2014).

Following the validation of the DTBIA protocol, the DTBIA assay was used in a large scale *S. citri* monitoring in Algerian citrus groves and allowed the detection of *S. citri*. Relatively,

the local isolates were then studied molecularly through PCR assays using three specific primer pairs. In this context, the partial spiralin gene nucleotide sequence, retrieved from the Algerian isolate "G" (Accession N°LN713947.1), revealed high percentage of nucleotide homology (99%) with the Iranian Fasa I strain (GenBank Accession No.FJ755921.1). Accordingly, Khanchezar *et al.* (2010) reported that the isolate Fasa I formed a group close to the Syrian Palmyre isolate. The relative phylogenetic tree evidenced that obtained Spiralin sequence from the Algerian strain was genetically closer to the Iranian Fasa strain I than to the other Mediterranean strains. Relatively to this gene sequence, Duret *et al.* (2003) reported that spiralin, seems more likely to be involved in specific spiroplasma–insect cell interactions rather than playing a role in protecting the spiroplasma cell membrane within the insect. Indeed, spiralin is acting as a lectin that is able to bind insect vector glycoproteins (Castano *et al.*, 2002; Killiny *et al.*, 2005).

Otherwise, the Algerian nucleotide sequence targeting the putative adhesin protein P89, revealed a high percentage of nucleotide homology (96%) with the Spanish strain (273-14) of *S. citri* isolated from carrots. The local isolate, was phylogenetically clustered into two groups revealing that the Algerian, Spanish and Mexican isolates of *S. citri* were genetically close to each other comparing to the Italian, Egyptian and American isolates. It's important to highlight that the *S. citri* putative adhesion-related protein P89, is present on a plasmid as well as the pathogen genome (Fletcher *et al.*, 1996; Yu *et al.*, 2000; Berho *et al.*, 2006b), and is implicated in adherence of spiroplasmas to cells from the leafhopper vector, probably involved in the interactions of the spiroplasma with the insect midgut epithelial or salivary gland cells *in vivo* during the transmission process (Yu *et al.*, 2000; Breton *et al.*, 2010; Duret *et al.*, 2010). Considering the similarities of the Algerian strain with the Spanish strain isolated from carrots, Mello *et al*. (2008) reported that, one RAPD primer (OPA-13) differentiated between carrot and citrus strains of *S. citri.* This result was different from an earlier report that showed no significant genetic differences among citrus and carrot strains but since this first study used the 16S rDNA region that is a more taxonomy marker than RAPD these results are not comparable (Lee *et al.*, 2006).

Beside these primary results, the Algerian isolate "G", also reacted positively with the primer pairs targeting the *TraG* gene, which is essential for insect transmission; confirming the presence of the pSci6 plasmid in the genome. The *TraG* gene is present in two identical copies on the pSci6 plasmid but is absent on pSciA, pSci1-5 and pSKU146 of *S. kunkelii*. The 0.9-kbp *TraG* fragment, encodes determinants that are critical for insect transmission of *S. citri* by its natural vector *C. haematoceps (*Berho *et al.,* 2006a*).* Mentioning that in mycoplasmas,

attachment to the host cells is by a specialized structure, which is formed through the recruitment of several proteins that interact between the mycoplasma and the host cell (Balish and Krause, 2005). Similar structures have been observed in cells of *S. kunkelii* attached to the gut epithelium of the leafhopper vector (Özbek *et al.*, 2003; Ammar *et al.*, 2004).

Considering the potential insect transmissibility of the Algerian strain by putative leafhopper vectors and following the finding that the identified Algerian isolate was carrying the *TraG* gene responsible for the insect transmission of the pathogen, the presence of potential vector was investigated in the studied area. Considering that the detection of *S. citri* in leafhoppers is dependent on the collection time of the year (Fos *et al.*, 1986), our monitoring was carried out according to the environmental and climatic considerations. The relative survey evidenced that among 23 species captured during the four months study, *Neophilenus campestris* was the most abundant specie in citrus groves in the Mitidja province and adults were particularly abundant in summer. Our results also showed the absence in 2016 of *Neoaliturus haematoceps*, the main insect vector of *S. citri* in this area. The eudominant leafhopper specie identified was *N. campestris* that is not identified as a *S. citri* vector . Taking in consideration that Fos (1986), reported that among the many leafhoppers in Morocco, Turkey, Syria and France (Corsica), only the species *Neoaliturus haematoceps* was found to be infected with *Spiroplasma citri*.

Among the collected insects, the specie *Macrostelles sp.* was identified and represented as a sub-rare specie. The specie *M. fascifrons (aster leafhopper)* was reported for the experimental transmission of *S. citri* after feeding on plants infected with the pathogen, *M. fascifrons* transmitted the spiroplasma from aster to aster and horseradish, from yellow rocket (*Barbarea vulgaris*) to aster, and from turnip (*Brassica rapa*) to turnip (O'Hayer *et al.*, 1983). Moreover, the specie *Euscelis alsius* was also identified in selected Algerian citrus groves as a sub-rare species during our survey. In comparison with previous works another Euscelis specie, *Euscelis plebejus*, displayed experimental transmissibility; *S. citri* multiplied in all leafhoppers injected; low transmission rate of *S. citri* by *E. plebejus* could not be correlated with the failure of organisms to multiply in the leafhoppers or to invade the salivary gland (Townsend *et al.*, 1977a). In addition, *Euscelidius sp*. represented also a sub-rare species in Algerian citrus groves, the *Euscelidius* gender (*Euscelidius variegatus)* showed possible transmissibility of *S. citri* when *E. variegatus* infected with strain AsP-1 of *S. citri* infected five of 21 bean plants (Townsend *et al.*, 1977b).

Our findings through this preliminary survey evidenced the absence of the known *S. citri* insect vectors in Algerian citrus groves. However, additional research is needed to investigate

if the eudominant leafhoppers species identified are potential *S. citri* vector. Moreover, it is important to know which environmental conditions influence movement of vectors from other plants (in particular from herbaceous plants). Finally, actions need to be taken in order to avoid a deterioration of the citrus industry in the main Algerian citrus growing areas. In this context, it is of utmost importance to strength the quarantine measures and schedule periodic surveys on the other main emerging diseases i.e. HLB, citrus variegated virus, sudden death, stem pitting tristeza and their insect vectors.

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Chapter III

Development and application of Loop Mediated Isothermal Amplification technique (LAMP) for the detection of *Spiroplasma citri*, causal agent of stubborn disease

The research project described in this chapter was mostly carried out under the supervision of Dr. Raymond Yokomi in the San Joaquin Valley Agricultural Sciences Center, USDA-ARS (California) in the context of a research internship abroad as intended in the doctoral program. Part of this experiment was also carried out in the CIHEAM-Bari (Italy).

Introduction

Spiroplasma citri is a helical plant-pathogenic bacterium responsible for the "stubborn" disease of citrus (Saglio *et al.*, 1973; Carle *et al.*, 2010). This leafhopper-transmitted, phloem-limited spiroplasma, belongs to the class of Mollicutes, a group of wall-less microorganisms phylogenetically related to low-G+C-content, Gram-positive bacteria (Weisburg *et al.*, 1989). Isolation and culturing of *S. citri* is technically demanding and time consuming. *S. citri* is typically low in titer and unevenly distributed in citrus. Typical symptoms of CSD can vary with season, citrus cultivar, and disease severity. CSD-affected trees show stunted growth of canopy with shortened internodes. Small leaves and bunchy upright growth, leaves are usually mottled. Most fruit drop while very small. The fruits that could reach maturity are usually misshapen and abnormally matured with aborted seed (Bové and Garnier, 2000). Significant yield reduction occurred only in severely symptomatic trees in which *S. citri* was broadly distributed within the tree canopy. The significant reductions in fruit yield and quality associated with S. *citri* infection validate the concern among citrus growers in California's Central Valley that CSD is a significant constraint to production and marketability. Moreover, entire groves have been destroyed as a CSD management strategy in central California; therefore, economic damages due to CSD can be substantial (Calavan, 1969; Kyriakou *et al.*, 1996; Mello *et al.*, 2010; Yokomi *et al.*, 2011).

The control of the disease is greatly dependent on preventive and roughing measures based on accurate and early detection because once a tree becomes infected, there is no practical cure for CSD (Shi *et al.*, 2014). As such, new diagnostic methods that can robustly detect CSD are required for field surveys and quarantine practices to guarantee clean source trees for the industry. Definitive diagnostic methods include bio-indexing and culturing in artificial media that are both time consuming and laborious. DNA-based molecular diagnostic with several molecular techniques including Polymerase chain reaction (PCR) detection of *S. citri* are used using primers amplifying the highly abundant spiralin gene or multicopy genes encoding membrane proteins (Yokomi *et al.*, 2008) and phage or prophage sequences of *S. citri* were used for detection (Lee *et al.*, 2006). Php-orf1 and Php-orf3 from conserved prophage sequences in the *S. citri* genome were also used in SYBR Green-based quantitative PCR (qPCR)

assays (Wang *et al.*, 2015). Digital droplet PCR was also developed; it is an approach to measure the absolute copy number of nucleic acid targets without the need of external standards. ddPCR methodology was more robust for diagnosis of CSD and the area under the curve was significantly broader compared to qPCR (Maheshwari *et al.*, 2017). These techniques offer reliable detection of the pathogen from infected plants; however, all the PCR-based methods require purified DNA from plant sample before being analyzed.

Isothermal nucleic acid amplification technologies have a significant advantage over PCR based methods, they can be applied at a constant temperature in a single step process (Li and Macdonald, 2015), without the need of thermal cycling, it allows diagnostics to be carried out using small and portable instruments (Chang *et al.*, 2012). Loop-mediated isothermal amplification (LAMP) is a very simple, cost-effective and sensitive technique for detection of specific DNA sequences, first described by Notomi *et al*. (2000). LAMP amplification relies on a use of four to six primers, specially designed to recognize six to eight distinct regions of a target gene, resulting in high specificity and efficiency (Tomita *et al.*, 2008). The procedure is rapid and is able to amplify from a single copy to 10^9 in one hour at constant temperature; typically in the range of 60–70°C (Notomi *et al.*, 2000). LAMP is also less sensitive to PCR inhibitors allowing the use of crude DNA extracts (Kogovšek *et al.*, 2015), a simple and rapid sample preparation methods without DNA purification steps are sufficient (Kaneko *et al.*, 2007). LAMP has been optimized for visualization using fluorescent assimilating probes that allow the monitoring of the isothermal LAMP reaction in real-time (Kubota *et al.*, 2011) and being able to be run directly on portable devices (Jenkins *et al.*, 2011). Several LAMP based techniques for the detection of plant pathogens has been successfully developed and applied (Varga and James, 2006; Boubourakas *et al.*, 2009; Harper *et al.*, 2010; Tomlinson *et al.*, 2010; Keremane *et al.*, 2015; Kogovšek *et al.*, 2015).

The aims of this study were to (i) Develop a loop mediated isothermal technique for the detection of *S. citri (ii)* Validate the technique for Mediterranean and American strains (iii) Develop a rapid crude plant extract method for on-site diagnostic using a BioRanger platform.

Material and methods:

II.1.1 Pathogen isolation and cultivation

Petiole leaf midribs or fruit columella collected from infected field plot near Ducor in California, were excised, surface sterilized, and diced with a sterile razor blade in 5 ml of LD8 broth medium (Lee and Davis, 1983), passed through a 0.45-µm filter and incubated at 30°C. Presence of *S. citri* was confirmed after 3 to 14 days by examining 10 µl of culture medium under dark-field microscopy at ×400 to 1,000 for the presence of motile, helical spiroplasmas.

II.1.2 Pathogen isolates and DNA extraction

DNA of thirty different spiroplasma strains and five other bacterial species were provided from different sources listed in Table 4. DNA of the different selected bacterial strains was quantified using a NanoDrop and kept at -20 ºC for the following sensitivity and specificity tests. Total DNA from *S. citri* cells culture was isolated following the CTAB protocol (Doyle, 1990). Plant DNA extracts was used for LAMP assay, mainly to validate the LAMP protocol and to compare the results with qPCR assays conducted from the same extractions.

II.1.3 LAMP primers design

Sequences of the Spiralin region of *S. citri* were compared to identify potential specific conserved regions to *S. citri*. The LAMP target sequence was selected in the spiralin region. Three LAMP set candidates were designed using the online software Primer ExplorerV4 (Eiken Chemicals, Tokyo, Japan), their analytical sensitivity was compared in order to choose the best performing LAMP primers set. Additionally, the Loop Backward (LB) primer was designed manually. Primers for LAMP assay were designed from the conserved region in the partial spiralin sequences identified by multiple sequence alignment of representative *Spiroplasma* isolates and a range of other bacteria. A set of three primers for LAMP assay comprising two outer primers, two internal primers and one loop primer was designed manually.

The FIP was made with the complementary sequence of F1 (F1c) and F2, and the BIP made with the complementary sequence of (B1c) and B2 (Table 5).

The organization and position of the LAMP primers and their complementarity to target DNA used in this study are shown in Figure 11. In order to avoid the false positive reaction by cross reactivity within the designed primers, the last were nBLAST searched on NCBI for analyzing the sequence specificity and to evaluate the possible cross-reaction with other sibling species. The secondary structures (hairpin, self-dimer and hetero-dimer) of the primers and their

corresponding stability were reviewed in OligoAnalyzer 3.1 tool. Whereas, the LAMP primers were synthesized by Invitrogen, CA (USA).

II.1.4 DNA Amplification and reaction optimization

In order to determine the right conditions for amplification, LAMP assays were carried out at 63°C and 65°C. The temperature at which the fastest positive signal was obtained and the reactions gave specific amplification, was selected. The LAMP assays for *S. citri* detection were performed in 25 µL reaction mixtures, including ISO-001 master mix (Optigene® Limited, West Sussex, United Kingdom) for LAMP assays. This master mix contains an engineered GspSSD LFDNA polymerase (Geobacillus sp. SSD, large fragment DNA polymerase) with strand displacement and reverse transcriptase activities and without any exonuclease activity. Detection of fluorescence of the amplification product was achieved using the BioRanger™ device. The enzyme mix used for the assays also has a recombinant pyrophosphatase from Aeropyrum pernix and hence the reaction does not produce adequate quantities of inorganic phosphate in the buffer. The optimized reaction mixtures contained 1.6 µM FIP and BIP, 0.2 µM of the F3 and B3 primers, 0.8 µM of the loop primer LB and 1.5-3µl template DNA.

II.1.5 Specificity of the LAMP assay

The specificity of the LAMP assay was tested with several strains, including 26 *S. citri* strains, 4 isolates of other spiroplasma from other hosts, 5 isolates of other pathogens (Table 4) and one no-template control consisting of nuclease free water. Finally, a dilution of 11ng/µl. was performed on each DNA belonging to the different analyzed strains, those in order to harmonize the quantity of the tested DNA.

II.1.6 *In vitro* sensitivity tests

The sensitivity of the LAMP assay was assessed by using 10-fold serial diluted *S. citri*, a synthetic clone was constructed containing a 674 bp, the fragment of the spiralin gene in pUC57 vector. The plasmid restriction was performed by using the SpeI-HF restriction enzyme in addition to the CutSmart® buffer (New England Biolabs) while the DNA was purified using the Nucleospin DNA purification kit and the concentration of the plasmid construct was measured three times using Quant-iT™ Qubit™ dsDNA HS Assay Kit (Invitrogen™).

The LAMP experiments were conducted in triplicate (repetitions) and the average tp (time of positivity) values were plotted. To assess the efficiency of the amplification, an alternative qPCR amplification efficiency was used. Tenfold serial dilutions of the plasmid DNA were utilized for qPCR and LAMP assays.

Table 4. *Spiroplasma* species strains and other bacterial species used for sensitivity and specificity tests of the LAMP assay

Genus	Specie	Strain code	Host specie	Country/Source	LAMP
Pseudomonas	*savastanoi pv. savastanoi*	CFBP 1670	Olive	ex-Yougoslavia	-
Agrobacterium	*tumefasciens*	CFBP42	Tomato	/	-
Erwinia	*amylovora*	EAI205	Pear	Italy	-
Pseudomonas	*syringae pv. syringae*	LMG 1247	Lilac	United Kingdom	-
Xylella	*fastidiosa subsp. pauca*	Xf	Olive	Italy	-
Spiroplasma	*floricola*	Sf	Tulips	(Courtesy of Saillard C.)	-
Spiroplasma	*phoeniceum*	Sp	Periwinkle	(Courtesy of Saillard C.)	-
Spiroplasma	*melliferum*	Sm	Honey bee	(Courtesy of Saillard C.)	+
Spiroplasma	*kunkelii*	Sk	Corn	(Courtesy of Saillard C.)	-
Spiroplasma	*citri*	SP3	Citrus	Egypt	+
Spiroplasma	*citri*	Sp4	Citrus	Syria	+
Spiroplasma	*citri*	Sp5	Citrus	Algeria	+
Spiroplasma	*citri*	Sp6	Citrus	Egypt	+
Spiroplasma	*citri*	Sp15	Citrus	Italy	+
Spiroplasma	*citri*	Sp18	Citrus	Turkey	+
Spiroplasma	*citri*	Sp20	Citrus	Turkey	+
Spiroplasma	*citri*	F1G	Citrus	Algeria	+
Spiroplasma	*citri*	F3P1	Citrus	Morocco	+
Spiroplasma	*citri*	BLH M&B	Beet leaf hopper	USA	+
Spiroplasma	*citri*	181	Citrus	USA	+
Spiroplasma	*citri*	LB10	Citrus	USA	+
Spiroplasma	*citri*	LB319	Citrus	USA	+
Spiroplasma	*citri*	163	Citrus	USA	+
Spiroplasma	*citri*	164	Citrus	USA	+
Spiroplasma	*citri*	FBLH13	Beet leaf hopper	USA	+
Spiroplasma	*citri*	FBLH15	Beet leaf hopper	USA	+
Spiroplasma	*citri*	FBLH23	Beet leaf hopper	USA	+

Spiroplasma	*citri*	DF76	Citrus	USA	+
Spiroplasma	*citri*	DF551	Citrus	USA	+
Spiroplasma	*citri*	189	Citrus	USA	+
Spiroplasma	*citri*	160	Citrus	USA	+
Spiroplasma	*citri*	BR12		USA	+
Spiroplasma	*citri*	D4-2	Diakon	USA	+
Spiroplasma	*citri*	CC-2	Chinese cabbage	USA	+
Spiroplasma	*citri*	LB7	Citrus	USA	+

Table 5. LAMP primers used in this study for *Spiroplasma citri* isothermal detection

Primers	**Sequence (5′ → 3′)**	**Length (bp)[a]**
F3	ACAGCAAACCCAAAACAAG	19
B3	CAACAGTTTTATCTTTTGCTGGAG	24
FIP [b]	CTGCTGTTGCTGTTTTTACAACTCTTTTGCTGAAATTAAAACAGCGTTAGAAGC	54
BIP [c]	CAATTTGATGTTTATCAAGACAACTTTTACTTCAACGTTACCTCCTT	47
LB	GGTRMATCATTAACAACAAT	20

[a] bp, base pair

[b] Forward internal primer, FIP consists of two fragments, F1c and F2, separated by a TTTT spacer in bold. They are in reverse (F1c) and forward (F2) orientations.

[c] Backward internal primer, BIP consists of two fragments, B1c and B2, separated by a TTTT spacer in bold. They are in forward (B1c) and reverse (B2) orientation

DNA ranged from 10 ng to 0.1 fg. Reaction mixture without DNA template was included as a negative control. The detection limit in the LAMP assay was defined as the last reaction with the positive reaction.

Similarly, the detection limit of the LAMP assay was determined using serial dilutions of pure genomic DNA obtained from *S. citri* cultures, incorporated into non-diseased crude plant extract samples for monitoring plant extract inhibition within this assay. DNA decimal dilutions ranging from 10ng/µl to 10fg/µl were prepared in sodium acetate buffer (50mM) and healthy crude plant extract; For the LAMP reaction and the fluorescence was measured each 30s. A cut-off Tp value, to determine whether samples were positive or negative, was defined based on the results of the detection limit and tests with infected plant materials.

II.1.7 qPCR reactions

To compare the sensitivity of the LAMP assay, qPCR was performed with the extracted DNA from *S. citri* strains using Spiralin primers (SP1) (Yokomi and Sisterson, 2011). The qPCR assay was performed in the CFX96 Real-Time System (Bio-Rad) in a final volume of 20 µl reaction mixture, including 10 µl of SsoAdvanced™ Universal Probes Supermix (Bio-Rad), 900 nM forward and reverse primers and 250 nM probe, 2 µl of *S. citri* DNA and final volume made up with double distilled water. The thermal cycling conditions consisted of initial denaturation at 95°C for 5 min, then 40 cycles of denaturation at 95°C for 10 s, and annealing at 59°C for 30 s. a negative (non-template) control was included.

II.1.8 BioRanger platform

The BioRanger platform tool from Diagenetix, Inc was used during our experiments, the BioRanger improves upon the previous Smart-DART products. The platform includes a portable and battery operable device, able to test eight samples simultaneously and periodically measures fluorescence for real-time detection of the isothermal amplification. The BioRanger platform was developed for an easy use with Bluetooth enabled Android devices. The analysis starts automatically, with data displayed graphically in real-time as the reaction progresses. Fluorescence readings were recorded using the channel optimized for fluorescein each 30s. LAMP reaction for the detection was carried out at 65ºC for 30 min and 80º C for 5 min. The results are whether e-mailed from the Android device or saved to view later (Figure12).

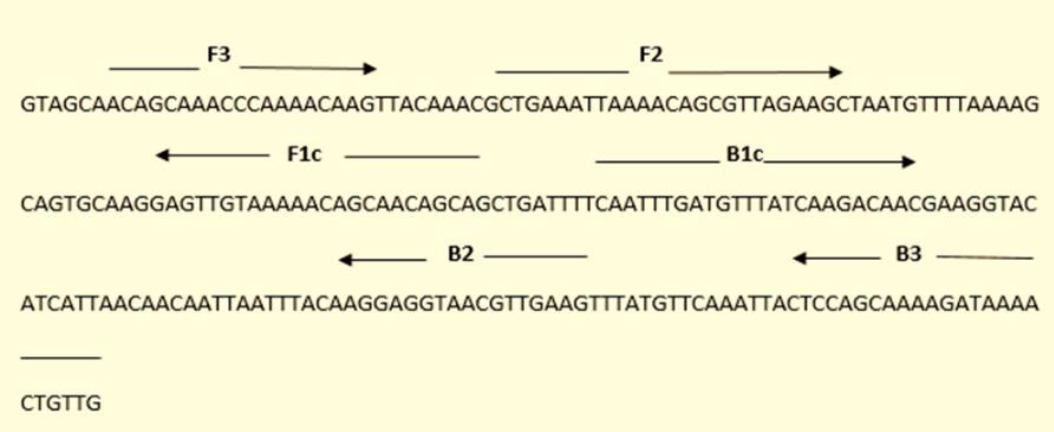

Figure 11. Spiralin target DNA fragment. This sequence was used to design LAMP primers, the arrows are showing the position and orientation of the primers.

Figure 12. BioRanger device and the android tablet used for the LAMP assay in this study

II.1.9 Crude plant extracts preparation method

The first optimization of the protocol concerned the crude plant extract homogenization, where different buffers were compared. Among these buffers which were used with leaf petioles, the one mentioned as ELISA buffer by Kogovšek *et al.* (2015), including 264 mM Tris (236 mM Tris-HCl, 137 mM NaCl, 2% PVP K-25, 2 mM PEG 6000, 0.05% Tween 20, pH 8.2), 50 mM Na acetate buffer, pH 5.5, Tris-EDTA (20 mM Tris, pH 8.0 containing 2 mM EDTA and 1% TritonX100®) (Keremane *et al.*, 2015) and PEP buffer (PBS, PVP10, Tween 20) were used during this experiment. The protocol was carried out by using 2–5 ml of buffer mixed with leaf petioles samples. The crude plant extracts were used as an alternative to DNA extraction, because of the low titer of this pathogen, we maximized the quantity of plant tissue to be used by taking a ratio of 1:10 of plant tissue and extraction buffer was selected. 500 mg leaf petioles were crashed in 5 ml of extraction buffer, sodium acetate buffer (50mm), in a mortar then heated for 10 min at 95ºC. The samples were centrifuged for 30s and the lysate was diluted 1:10 in water. The obtained crude plant extract was used for the LAMP reactions; for later use plant petioles were also conserved at -20 °C in paper bags.

II.1.10 Field validation of the LAMP assay

In total 25 citrus leaf samples were collected from a field located at Liberty Bell area in Fresno, California, in which the stubborn disease was already reported. The collected samples were with or without visible symptoms associated to the stubborn disease, Therefore, it was advisable to collect the samples from the four cardinal points of the tree, considering that in the case of stubborn disease detection, uneven distribution in plant material poses the most significant concern;

In the case of leaf petioles testing, 0.5g of material were used, this amount equal to 12 petioles collected from the four parts of the citrus tree. The collected leaves were placed in plastic bags upon using. The petioles were excised from the leaves and were processed following the above-mentioned crude plant extraction procedure method. The LAMP assays were conducted using the BioRanger device. Meanwhile, for the LAMP assay, healthy crude plant extracts samples were also included in the reaction as negative controls.

In order to determine the reliability of the assay, the same selected samples were processed by qPCR; relatively, the DNA was extracted from the selected following the CTAB protocol (Doyle, 1990), then the DNA aliquots were used for processing the qPCR reactions.

Results

II.1.11 Specificity of the LAMP Assay

During the trials, the developed LAMP protocol, detected successfully the samples infected with *S. citri;* however, it evidenced a cross reaction with the taxonomically closely related species *S. mellifferum*, and no cross reaction occurred with the other spiroplasma and pathogens tested (Table 4)

II.1.12 Sensitivity of the LAMP assay

The sensitivity of the optimized isothermal assays was determined using *S. citri* clone target and pure DNA serial dilutions ranging from 10 ng/ul to 10 fg/ul. The limit of detection of the LAMP assay was 70fg/ul for the pure plasmid DNA and 100fg/ul for the pure DNA incorporated in healthy plant extract. *S. citri* detection occurred at all concentration up to 70 fg/ul (Figure 13).

It is important to highlight that the analytical sensitivity of the LAMP assay was estimated to be nine-times lower than that of the qPCR and all positive reactions were observed before 30 min of amplification (Table 6), therefore a run reaction time of 30 min was sufficient to achieve the result.

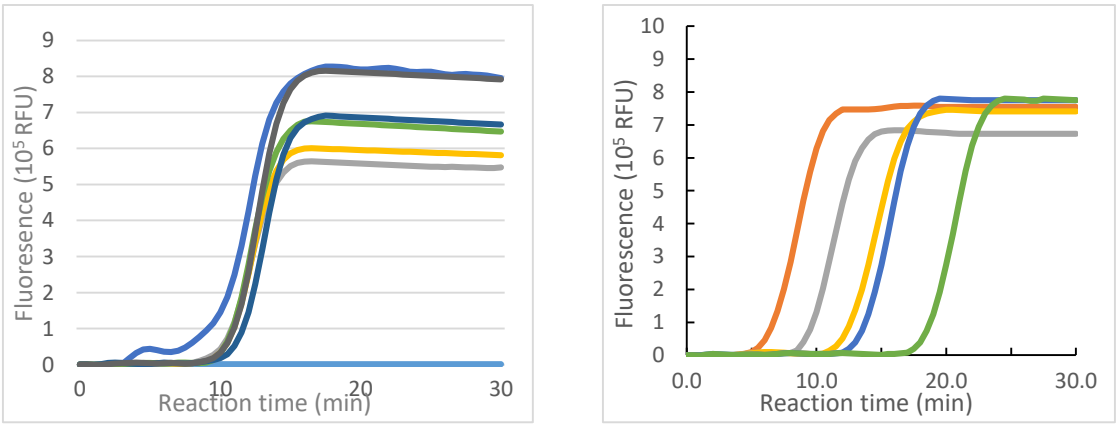

Figure 13. (A) Amplification curve generated in the LAMP assay using different *Spiroplasma* isolates at a concentration of 11ng/µl, (B) Sensitivity of LAMP assay and amplification curve generated in the LAMP assay using 10 fold serial dilutions of *S. citri* pure DNA ranging from 10 ng/ul to 10fg/ul

II.1.13 Crude plant extracts preparation method

The appropriate buffer among the four buffers tested to be used have been determined by homogenizing selected true positives and true negatives in the different buffers. The buffer that evidenced the best analytical properties during the LAMP analysis was selected for further investigations. In this context, the comparison between different buffers revealed that sodium acetate buffer (50mM) was the most efficient and accurate by detecting 3/3 positive samples and 0/3 negative controls. Whereas the ELISA buffer and Tris-EDTA buffer gave 3/3 positive samples and 2/3 of the negative controls that reacted positively. PEP buffer in the other hand gave false positives 3/3 negatives reacted positively**.** Moreover, concerning the on-site application of the *S. citri* testing protocol using the LAMP assay, the sample preparation was simplified and shortened by omitting the DNA extraction step and the crude homogenate was found to be suitable for LAMP testing.

II.1.14 Field validation of the LAMP assay

The reliability of the assay and the crude plant extract procedure was determined on a total of 25 citrus leaf samples. Among this tested samples, the LAMP assay reported 3 true positive and 22 true negative samples. The accuracy of the whole procedure developed was compared to the standard qPCR detection of *S. citri*. It was shown that the results were in accordance with the qPCR results (Table 6)

Table 6. Comparative analysis of LAMP and qPCR for the field validation test. Tp values >22min and Ct values >30 are considered negatives.

Row	Tree n°	LAMP results	LAMP Tp (min)	q PCR (ct)
R30	8	-		39.09
	7	-		34.12
	6	-		37.21
	5	-	24	36.3
	4	-	23	35.01
R31	10	+	**8.5**	26.1
	9	-		36.55
	8	-		36.19
	7	+	**13.5**	26.88
	6			25.12
R32	23			36.78
	24	-	23.5	35.06
	25	-		36.36
	26	-		35.3
	28	-		35.51
R33	42	-		36.31
	41	-		38.71
	40	-		34.98
	39	-		37.91
	38	-		34.79
R34	55	+	**15**	29.05
	56	-	23	34.73
	57	-	23	35.84
	58	-		36.08
	59	-		36.38

Discussion

The obtained results during this research will surely improve the current management of this disease. In fact, this study allowed the development for the first time of the LAMP assay, which is considered as an innovative tool with cost-effective, and user- friendly method for *S. citri* detection in plant. It is important to highlight that the LAMP isothermal technologies are efficiently able to generate billions of DNA copies within 40 to 60 min, they can produce large quantities of amplicons that have a complex tertiary structure that is highly stable and capable of self-replication (Notomi *et al.*, 2000; Li and Macdonald, 2015).

Since currently the most sensitive and reliable methods for detecting *S. citri* are based on DNA detection methodologies, these assays usually require a DNA extraction step followed by PCR thus, can be prohibitively expensive for running large-scale screenings. This study allow to develop and validate a loop mediated isothermal technique for the detection of different *S. citri* strains. In addition, the experimental trials allow the development of a rapid crude plant extract preparation method for the on-site diagnostic using the BioRanger platform.

The primers designed targeted the Spiralin gene, which is considered as the main membrane protein. The targeted spiralin region is known to be a conserved region (Bové *et al.*, 1993; Foissac *et al.*, 1996) and present as a single copy in the chromosome of all *S. citri* strains. During the trials on the specificity of the developed LAMP assay, the technique presented a limited cross-reaction with the closely related specie *S. melliferum*. These results are considered of minor consequence, as this specie do not infect citrus. Furthermore, the assay developed did not react neither with the other bacterial strains tested nor with DNA extracted from healthy citrus host plants.

While, the LAMP primers readily detected all *S. citri* DNA and was consistent in different samples from various sources (Table 1). The limit of detection of the developed LAMP assay was 70 fg/µl for the pure plasmid DNA and 100 fg/ µl for the pure DNA incorporated in healthy plant extract. The LAMP assay developed during this study, was about 9 times less sensitive than standard qPCR technique targeting the Spiralin gene.

The detection limit for spiralin primers in qPCR was previously estimated to 6.4fg (Wang *et al.*, 2015). Hence, LAMP technique likely detected the bacterium in low-titer concentrations; within the obtained limit of detection, after 22 min. of LAMP reaction a slight DNA amplification background was observed from some healthy crude plant extracts.

For such tests, that produce a quantitative output, the selection of a cutoff can be somewhat arbitrary and is based on the type of error, which can be, minimize and eliminate false reading. Relatively, it is important to avoid false negative decisions, selecting a threshold with a high sensitivity would accomplish the goal but at a cost of a greater false positive rate. Hence, the analysis conducted offers the reader the opportunity to analyze the outcome over a range of combinations (Wang and Turechek, 2016). In this context and based on the results obtained, a cut-off value of 22min for LAMP assays was used and any amplification that occurred beyond this limit was considered negative.

Considering that DNA extraction protocols revealed a high efficiency (Yokomi *et al.*, 2008) without being considered as suitable for immediate analysis. A simplified procedure was developed where the DNA extraction step was replaced and crude extract could be directly used for analysis. Due to limitations in low spiroplasma titer and hard leaf petioles (Gumpf and Calavan, 1981), homogenization was considered as limiting factor for applying in field conditions. Recently new approaches were also developed for other pathogens, consisting on the maceration for few minutes of a portion of leaf or shoots, as an extraction step (Yaseen *et al.*, 2015; Si Ammour *et al.*, 2017), this method was not suitable in the case of *S. citri*, due to its uneven distribution in the plant. Relatively, the only efficient procedure was the manual homogenization of leaf petioles with mortar and pestle, which enables the release of the bacterial cells of *S. citri*. In the same context, Kogovšek *et al.* (2015), reported that the on-site applicable homogenization method for Flavescence dorée detection compared to the fastprep-assisted homogenization used in the in-lab DNA extraction protocol, revealed higher efficiency of the latter; however, this was not suitable for fast on-site analysis.

The LAMP reactions were successfully carried out in the BioRanger (Diagenetix, Inc., Hawaii, USA) device testing the crude plant extract procedure. The validation of the developed LAMP assay which was performed on field samples, highlighted its suitability and provided information on the reliability of the assay for field diagnostic. The LAMP assay detection diagnostic reliability of the on-site procedure was confirmed by qPCR assay which evidenced the same results obtained previously.

Due to its fast process, which is almost 10 times quicker than qPCR method, one hour is needed from sampling to the result obtention with LAMP, whereas almost 10 hours are needed for the

qPCR-based assay. The developed LAMP assay can be applied for in-laboratory use as well as for field application.

Meanwhile, since the reactions are carried out at a constant temperature, it does not require stringent thermal cycling nor an expensive qPCR machine, which make it more cost-effective, applicable to small on-site laboratories and the potential for use by growers or crop consultants using hand-held LAMP devices. In this sense, the availability of easy to operate, cost effective testing kits that can give access to instant test results would enable the growers to implement timely orchard management practices.

Furthermore, it would be of a great interest to investigate the potential use of isothermal technologies for the development of a field detection kit, for testing leafhoppers vectors of *S. citri.* This technology was described by Keremane *et al.* (2015), on the development of field detection system for *Candidatus* Liberibacter asiaticus from the psyllid vector *Diaphorina citri*, and described by Yaseen *et al* (2015), that also developed the on-site detection of *Xylella fastidiosa* in host plants and in "spy insects" using the LAMP method.

Given that the large scale testing can be helpful for effective disease management, the LAMP technology lends itself well in such situations. Actually, as more tools become available more informed stubborn disease management decisions could be taken.

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Chapter IV

Genetic diversity within American and Mediterranean strains of *S. citri* causal agent of Citrus Stubborn Disease (CSD)

The research project described in this chapter was mostly carried out under the supervision of Dr. Raymond Yokomi in the San Joaquin Valley Agricultural Sciences Center: USDA-ARS (California) in the context of a research internship abroad as intended in the doctoral program. Part of this experiment was also carried out in the CIHEAM-Bari (Italy).

Introduction

S. citri, the causal agent of citrus stubborn disease (CSD) (Saglio *et al.*, 1973) and horseradish brittle root disease (Fletcher *et al.*, 1981), is the first-cultured and most-studied spiroplasma and is transmitted in a circulative–propagative manner by several species of the leafhoppers such as *Circulifer tenellus* in California (Oldfield *et al.*, 1976; Liu *et al.*, 1983) and *Circulifer haematoceps* in the Mediterranean area (Fos *et al.*, 1986). It infects many plant species other than citrus, including Madagascar periwinkle, carrots and horseradish in which it induces stunting, leaf yellowing, and wilting.

Genetic investigations of the *S. citri* were carried out on a circular chromosome, on plasmids and within sequences of inserted latent viruses in the genome (Melcher and Fletcher, 1999; Carle *et al.*, 2010). Spiralin, the most abundant membrane protein, is considered as one of the most thoroughly characterized *S. citri* membrane proteins (Beven *et al.*, 1996; Foissac *et al.*, 1996; Wroblewski *et al.*,1977). Spiralin represents more than 20% of the total membrane protein. It has been purified to homogeneity and shown to have a molecular weight of about 26,000. The spiralin gene has been identified, cloned, and expressed in *Escherichia coli* cells (Mouches *et al.*, 1984). Several forms of spiralin were produced in *E. coli*, including a major form with a slightly larger molecular mass than that of spiralin in *S. citri*. Among the six open reading frames (ORF) present on the *S. citri*, DNA insert, the ORF corresponding to the spiralin gene was identified by the size and amino acid composition of its sequence-deduced translational product (Chevalier *et al.*, 1990). The comparison of the spiralin genes belonging to *S. citri* and *S. melliferum* has shown that the two genes have a conserved N-terminal end.

Several *S. citri* protein candidates have been identified as involved in transmission and, for a few of them, in an interaction with leafhopper vector proteins. Spiralin, was suspected to be involved in the transmission for two reasons: (i) a *S. citri* spiralinless mutant was less effective in its transmissibility (Duret *et al.*, 2003); (ii) spiralin acted *in vitro* as a lectin able to bind to

glycoproteins of insect vectors and therefore might function as a ligand able to interact with leafhopper receptors (Killiny *et al.*, 2005).

In order to further investigate the spiralin gene of *S. citri*, the objective of this study was to assess the genetic diversity of the spiralin gene from *S. citri* isolates originating from different geographical regions.

Materials and Methods

III.1.1 Pathogen isolation and culture

S. citri strains which were included during this investigation, belong to various locations and collected in different years (Table 7). The pathogen isolation consist on the use of petiole leaf midribs or fruit columella collected from field plot which were excised, surface sterilized, and diced with a sterile razor blade in 5 ml of LD8 broth medium, then passed through a 0.45-µm filter and incubated at 30˚C. The presence of *S. citri* was confirmed after 3 to 14 days by examining 10 µl of culture medium under dark field microscopy at ×400 to 1,000 magnification for the presence of motile helical spiroplasmas.

III.1.2 DNA Extraction

Total DNA were extracted from cells cultures of 8 Americans and 3 Mediterranean's isolates, (Table 1) by following the CTAB protocol (Doyle, 1990). The collected pellets of the cells culture were re-suspended in CTAB buffer and DNA extraction was accomplished via standard procedures, then the DNA of the different isolates was quantified using a NanoDrop and kept at -20 ºC.

III.1.3 Thermal gradient optimisation

In order to determine the optimal annealing temperature, experiments were performed with a range temperature of 50, 49.4, 48.1, 46.2, 43.9, 42, and 40˚C in the S100 Thermal cycler, this by using the same amount of *S. citri* DNA and primers concentrations (900nM/250nM).

III.1.4 Polymerase Chain Reaction (PCR) and Cloning

Primer pairs F1/R1 were used for the amplification of spiralin gene (Khanchezar *et al.*, 2014) (Table 8). The PCR assay was carried out in 25 µl reaction mixtures, including 14.5 µl deionized water, 2.5 µl DNA template, 2 µl of 10 mM dNTPs, 2.5 µl of 10xPCR reaction buffer, 2 µl of each 10 µM primer, 0.25 µl of 5 U/µl Taq DNA polymerase (Takara). Whereas, the PCR amplification was performed as follows: initial denaturing at 94° C for 4 min, 35 cycles of 94° C for 30 s, annealing temperature (46° C for F1/R1) for 40 s and 72° C for 1 min. Final extension at 72° C was for 10 min

Table 7. List of the *S. citri* isolates used in this study.

Samples	Host	Location
BLH M&B	Beet leaf hopper	Fowler,Ca
181	Citrus	Strathmoore, Ca
LB10	Citrus	Ducor, Ca
163	Citrus	San emidio, Ca
FBLH13	Beet leaf hopper	Mettler,Ca
DF76	Citrus	Heron,Ca
189	Citrus	UCR,Ca
160	Citrus	Kings163,Ca
Sp3	Citrus	Egypt
Sp4	Citrus	Syria
Sp15	Citrus	Italy

Table 8. List of the primers used in this study

Gene	Primers	Primer sequence	Length (bp)	Position	Ref
Spiralin	SCf1	TAATTTTAATAACATTTG CTT	817,or1,135 bp[b]	-62 to -41[a]	Khanchezar *et al.*, 2014
	SCr1	TTTGTTCTAAATAAGAAAAAG		8–29[c]	

[a] Upstream position of nucleotide from the adenine nucleotide at starting ATG codon
[b] Size of PCR product varied based on different isolates of *S. citri*
[c] Downstream of the stop codon

The gel electrophoresis was carried out on 1% agarose gel at 100 volt for 45 minutes. At the end of the running gel, amplicons were extracted from agarose gels and purified with QIA quick PCR Purification Kit (Qiagen, Inc.). DNA was inserted in plasmid vector pUC18 vector (Agilent Technologies StrataClone PCR Cloning Kit) and cloned into *Escherichia coli* DH5α. Plasmids from successfully transformed colonies were extracted by a small-scale preparation alkaline lysis and cleaned with chloroform/phenol separation, and dissolved in water. The target inserts were confirmed by restriction digests with EcoRI according to product specifications (Promega, WI).

III.1.5 Sequence analysis

The nucleotide sequences of PCR products were subjected to BLAST in NCBI. MEGA 6 software was used for multiple sequence alignment and phylogenetic tree construction. The sequences of all isolates were putatively translated and amino acid sequences were aligned by MEGA 6 software.

Results

III.1.6 PCR Amplified Spiralin Genes of *S. citri* strains

Spiralin gene amplification with primer pair F1/R1 was performed on the 11 DNA extracted from different *S. citri* strains (Table 7). In the reference strain GII-3, primer F1 is located about 62 bp upstream of the spiralin gene start codon and primer R1 is located 29 bp downstream of the spiralin stop codon and amplification leads to a product of 817 bp length.

III.1.7 Sequence and phylogenetic analysis of *S. citri* Isolates

Nucleotide sequence alignments of PCR products (817 bp) from the American and Mediterranean strain showed that both of the Egyptian strain SP3 and Italian strain sp15 showed 98% of nucleotide homology with the Iranian isolate SPF1 (GenBank Accession No KT834818.1) and Moroccan *S. citri* reference strain GII-3 (GenBank Accession No. AM285305.1). The Syrian isolate SP4 showed 98% of nucleotide homology with the Moroccan strain R8A2 (GenBank Accession N° U13998.1). The spiralin sequence of the American isolates showed 99% of nucleotide homology with the strain R8A2 (GenBank Accession N° U13998.1). The bootstrapped phylogenetic tree (Figure 14) indicated that spiralin genes from the Egyptian and the Italian strains were distinct from the others, forming a different and separate cluster. In that cluster (Figure 14), these two isolates (SP3 and SP15) were identical or strikingly similar. Whereas the American strain DF76 isolated from Heron California share the same cluster with the studied Syrian strain (SP4).

III.1.8 General features of spiralins

All eleven spiralin deduced sequences from our Mediterranean and American isolates were mostly identical for their N-terminal lipoprotein signal sequence to that of *S. citri* GII-3, the reference *S. citri* isolate. Conserved repeated motifs were evidenced and located around positions 50 and 160 in *S. citri*. These repeated elements were identified in all our isolates. In all spiralins the presence of two highly positively charged lysine residues within the first three amino acids (n-region of the signal peptide), and the hydrophobic stretch of 17 amino acid length (h-region) are features recognized in lipid modification of the cysteine at position 24 with a diacylglycerol. In addition, the distinct sequence (VVAC) at the C-terminal end of the signal peptide corresponding to a lipobox was identified in all spiralins of our isolates (Figure 15, 16). For all *S. citri* studied strains, four amino acids residues, Ala, Val, Lys, and Thr, represent as much as 50% of all residues, while only a small.

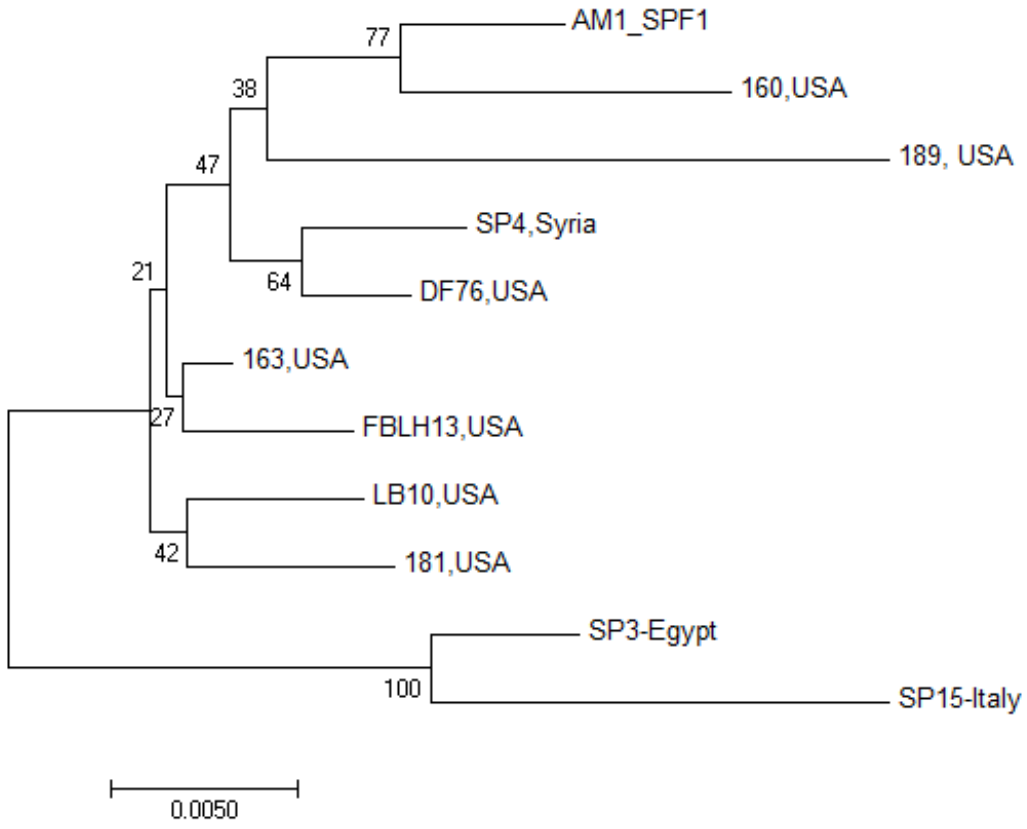

Figure 14. Phylogenetic tree based on Spiralin sequences from different *S. citri* strains. The neighbor-joining method using MEGA 6.06 was utilized; Bootstrapping consensus values are shown on trees.

Figure 15. Amino acid sequences of spiralin from various *S. citri* strains (70 to 100 A.A). Dashes correspond to amino acid identity

Figure 16. Presence of two highly positively charged lysine residues within the first three amino acids (n-region of the signal peptide), and the hydrophobic stretch of 17 amino acid length (h-region) are features recognized in lipid modification of the cysteine at position 24 with a diacylglycerol

number of Cys, Arg, Tyr and Phe residues are present. Neither spiralin from our isolates contains Tryptophan

Discussion

Genetic diversity in bacteria can be assessed by examining specific restriction sites, the amplicons produced by random primers, repetitive elements and genome sequences (Louws *et al.*, 1999). Moreover, RAPD-PCR, when optimized for a particular application, can provides effective discrimination among species and strains (Qin *et al.*, 2001).

In this present study, the genetic diversity of Mediterranean and American strains of *S. citri* was assessed. *S. citri* studied isolates yielded differential amplicons and indicated that these isolates were separated into two main branches of the established phylogenetic tree. It showed that significant variability apparently exists between the cluster that includes the Egyptian (SP3) and Italian isolates (SP15) of *S. citri* in comparison to the other cluster including the American and Syrian isolates (SP4).

It is challenging to evaluate the significance of the differences among our strains and clusters in the generated phylogenetic tree from the spiralin gene (Figure 14). One significant measure can be generated by analyzing cases in which several different *S. citri* lines are derived from a common parent. Such comparisons are useful because, since we know the derivation histories and some of the genomic information about some strains, we can apply that information to inform our interpretation of the phylogenetic results in this study. In this context, Yokomi *et al.* have shown the presence of two genetically different populations of *S. citri* in field trees from central California and from historical strains collected from southern California in the 1960s (Yokomi *et al.*, 2008).

The characterization of the spiralin gene indicated that the truncated sequence of the Syrian isolate (SP4) clustered with strain belonging to the third cluster, which include the American strain DF76 isolated from citrus tree located in Heron California; this can suggest that this isolate might have originated from this group.

Similar repeated elements than those reported by Foissac *et al*. were identified in all our isolates, these motifs are located around the positions 50 and 160 in all *S. citri* isolates. These reported

conserved repeated motifs are identical in spiralins from the different spiroplasma species. For our Mediterranean isolates, repeated motifs containing substitutions to amino acids and sharing similar physico-chemical properties were identified. In addition the distinct sequence (VVAC) at the C-terminal end of the signal peptide corresponding to a lipobox (Madan Babu and Sankaran, 2002) and which constitutes the cleavage site for the lipoprotein-specific SPase were also identified in all spiralins of our studied isolates. Among all *S. citri* membrane proteins, spiralin is the most abundant in *S. citri*. As evidenced in our isolates, this protein has an unusual chemical makeup, lacking, Tryptophan (Bové *et al.*, 1993). Our findings confirm that the gene that encodes spiralin usually has a very conserved sequence in the first 24 amino acids at the N-terminus. Spiralins of different strains of *S. citri* have the same number of amino acids but are usually polymorphic along the sequence. As reported previously, *S. citri* spiralin is suspected to act as a bacterial sugar-binding adhesin (lectin) (Killiny *et al.*, 2005). Repeated regions in lectins and adhesins have been described for several Gram positive bacteria (Dramsi *et al.*, 1993; Vengadesan and Narayana, 2011). Repetitive elements present in diverse adhesins of mollicutes have been also shown to allow the bacterial binding to eukaryotic cells (Minion *et al.*, 2000; Béven *et al.*, 2012).

The occurrence of a region rich in repeats and strands in spiralin could support the hypothesis of the involvement of this lipoprotein in the binding to insect vector glycoproteins through repeated elements (Killiny *et al.*, 2005). It was reported that some proteins may be important in *S. citri* adherence to cells of its vector during transmission (Yu *et al.*, 2000). According to our findings, general common features of spiralins from the different groups included the presence of motifs responsible for N-terminal lipoylation and of internal repeats. Khanchezar *et al.* reported that the analyses of spiralin sequences for repetitive elements indicated that *S. citri* spiralins contained 29-amino acids long degenerate repetitive elements. In addition neither spiralin of our isolates contains Tryptophan, this property is of particular importance. Indeed, in *Spiroplasma* spp., TGA is not a termination codon but codes for tryptophan, in addition to the universal tryptophan codon TGG (Renaudin *et al.*, 1987). Therefore, the absence of TGA codons (read as tryptophan in spiroplasmas, but as stop codons in *E. coli*) accounts for the full expression of spiralin in the bacterial transformant (Mouches *et al.*, 1984). Spiralin seems to occur as dimers, tetramers, and large oligomers in the spiroplasmal membranes. Disulfide bonds involving cysteine have been implicated in the formation of oligomers (Wróblewski, 1981).

Our findings showed also a variation in the number and/or in the location of serine and threonine residues occuring in the different studied *S. citri* spiralins and this could result in variation in the number of these hypothetical post-translational sites of acylation. Such acylation could also be affected by modifications of the spatial arrangement of spiralin because of amino acid changes other than those affecting serine and threonine. Therefore, variation in the number of such post translational acylations could explain the spiralin variations.

It is important to mention that various proteins on the tip structure may participate in the spiroplasma orientation and adherence to insect cell surface proteins, as spiralin covering the entire surface of the spiroplasma could not play a role in vivo in the adherence mediated via the terminal tip structure. It could be involved in the interaction between the pleïomorphic forms of spiroplasma and the membranous vesicles within the cytoplasm of the leafhopper midgut or salivary glands epithelial cells observed by Wayadande and Fletcher, 1995.

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Chapter V

Conclusion

In order to control efficiently some emergent and re-emergent citrus diseases, there is a strong need to develop new detection techniques for pathogen diagnosis. Developing new detection techniques for rapid and accurate detection of multiple plant pathogens and their associated variants is essential. The usage of on-site diagnostic tools will surely facilitate the design of proper pest distribution strategies to prevent the spread of diseases; it will also improve the design of cultivar choice. This all implies the importance of early detection of pathogens at the point of inspection using on-site detection tools before it causes restrictive import requirements on products. On-site detection is increasingly important for rapid plant disease diagnosis and the need for on-site detection motivates the production of test devices and kits for their use in-field for the plant pathogen/pest detection, even by growers themselves.

The present study indicates that the developed Direct Tissue Blot ImmunoAssay protocol is a reliable technique for the mass detection of *S. citri* in citrus orchards and an alternative to PCR assays. This technique does not require a sophisticated equipment or highly skilled operators; thus, reducing time processing and costs. This technique allowed the detection of *S. citri* in Algerian citrus groves (Chapter II); relatively, the local isolates were then studied molecularly and evidenced that the spiralin nucleotide sequence obtained from the Algerian strain was genetically closer to the Iranian Fasa I strain than to the other Mediterranean strains. This Algerian strain also reacted positively with the primer pairs targeting the *TraG* gene, which is essential for insect transmission; confirming the presence of the pSci6 plasmid in the genome. Following our findings, a preliminary insect vector survey was carried out evidencing that *N. hematoceps* the main vector in the Mediterranean region was not present but additional research is needed to investigate if the eudominant leafhoppers species identified are potential *S. citri* vectors.

Since currently the most sensitive and reliable methods for detecting *S. citri* are based on DNA detection methodologies, that, can be prohibitively expensive for running large-scale screenings. A LAMP assay was also developed, in order to obtain a cost-effective and user- friendly method for *S. citri* detection in plant. Following the development of this detection method, an evaluation of the performance of the assay was compared with real-time PCR, an already established “gold standard” (Chapter III); the comparison of the two approaches enables us to confirm the results obtained from the developed assay. It’ is also important to highlight that the LAMP can be run

directly on portable devices and the reactions were successfully carried out in the BioRanger (Diagenetix, Inc., Hawaii, USA) device testing the crude plant extract procedure.

These small, handy instruments are potentially accessible, and provide the benefit of real-time detection. Ideally, an adequate testing of the accuracy and reproducibility of the method was performed with a significant number of field samples. The validation of our developed LAMP assay was performed on-site, which highlighted its suitability and provided information on the reliability of the assay for field diagnostic.

Finally, our study concern also an investigation on the genetic diversity of Mediterranean and American strains of *S. citri*. The study enabled to confirm the presence in our different isolates of a conserved sequence in the first 24 amino acids at the N-terminus in the gene that encodes spiralin. however, it shows that even if Spiralins of different strains of *S. citri* have the same number of amino acids, they are usually polymorphic along the sequence. Considering that *S. citri*, spiralin acts as lectin-binding insect glycoproteins and this binding activity is suspected to allow the spiroplasma to reach the salivary glands for a fully efficient transmission of the bacteria to the plant host by the vector insect. Future studies will be conducted with the aim at determining whether mutations in spiralin N- or C-terminal parts have an impact on the lectin-binding activity.

Stubborn disease is a re-emergent citrus disease, the control of the causal agent *S. citri* is very difficult, and it is mainly based on preventive disease control measures; it is of primary importance to use healthy propagating material. At this point, the early detection of the pathogen will surely will be useful, since the goals of developing on-site detection methods are to increase efficiency of testing and to broaden the range of pathogen detection abilities.