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Investigation and characterization of viruses and phytoplasmas infecting fig trees

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Dedication

*My deepest gratitude goes to **my family** who taught me that the best kind of knowledge to have is that, which is learned for its own sake and even the largest task can be accomplished if it is done one step at a time. For their unflagging love and support throughout my life; this dissertation is simply impossible without them.*

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Love & forbearance.

Contents

List of figures

List of tables

List of Annexes

List of Abbreviations

%	Percentage
µ	Micro
µg	Microgram
µl	Microliter
µM	Micromolar
16S rDNA	16S ribosomal DNA
16S rRNA	16S ribosomal RNA
A	Adenine
B.C.	Before Christ
Bp	Base pair
C	Cytosine
C°	Degree Celsius
ca.	Circa
Ca. P.	'*Candidatus* Phytoplasma'
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CP	Coat protein
CPm	Minor coat protein
CTAB	Cetyl trimethylammonium bromide
DAPI	4′,6-diamidino-2-phenylindole
DMB	Double membrane bodies
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EDTA	Ethylene diamino tetra acetic acid (disodium salt)
EFSY	European stone fruit yellows

EM	Electron microscopy
EtOH	Ethanol
FCV-1	Fig cryptic virus
FLMaV-1	Fig leaf mottle-associated virus 1
FLMaV-2	Fig leaf mottle-associated virus 2
FLV-1	Fig latent virus 1
FMD	Fig mosaic disease
FMMaV	Fig mild mottle-associated virus
FMV	Fig mosaic virus
G	Guanine
G	Gram
H	Hour
HCl	Hydrochloridric acid
HEL	Helicase
HSP70	Heat shock protein 70
Kbp	Kilo base pair
KCl	Potassium chloride
KDa.	Kilo Dalton
L	Liter
LB	Luria Bertani
M	Molar
mA	Milliampère
Mg	Milligram
mg/ml	Milligram per milliliter
$MgCl_2$	Magnesium chloride
$MgCl_2$	Magnesium chloride
Min	Minute
miRNA	Micro RNA
Ml	Milliliter
MLO	Mycoplasma-like organisms

mM	Millimolar
M-MLV	Moloney-murine leukemia virus
M-MLV	*Moloney Leukaemia virus* transcriptase
Na_2SO_3	Sodium sulfite
NaCl	Sodium chloride
$NaCa_3$	Sodium carbonate
NaI	Sodium iodide
NaOAc	Sodium acetate
Ng	Nanogram
ng/ml	Nanogram per millilitre
NH4oAC	Ammonium acetate
NLS	N-lauryl sarkosyl
nM	Nano molar
nt(s)	Nucleotide(s)
ORF	Open reading frame
PCR	Polymerase chain reaction
Pmol	Pico molar
Prb	Probe
PRO	Protease
RdRp	RNA dependent RNA polymerase
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAse	Ribonuclease
Rpm	Revolutions per minute
RT-LAMP	Reverse transcription-loop-mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
SDW	Sterile distilled water
Sec	Second
spp.	Species
ssRNA	Single stranded ribonucleic acid

STE	Sodium chloride Tris-EDTA
T	Thymine
TAE	Tris-acetate-EDTA
Taq	*Thermophilus aquaticus*
TBE	Tris-borate-EDTA
TNA	Total nucleic acid
Tris	Tris (hydroxymethyl) aminomethane
U	Enzymatic unit
UV	Ultraviolet
V	Volt

General introduction

The common fig (*Ficus* carica L.) is a member of *Moraceae* family, which encloses 60 genera and possibly more than 850 species of trees, shrubs and herbs (Somashekhar *et al.*, 2013). It is the only member of its genus cultivated for thousands of years for its dry and fresh fruit consumption worldwide. The southwest Asia and eastern Mediterranean are considered to be the best areas for common fig cultivation (Hanelt, 2001). The Food and Agriculture Organization (FAO) of the United Nations reports a world production of figs over one million ton/year, 90% of which is concentrated in the Mediterranean basin and Middle East. Turkey is the largest fig-producer in the world (285,000 tons) followed by Egypt (170,000 tons) and other Mediterranean countries. In addition, United States, Brazil, India, Japan, and China are also considered as important players in the fig production market.

Fig is a sustainable crop, nevertheless it is vulnerable to the attack by several diseases, pests and disorders. Fig diseases have been recorded as early as the time of Theophrastus in the 3rd century B.C. Figs are prone to fig mosaic, an endemic disease that is widely distributed in most varieties and countries where figs are cultivated. This disease, first described by Condit and Horne in 1933, mainly affects leaves, young branches and fruits (Serrano *et al.*, 2005). It is present all over the world and causes real damages in fig orchards.

Mosaic is considered the main infectious disease of fig, FMD (fig mosaic disease)-associated symptoms are extremely variable (Condit and Horne, 1933; Martelli *et al.*, 1993). It can be transmitted by grafting and not by seed (Condit and Horne, 1933) and, it is spread by the eriophyid mite *Aceria ficus* in a semi-persistent manner (Flock, 1955a; Proeseler, 1972).

The adoption and diffusion of molecular techniques has allowed to dig deeper in the sanitary status of fig. To date, at least 10 viruses have been reported to infect fig trees, the genomes of eight of which, classified as definitive or tentative species of the genera *Closterovirus*, *Ampelovirus, Trichovirus, Alphacryptovirus, Emaravirus, Maculavirus, Luteovirus, Umbravirus and Badnavirus* have been sequenced completely or partially (Elbeaino *et al.*, 2006, 2007, 2009c, 2010, 2011a, 2011b; Gattoni *et al.*, 2009; Tzanetakis *et al.*, 2010; Laney *et al.*, 2012). This list comprises fig mosaic virus (FMV), fig leaf mottle-associated virus 1

(FLMaV-1), fig leaf mottle-associated virus 2 (FLMaV-2), fig mild mottling-associated virus (FMMaV), Arkansas fig closterovirus-1 (AFCV-1), Arkansas fig closterovirus-2 (AFCV-2), fig latent virus 1 (FLV-1), fig cryptic virus 1 (FCV-1), fig fleck-associated virus (FFkaV), fig badnavirus 1 (FBV-1) and strawberry latent ringspot virus (SLRSV). Among these viruses, FMV is the most relevant and is considered the agent of the worldwide spread fig mosaic characterized by various degree of discoloration and malformation of leaves and fruits (Martelli *et al.*, 1993). Besides to these viruses, three viroids have also been detected in fig: Apple dimple fruit viroid (ADFVd) (Chiumenti *et al.*, 2014), Citrus exocortis viroid (CEVd) and Hop stunt viroid (HSVd) (Yakoubi *et al.*, 2007).

A few pests have been described in fig plants. The nematodes belonging to *Meloidogyne* genus are the most common and widely distributed pests (McBeth, 1949); arthropod pests, like the coleopteran *Carpophillus hemipterous* and lepidopteran *Ephestia figulilezza* (Ferguson *et al*., 1990), have been also identified. Fig can also be infected from many fungal diseases caused by *Alternaria, Aspergillus, Botrytis,* and *Penicillium* fungi (Tous and Ferguson, 1996).

To date, the main available diagnostic technique to detect fig viruses is RT-PCR, a molecular technique which necessitates high manipulation. The use of serology in the diagnosis of fig viruses is severely limited due to the high content of milky substances in fig tissues that prevent their transmission onto herbaceous hosts and the purification from woody tissues. All these limiting factors have led to the development of a sensitive, fast, and reliable diagnostic technique that could be used in screening programs of a large number of fig samples. The TaqMan RT-PCR seems to be for this aim a perfect diagnostic tool that could compensate all these constraints. Accordingly, this study aimed at the development of singleplex and Multiplex-TaqMan RT-PCR assays to detect single and multiple infections of the main fig viruses, i.e. FMV, FLMaV-1, FLMaV-2, FMMaV, FCV-1, FLV-1 and FFkaV.

Nothing is known regarding the phytoplasma infections in fig. Phytoplasmas are associated with several hundred plant diseases worldwide, many of which have an important economic impact, in particular those affecting woody plant, *i.e.,* coconut lethal yellowing, peach X-disease, grapevine yellows, and apple proliferation. Herbaceous plants are also severely affected by phytoplasma diseases (Bertaccini and Duduk, 2009). Initially the phytoplasmas

detection was made difficult by their low concentration, especially in infected woody plants, and their erratic distribution in the sieve tubes. Electron microscopy and graft-transmission were the only available techniques to index phytoplasmas. The DNA-specific dye DAPI was also applied for the detection. All these techniques are unable to differentiate phytoplasmas. In addition, serological detection techniques had a little contribution in this field due to the difficulties faced in the production of antisera. Currently, the detection of phytoplasmas is carried out by nucleic acid-based techniques, in particular the polymerase chain reaction (PCR) assay, which is addressed on their 16S rRNA gene (Ahrens and Seemüller, 1992; Lee *et al.*, 1993a; Namba *et al.*, 1993a; Smart *et al.*, 1996; Firrao *et al.*, 2005). PCR is the method of choice for detection of phytoplasmas in plant tissues and insect vectors. In addition, both RFLP of PCR-amplified and phylogenetic analysis of 16S rDNA are employed to differentiate, characterize and classify phytoplasmas (Seemüller *et al.*, 1998; Lee *et al.*, 2000, 2007).

In general, plants infected with phytoplasmas show symptoms of virescence/phyllody, sterility of flowers, proliferation of axillary buds resulting in witches' broom, abnormal internode elongation and generalized stunting (Bertaccini, 2007). Similarly, to several other crops and cultivated or wild plant species, it is likely that also fig is affected by phytoplasma diseases. Phytoplasma-like symptoms, *i.e.* yellowing, deformed leaves and short internodes, were recurrently observed on a number of fig trees at Locorotondo (Apulia region, southern Italy). Accordingly, a deeper investigation by field surveys and laboratory analyses was conducted on these symptomatic fig plants aiming to identify the possible responsible agents. The results of this study are following reported.

Chapter 1

Viruses associated with fig mosaic disease

Abstract

Singleplex and multiplex TaqMan RT-PCR assays were developed to detect fig-infecting viruses, *i.e.* fig leaf mottle-associated virus 1 (FLMaV-1), fig leaf mottle-associated virus 2 (FLMaV-2), fig mild mottle-associated virus (FMMaV), fig mosaic virus (FMV), fig latent virus 1 (FLV-1), fig cryptic virus 1 (FCV-1) and fig fleck-associated virus (FFkaV). The sensitivity of the newly developed assays was compared with the conventional RT-PCR using 10^0 to 10^{-6} serial dilutions of cDNA. Results showed that TaqMan RT-PCR was in general from 10^2 to 10^3 times more sensitive than RT-PCR, except for the case of FLV-1 detection. In Multiplex PCR up to five viruses were detected in naturally infected figs, regardless of the combination of virus-specific probes and primers used. The application of both RT-PCR and TaqMan RT-PCR in a large-scale survey on fig trees in Egypt showed the presence at different extent of all 7 viruses under study, mostly in mixed infection (63%). The prevailing viruses were FMV and FFkaV (62% and 59% of samples, respectively), followed by FLMaV-2 (32%), FLV-1 (16%), FLMaV-1 (14%), FCV-1 (7%) and FMMaV (4%). FMV was constantly associated to mosaic-diseased trees, with very few exceptions. Conversely, some mosaic affected fig trees were found free from FMV, but infected by mixture of two or more of the other viruses under study.

Keywords: Fig, mosaic, viruses, single and multiple detection, RT-PCR and TaqMan RT-PCR.

1.1 Introduction

History

The fig mosaic disease (FMD), described by (Condit I.J. and Horne W.T., 1933) for the first time, is the major disease affecting fig trees worldwide. Its etiological agent was thought to be of viral origin until the ultrastructural observation revealed the presence of intracytoplasmic enveloped spherical bodies in infected fig cells, 90 to 200 nm in diameter, that were called double membrane bodies (DMBs) (Bradfute *et al.*, 1970; Plavšic and Milicic, 1980; Appiano *et al.*, 1990; Martelli *et al.*, 1993).

Fig infecting viruses: state of the art

All known fig viruses were found associated with mosaic-diseased fig trees and their molecular description was made possible due to the recovery of their viral replicative form, double-stranded RNA (dsRNA), from tissue extracts (Elbeaino *et al.*, 2006, 2007, 2009b; Walia *et al.*, 2009). FMD, the most widespread infectious disorder of this species, has an extremely variable symptomatology (Condit and Horne, 1933; Martelli *et al.*, 1993). The transmission of this disease is mediated by infected plant material, grafting and eriophyid mites but not by seeds (Martelli *et al.*, 1993). A single-stranded negative sense RNA virus, belonging to the family *Bunyaviridae* (now *Fimoviridae*) has been identified as the etiological agent of this disease (Elbeaino *et al.*, 2009b, 2010). To date, at least ten different viruses and three viroids have been found associated with FMD (Table 1).

Table 1: Viruses and viroids of fig.

Agents	Genus	References
Fig leaf mottle-associated virus 1 (FLMaV-1)	*Closterovirus*	(Elbeaino *et al.*, 2006)
Fig leaf mottle-associated virus 2 (FLMaV-2)	*Ampelovirus*	(Elbeaino *et al.*, 2007)
Fig mosaic emaravirus (FMV)	*Emaravirus*	(Elbeaino *et al.*, 2009b)
Fig latent virus 1 (FLV-1)	*Trichovirus*	(Gattoni *et al.*, 2009)
Fig mild mottle-associated virus (FMMaV)	*Closterovirus*	(Elbeaino *et al.*, 2010)
Fig cryptic virus (FCV-1)	*Alphacriptovirus*	(Elbeaino *et al.*, 2011b)
Fig fleck-associated virus (FFkaV)	*Maculavirus*	(Elbeaino *et al.*, 2011a)
***Fig badnavirus* 1** (FBV-1)	*Badnavirus*	(Laney *et al.*, 2012)
Strawberry latent ringspot virus (SLRSV).	*Sadwavirus*	(Elbeaino *et al.*, 2015)
Apple dimple fruit viroid (ADFVd)	*Apscaviroid*	(Chiumenti *et al.*, 2014)
Citrus exocortis viroid (CEVd)	*Pospiviroid*	(Yakoubi *et al.*, 2007)
Hop stunt viroid (HSVd)	*Hostuviroid*	(Yakoubi *et al.*, 2007)

Fig mosaic virus (FMV)

Fig mosaic is the main viral disease infecting fig trees worldwide that can be transmitted by grafting (Condit and Horne, 1933) and by the eriophyid mite *Aceria ficus* (Flock, 1955b). The first record of this disease was from California (Condit and Horne, 1933). FMD has an extremely variable symptomatology; infected fig trees exhibit foliar discolorations (green yellowish mottling, mosaic, ring and line patterns) (Li *et al.*, 2012) and malformation (Rubio-Somoza and Weigel, 2011). Leaves with mild or severe deformation display a tremendous variety of shapes and sizes, *i.e.* twisted, puckered and rosetted. Discolorations are common, consisting of various patterns of chlorotic mottling, blotching, banding, clearing, feathering of the veins and chlorotic-necrotic ringspots and line patterns. In some leaves chlorotic spots are relatively small and uniformly scattered on the blade surface. Some infected trees may grow vigorously, whereas others have a reduced size. Severely affected trees may have fruits smaller than normal, distorted and showing yellowish mottling, longitudinal stripes and ring spots. Symptomatic fruits may drop prematurely.

FMV belongs to *Emaravirus* genus and has spherical particles known as double membrane bodies (DMB). The genome of FMV is a multipartite, consisting of six single-stranded, negative sense RNA (Figure 1) (Elbeaino *et al.*, 2009a, 2009b). Each segment consists of a

single open reading frame (ORF) that encodes the following proteins in the order: RNA-dependent RNA polymerase (p1, 264 kDa), a putative glycoprotein (p2, 73 kDa), a putative nucleocapsid protein (p3, 35 kDa) and a protein with unknown function (p4, 40.5 kDa). RNA-5 expresses a polypeptide of 502 amino acids (*ca.* 59 kDa), whereas RNA-6 codes for a polypeptide of 188 amino acids (21.5 kDa) (Elbeaino *et al.*, 2012), both with unknown function. FMV resembles very much the molecular structure of members of the family *Fimoviridae* (Elbeaino *et al*., 2018). This virus is considered as a cosmopolitan virus, present in all places where fig is grown.

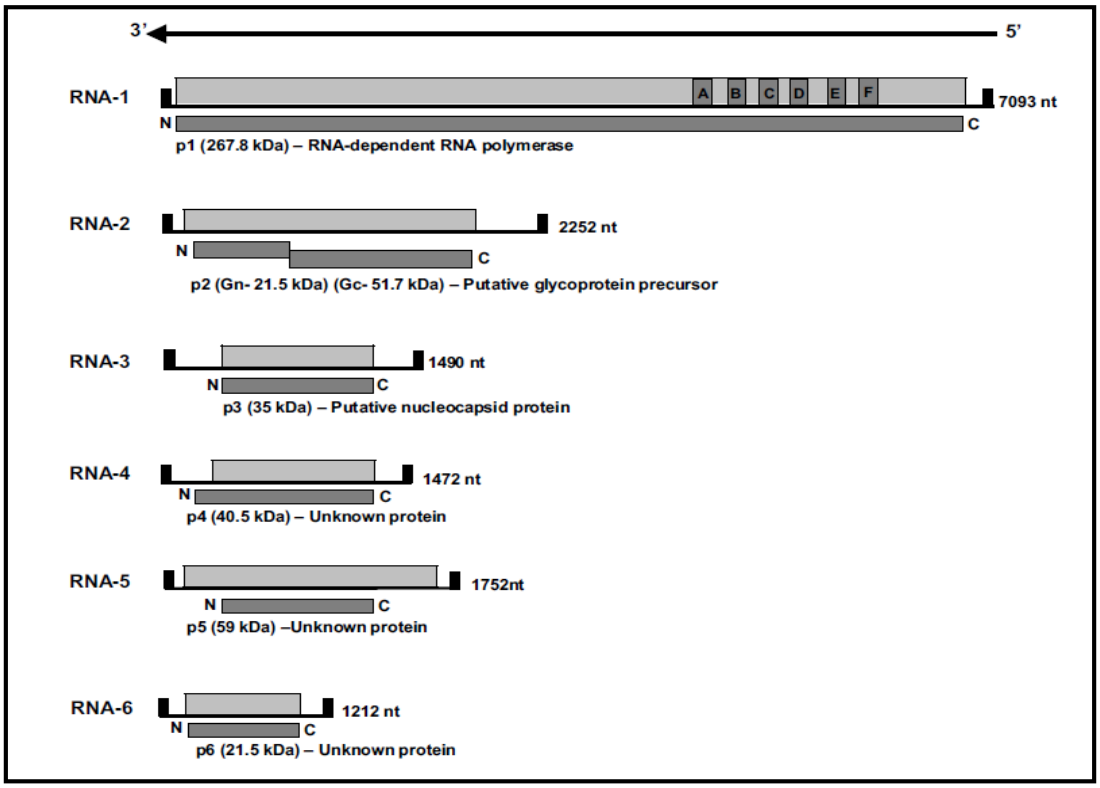


Figure 1: Schematic representation of the organization of the six RNA segments constituting the putatively complete genome of FMV. The 13 nucleotides conserved at the 5' and 3' termini are indicated as black boxes on each segment. Letters (A-F) represents the conserved motifs of the RNA dependent RNA polymerase RdRp (RNA-1) gene. Expression products of each RNA (p1 to p6) are represented as dark grey boxes. The function and estimated molecular weight of each protein are reported (Elbeaino *et al.*, 2012).

Fig leaf mottle-associated virus 1 (FLMaV-1), Arkansas fig closterovirus 1 (AFCV-1) and Arkansas fig closterovirus 2 (AFCV-2)

FLMaV-1 is reported in several countries including Italy. It belongs to the family *Closteroviridae* with filamentous virus particles about 1,800 nm long. The complete genome sequence is not yet completely available but most likely it has a genome organization similar to that of species of the genus *Closterovirus,* whose *Beet yellows virus* is the type species (Figure 2). FLMaV-1 is not mechanically transmissible to herbaceous hosts (Elbeaino *et al.*, 2006). FLMaV-1 is associated to symptoms of mottling, vein clearing, blotching, and various patterns of chlorotic mottling and discoloration of the second and third veins of fig leaves (Elbeaino *et al.*, 2006; Elbeaino *et al.*, 2007; Elçi *et al.*, 2012).

Two additional closteroviruses, *i.e*. Arkansas fig closterovirus 1 and 2 (AFCV-1 and 2) have been also reported from mosaic-diseased fig plants in Arkansas, and shown to be closely related to FLMaV-1 (Tzanetakis *et al.*, 2010). However, little molecular information are available on AFCV-1 genome (311 nucleotides, accession number JN882588), whereas no sequences are reported for AFCV-2 (Tzanetakis *et al.*, 2010) suggested that both are variants of FLMaV-1, 2 and FMMaV.

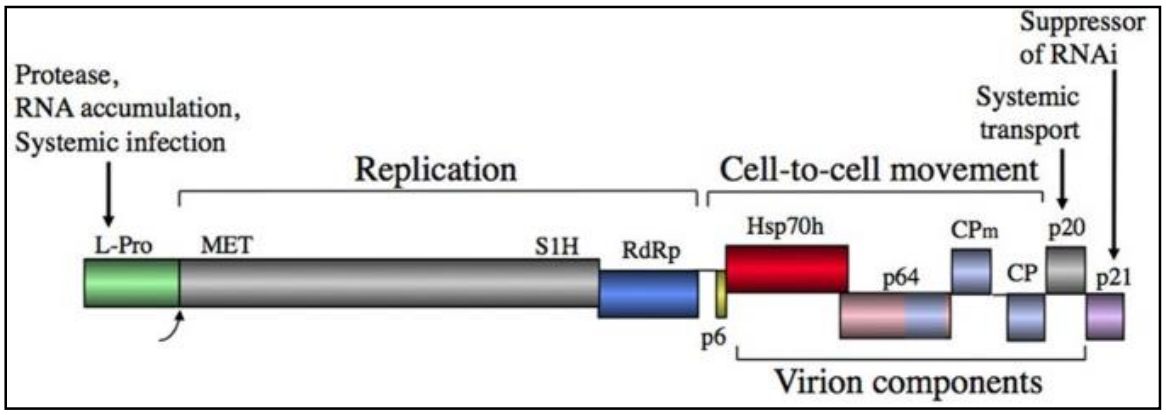


Figure 2: Diagram of *Beet yellows virus* (BYV) genome with gene functions shown, the type species of the genus *Closterovirus*. L-Pro, papain-like leader protease; MET, methyl transferase; S1H, superfamily I helicase; RdRp; p6, 6 kDa protein; HSP70h, HSP70 homolog; p64, 64 kDa protein; CPm, minor capsid protein; CP, capsid protein; p20, 20 kDa protein; p21, 21 kDa protein (Dolja *et al.*, 2006).

Fig leaf mottle-associated virus 2 (FLMaV-2)

Fig trees host an additional member of the family *Closteroviridae*, tentatively identified as a putative species of the genus *Ampelovirus*, denoted FLMaV-2. This virus was recovered from an infected fig tree of Algerian origin displaying chlorotic mottling of the leaves and clearing of the second and third veins (Elbeaino *et al.*, 2007). FLMaV-2 is not transmissible mechanically to herbaceous hosts, similarly to FLMaV-1. FLMaV-2 has a particle exceeding 2000 nm in length, which is congruent to that of the family *Closteroviridae* (Elbeaino *et al.*, 2007) and has a double-stranded RNA of about 20 kb. Its genome organization is most likely similar to the type-species of the genus *Ampelovirus*, *i.e. Grapevine leafroll-associated virus* 3 (GLRaV-3) (Figure 3). Sequence analysis of HSP70 gene showed an amino acids identity of ca. 45% to closteroviruses. Specific primers were designed on the basis of HSP70 and successfully used in RT-PCR to detect this virus in infected fig trees. FLMaV-1 and FLMaV-2 occur in fig growing areas of six Mediterranean countries (Albania, Algeria, Lebanon, Syria, Tunisia and Italy) with different extent of infections (Elbeaino *et al.*, 2010).

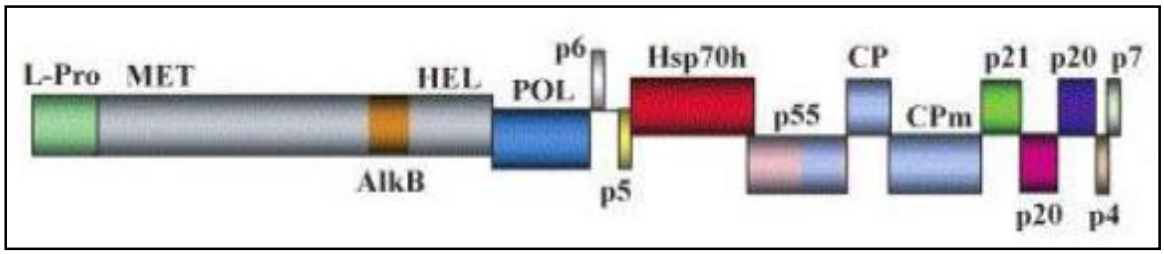


Figure 3: Diagram of *Grapevine leafroll-associated virus 3* (GLRaV-3) genome, the type species of the genus *Ampelovirus*. L-Pro: Leader Papain-like protease, MET, Hel, POL: RdRp, p6: 6 KDa protein, p5: 5 KDa protein, HSP70h: Homologue of the heat shock-proteins from the HSP70 family, p55: 55 KDa protein, CP, CPm, p21: 21 KDa protein, p20: 20 KDa protein, p4: 4 KDa protein, p7: 7 KDa protein (Dolja *et al.*, 2006).

Fig mild mottle-associated virus (FMMaV)

FMMaV is a tentative member of the *Closterovirus* genus, found associated with symptoms of light mottling with little or no malformation of the leaves, generally milder than those displayed by mosaic-affected fig plants (Elbeaino *et al.*, 2010). Long filamentous particles with distinct cross banding, resembling those of closterovirus virions, were observed by electron microscopy. Seven open reading frames (ORFs), *i.e.* an incomplete ORF1b encoding

the putative RNA-dependent RNA polymerase (RdRp), a 25 kDa protein with unknown functions, a 6 kDa protein with putative nucleotide-binding properties, a 63 kDa homologue of the heat-shock protein 70 (HSP70) that showed 30% sequence divergence compared to HSP70 of (FLMaV-1) (Elbeaino *et al.*, 2009c), a 64 kDa protein, the minor coat protein (CPm) of 26 kDa in size, and the incomplete coat protein (CP) form the genes till now identified. The genome organization of FMMaV is the same as that of members of the genus *Closterovirus* (Figure 4). This classification was confirmed by comparative analyses of the RdRp, HSP70h and (CPm) amino acid sequences of members of the genus (Elbeaino *et al.*, 2010).

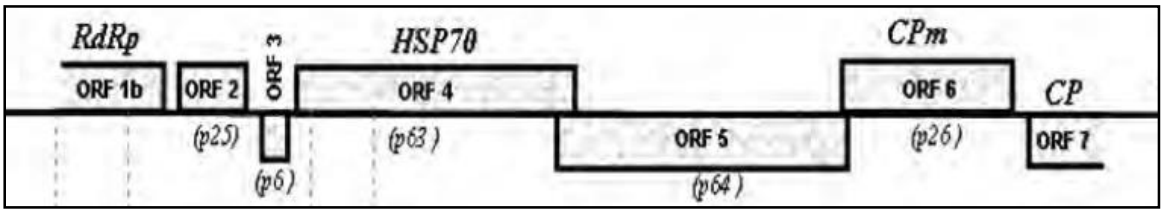


Figure 4: Schematic representation of partial genome of FMMaV. Boxed regions correspond to ORFs and boxes with open ends correspond to incomplete ORFs. The sequenced genome encompasses seven open reading frames (ORFs) (Elbeaino *et al.*, 2010).

Fig Badnavirus 1 (FBV-1)

Badnaviruses are among the most significant viruses because of the severity of symptoms they induce and for their common integration in the plant genomes (Chiumenti *et al.*, 2013). *Badnaviruses* are dsDNA *Pararetroviruses* and capable of episomal replication. They have circular genomes of 7-8 kb, encapsidated in non-enveloped bacilliform virions. FBV-1 was detected in both fig mosaic symptomatic and asymptomatic trees (Laney *et al.*, 2012). Four ORFs were determined in the genome of FBV-1. The largest ORF III polyprotein product carries the movement protein, the virus coat protein, the aspartic protease, the reverse transcriptase and the ribonuclease H domains (Figure 5). FBV-1 is the only reported DNA virus in fig (Laney *et al.*, 2012; Chiumenti *et al.*, 2013). It can be transmitted to seedlings without inducing symptoms. Mealybugs and aphids are vectors of *Badnaviruses*, thus FBV-

1 may share vectors with the closteroviruses that infect this crop (Tzanetakis *et al.*, 2010). FBV-1 seems to be simply transmissible by mechanical means.

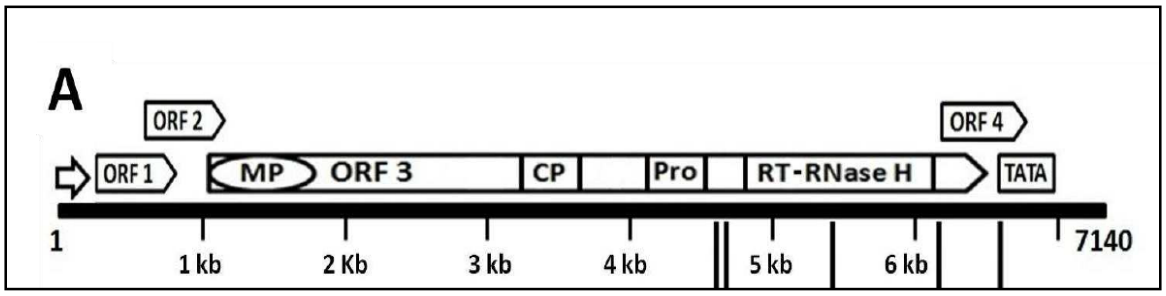


Figure 5: Linear representation of *Fig badnavirus-1* genome (A) showing tRNA MET primer-binding site (denoted by an arrow); TATA-box (denoted by a box); ORF 1; ORF2; ORF 3 with movement protein (MP), capsid protein zinc-finger domain (CP), pepsin like aspartate protease (Pro), reverse transcriptase (RT) and RNase H (RNase H) motifs; and ORF 4 (Laney *et al.*, 2012).

Fig cryptic virus 1 (FCV-1)

FCV-1 is a bipartite dsRNA virus reported from Italy. It is a tentative species of the genus *Alphacryptovirus* in the family *Partitiviridae*. It is one of viruses known to infect woody crops in nature (Elbeaino *et al.*, 2011b; Elçi *et al.*, 2012).

Members of the family *Partitiviridae* are transmitted through seeds and pollen but not by grafting or mechanical inoculation. FCV-1 does not induce symptoms.

Virions of this family are isometric, with two monocistronic double-stranded RNA. As it is shown in (Figure 6), viral dsRNAs are 1696 bp (RNA-1) and 1415 bp (RNA-2) in size. RNA-1 contains a single ORF (1419 nts) potentially encoding a 54 kDa protein (Elbeaino *et al.*, 2011b). The presence of FCV-1 has been reported in fig trees from six different Mediterranean countries (Elbeaino *et al.*, 2011b; Elçi *et al.*, 2012).

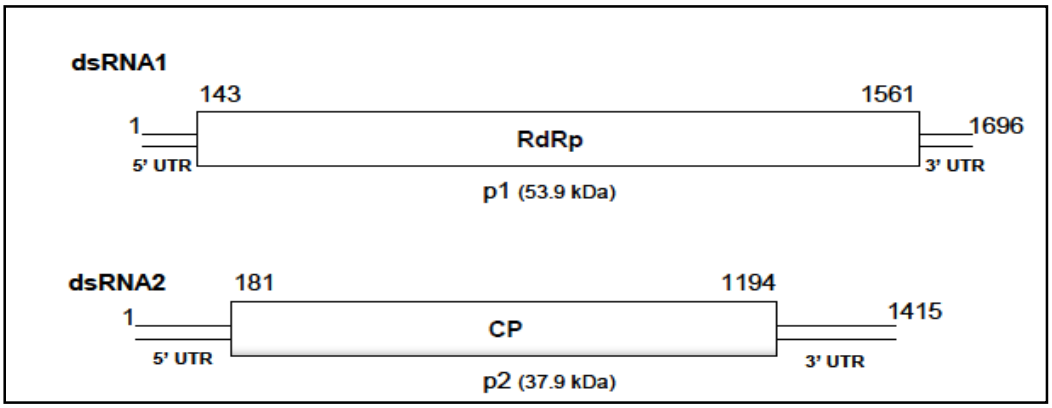


Figure 6: Schematic representation of the genome of FCV-1. DsRNA-1 contains the RdRp ORF (nt position 143-1561) and dsRNA-2 codes for a putative capsid protein (nt position 181-1194). The RdRp and the putative CP genes are represented by rectangular boxes and their estimated molecular weights are indicated between brackets. Ends of both segments contain not translated regions (5'and 3'UTR).

Fig fleck-associated virus (FFkaV)

FFkaV is a positive sense, single stranded RNA virus, recognized as one of putative FMD associated viruses (Elbeaino *et al.*, 2011a). It belongs to the family *Tymoviridae.* Members of this family are known to infect cultivated and wild monocotyledonous and dicotyledonous plants and their genome harbors a large polyprotein needed for viral replication (Dreher *et al.*, 2005). This virus is not transmissible mechanically to herbaceous hosts. Sequence analysis showed that the ssRNA genome comprises 7,046 nucleotides in size, excluding the 3'-terminal poly (A) tract, and contains two open reading frames (Figure 7). ORF1 encodes the replication-associated polyprotein RP containing the signatures of MTR, PRO; Hel; RdRp and the CP cistron. ORF2 (lower box) encodes the putative movement protein (MP) (Elbeaino *et al.*, 2011a).

FFkaV symptoms consist of discrete clearing (flecking) of some veinlets. It occurs in field-grown fig trees in six Mediterranean countries.

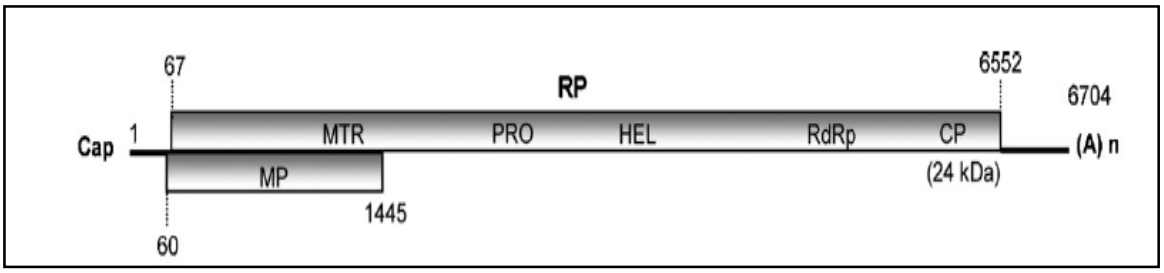


Figure 7: Schematic representation of FFkaV genome. ORF1 (upper box) codes for the replication-associated polyprotein RP containing the signatures of MTR, PRO; Hel, RdRp and the CP cistron. ORF2 (lower box) encodes the MP.

Fig latent virus 1 (FLV-1)

FLV-1 is widespread in Apulian (Southern Italy) fig orchards, in trees showing or not mosaic symptoms FLV-1 is a putative member of the genus *Trichovirus* in the family *Flexiviridae*, with filamentous particles *ca.* 700 nm long and the viral genome is a single-stranded positive-sense RNA with an estimated size of ca. 8,000 nt (Gattoni *et al.*, 2009).

This virus has a relevant difference with all members of the genus regarding the size of the coat protein subunits (46 versus 22-27 kDa) and the presence of four ORFs (Figure 8). It is transmitted by seeds at high efficiency rate (from 80 to 100%), a feature that differentiates FLV-1 from most of the other seed-borne plant viruses and from other *Trichoviruses*, none of which result to be transmitted through seeds. It is mechanically transmitted by sap inoculation to a limited number of indicator plants without inducing symptoms (Gattoni *et al.*, 2009). Immunosorbent electron microscopy (ISEM) (Milne, 1984) using specific viral antiserum was found useful to ascertain the presence of FLV-1. RT-PCR applied on silica-extracted TNAs from leaf tissues or cortical scraping was also found reliable in detecting FLV-1 (Gattoni *et al.*, 2009).

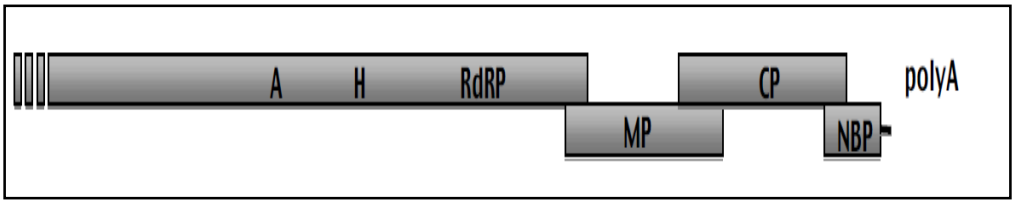


Figure 8: Diagrammatic representation of the FLV-1 genome. A domain; H, helicase; RpRd, MP, CP and NBP, nucleotide-binding protein (Gattoni *et al.*, 2009).

Fig mosaic general symptoms

The range of symptoms varies from tree to tree. Symptoms are characterized by foliar discolorations (green-yellowish mottling, mosaic, ring and line patterns) due to disrupted photosynthetic pathway consequent to chloroplast modifications mediated by unregulated micro RNAs (miRNA) (Li *et al.*, 2012), malformation due to variations of hormone-dependent metabolic pathways, or a localized unbalanced miRNAs which play a critical role in leaf shaping and development (Rubio-Somoza and Weigel, 2011). Tree decline symptoms are observed in some case with severe infections.

Geographical distribution of viruses associated with fig mosaic disease (FMD)

Although the history of fig viruses is relatively recent, thanks mainly to molecular diagnostic techniques it has been possible to detect their presence in many fig-growing countries. FCV-1, FFkaV, FMMaV and FMV were detected In Iran (Ale-Agha and Rakhshandehroo, 2014; Norozian *et al.*, 2014). FLMaV-1 and FMV were reported as causal organisms of FMD in Saudi Arabia (Alhudaib, 2012). FLMaV-1 (predominant), FMV, FLMaV-2 and FMMaV were detected In Egypt (Elbeshehy and Elbeaino, 2011), as well as FMMaV and FLV-1 in Tunisia (El-Air *et al.*, 2013). In USA various fig cultivars were found infected with FBV-1 (Laney *et al.*, 2012). FMMaV, FMV and FBV-1 were recently reported from Montenegro (Latinovic *et al.*, 2019), whereas FCV-1 in fig trees from Albania, Algeria, Italy, Lebanon, Syria Tunisia (Elbeaino *et al.*, 2011b) and Turkey (Elci *et al.*, 2017). FLMaV-1 and FLMaV-2 were reported from Tunisia (Nahdi, 2007), California (Angelini *et al.*, 2007), Mexico, South Africa (Castellano *et al*., 2007), western Saudi Arabia (Aldhebiani *et al.*, 2015), Bosnia and Herzegovina (Delić *et al.*, 2016), Montenegro (Perović *et al.*, 2016), Albania, Algeria, Lebanon, Syria and Italy with different extent of infections (Elbeaino *et al*., 2010).

Detection methods of fig viruses

- **Molecular methods**

➢ The analysis of double-stranded RNAs is often used to detect fig viruses, but it is still considered as a non-reliable technique due to the following reasons: dsRNA is an intermediate product of the replication of viral ssRNA in plant cells and is consistently found in plants infected with ssRNA viruses, which represent approximately 90% of all described plant viruses, regardless of the host (Valverde *et al.*, 1990); virus concentration is variable during seasons and the presence of inhibitors and detriments (latex, polysaccharides) can inhibit their extraction. This technique cannot be applied routinely because it is laborious, time consuming and aspecific.

➢ RT-PCR was developed to detect RNA viruses, employing reverse transcriptase which is added at the reverse transcription step before PCR (Webster *et al.*, 2004; Lopez *et al.*, 2008). This technique shows high efficiency to detect virus presence during different periods of the year and from different plant tissues. This tool is the more practical alternative to dsRNA due to its higher sensitivity and specificity.

- SybrGreen-based- TaqMan RT-PCR and RT-LAMP diagnostic method are available for FLV-1 (Chiumenti *et al.*, 2012) and FMV detection (Ishikawa *et al.*, 2015), respectively.

- **Serological methods**

➢ Only a few applications of serological techniques in the fig virus detection have been made out due to the not transmissible nature of fig viruses onto herbaceous hosts and the difficulties to purify them from highly milky fig tissues. These limitations have hindered their identification and the production of specific antisera, except for FLV-1, for which a polyclonal antiserum produced starting from infected root tissues is only reliable in Western blot and Immunosorbent electron microscopy (ISEM) assays (Gattoni *et al.*, 2009). Lately, a polyclonal antiserum has been produced for the most important virus of fig, FMV, through the recombinant protein technology (Shahmirzaie *et al.*, 2019). This polyclonal antiserum could be largely employed in Western blot (WB), Dot Immunobinding assay (DIBA) and Immunosorbent electron microscopy (ISEM).

1.2 Objectives

The sanitary certification of plant propagating material seems to be the most effective measure to produce mosaic free plant material and counter the spread of viruses in fig. For this purpose, the development of higher sensitive and reliable detection methods than those currently available could be useful.

Aim of this study was to overcome the constraints normally encountered in the diagnosis of fig viruses by developing a singleplex and a Multiplex TaqMan® RT-PCR. This molecular approach is highly sensitive, relatively quick, extremely efficient, and able to detect viruses in single and multiple infections, as well as a wide range of virus isolates.

This objective will be pursued by:

- Designing sets of virus specific primers and probes, based on nucleotide sequence alignments of several isolates of each virus species, taking into account the highly conserved regions.
- Labeling all probes with different fluorophores to be used singularly and simultaneously in singleplex and multiplex TaqMan® RT-PCR for detecting single and multiple infections, respectively.
- Evaluating the sensitivity of both assays in comparison with conventional RT-PCR usually used in the detection and identification of fig viruses.

1.3 Materials and methods

Evaluation of the sanitary status of fig in Egypt

1.3.1 Virus sources

Young leaves collected in June 2017 from a total of 67 fig accessions from different Mediterranean countries, maintained in a screen house at the Mediterranean Agronomic Institute of Bari (CIHEAM-IAM Bari, Italy) and containing a minimum of 5 isolates for each of the 7 viruses in study (FLMaV-1, FLMaV-2, FMMaV, FMV, FLV-1, FCV-1, FFkaV), were used for the validation of the TaqMan RT-PCR assays. The sensitivity for the fig virus detection of the developed assays was compared to that of RT-PCR. The collection of samples from Egyptian fig trees was conducted in two different periods (May-June and November-December 2016), thus 100 samples were gathered in each period using the same fig plants for a further sensitivity comparison between the two molecular detection techniques. The plant tissues for this study consisted of leaves and lignified dormant cuttings collected in two different periods (May-June and November-December 2016, respectively) from the same fig trees of cultivars Sultany, El-Adsy, Abode and Komethery, distributed in five fig-growing areas of Egypt, *i.e.* Marsa Matrouh, Fayium, Ismailia, North Sinai and Giza. The survey involved 13 asymptomatic and 87 mosaic affected fig trees with symptoms of chlorotic blotches, vein clearing, vein banding, chlorosis, chlorotic ring spot and mosaic. Symptoms of each tree were accurately annotated and afterward correlated with the viruses detected in laboratory.

1.3.2 Total nucleic acid extraction

Total nucleic acid was extracted following the protocol of (Foissac *et al.*, 2001) consisting in the use of 0.1 g of tissue from cambial scrapings of mature fig cuttings (in autumn) and from leaf veins (in spring) that was ground in a sterile mortar in the presence of liquid nitrogen. The powder was homogenized with 1 ml of grinding buffer (Annex 1) to help in breaking the cell walls and the membranes of the plant cells.

Then, 100 µl of N-Lauryl sarcosine sodium salt (NLS), used in cell lysis to inhibit the initiation of DNA transcription (Johnson, 2013), was added on virus suspension and gently vortexed. The samples were incubated at 70°C for 7 min and cooled on ice for 5 min. After a centrifugation at 13,000 rpm for 10 min, 300 µl of the supernatant were transferred to new Eppendorf tube, 150 µl of absolute ethanol were added to preserve the integrity of the nucleic acids and to remove alcohol-soluble salts. The purity of total nucleic acid was protected by adding 300 µl of NaI, after that 35 µl of silica (Annex 1) were added to capture the free nucleic acid in the suspension. The mixture was stirred for 25 min at room temperature and centrifuged at 6,000 rpm for 1 min. The silica-pellet was washed three times with 500 µl of washing buffer to remove possible salt residues. The pellet was dried and then resuspended in 120 µl of sterile water, incubated at 70°C for 4 min and centrifuged for 3 min at 13,000 rpm. Then 120 µl of the supernatant were recovered, placed in a new Eppendorf tube and stored at -20°C (Annex 1). To evaluate the quantity and quality of extracted total nucleic acid (TNA), 10 µl of each sample were loaded in a 1.2% agarose gel in TBE buffer 1X (Annex 2) and visualized by staining GelRed nucleic acid dye (Biotium) 0.5 µg/ml and UV illumination. The purity of the extracted nucleic acid was measured by Nanodrop at A260/280, with a ratio of ~2.0 for almost all samples.

1.3.3 Design of primers and TaqMan probes

For TaqMan RT-PCR assays, different sets of sense and antisense virus-specific primers/probes were manually designed based on the alignment of the genomic nucleotide sequences of numerous viral isolates for each species retrieved from GenBank (Table 2), to identify the most conserved nucleotide stretches on which universal primers and probes were designed to detect the majority possible of virus isolates.

Table 2: Accession numbers of sequences of virus isolates used in the CLUSTAL alignment for the identification of conserved genomic regions.

Virus	GenBank accession number
FLMaV-1	MG407556/ KU198352/ KU198380/ KU198382/KU198383/ KU198384/ KU198385/KU198386/ LN873219/ AM113547/ KX397035/ LN850109/ LN850110/ LN850111/ KU198378/ KU198379
FLMaV-2	FJ473383/ AM286422
FMMaV	FJ611959
FMV	KX397602/ KX397603/ KX397604/ KC295760/ KC295761/ KC295754/ KC295746/ KC295747/ KC295749/ KC295756/ KC295757/ AM941711
FCV-1	FR776004/ FR776005/ FR776006/ FR776007 /FR776008/ FR776009/ FR687854
FLV-1	MG407553/ KM516762/ KM516763/ FN377573
FFkaV	FR821254/ FR821255/ FR821256/ FR821257/ FR821258/ FM200426

Nucleotide sequences were aligned for homology using Clustal X program (Thompson *et al.*, 1997). Firstly, these primers were used in RT-PCR to evaluate their capacity to detect large numbers of virus isolates from different geographical origin. Primers pairs that showed the broadest spectrum of detection for each virus were chosen and adopted in TaqMan RT-PCR assays (Table 3). The viral nature of the amplicons generated from different PCRs was ascertained by sequencing (Eurofins Genomics, Germany).

Table 3: List of primers and TaqMan probes used in RT-PCR and TaqMan RT-PCR for the detection of seven fig viruses.

Virus	Primer	Target gene	Primer sequence (5'-3')	PCR size (bp)	References
FLMaV-1	N17s N17a	RT-PCR HSP70h	CGTGGCTGATGCAAAGTTTA GTTAACGCATGCTTCCATGA	352	(Elbeaino *et al.*, 2006)
FLMaV-2	F3s F3a	RT-PCR HSP70h	GAACAGTGCCTATCAGTTTGATTTG TCCCACCTCCTGCGAAGCTAGAGAA	360	(Elbeaino *et al.*, 2007)
FMMaV	LM3s LM3a	RT-PCR HSP70h	AAGGGGAATCTACAAGGGTCG TATTACGCGCTTGAGGATTGC	311	(Elbeaino *et al.*, 2010)
FMV	E5s E5a	RT-PCR RdRp	CGGTAGCAAATGGAATGAAA AACACTGTTTTTGCGATTGG	302	(Elbeaino *et al.*, 2009a)
FLV-1	ff-up ffdo	RT-PCR RdRp	CGCTTTGCCCCAATGTGCAGAT TCGAAGGCCAGAGTTGATGCA	193	(Gattoni *et al.*, 2009)
FCV-1	R1s R1a	RT-PCR RdRp	TCGGATTGTCTTTGGAGAGG CGCATCCACAGTATCCCATT	353	(Elbeaino *et al.*, 2011a)
FFkaV	d8s d8a	RT-PCR RdRp	TCAATCCCAAGGAGGTGAAG ACACGGTCAATGAGGGAGTC	270	(Elbeaino *et al.*, 2011b)
FLMaV-1	LM1s LM1a Probe	TaqMan HSP70h	ACTTCAACCTCACTATAACGTGGAA GACTAGGCGTAAACAATATAATGA **FAM-TGCATATAACTCGTGGGGCACGAAGT-BHQ1**	140	This study
FLMaV-2	LM2s LM2a Probe	TaqMan CPm	CGGACGGTAAGGTGGAAACTG GATAGGCACTGTTCGCAGCTTGT **ROX-TTACCCACGGTTATTTACTTGCGACC-BHQ2**	141	
FMMaV	MMs MMa Probe	TaqMan CPm	CGGAAACGCTGACTTACAC GAAAGAACGCACGAACAAC **HEX-AGTCTTAGTTGGCGGCTCTAGCGCACTC-BHQ1**	78	This study
FMV	FMVs FMVa Probe	TaqMan RdRp	AGGTGCTTTCATGGTTTACTC GGATTATTGACACTGATGCCTG **CalGold540-CAACGCAGCTTAAGGTGATCAACAAAAAC-BHQ1**	161	
FLV-1	LVs LVa Probe	TaqMan CP	GCCCCAATTGCAGATACATAG GTCCCCTGAGAAGTGATC **TAM-AGTTTTTAGTCTCCAATGCCCCAGCAA -BHQ2**	121	
FCV-1	R1s R1a Probe	TaqMan RdRp	TCCCACAAGTTCAATCATTACC TGTTACCTTCACCCTTCTTACC **TEXAS RED ATTTGAAGGAGGTTTTGACCACGTCCCATTT-BHQ2**	108	
FFkaV	FKs FKa Probe	TaqMan CP	CCACCTACAAAGACACCCCAAG AGGACGCCGCGAATAATCAC **CY5-TTGGCATCAACGCACCAATTTCCGGCTC-BHQ3**	112	

1.3.4 Viral complementary DNA synthesis (cDNA)

The process of reverse transcription was performed using random hexamers to generate a cDNA pool, following the protocol of (Gubler and Hoffman, 1983). TNA solution (from 8 to 10 µl) was mixed in the presence of 1 µl random hexamers primer (Boehringer Mannheim, GbmH, Germany) (0.5 µg/µl) and 1.5 µl sterile distilled water (SDW), denatured at 94°C for 5 min and kept in ice for 5 min. Denatured TNA was reverse transcribed for 1h at 39°C in 4 µl M-MLV buffer 5x (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 2 µl DTT (10 mM), 1 µl dNTPs (0.5 mM), and 0.5 µl *Moloney Murine Leukaemia virus* (M-MLV) (200 U/µl) reverse transcriptase (Invitrogen Laboratories, USA) in a final volume of 20 µl.

1.3.5 Reverse-transcription polymerase chain reaction (RT-PCR)

The reverse-transcription polymerase chain reaction (RT-PCR) was carried out on reverse transcribed TNA, using seven primer pairs, known as virus-specific primers which were reported previously in literature to detect FLMaV-1, FLMaV-2, FMMaV, FMV, FLV-1, FCV-1 and FFkaV.

An amount of 2.5 µl of reverse transcribed TNA mixture (cDNA) from each sample served to perform a PCR with an additional mixture of 2.5 µl *Taq* polymerase buffer 10X (Promega corporation, Madison, WI, USA), 0.5 µl of dNTPs (10 mM), 0.5 µl of forward primer (10 µM), 0.5 µl of reverse primer (10 µM) and 0.25 µl of *Taq* polymerase enzyme (5 U / µl) in a final volume 25 µl adjusted with sterile water. PCR amplification was performed in Bio-Rad C1000 thermal cycler by applying an initial denaturation at 94°C for 4 min, followed by 40 cycles with an initial denaturation temperature of 94°C for 30 sec, primer annealing at 55°C for 35 sec, elongation at 72°C for 35 sec and a final elongation at 72°C for 5 min. RT-PCR products were analyzed by electrophoresis on 1.2% agarose gel in TAE buffer 1 X (Annex 2), stained with GelRed dye (Aurogen, Italy) 0.5 µg/ml and visualized under UV illumination.

1.3.6 TaqMan RT-PCR

For TaqMan RT-PCR approach, reactions were replicated three times and conducted in 2.5 µl cDNA, 10 µl SsoFast Probe Supermix buffer 2 X (Promega, Milan, Italy), 0.5 µl of sense and antisense primers (10 µM), 0.5 µl of probe (5 µM), and 6 µl RNAse-free water in a final volume of 20 µl.

Amplification process was conducted in CFX1000 Real-time PCR apparatus (BioRad, Hercules, CA) with the following thermocycling conditions: initial denaturation step at 94°C for 10 sec, primer annealing temperature at 94°C for 30 sec and a final elongation step at 56°C for 40 sec. Each run included a negative control (virus-free fig material), a positive control (virus-infected fig material) and a non-template control (NTC).

Trials of using different primers and probes concentration were conducted to stabilize the best amplification curves in TaqMan RT-PCR. Given that no differences were observed, when comparing RT-PCR results using the same concentration with those of TaqMan RT-PCR; same concentration used in RT-PCR was adopted also in TaqMan RT-PCR.

Forty PCR cycles were performed, and data were collected during the 58°C stage and analyzed using the manufacturers' software (GelDoc EZ system, v. 6.0.1, BioRad). Amplicons generated were analyzed by electrophoresis in 1.5% agarose gel to check those primers (together with probes) that amplified the exact size of expected viral DNA. PCR amplification conditions were the same as described previously.

1.3.7 Multiplex TaqMan RT-PCR

This diagnostic technique allows the simultaneous and sensitive detection of different RNA targets (as in the case of multiple infections) in a single reaction. In order to take further advantage of the newly designed primers and probes, the multiplex TaqMan RT-PCR approach was also applied for the simultaneous detection of different fig viruses in a single reaction. To this aim, up to five virus-specific primers with their corresponding probes were mixed in one reaction tube for the detection of the viruses under study. The interpretation of results was eased by the colorimetric aspect of the TaqMan probes designed with different fluorescence (Table 3). The reactions were performed in a final volume of 20 µl, consisting

of 2.5 µl cDNA, 10 µl SsoFast Probe Supermix buffer 2 X (Promega, Milan, Italy), 0.5 µl of sense and antisense primers (10 µM), 0.5 µl of probe (5 µM) and 6 µl SDW added to adjust the final volume. The cycling conditions were as follows: initial denaturation step at 94°C for 10 sec, primer annealing temperature at 94°C for 30 sec and a final elongation step at 56°C for 40 sec. Data were collected as described for the TaqMan RT-PCR.

1.3.8 Comparison of sensitivity between RT-PCR and TaqMan RT-PCR

To analyze the differences in sensitivity between TaqMan RT-PCR and RT-PCR, identical amounts of cDNA were used for both techniques. The serial dilutions of the cDNA synthesized from TNA extracted from infected and virus-free fig materials were tested. Since the virus concentration in the original plant material is unknown, the limit of detection was expressed as relative value. The relative limit of detection was defined based on the CT average of the amplification curves (Ct < 32). The concentration of cDNA used in all PCR assays was estimated to be 50 ng/µl. Series of ten-fold dilutions (from 10^{0} to 10^{-6}) of the synthesized cDNA templates, originated from healthy and infected fig plants, were prepared for each virus species. Three replicates of each dilution were amplified following the conditions and protocols described previously.

1.3.9 Validation of the TaqMan RT-PCR technique

To validate the TaqMan RT-PCR developed for detecting fig viruses, infected samples from several fig growing areas in Egypt, collected in two different periods of the year (early summer and autumn), were analyzed in comparison with the RT-PCR and the results generated from both techniques were evaluated.

1.4 Results

1.4.1 Validation of TaqMan RT-PCR assays for the detection of fig viruses

The primers and TaqMan probes designed in this study, which were used primarily in singleplex approaches to evaluate their efficacy in TaqMan RT-PCR, enabled the detection of fig viruses in sources from different geographical origins. In the cases of FLMaV-1, FLMaV-2, FMV and FFkaV, these primers and probes proved to be highly performing and to possess a large detection spectrum by amplifying a higher number of isolates than the RT-PCR (Table 4). The only exception was in the FLV-1 detection, for which the primers and probes used in TaqMan RT-PCR (Table 3) were not able to amplify all FLV-1 isolates analyzed, contrarily to the RT-PCR assay (Table 4). This result was somehow predicted, considering the high sequence variability reported in FLV-1 genome (Minafra *et al.*, 2009) and the use of an additional stretch of nucleotide sequences (probe TaqMan) needed for the overall TaqMan RT-PCR amplification process likely influenced the success of this method in this case.

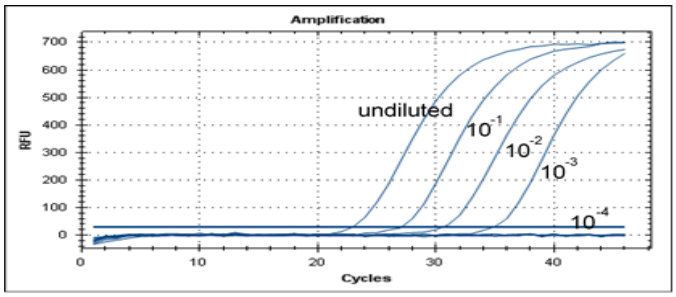
Table 4: Results of RT-PCR and TaqMan RT-PCR assays conducted on leaf samples of fig accessions from Mediterranean origins, maintained under screen house and\or in the field, with single and multiple virus infections, used as positive controls reactions. Some of the isolates were infected with up to five viruses. Shadowed numbers represent divergent results obtained with the two different diagnostic techniques.

Source	Sample No.	FLMaV-1		FLMaV-2		FMMaV		FMV		FCV-1		FLV-1		FFkaV	
		RT-PCR	TaqMan RT-PCR	RT-PCR	TaqMan RT-PCR	RT-PCR	TaqMan RT-PCR	RT-PCR	TaqMan RT-PCR	RT-PCR	TaqMan RT-PCR	RT-PCR	TaqMan RT-PCR	RT-PCR	TaqMan RT-PCR
Albania	8	1	1	-	-	-	-	2	2	-	-	-	-	1	1
Algeria	6	1	1	3	3	-	-	1	1	-	-	-	-	2	2
Egypt	11	2	3	2	2	1	1	3	4	-	-	3	1	2	3
France	6	2	3	-	-	-	-	2	3	-	-	-	-	1	1
Italy	14	4	4	3	4	2	2	6	6	3	3	3	1	3	5
Lebanon	11	4	4	4	5	3	3	7	7	1	1	4	2	4	5
Saudi Arabia	4	3	3	-	-	-	-	3	4	-	-	-	-	1	1
Tunisia	10	4	5	2	2	2	2	7	8	1	1	3	1	5	6
Turkey	7	1	1	1	1	1	1	2	2	-	-	1	-	3	3
Total	**67**	**22**	**25**	**15**	**17**	**9**	**9**	**33**	**37**	**5**	**5**	**14**	**5**	**22**	**27**
Infection %		**32.8**	**37.3**	**22.3**	**25.3**	**13.4**	**13.4**	**49.2**	**55.2**	**7.4**	**7.4**	**20.8**	**7.4**	**32.8**	**40.2**

1.4.2 Sensitivity comparison between TaqMan RT-PCR and RT-PCR

A further validation of TaqMan RT-PCR performance was conducted through a sensitivity comparison between the newly developed methods and the conventional RT-PCR reported in the literature for the detection of fig viruses. Ten-fold serial dilutions of viral cDNA were prepared to compare the detection limits, sensitivity and reliability of RT-PCR and TaqMan RT-PCR techniques. Differences in detecting fig-viruses in TaqMan RT-PCR were noticed directly after the second dilution, once compared with the RT-PCR. In general, the RT-PCR assays showed the lowest detection limit with a maximum detection power of 10^{-2} dilution, differently from TaqMan RT-PCR, which was able to detect all fig viruses at higher dilutions. In addition, the detection of six viruses in TaqMan-PCR was nearly at the same level of sensitivity. The level of sensitivity in the detection of each single virus is shown in the Figures 9-14. For FLMaV-1 detection, TaqMan RT-PCR showed to be slightly more sensitive than the RT-PCR since it was able to detect the virus up to 10^{-3} dilution, compared to 10^{-2} dilution with RT-PCR (Figure 9, A and B).

(A)



(B)

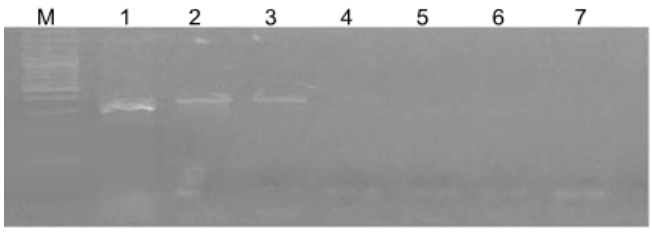
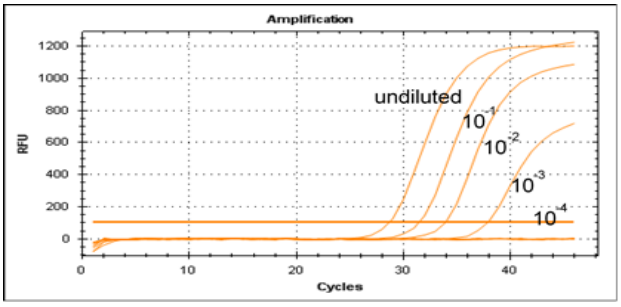


Figure 9: Sensitivity comparison between RT-PCR and TaqMan assay for the detection of FLMaV-1 in ten-fold dilution series, starting from purified cDNA of infected fig plants (A and B). (A) Curves of fluorescence accumulation through amplification cycles of serial cDNA dilutions showing the dynamic range of detection of real-time PCR assay. (B) Agarose gel showing the amplification products obtained by RT-PCR. M: 100 bp DNA ladder, lane 1 cDNA: undiluted cDNA, lane 2: 1: 10 dilution, lane 3: 1: 10^2 dilution, lane 4: 1: 10^3 dilution, lane 5: 1: 10^4 dilution, lane 6: 1: 10^5 dilution and lane 7: 1: 10^6 dilution.

More significant was the difference in the sensitivity of FLMaV-2 detection by TaqMan RT-PCR (up to 10^{-3} dilution) in comparison with the detection limit of conventional RT-PCR (only the undiluted cDNA showed an amplified band) (Figure 10, A and B).

(A)



(B)

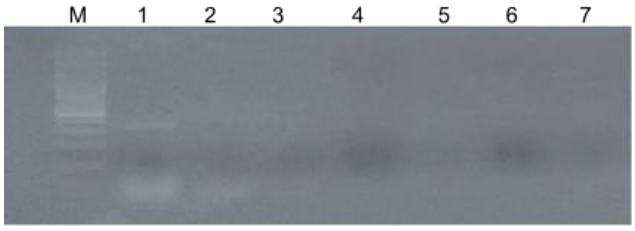
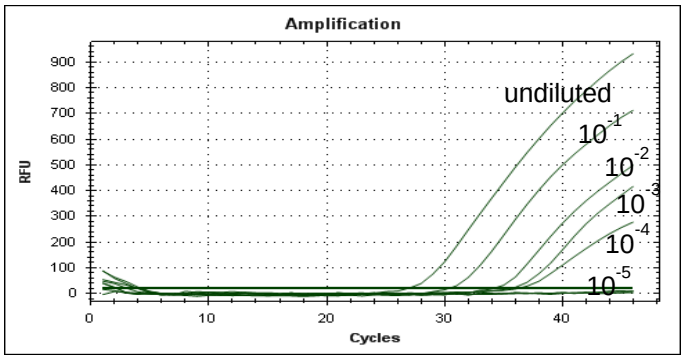


Figure 10: Sensitivity comparison between RT-PCR and TaqMan assay for the detection of FLMaV-2 in ten-fold dilution series started from purified cDNA of infected fig plants (A and B). (A) Curves of fluorescence accumulation through amplification cycles of serial cDNA dilutions showing the dynamic range of detection of real-time PCR assay. (B) Agarose gel showing the amplification products obtained by RT-PCR. M: 100 bp DNA ladder, lane 1 cDNA: undiluted cDNA, lane 2: 1: 10 dilution, lane 3: 1: 10^2 dilution, lane 4: 1: 10^3 dilution, lane 5: 1: 10^4 dilution, lane 6: 1: 10^5 dilution and lane 7: 1: 10^6 dilution.

The higher sensitivity of TaqMan RT-PCR was also confirmed for FMMaV, which detected the virus at a dilution of 10^{-4} compared to 10^{-1} dilution of conventional PCR (Figure 11, A and B).

(A)



(B)

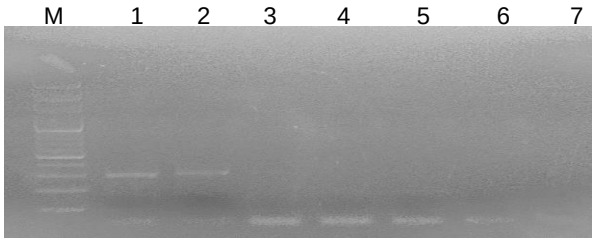
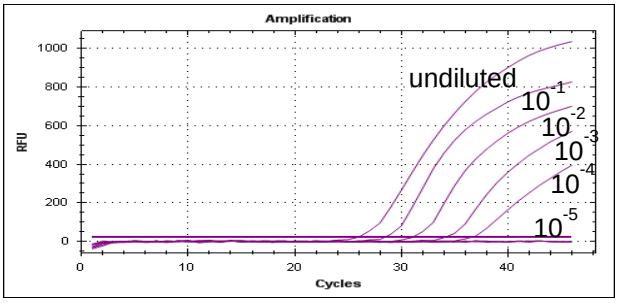


Figure 11: Sensitivity comparison between RT-PCR and TaqMan PCR assay for the detection of FMMaV in ten-fold dilution series started from purified cDNA of infected fig plants (A and B). (A) Curves of fluorescence accumulation through amplification cycles of serial cDNA dilutions showing the dynamic range of detection of Real-time PCR assay. (B) Agarose gel showing the amplification products obtained by RT-PCR. M: 100 bp DNA ladder, lane 1 cDNA: undiluted cDNA, lane 2: 1: 10 dilution, lane 3: 1: 10^2 dilution, lane 4: 1: 10^3 dilution, lane 5: 1: 10^4 dilution, lane 6: 1: 10^5 dilution and lane 7: 1: 10^6 dilution.

Similar results were obtained in the detection of FFkaV, which was detected up to a dilution of 10^{-4} by TaqMan RT-PCR compared to 10^{-2} dilution with the conventional PCR (Figure 12, A and B)

(A)



(B)

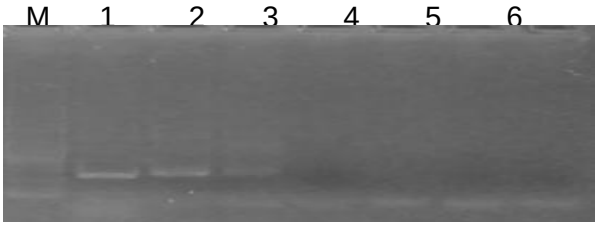
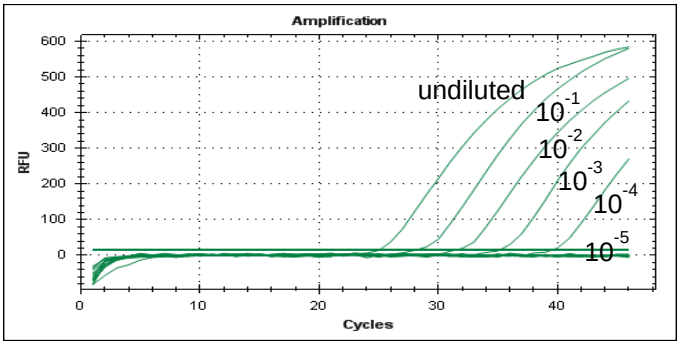


Figure 12: Sensitivity comparison between RT-PCR and TaqMan PCR assay for the detection of FFkaV in ten-fold dilution series started from purified cDNA of infected fig plants (A and B). (A) Curves of fluorescence accumulation through amplification cycles of serial cDNA dilutions showing the dynamic range of detection of Real-time PCR assay. (B) Agarose gel showing the amplification products obtained by l RT-PCR. M: 100 bp DNA ladder, lane 1 cDNA: undiluted cDNA, lane 2: 1: 10 dilution, lane 3: 1: 10^2 dilution, lane 4: 1: 10^3 dilution, lane 5: 1: 10^4 dilution, lane 6: 1: 10^5 dilution and lane 7: 1: 10^6 dilution.

PCR with the TaqMan probe presented a higher sensitivity than RT-PCR also in the detection of FMV targets (10^{-4} *vs* 10^{-2} dilutions) (Figure 13, A and B).

(A)



(B)

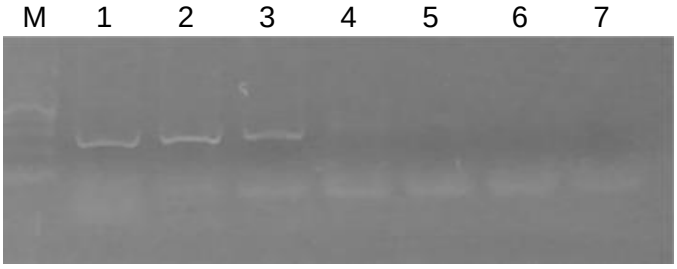
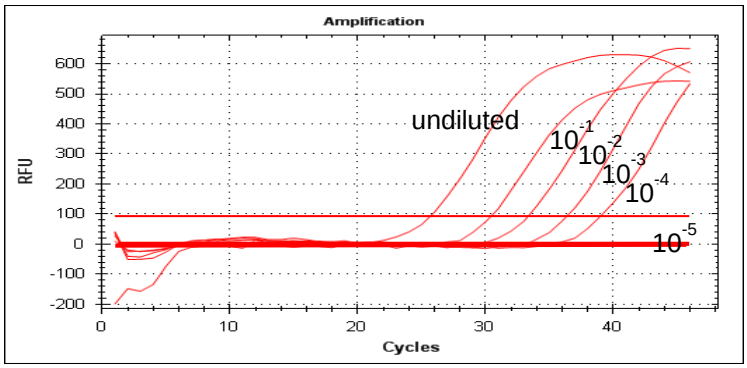


Figure 13: Sensitivity comparison between RT-PCR and TaqMan PCR assay for the detection of FMV in ten-fold dilution series started from purified cDNA of infected fig plants (A and B). (A) Curves of fluorescence accumulation through amplification cycles of serial cDNA dilutions showing the dynamic range of detection of Real-time PCR assay. (B) Agarose gel showing the amplification products obtained by RT-PCR. M: 100 bp DNA ladder, lane 1 cDNA: undiluted cDNA, lane 2: 1: 10 dilution, lane 3: 1: 10^2 dilution, lane 4: 1: 10^3 dilution, lane 5: 1: 10^4 dilution, lane 6: 1: 10^5 dilution and lane 7: 1: 10^6 dilution.

The endpoint sensitivity of FCV-1 was estimated to a dilution of 10^{-4} in TaqMan RT-PCR, whereas the RT-PCR assays showed lower levels of sensitivity with a maximum detection power of 10^{-1} dilution (Figure 14, A and B).

(A)



(B)

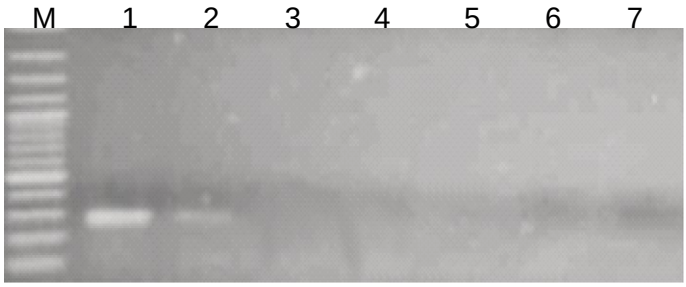


Figure 14:Sensitivity comparison between RT-PCR and TaqMan PCR assay for the detection of FCV-1 in ten-fold dilution series started from purified cDNA of infected fig plants (A and B). (A) Curves of fluorescence accumulation through amplification cycles of serial cDNA dilutions showing the dynamic range of detection of Real-time PCR assay. (B) Agarose gel showing the amplification products obtained by RT-PCR. M: 100 bp DNA ladder, lane 1 cDNA: undiluted cDNA, lane 2: 1: 10 dilution, lane 3: 1: 10^2 dilution, lane 4: 1: 10^3 dilution, lane 5: 1: 10^4 dilution, lane 6: 1: 10^5 dilution and lane 7: 1: 10^6 dilution.

1.4.3 Multiplex TaqMan RT-PCR

PCR TaqMan probes and primers, when mixed together in one tube PCR reaction, *i.e.* multiplex TaqMan RT-PCR, showed to be efficient to detect contemporarily up to five fig viruses, in natural mixed infections (Figure 15). This result was obtained independently from the combination of the viral species involved (data not shown), demonstrating the lack of interference among TaqMan RT-PCR primers and probes during the multiplex reactions. No differences were recorded in the specificity of singleplex *versus* multiplex in the detection of fig viruses. Results of multiplex TaqMan RT-PCR assays showed that the CT values of different amplifications increases with the number of viruses present in the tested sample, ranging between 20 and 40 when passing from single to multiple infections. This delay in the increment of CT value is most likely due to the low performance of the polymerase enzyme once engaged in amplifying simultaneously more than one template in one reaction. The

feasibility of multiplex RT-PCR based on an accurate design of primers and probes allowed the simultaneous and sensitive detection of different viral RNA targets (Figure 15, a, b, c and d).

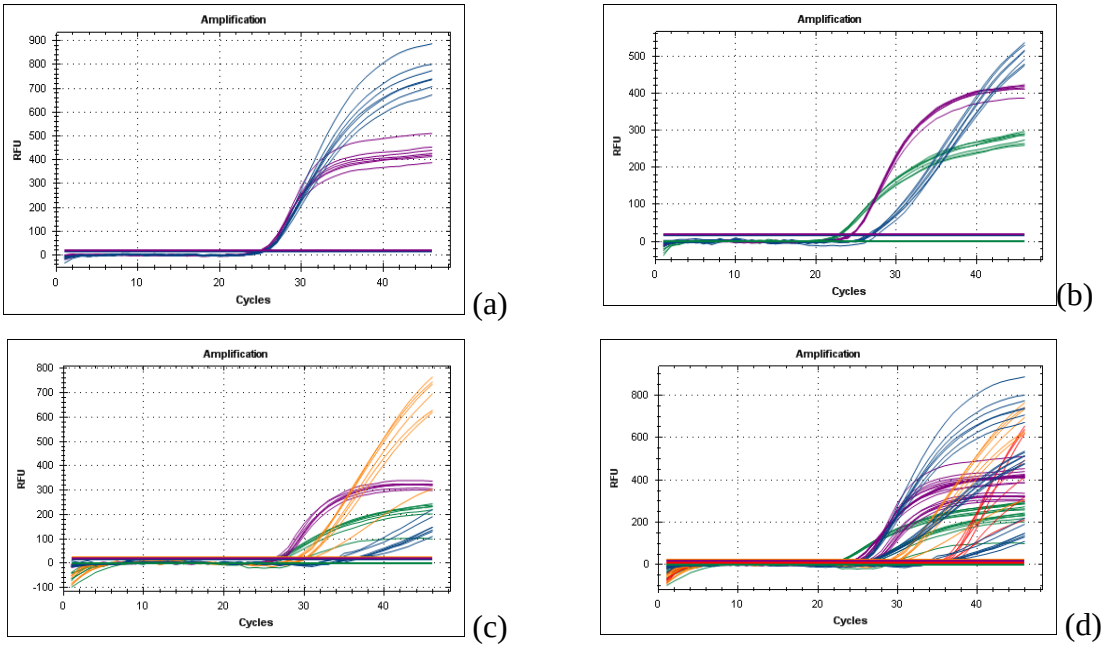


Figure 15: Multiplex qRT-PCR amplifications of: a) FLMaV-1 and FFkaV; b) FLMaV-1, FMV and FFkaV; c) FLMaV-1, FLMaV-2, FMV and FFkaV; d) FLMaV-1, FLMaV-2, FMMaV, FMV and FFkaV from purified cDNA of infected fig plants. Curves of the cDNA against the threshold cycles values showing the dynamic range of detection assay. (FLMaV-1 = blue; FFkAV= purple; FMV= green; FLMaV-2= orange; FMMaV= red).

1.4.4 Comparative detection of fig viruses by RT-PCR and TaqMan RT-PCR assays on field collected fig samples

The RT-PCR and TaqMan RT-PCR assays applied on two types of fig plant material, *i.e.* TNA extracted from of leaf tissues during May-June and that from phloem tissues during November-December 2016, showed that all viruses checked in this study were present in the Egyptian fig trees at different extent.

Out of 100 samples tested, 97 (97%) were infected by at least one virus, with mixed infections (63%) prevailing on single infections (34%). FMV (62%) and FFkaV (59%) were the prevailing viruses, followed by FLMaV-2 and FLV-1, with infections rates of 32% and 16%,

respectively. FLMaV-1, FCV-1 and FMMaV were less detected, with infection rates of 14%, 7% and 4%, respectively (Figure 16). All these results were obtained by applying TaqMan RT-PCR assay, which showed marked differences from those obtained with RT-PCR (Figure 16). In fact, once again the comparison of the two diagnostic techniques showed that the TaqMan® RT-PCR assay was almost always more efficient to detect the fig viruses than RT-PCR (on average 8.7% more positives). In particular, the TaqMan RT-PCR revealed to be more efficient than the RT-PCR in the detection of FCV-1 (>71.4%), FMMaV (>25%), FLMaV-2 (>15.6%), FFkaV (>13.6%), FMV (>11.3%) and FLMaV-1 (>7.1%), but less efficient in the detection of FLV-1 (<25%). These differences were even more marked in the comparative tests carried out at the end of autumn (Figure 16). All PCR amplicons which were positive uniquely with the TaqMan RT-PCR assay, but negative in RT-PCR, were cloned, sequenced and their viral nature were ascertained. Given the small size of the TaqMan RT-PCR amplicons, ranging between 78 and 140 bp, their sequences were considered insufficient to draw a phylogenetic scenario on the tested isolates, moreover since the variations for all viruses ranged from 1% to 3% (too small) the submission to the GenBank was not carried out.

In the detection of the fig viruses tested in this study, the assays carried out on leaf tissues in the spring time were significantly more sensitive and effective than those carried out in late autumn on phloem tissues (Figure 16). Not a single fig tree was found free from the viruses under study in the most important cv. Sultany, which was also the cultivar with the highest number of mixed infections (80%). Only one plant for each of the other three cultivars (Adobe, El Adsy and Komthery) was found virus-free (Table 5) and the rate of mixed infections ranged from 52% (cv. Adobe) to 64% (cv. El Adsy).

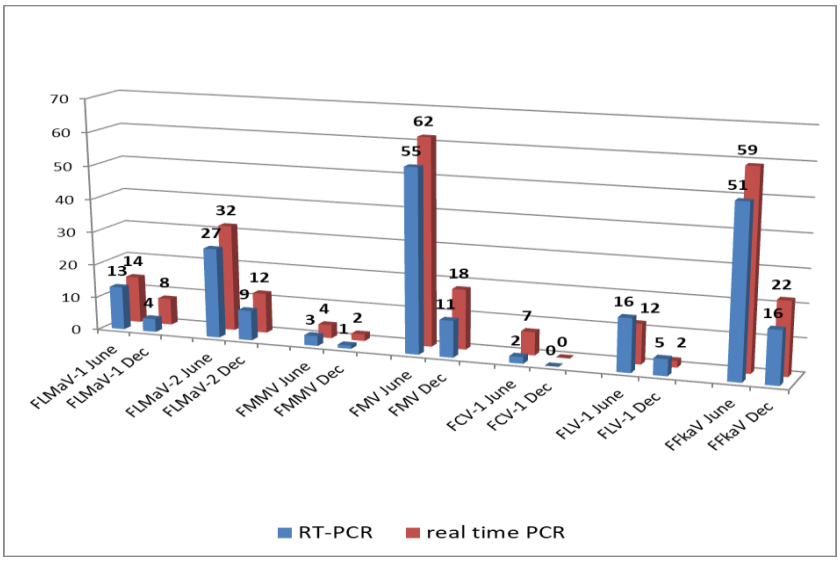


Figure 16: Differences in infection rates for each virus through the application of the RT-PCR and TaqMan RT-PCR assays on fig samples collected in June (back rectangles) and December (front rectangles) 2016.

Table 5: Virus infections found in four main fig varieties in Egypt.

Varieties	Tested trees	Infected trees		FMV		FFkaV		FCV-1		FLV-1		FMMaV		FLMaV-1		FLMaV-2	
	No.	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Sultany	25	25	100	16	64	16	64	4	16	7	28	2	8	3	12	8	32
Al-Adsy	25	24	96	14	54	16	64	1	25	6	24	0	0	6	24	8	32
Adobe	25	24	96	16	64	14	54	1	25	1	4	1	4	4	16	7	28
Komethery	25	24	96	16	64	13	52	1	25	2	8	1	4	1	4	9	36
Total	**100**	**97**	**97**	**62**	**62**	**59**	**59**	**7**	**7**	**16**	**16**	**4**	**4**	**14**	**14**	**32**	**32**

1.5 Discussion

The TaqMan® RT-PCR assay was developed to enhance virus diagnostic sensitivity in fig trees in order to implement large-scale surveys. Accordingly, this newly developed assay is strongly recommended to diagnostic laboratories for the following reasons: (i) it is up to 10^2 - 10^3 fold more sensitive than the RT-PCR for fig viruses detection, thus it allows to attenuate the seasonal fluctuation of the titer of most viruses that makes them undetectable; (ii) it is less aspecific and more able to detect a broad range of virus isolates from different geographical regions; (iii) it reduces cross contamination; (iv) it eliminates any post-PCR manipulations, *i.e.* the need for gel electrophoresis and documentation. Undoubtedly, this newly developed assay showed to be more sensitive than the RT-PCR previously used for the detection of fig viruses. These findings were in agreement with previous studies which showed higher sensitivity of TaqMan RT-PCR than RT-PCR in detecting several plant viruses (Olmos *et al.*, 2005; Loconsole *et al.*, 2010; Shiller *et al.*, 2010; Harper *et al.*, 2011). The Multiplex- TaqMan® RT-PCR was able to detect multiple infections based on an accurate primer design for the identification of several viruses by one reaction. This technique can be recommended to be a first-choice diagnostic method in fig since it was able to detect simultaneously up to five viruses without leaving any doubt on its efficacy to detect more isolates (also of different origin) due to the modality with which primers and probes were designed. This technique can efficiently replace RT-PCR especially for large scale surveys, since it is more sensitive, cheaper and less time consuming. Accordingly, this technique could be extremely useful to detect at large spectrum the fig viruses and recommended to be applied on plant material for the certification program of fig. According to previous studies, multiplex real-time PCR assays using TaqMan probe revealed to be effective to detect simultaneously up to three *Potyviruses* in tobacco plants (Dai *et al.*, 2013) and three viruses in pome fruit trees (Malandraki *et al.*, 2017).

The application of PCR techniques for a preliminary evaluation of the sanitary status of fig trees in five provinces of Egypt highlighted that all the seven viruses under study, *i.e.* FLMaV-1, FLMaV-2, FMMaV, FMV, FCV-1, FLV-1, and FFkaV, were present in the country, with infection rates from 62% (FMV) to 4% (FMMaV). Although limited to only

100 fig trees, the results of this survey clearly indicate how severely degraded is the sanitary status of fig crop in Egypt (97% of infected trees). Particularly worrying is the incidence of FMV (62%), the unique virus clearly correlated with the fig mosaic disease. The several FMV-infected samples found in association with most of the mosaic symptoms in field further confirms what previously reported regarding the etiology of FMV (Elbeaino *et al*., 2009a). Nevertheless, in two cases FMV was detected in symptomless fig trees. Whether this is due to the presence in the country of some mild virus strains or to the biological response of some Egyptian fig ecotypes to FMV infection remains to be determined.

In the same context, the presence of mosaic-like symptoms in 27 fig trees PCR-negative for FMV, but that contained mixtures of three or four of the other viruses tested, was taken as an evidence that a viral complex (FMV-free) can induce mosaic symptoms in fig trees similar to those of FMV. Finally, to the best of our knowledge, this study reports for the first time the presence of FLV-1, FCV-1 and FFkaV in the Egyptian fig orchards.

Chapter 2

Identification and characterization of phytoplasmas infecting fig plants

Abstract

In July 2017, a survey was conducted in a fig collection plot at Locorotondo (south of Italy) to investigate the possible presence of phytoplasmas in fig trees showing yellowing, deformed leaves, short internodes, mottling and mosaic. Samples were collected from symptomatic plants and tested by nested PCR assays using universal and specific primers to amplify the 16S rDNA of these prokaryotes. PCR results detected the presence of phytoplasma sequences in twenty fig samples that clustered close to two phylogenetically distinct phytoplasmas, *i.e.* '*Candidatus* Phytoplasma asteris' and '*Ca.* P. solani' affiliated to 16SrI and 16SrXII ribosomal groups, respectively. The presence of phytoplasmas belonging to both ribosomal groups was confirmed with group specific Real-time PCR and RFLP assays on 16S ribosomal amplicons. Results of this study indicate for the first time the occurrence of phytoplasmas in fig, however more work should be carried out to verify their association with the symptoms observed on diseased fig plants.

Keywords: Fig, phytoplasma, PCR, Real-time PCR, RFLP, sequence analysis

2.1 Introduction

Phytoplasmas, previously known as mycoplasma-like organisms, belong to the class *Mollicutes*. They are single-celled sub-microscopic microorganisms, similar to bacteria but much smaller, with a diameter normally less than 1 μm. Their cells are composed of cytoplasm containing DNA and small ribosomes (70S). Phytoplasmas are wall-less prokaryotes with variable shapes (pleomorphic organisms) (Figure 17) that colonize the phloem cells of the host plants (Doi *et al.*, 1967; Whitcomb and Tully, 1989).

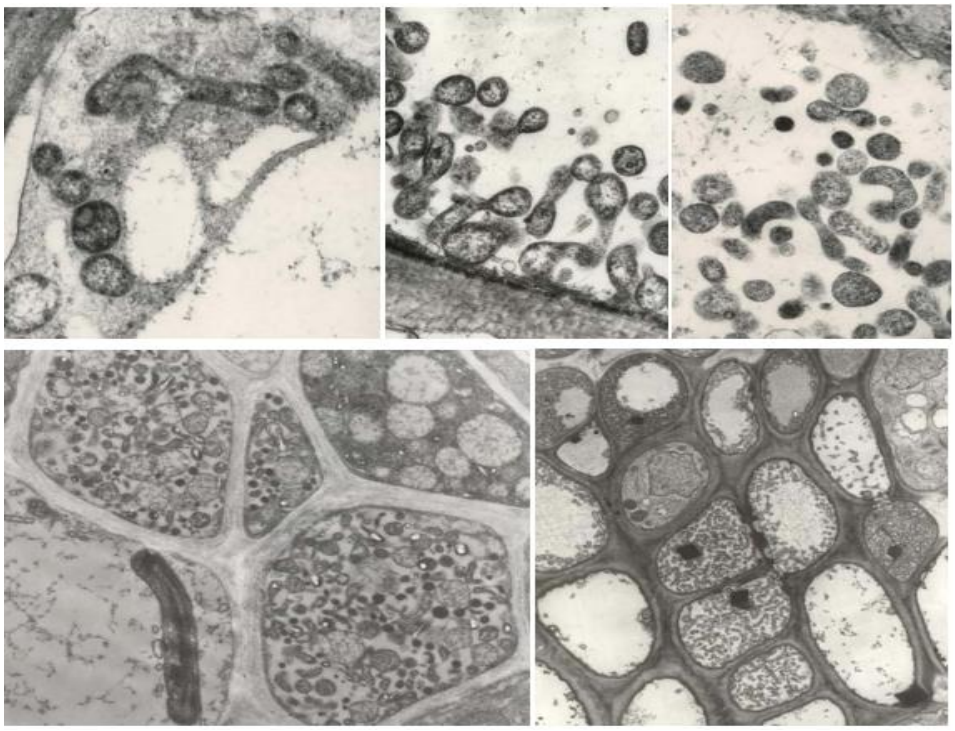


Figure 17: Electron micrographs of sieve tubes cross sections showing the polymorphism in shape and dimensions of phytoplasma cells.

The development of molecular tools has facilitated the detection and identification of phytoplasmas based on the 16S rRNA gene (Namba *et al.*, 1993b; SEEMüLLER *et al.*, 1994). In addition, several other techniques that utilize specific antibodies, DNA probes and analyses RFLP profiles have become extremely helpful in detection, identification, classification (Davies and Clark, 1991; Firrao *et al.*, 1996).

History

Phytoplasma diseases have been spotted globally. In Japan, mulberry dwarf disease had resulted in severe damage to mulberry plants, the only source of food for silkworms, since the Tokugawa period (1603–1868) (Okuda, 1972). In 1890, Shirai reported that this disease was transmissible by grafting, despite its causal agent remained unknown. Other phytoplasma diseases encompassing paulownia witches' broom disease (Kawakami, 1902) and rice yellow dwarf disease (Arashida *et al.*, 2008) were reported in Japan. Aster yellows disease was reported in the United States in the early 20th century (Kunkel, 1926). At the beginning, these diseases were ascribed to plant viruses due to the presence of virus-like symptoms and their transmissibility by insects. (Teranaka and Asuyama, 1967) discovered a new plant pathogen characterized by small pleomorphic bodies similar to that of mycoplasmas (bacterial pathogens of humans and animals) by observing ultrathin sections of phloem tissues of dwarfed mulberry plants, of paulownia affected by witches' broom, and aster with yellowing. These agents were named mycoplasma-like organisms (MLOs) due to their morphological similarity to mycoplasmas, as well as their sensitivity to tetracycline antibiotics (Ishiie and Asuyama, 1967). The detection of these novel plant pathogens, MLOs, was confirmed in successive studies (Granados *et al.*, 1968; Hull *et al.*, 1969).

Geographic distribution of phytoplasma diseases and their economic importance

Several plant species are affected by phytoplasma diseases, *i.e.* vegetables, fruit crops, ornamental plants, timber and shade trees, as well as many other important crops are exposed to mild/severe phytoplasma infections all over the world. Severe infections of herbaceous and woody plants were reported by (Bertaccini, 2006). Aster yellows phytoplasma causes major economic losses of vegetable crops (lettuce, carrot, and celery) and ornamental plants (gladiolus, hydrangea, China aster, and purple coneflower) in North America and Europe. During the 1990s, peach yellows and X-disease resulted in heavy losses in peach and cherry orchards in the United States. As well, citrus phytoplasma diseases were described in several regions of the Middle East, as in the case of the lime witches' broom, which resulted in severe damages of traditional lime production in the Sultanate of Oman and in Iran. In addition to many other diseases, the rice yellow dwarf disease in several regions of southeastern Asia,

potato witches' broom and maize bushy in central and South America, sweet potato witches' broom and associated diseases in Asia and Australia, grapevine yellows in Europe and in Australia, pear decline, apple proliferation, European stone fruit yellows and other fruit declines related with phytoplasmas have reduced the production and quality of fresh fruit in Europe. Legume diseases such as peanut witches' broom, sesame and soybean phyllody cause significant losses of these crops in Asia (Bertaccini and Duduk, 2010). Some of the phytoplasma diseases were described as destructive of forest trees in a number of continents, *i.e.* paulownia witches' broom, coconut lethal yellowing, and mulberry dwarf. Elm yellows and witches' broom have almost eradicated historical and new elm plantations in Europe and North America (Lee *et al.*, 2000).

Movement of phytoplasmas within the plant

The movement of phytoplasmas within the sieve tubes occurs from source to sink. Based on several previous studies, phytoplasmas have unequal distribution in host plants, and seasonal variations in woody plants. In general, low titer was found in the roots (sink organ) and moderate in the stems, whereas the highest titer was found in mature leaves (source organs) (sometimes with a titer ca 40 times higher than that of the roots). In sink leaves, phytoplasma concentration is low or below the limit of detection (Christensen *et al.*, 2004). Phytoplasmas infecting deciduous woody plants are shown to be absent from the aerial parts of trees during the winter and survive in the root system to re-colonize the stem and branches in spring (Seemüller. *et al.*, 1984; Guthrie *et al.*, 1998). Phytoplasmas have been detected by (Waterworth and Mock, 1999) in dormant scions and in the aerial parts of pear and *Prunus* spp. trees during winter (Jarausch *et al.*, 1999; Errea *et al.*, 2002).

Phytoplasma virulence proteins

Type III secretion systems (T3SSs), which enable several Gram negative pathogens to inject virulence effector proteins into host cells (Cornelis and Van Gijsegem, 2000), are absent in the Gram positive phytoplasmas. On the other hand, the same genes encoding SecA, SecY, and SecE, needed for protein translocation in *Escherichia coli* (Economou, 1999), were also detected in the strain OY-M of '*Ca*. P. asteris' (Kakizawa *et al.*, 2001; Kakizawa *et al.*, 2004)

and in three other phytoplasma genomes (Bai *et al.*, 2006; Kube *et al.*, 2008; Tran-Nguyen *et al.*, 2008). Previous studies confirmed the expression of SecA protein in plants infected with five phytoplasma species (Wei *et al.*, 2004), thus revealing the highly conserved nature of SecA system among phytoplasmas. Virulence phytoplasma proteins secreted via the Sec system can cause changes in plant morphology. TENGU encodes a very small protein (4.5 kDa) and it is one of the secreted proteins reported from '*Ca*. P. asteris' OY-M strain, which induces symptoms similar to phytoplasma infection, including witches' broom (development of numerous shoot branches) and dwarfism (Hoshi *et al.*, 2009; Sugawara *et al.*, 2013). Based on microarray analysis, TENGU was identified to play a significant role in down regulation of many auxin- related genes in tengu-transgenic plants, suggesting that TENGU suppresses auxin signaling or biosynthesis pathways (Hoshi *et al.*, 2009; Denancé *et al.*, 2013). More than 56 genes encoding secreted proteins were detected in the '*Ca*. P. asteris' AY-WB strain genome. SAP11 contains eukaryotic nuclear localization signals and is mainly localized in plant cell nuclei (Bai *et al.*, 2009). SAP11 expression induces crinkled leaves and many stems in infected plants and the fecundity of insect vectors was raised on SAP11-expressing versus normal plants (Sugio *et al.*, 2011). Further to SAP11, SAP54 of '*Ca*. P. asteris'AY-WB strain was found to target flower organ development in *Arabidopsis thaliana* and it induces some morphological changes similar to those observed in phytoplasma-infected plants (MacLean *et al.*, 2011). The symptoms induced in phytoplasma-infected plants may be due to the side effects of infection, because phytoplasmas consume the metabolites of infected plants due to their lack of several metabolic pathways (Oshima *et al.*, 2004). This hypothesis has been completely changed after the identification of TENGU, SAP11, and SAP54, effector proteins which could aggressively induce symptoms and regulate the plant-gene activity (Hoshi *et al.*, 2009; Himeno *et al.*, 2011; Sugio *et al.*, 2011).

Molecular classification and taxonomy of phytoplasmas

MLOs nomenclature was based on the natural plant host and symptomatology. In the early 1990s, the 16S rRNA gene sequences of MLOs were compared with each other, as well as with *Acholeplasma laidlawii*, *Spiroplasma citri*, and several mycoplasmas (Kuske and Kirkpatrick, 1992; Tsuchizak12, 1993). According to these analyses, MLOs formed a monophyletic group within the class Mollicutes which was closer to *Acholeplasma* spp. than to *Spiroplasm*a spp. and animal mycoplasmas.

The progress accomplished in the field of serological (Lee *et al.*, 1993b) and nucleic acid-based (Lee and Davis, 1988; Lee *et al.*, 1992) techniques has widely contributed to better understand the diversity and genetic interrelationships of phytoplasmas. Molecular tools, such as PCR/RFLP and nested-PCR based on the conserved (16S rDNA) region, were employed to produce a general and reliable system of phytoplasma detection, identification and classification (Lee *et al.*, 1998; Seemüller *et al.*, 1998). Updated classification of phytoplasmas is shown in Table 6 (Bertaccini and Lee, 2018). For more accurate differentiation of phytoplasmas, besides to 16S rDNA, additional non ribosomal genes were suggested to be analyzed (Lee *et al.*, 1994 2004a, 2004b; Smart *et al.*, 1996; Schneider and Gibb, 1997; Martini *et al.*, 2002, 2007).

In 1994, the name phytoplasmas was formally adopted by the Phytoplasma Working Team at the 10th Congress of the International Organization of Mycoplasmology to collectively denote MLOs. In 2004, phytoplasmas were suggested to be within a novel genus '*Candidatus* Phytoplasma' (IRPCM, 2004). To accommodate phytoplasmas as a novel '*Ca.* Phytoplasma' the following properties must be pursued: single, unique 16S rRNA gene sequence (>1200 bp) and 16S rRNA gene sequence has less than 97.5% similarity to that of any previously described '*Ca.* Phytoplasma' species. Several ecologically or biologically distinguished phytoplasmas supposed to represent a new taxon may fail to fulfill the requirement of sharing <97.5% sequence similarity with existing '*Ca.* Phytoplasma' due to the highly conserved nature of the 16S rRNA gene. In this condition, additional unique biological properties are highly needed for speciation such as antibody specificity, host range and vector transmission specificity as well as other molecular criteria (gene) (Seemüller and Schneider, 2004).

Table 6: Classification of phytoplasmas based on RFLP analyses of 16S rRNA gene.

16Sr group	Phytoplasma strain	GenBank Acc. no.	Reference
16SrI	**Aster yellows**		
I-A	Aster yellows witches' broom (AYWB)	NC_007716	(Bai *et al.*, 2006)
I-B	'*Ca.* P. asteris'	M30790	(Lee *et al.*, 2004a)
I-C	Clover phyllody (CPh)	AF222065	(Lee *et al.*, 2004a)
I-D	Paulownia witches' broom (PaWB)	AY265206	(Lee *et al.*, 2004a)
I-E	Blueberry stunt (BBS3)	AY265213	(Lee *et al.*, 2004a)
I-F	Aster yellows from apricot (A-AY)	AY265211	(Lee *et al.*, 2004a)
I-I	Strawberry witches' broom (STRAWB1)	U96614	(Jomantiene *et al.*, 1998a; Jomantiene *et al.*, 1998b)
I-K	Strawberry witches' broom (STRAWB2)	U96616	(Jomantiene *et al.*, 1998a; Jomantiene *et al.*, 1998b)
I-L	Aster yellows (AV2192)	AY180957	(Lee *et al.*, 2003)
I-M	Aster yellows (AVUT)	AY265209	(Lee *et al.*, 2004a)
I-N	Aster yellows (IoWB)	AY265205	(Lee *et al.*, 2004a)
I-O	Soybean purple stem (SPS)	AF268405	(Lee *et al.*, 2002)
I-P	Aster yellows from *Populus*	AF503568	(Šeruga *et al.*, 2003)
I-Q	Cherry little leaf (ChLL)	AY034089	(Valiunas *et al.*, 2005)
I-R	Strawberry phylloid fruit (StrawbPhF)	AY102275	(Jomantiene *et al.*, 2002)
I-S	Pepper little leaf (PeLL)	DQ092321	(Santos-Cervantes *et al.*, 2008)
I-T	Tomato little leaf (ToLL)	DQ375238	(Santos-Cervantes *et al.*, 2008)
I-U	Mexican potato purple top (JAL8)	FJ914650	(Santos-Cervantes *et al.*, 2010)
I-V	Mexican potato purple top (SON18)	FJ914642	(Santos-Cervantes *et al.*, 2010)
I-W	Peach rosette-like disease (PRU0382)	HQ450211	(Arocha-Rosete *et al.*, 2011)
I-X	Papaya bunchy top (BTS)	JF781308	(Acosta *et al.*, 2013)
I-Y	'*Ca*. P. lycopersici'	EF199549	(Arocha *et al.*, 2007)
I-Z	Papaya bunchy top (BTS)	JF781311	(Acosta-Pérez *et al.*, 2017)
16SrII	**Peanut witches' broom**		
II-A	Peanut witches' broom (PnWB)	L33765	(Gundersen *et al.*, 1994)
II-B	'*Ca.* P. aurantifolia' (WBDL)	U15442	(Zreik *et al.*, 1995)
II-C	Faba bean phyllody (FBP)	X83432	(Schneider *et al.*, 1995)
II-D	'*Ca*. P. australasia'	Y10096	(White *et al.*, 1998)
II-E	*Pichris echioides* phyllody (PEY)	Y16393	(Seemüller *et al.*, 1998)
II-F	Cotton phyllody (CoP)	EF186827	(Martini *et al.*, 2007)
II-G	Cactus witches' broom (CWB)	EU099568	(Cai *et al.*, 2008)
II-J	Cactus witches' broom (CWB)	EU099552	(Cai *et al.*, 2008)
II-H	Cactus witches' broom (CWB)	EU099569	(Cai *et al.*, 2008)
II-K	Cactus witches' broom (CWB)	EU099572	(Cai *et al.*, 2008)
II-I	Cactus witches' broom (CWB)	EU099551	(Cai *et al.*, 2008)
II-L	Cactus witches' broom (CWB)	EU099546	(Cai *et al.*, 2008)
II-M	Potato purple top	FJ914643	(Yadav *et al.*, 2014)
II-N	Papaya BTSp	JF781309	(Acosta *et al.*, 2013)
II-O	Tabebuia witches' broom	EF647744	(Mafia *et al.*, 2007)
II-P	Cuban papaya phytoplasma	DQ286948	(Pérez-López *et al.*, 2016)
II-Q	Papaya bunchy top	JF78131	(Pérez-López *et al.*, 2016)
II-R	*Echinopsis* yellow patch	DQ535900	(Pérez-López *et al.*, 2016)
II-S	*Amaranthus hypocondriacus*	FJ357164	(Pérez-López *et al.*, 2016)
II-T	Tomatillo witches' broom	U125185	(Pérez-López *et al.*, 2016)

II-U	Papaya little leaf	KP057205	(Yang *et al.*, 2016)
II-V	*Praxelis clematidea* phyllody	KY568717	(Yang *et al.*, 2017)
II-W	*Crotalaria* witches' broom	KY872734	(Al-Subhi *et al.*, 2017)
16SrIII	**X-disease**		
III-A	'*Ca*. P. pruni'	AF533231	(Liefting and Kirkpatrick, 2003)
III-B	Clover yellow edge (CYE)	L33766 8	(Gundersen *et al.*, 1994)
III-C	Pecan bunch (PB)	EF186807	(Martini *et al.*, 2007)
III-D	Goldenrod yellows (GR1)	EF186810	(Martini *et al.*, 2007)
III-E	Spiraea stunt (SP1)	AF190228	(Davis *et al.*, 2013)
III-F	Milkweed yellows (MW1)	AF510724	(Davis *et al.*, 2013)
III-G	Walnut witches' broom (WWB)	AF190226/ AF190227	(Davis *et al.*, 2013)
III-H	Poinsettia branch-inducing (PoiBI)	AF190223	(Davis *et al.*, 2013)
III-I	Virginia grapevine yellows	AF060875	(Davis *et al.*, 1998)
III-J	Chayote witches' broom	AF147706/ AF1477067	(Montano *et al.*, 2000)
III-K	Strawberry leafy fruit	AF274876	(Jomantiene *et al.*, 1998b)
III-L	Cassava frog skin disease	EU346761	(Alvarez *et al.*, 2009)
III-M	Potato purple top (MT117)	FJ226074	(Davis *et al.*, 2013)
III-N	Potato purple top (AKpot6)	GU004365	(Davis *et al.*, 2013)
III-O	Dandelion virescence (Dan Vir)	AF370120	(Jomantiene *et al.*, 2002)
III-P	Dandelion virescence (Dan Vir)	AF370119/ AF370120	(Jomantiene *et al.*, 2002)
III-Q	Black raspberry witches' broom	AF302841	(Davis *et al.*, 2001)
III-R	Cirsium white leaf (CirWL)	AF373105	(Zhao *et al.*, 2009b)
III-S	Western peach X-disease (WX)	L04682	(Zhao *et al.*, 2009b)
III-T	Sweet and sour cherry (ChD)	FJ231728	(Valiunas *et al.*, 2009)
III-U	Cirsium white leaf (CWL)	AF373105/ AF373106	(Jomantiene *et al.*, 2002)
III- V	Passion fruit phytoplasma	GU292082	(Davis *et al.*, 2012)
III-W	*Heterothalamus* little leaf (HetLL)	KC412029	(Galdeano *et al.*, 2013)
III-X	*Conyza* witches' broom	KC412026	(Galdeano *et al.*, 2013)
III-Y	Cranberry false blossom	KF62652	(Lee *et al.*, 2014)
III-Z	Broccoli stunt strain BSP-21	JX626327	(Pérez-López *et al.*, 2016)
16SrIV	**Coconut lethal yellows**		
IV-A	Coconut lethal yellowing (LYJC8)	AF498307	(Harrison *et al.*, 2002)
IV-B	Yucatan coconut lethal decline	U18753	(Harrison *et al.*, 1994)
IV-C	Tanzanian coconut lethal decline	X80117	(Harrison *et al.*, 1994)
IV-D	Texas phoenix decline (TPD)	AF434969	(Harrison *et al.*, 2008)
IV-E	Coconut lethal yellowing	DQ631639	(Martinez *et al.*, 2008)
IV-F	*Washingtonia robusta* decline	EU241512	(Harrison *et al.*, 2008)
16SrV	**Elm yellows**		
V-A	'*Ca.* P. ulmi' (EY)	AY197655	(Lee *et al.*, 2004b)
V-B	'*Ca.* P. ziziphi' (JWB-G1)	AB052876	(Jung *et al.*, 2003a)
V-C	"Flavescence dorée" (FD-C)	X76560	(Daire *et al.*, 1992)
V-D	"Flavescence dorée" (FD-D)	AJ548787	(Marta Martini., 1999)
V-E	'*Ca*. P. rubi' (RuS)	Y16395	(Seemüller *et al.*, 1998)
V-F	'*Ca*. P. balanite' (BltWB)	AB689678	(Win *et al.*, 2013)
V-G	Jujube witches' broom	AB052879	(Jung *et al.*, 2003a)
V-H	*Bischofia polycarpa* witches' broom	KJ452547	(Lai *et al.*, 2014)
V-I	Rubus stunt (BlackPort)	KR233473	(Fránová *et al.*, 2016)

16SrVI	**Clover proliferation**		
VI-A	'*Ca.* P. trifolii' (CP)	AY390261	(Hiruki and Wang, 2004)
VI-B	*Fragaria multicipita*	AF036354	(Jomantiene *et al*., 1998a)
VI-C	Illinois Elm Yellows (ILEY)	AF409069	(Jacobs *et al.*, 2003)
VI-D	Periwinkle little leaf (PLL-Bd)	AF228053	(Siddique *et al*., 2001)
VI-E	*Centaurea solstitialis* virescence	AY270156	(Faggioli *et al*., 2004)
VI-F	Catharanthus phyllody (CPS)	EF186819	(Martini *et al.*, 2007)
VI-H	Portulaca little leaf (PLL-Ind)	EF651786	(Samad *et al*., 2011)
VI-I	'*Ca.* P. sudamericanum' (WB-Br4)	GU292081	(Davis *et al.*, 2012)
16SrVII	**Ash yellows**		
VII-A	'*Ca.* P. fraxini' (AshY)	AF092209	(Griffiths *et al.*, 1999)
VII-B	Erigeron witches' broom (ErWB)	AY034608	(Barros *et al*., 2002)
VII-C	Argentinian alfalfa witches' broom	AY147038	(Conci *et al*., 2005)
VII-D	Erigeron witches' broom	KJ831066	(Flôres *et al*., 2015)
16SrVIII	**Loofah witches' broom**		
VIII-A	'*Ca*. P. luffae' (LufWB)	AF086621	(Ho *et al.*, 2001)
16SrIX	**Pigeon pea witches' broom**		
IX-A	Pigeon pea witches' broom	AF248957	(Gundersen and Lee, 1996)
IX-B	'*Ca.* P. phoenicium'	AF515636	(Verdin *et al*., 2003)
IX-C	Naxos periwinkle virescence (NAXOS)	HQ589191	(Heinrich *et al.*, 2001)
IX-D	Almond witches' broom (AlWB)	AF515637	(Verdin *et al*., 2003)
IX-E	Juniper witches' broom (JunWB)	GQ925918	(Davis *et al.*, 2010)
IX-F	Almond and stone witches' broom	HQ407532	(Molino Lova *et al.*, 2011)
IX-G	Almond and stone witches' broom	HQ407514	(Molino Lova *et al.*, 2011)
IX-H	Sarson phyllody	KU892213	(Ahmad *et al.*, 2017)
16SrX	**Apple proliferation**		
X-A	'*Ca.* P. mali'	AJ542541	(Seemüller and Schneider, 2004)
X-B	'*Ca.* P. prunorum'	AJ542544	(Seemüller and Schneider, 2004)
X-C	'*Ca.* P. pyri'	AJ542543	(Seemüller and Schneider, 2004)
X-D	'*Ca.* P. spartii'	X92869	(Marcone *et al.*, 2004a)
X-E	Black alder witches' broom BAWB	X76431	(Seemüller *et al.*, 1994)
16SrXI	**Rice yellow dwarf**		
XI-A	'*Ca.* P. oryzae'	AB052873	(Jung *et al.*, 2003b)
XI-B	Sugarcane white leaf (SCWL)	X76432	(Lee *et al.*, 1997)
XI-C	Leafhopper-borne (BVK)	X76429	(Seemüller *et al.*, 1994)
XI-D	Sugarcane white leaf (SCWL)	KR020685	(Zhang *et al.*, 2016)
XI-E	'*Ca.* P. cirsii'	KR869146	(Šafárová *et al.*, 2016)
XI-F	Sugarcane grassy shoot (SCGS)	HF586648	(Yadav *et al.*, 2017)
16SrXII	**"Stolbur"**		
XII-A	'*Ca*. P. solani' STOL11	AF248959	(Quaglino *et al.*, 2013)
XII-B	'*Ca.* P. australiense' (AUSGY)	L76865	(Davis *et al.*, 1997)
XII-C	Strawberry lethal yellows	AJ243045	(Padovan *et al.*, 2000)
XII-D	'*Ca.* P. japonicum'	AB010425	(Sawayanagi *et al.*, 1999)
XII-E	'*Ca.* P. fragariae' (StrawY)	DQ086423	(Valiunas *et al.*, 2006)
XII-F	"Bois noir" (BN-Op30)	EU836630	(Quaglino *et al.*, 2009)
XII-G	"Bois noir" (BN-Fc3)	EU836647	(Quaglino *et al.*, 2009)
XII-H	'*Ca.* P. convolvuli' (BY-S57/11)	JN833705	(Martini *et al.*, 2012)
XII-I	Potato strain China (169/Hezuo88)	EU338445	(Cheng *et al.*, 2015)
16SrXIII	**Mexican periwinkle virescence**		
XIII- A	'*Ca*. P. hispanicum'	AF248960	(Davis *et al.*, 2016)
XIII-B	Strawberry green petal	U96616	(Jomantiene *et al.*, 1998b)

XIII-C	Chinaberry yellows (CBY1)	AF495882	(Harrison *et al.*, 2002)
XIII-D	Mexican potato purple top (SINPV)	FJ914647	(Santos-Cervantes *et al.*, 2010)
XIII-E	Papaya apical curl necrosis (PACN)	EU719111	(Melo *et al.*, 2013)
XIII-F	Strawberry red leaf	KJ921641	(Fernández *et al.*, 2015)
XIII-G	'*Ca*. P. meliae' (ChTY)	KU850940	(Fernández *et al.*, 2016)
16SrXIV	**Bermudagrass white leaf**		
XIV-A	'*Ca.* P. cynodontis'	AJ550984	(Marcone *et al.*, 2004b)
XIV-B	Bermuda grass white leaf Iran	EF444485	(Salehi *et al.*, 2009)
XIV-C	Bermuda grass white leaf	KP019339	(Mitrović *et al.*, 2015)
16SrXV	**Hibiscus witches' broom**		
XV-A	'*Ca*. P. brasiliense'	AF147708	(Montano *et al.*, 2001)
XV-B	Guazuma wiches' broom (GWB)	HQ258882	(Villalobos *et al.*, 2011)
16SrXVI	**Sugarcane yellow leaf syndrome**		
XVI-A	'*Ca*. P. graminis'	AY725228	(Arocha *et al.*, 2005)
16SrXVII	**Papaya bunchy top**		
XVII-A	'*Ca*. P. caricae'	AY725234	(Arocha *et al.*, 2005)
16SrXVIII	**American potato purple top wilt**		
XVIII-A	'*Ca*. P. americanum'	DQ174122	(Lee *et al.*, 2006)
16SrXIX	**Chestnut witches' broom**		
16SrXIX-A	'*Ca*. P. castaneae'	AB054986	(Jung *et al.*, 2002)
16SrXX	***Rhamnus* witches' broom**		
XX-A	'*Ca*. P. rhamni'	X76431	(Marcone *et al.*, 2004a)
16SrXXI	***Pinus* phytoplasma**		
XXI-A	'*Ca.* P. pini' (Pin127S)	AJ632155	(Schneider *et al.*, 2005)
16SrXXII			
16SrXXII-A	'*Ca.* P. palmicola' LDN	Y14175	(Tymon *et al.*, 1998)
16SrXXII-B	'*Ca.* P. palmicola' (LYDM)	KF751387	(Harrison *et al.*, 2014)
16SrXXIII			
XXIII-A	Buckland valley grapevine yellows	AY083605	(Constable *et al.*, 2002)
16SrXXIV			
XXIV-A	Sorghum bunchy shoot	AF509322	(Blanche *et al.*, 2003)
16SrXXV			
XXV-A	Weeping tea witches' broom	AF521672	(Wei *et al.*, 2007)
16SrXXVI			
XXVI-A	Sugar cane phytoplasma D3T1	AJ539179	(Wei *et al.*, 2007)
16SrXXVII			
XXVII-A	Sugar cane phytoplasma D3T2	AJ539180	(Wei *et al.*, 2007)
16SrXXVIII			
XXVIII-A	Derbid phytoplasma	AY744945	(Wei *et al.*, 2007)
16SrXXIX			
XXIX-A	'*Ca*. P. omanense'	EF666051	(Al-Saady *et al.*, 2008)
XXIX-B	Bindweed witches' broom (RBiWB)	KY047493	(Esmailzadeh Hosseini *et al.*, 2016)
16SrXXX			
XXX-A	'*Ca*. P. tamaricis' (SCWB)	FJ432664	(Zhao *et al.*, 2009a)
16SrXXXI			
XXXI-A	'*Ca*. P. costaricanum'	HQ225630	(Lee *et al.*, 2011)
16SrXXXII			
XXXII-A	'*Ca*. P. malaysianum'	EU371934	(Nejat *et al.*, 2013)
XXXII-B	'*Ca*. P. malaysianum' (MYD)	EU498727	(Nejat *et al.*, 2013)
XXXII-C	'*Ca*. P. malaysianum' (MOP)	EU498728	(Nejat *et al.*, 2013)

16SrXXXIII	**Allocasuarina yellows diseases**		
XXXIII-A	'*Ca*. P. allocasuarinae' (AlloY)	AY135523	(Marcone *et al.*, 2004a)
16SrXXXVI			
XXXVI-A	'*Ca*. P. wodyetiae'	KC844879	(Neda Naderali *et al.*, 2017)
No group	'*Ca.* P. novoguineense' (BCS-Bo^{R})	LC228755	(Miyazaki *et al.*, 2018)

Symptoms induced by phytoplasma infection

Plants infected by phytoplasmas induce a wide range of symptoms as shown in Figure 18 that indicate their interference with plant system development. Typical phytoplasma symptoms are: witches' broom (loss of apical dominance) of developing tissues; phyllody (floral organs turning to the condition of leaves); virescence (green coloration of flower parts); bolting (growth of elongated stalks); formation of bunchy fibrous secondary roots; reddening of leaves and stems; generalized yellowing, decline and stunting of plants; phloem necrosis (McCoy *et al*., 1989). Photosynthesis, especially photosystem II, is blocked in many phytoplasma-infected plants. Yellowing is due to the degradation of chlorophyll and carotenoids (Bertamini and Nedunchezhian, 2001). Most plants show apical dominance but phytoplasma infection can cause the proliferation of axillary (side) shoots (Lee *et al*., 2000) and a decrease in size of the internodes and generalized stunting. In some cases sterility of flowers is described (Bertaccini, 2007). In addition, some other aspecific symptoms are recognized in phytoplasma-infected plants due to stress to which the infected plants are exposed.

Figure 18: Symptoms of phytoplasmas in different plant species. From left, periwinkle showing flower virescence infected with 16SrIX-C phytoplasmas (Duduk *et al*., 2008); carrot infected with aster yellows phytoplasmas (Duduk *et al*., 2009), lime witches' broom ('*Ca*. P. aurantifolia') on a lime tree, tomato infected with "stolbur" phytoplasmas (Duduk and Bertaccini, 2006).

Transmission of phytoplasmas

Insect vectors: in nature, the spread of phytoplasmas from plant to plant is mediated by phloem-feeding insects of the families Cicadellidae (leafhoppers), Fulgoridae (planthoppers) and Psyllidae in a persistent manner (McCoy, 1979). Accordingly, the host range of phytoplasmas are strongly based on their vectors. Analysis of phytoplasma genome indicated the presence of three types of immunodominant membrane proteins: immunodominant membrane protein (Imp) (Kakizawa *et al.*, 2009), antigenic membrane protein (Amp) (Kakizawa *et al.*, 2004), and immunodominant membrane protein A (IdpA) (Neriya *et al.*, 2011). Amp was a major antigenic protein that makes up the majority of their cell surface proteins (Kakizawa *et al.*, 2009). Positive selection of this gene has been clearly defined through several cloning attempts from various phytoplasma strains (Kakizawa *et al.*, 2006). It has been shown to interact with microfilament complexes of intestinal muscles of insect and is thought to be important for transmission and infection.

Phytoplasmas are found in most major organs of an infected insect body host. Phytoplasmas enter the insect's body through the stylet, move through the intestine, and are then absorbed into the hemolymph. Once inside hemolymph, they start to colonize the salivary glands, a process that can take up to some weeks. Transovarial transmission by insects was described for the following phytoplasma diseases: *Scaphoideus titanus*/aster yellows (Danielli *et al.*, 1996), *Hishimonoides sellatiformis*/mulberry dwarf (Kawakita *et al.*, 2000), *Matsumuratettix hiroglyphicus*/sugarcane white leaf (Hanboonsong *et al.*, 2002), *Cacopsylla pruni*'/*Ca*. P. prunorum' (Tedeschi *et al.*, 2006) and *C. picta*/'*Ca*. P. mali' (Mittelberger *et al.*, 2017).

Grafting: Phytoplasmas may also be transmitted from infected to healthy plant by grafting. For experimental trials, another possibility of transmission, when the graft is incompatible between two plant species, is through the use of parasitic plant species *Cuscuta* (dodder) which can form bridges between plants and allow the passage of phytoplasma from one plant to another.

Seed: no reports have shown the possibility of phytoplasmas' transmission by seeds (McCoy, 1979; Lee *et al.*, 2000) until recently. However, after the first doubtful epidemiological spreading of coconut lethal yellowing (Cordova *et al.*, 2003), a novel hypothesis has emerged and highlighted on the possibility of their transmission by seeds. This hypothesis has been

verified through several molecular analysis conducted on other crops including the severely affected alfalfa (*Medicago sativa*) and lime (*Citrus aurantiaca*) (Khan *et al.*, 2002; Botti and Bertaccini, 2006). Several probable cases of phytoplasma transmission through seeds have been described in literature *i.e.*, "stolbur" phytoplasma in pea, tomato, corn, winter oil seed rape (Zwolinska *et al.*, 2010; Calari *et al.*, 2011), herbaceous crops (Olivier C., 2008) and Bermudagrass white leaf phytoplasma in maize (Çağlar *et al.*, 2019).

Diagnostic methods for phytoplasma detection:

Transmission electron microscope (TEM): this method was adopted for phytoplasma detection until the early 1980s. This approach is still applicable in specialized and well equipped laboratories with a very expensive TEM equipment and it requires time for sample preparation consisting in ultrathin sections of phloem tissue (Maejima *et al.*, 2014).

Direct fluorescence detection (DFD) and DAPI: in the 1980s, florescent microscopy based diagnostic techniques were developed for the detection of phytoplasma, such as direct fluorescence detection (DFD) (Namba and Yamashita, 1981) and DAPI staining (Hiruki and da Rocha, 1986). The DFD method detects autofluorescence of necrotic phloem cells, and DAPI detects phytoplasma DNA. No plant DNA could interfere with the test due to its absence in the phloem (Schaper and Seemüller, 1982).

Serological techniques - enzyme-linked immunosorbent assay (ELISA)

ELISA, the most common method to diagnose viral diseases, was infrequently used to detect phytoplasma diseases in the 1980s, except few cases (Lin and Chen, 1985) due to difficulties faced to purify phytoplasma cells for specific antibody preparation. As phytoplasmas can only be propagated in plant hosts, the complete purification of phytoplasmas from plant material is almost impossible. It is possible that the plant material could interfere with ELISA test and cause false positive reactions in phytoplasma free samples. The use of monoclonal antibodies has ameliorated the specificity and sensitivity of ELISA (Lee and Davis, 1992). Several other serological tools such as immunofluorescence (Lherminier *et al.*, 1990), immunosorbent electron microscopy (Sinha, 1979; Sinha and Benhamou, 1983), dot blot or ELISA (Boudon-Padieu *et al.*, 1989) were used to detect phytoplasma in leafhopper vectors

or potential vectors. In other approaches, specific phytoplasma detection has been achieved through tissue blotting with direct or indirect coated antigen (Lin and Chen, 1985). Later on, the production of antibodies based on the partial sequences of the major immunodominant proteins of some phytoplasmas have been accomplished (Berg *et al.*, 1999; Blomquist *et al.*, 2001; Hong *et al.*, 2001).

Biological properties: the absence of symptoms after antibiotic (*i.e.* tetracycline) treatment has shown an evidence of the role of prokaryotic micro-organisms as agents of several plant diseases (Doi *et al.*, 1967; Lee and Davis, 1992). Classification and differentiation of uncultured phytoplasmas were based previously on their biological properties, such as specificity of plant and insect hosts, and symptoms of infected plants (Chiykowski and Sinha, 1990; Deng and Hiruki, 1991).

Molecular techniques

Polymerase chain reaction (PCR)

PCR amplification of the highly conserved 16S rRNA genes of phytoplasmas (Lee *et al.*, 1993a; Namba *et al.*, 1993a; Schneider *et al.*, 1995) has become a successful molecular detection tool. PCR has also been applied to inspect phytoplasma localization and dynamics in the plant (Nakashima and Hayashi, 1995; Sahashi *et al.*, 1995; Wei *et al.*, 2004). Universal and group specific PCR assays (Annex 3) are routinely used for the detection of phytoplasmas (Deng and Hiruki, 1991; Ahrens and Seemüller, 1992; Schaff *et al.*, 1992; Davis and Lee, 1993; Harrison *et al.*, 1996; Smart *et al.*, 1996; Jomantiene *et al.*, 1998b). PCR universal primers are useful for the preliminary identification of a wide range of phytoplasmas associated with plants and insects (Deng and Hiruki, 1991; Ahrens and Seemüller, 1992; Davis and Lee, 1993; Lee *et al.*, 1993a; Namba *et al.*, 1993a; Lorenz *et al.*, 1995; Schneider and Gibb, 1997; Lee *et al.*, 1998). However, PCR specific primers based on cloned DNA fragments (non-ribosomal DNAs) provide sensitive and specific phytoplasma detection tools (Bertaccini *et al.*, 1992; Gundersen and Lee, 1996; Schneider and Gibb, 1997). In addition, a barcode system has been widely used for the detection and identification of phytoplasmas in the last few years (Makarova *et al.*, 2012).

For more accurate phytoplasma detection, efficient and reproducible methods are required to access good quality of nucleic acids enriched with phytoplasma DNA, but this approach is hard to be achieved. Sample preparation is crucial and the target DNA should be available as much as possible for applying different molecular techniques. Approximately, the quantity of phytoplasma DNA is 1% of total DNA extracted from tissue (Bertaccini, 2007). Several laboratory protocols are available for total DNA extraction aimed at getting enough pure and concentrated phytoplasma DNA to perform molecular analysis at high precision and this could be accomplished through removal of DNA polymerase inhibitors, such as polysaccharides, phenolic compounds or humic substances from plants (Minsavage *et al.*, 1994; Hartung *et al.*, 1996; Wilson, 1997; Mumford *et al.*, 2006). Nested PCR assay was developed as an essential tool for detection of phytoplasma DNA with high sensitivity and specificity, either from samples with low titer due to seasonal variations, or in the presence of inhibitors that may interfere with the PCR (Gundersen *et al.*, 1994; Gundersen *et al.*, 1996; Leyva-López *et al.*, 2002; Jacobs *et al.*, 2003; Marzachì, 2004). Nested-PCR is carried out through two rounds of PCR either through the use of first universal primer pair followed by the second universal primer pair or through the use of universal primer pair followed by a group specific primer pair. Nested-PCR is capable of detection of dual or multiple phytoplasmas present in the infected tissues in case of mixed infection (Lee *et al.*, 1994). Restriction fragment length polymorphism (RFLP) is used for identification of all detected phytoplasmas since it is a technique that can differentiate among phytoplasmas that have homologous DNA sequences (Lee *et al.*, 1993a; Schneider *et al.*, 1993). As the RFLP patterns of each phytoplasma are conserved, the identification of unknown phytoplasmas is possible through the comparison of their RFLP profile with those of known phytoplasmas (Lee *et al.*, 1998; Wei *et al.*, 2007; Cai *et al.*, 2008; Wei *et al.*, 2008).

Quantitative PCR

Quantitative PCR (qPCR) is one of the most valuable methods for relative quantification of phytoplasmas. It is highly useful to analyze large number of samples in screening programs and since it is highly sensitive and reduces the risk of amplicon contamination. Universal phytoplasma qPCR assays have been developed based on 16Sr RNA gene (Christensen *et al.*, 2013) and 23S rRNA gene (Hodgetts J. *et al.*, 2009). In addition, specific, reliable , sensitive and fast group specific assays have been introduced to detect several phytoplasmas belonging to various ribosomal groups such as 16SrX (apple proliferation-AP), employing SYBR Green (Torres *et al.*, 2005) and TaqMan minor grove binding (MGB) probe technology (Baric and Dalla-Via, 2004; Baric *et al.*, 2006; Aldaghi *et al.*, 2007), "flavescence dorée" (FD) and "bois noir" (BN) phytoplasmas (Hren *et al.*, 2007), Colombia Basin potato purple top (16SrVI), aster yellows (16SrI), and pigeon pea witches' broom (16SrIX) (Crosslin *et al.*, 2006).

Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (Notomi *et al.*, 2000) is a rapid and reliable field-diagnostic system for phytoplasma diseases. LAMP is more sensitive and rapid than PCR amplification and does not need DNA purification or special equipment such as a thermal cycler (Tomlinson *et al.*, 2010; Sugawara *et al.*, 2012). Several successful applications of LAMP to detect phytoplasma presence have been reported for Napier stunt phytoplasma (16SrXI) (Obura *et al.*, 2011), grapevine yellows phytoplasmas (Kogovšek *et al.*, 2017) and '*Ca*. P. pyri' (Siemonsmeier *et al.*, 2019).

Phytoplasma cultivation in artificial media

Phytoplasmas originated from infected periwinkle shoots belonging to distinct ribosomal groups were used as phytoplasma source and successfully grown on specific commercially available media, known as PivL® (Phytoplasma in vitro) medium. Phytoplasma colonies were observed with binocular microscope and PCR assays were conducted to confirm the identity of phytoplasma DNA in cultured cells which served as a source of DNA template. Several benefits could be accompanied with this method, i.e. maintaining phytoplasma

strains through culture methods rather than by micropropagation techniques, genome sequencing and whole genome comparison could be applied as a master tool for phytoplasma identification in the near future (Contaldo *et al.*, 2012; Contaldo *et al.*, 2013). Extensive efforts have been progressively continued to optimize the culture system. In 2016, a new medium was developed for phytoplasma cultivation. This newly developed medium (CB), an Oxoid medium composed of tryptone and soya peptone enriched with horse serum, yeast extract, phenol red and antibiotics, supported the phytoplasma growth in the same manner as Piv medium, but with an additional advantage regarding the possibility to modify its composition in order to adapt to the diverse phytoplasmas species and stages of growth. Grapevine field-collected materials showing symptoms and infected by "flavescence dorée", "bois noir" and aster yellows were used as initial source of inoculum (Contaldo *et al.*, 2016). Very recently the media was used to growth colonies infected by phytoplasmas isolated from coconut with lethal yellowing disease, on these colonies some biochemical characterization was also achieved (Contaldo *et al.*, 2019).

Control of phytoplasma-associated diseases

The possible control measures that could be applied to limit the spread of phytoplasma diseases are: controlling the vectors, eliminating the pathogens from infected plants by meristem tip culture, antibiotics or other chemicals (Bertaccini, 2007). The use of pesticides to control insect vector has become the method of choice for limiting outbreaks of phytoplasma diseases, however, this approach is insufficient (Firrao *et al.*, 2007). The elimination of inoculum sources could be effective to reduce phytoplasma diseases only if phytoplasma transmission is mediated by monophagous vectors, as in the case of the disease/vector systems of "flavescence dorée"/*Scaphoideus titanus* and pear decline/ *Cacopsylla pyri*. This approach is hard to be accomplished efficiently when wild reservoir plants are sources of acquisition for polyphagous leafhoppers such as "stolbur"/*Hyalesthes obsoletus*/bindweed/nettle, or when reservoirs and/or vectors are unknown. The control of monophagous insects is easier than insects that are also able to live on wild plants. On the other hand, curing infected plants by antibiotics is often impossible because of high-cost, lack the efficacy for long period control and it is forbidden in many countries.

Several researches have been launched to understand better the biological features of phytoplasmas and their relationships with the host in order to find out new control measures which mainly interfere with the colonization of insects by phytoplasmas, or with the phytoplasma nutrient uptake in the plant phloem without resorting to pesticides. It is necessary first to determine the barriers of phytoplasmas' colonization of the insect body in order to reduce the infectivity of vector populations. On the other hand, if the mechanism of nutrient uptake by phytoplasma from host plant phloem will be clearly understood, this could be an alternative approach to reduce phytoplasma multiplication, and symptom expression in the host (Firrao *et al.*, 2007).

Till this moment, an integrated management of phytoplasma diseases through the control of insect vectors and the production of healthy propagating material to reduce their spread are still the prerequisite control methods. A novel attempt in the development of a non-chemical based method has been described by (Gross, 2017) to control phytoplasma vectors by semiochemicals through combining attractive compounds in traps and repellent compounds in dispensers which may be applied in push-and-pull strategies.

2.2 Objectives

Several fungal, viral and virus-like diseases have been reported to naturally infect fig trees. Fig mosaic is the most widespread viral disease all over the world. However, little information is available regarding phytoplasma infections. Further researches are needed to assess more precisely the possible presence of phytoplasmas in fig and characterize them. Accordingly, this study, carried out in south Italy, was aimed to detect and identify phytoplasmas infecting fig trees exhibiting symptoms resembling those associated with phytoplasmas. The objectives of this work were the detection of phytoplasmas infecting fig plants by PCR and Real-time PCR using universal and group specific primers and the identification and molecular characterization of detected phytoplasma strains.

2.3 Materials and methods

2.3.1 Source of plant material

In July 2017, a small-scale survey in a fig collection plot located in Locorotondo (Apulia, south of Italy) was conducted to investigate the possible presence of phytoplasmas. During this survey, 43 plants of different varieties (Abbondanza, Brogiotto nero, Canestrelle, Comunione, Lattarola, Nero di Sava, Paccia, Palazzo bianco, Ricotta, Ritonna, San Lorenzo, Verdesca, Zingarello nero) displaying symptoms of yellowing, deformed leaves, short internodes, mottling and mosaic (Figure 19) were selected for sampling laboratory analyses.

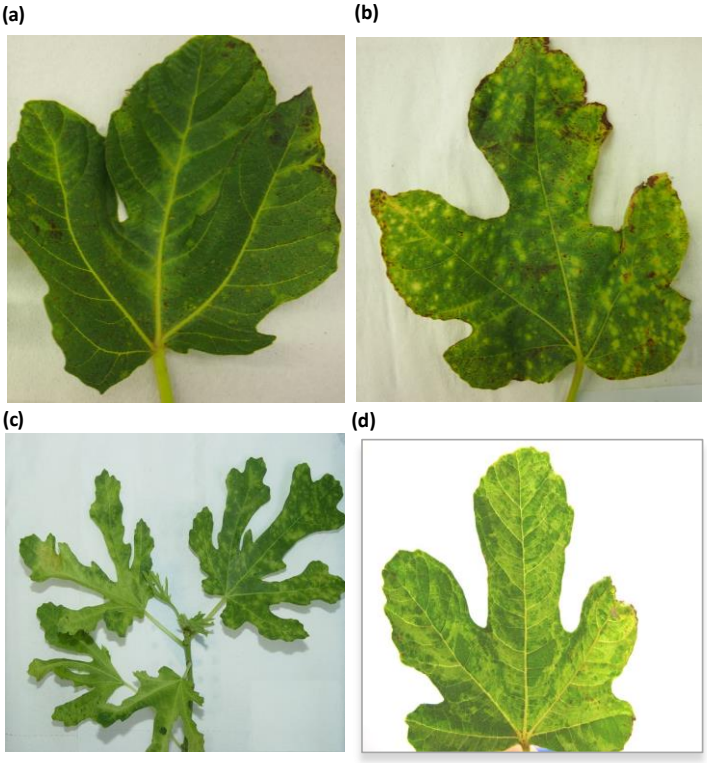


Figure 19: Leaves collected from diseased fig plants showing different types of symptoms: (a) Vein clearing and feathering, (b) chlorotic spots and mottling, (c) deformed leaves with short internodes, (d) mosaic.

2.3.2 Total nucleic acid extraction

The total nucleic acids were extracted using a CTAB buffer method (Murray and Thompson, 1980). Between 0.5 and 0.8 g of fresh leaf midribs were placed in Bioreba extraction bag with 2 ml of CTAB (Annex 1). Plant material was crushed with a hammer and homogenized. In each bag, additional 3 ml of CTAB were added; then, 1 ml of sap was transferred into a 2 ml microcentrifuge tube. Samples were heated at 65°C for 30 min, centrifuged at 10,000 rpm for 5 min and 1 ml was transferred to a new 2 ml micro-centrifuge tube. Then, 1 ml of chloroform - isoamyl alcohol (24: 1) was added and well mixed by shaking. After centrifugation at 13,000 rpm for 10 min, 750 µl supernatant from each sample were transferred into a 1.5 ml microcentrifuge tube and 450 µl (approximately 0.6 volumes) of cold 2-propanol were added. The mixture was mixed by inverting the tubes 2 times and incubated at -20°C for 30 to 60 min. The samples were centrifuged at 13.000 rpm for 10 min and the supernatant was discarded. The pellet was washed with 1 ml of 70% ethanol and centrifuged at 13.000 rpm for 10 min to eliminate any additional impurities. The pellet was then dried and re-suspended in 100 µl of RNAse and DNase-free water.

2.3.3 PCR, nested PCR and RFLP analysis of 16S rDNA

PCR and nested PCR assays were carried out using two primer pairs R16F1/R0 (Lee *et al.*, 1994) and R16F2n/R2 (Gundersen and Lee, 1996) (Table 8) to amplify a fragment of 16S rDNA from phytoplasma-infected samples. PCR reactions were performed in a volume of 50 µl: 3 µl sample DNA, 1×GoTaqFlexi Buffer (Promega, Madison, WI, USA) containing, 0.25 mM dNTPs, 5 pmol of each (forward and reverse primers) and 0.025 U\µl GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR cycling conditions were different depending on the primer set used. Using the first primer set (R16F1/R0), the denaturation step was carried out at 94°C for 4 min, and a total of 35 cycles were performed (1 min at 94°C for denaturation, 2 min at 50°C for annealing, 3 min at 72°C for extension). For nested PCR assays, 1 µl of direct reaction was diluted (1: 50) and added to the PCR mix containing R16F2n/R2 primers. PCR cycling conditions were: denaturation 4 min at 94°C, a total of 40 cycles were performed (1 min at 94°C for denaturation, 1 min at 54°C for annealing, 2 min

at 72°C for extension). R16F2n/R2 nested PCR products generated from fig samples were further used as templates for nested PCR with M1(=758f)/ M2(=1232r) and fU5/rU3 primers (Gibb *et al.*, 1995; Lorenz *et al.*, 1995). PCR mix in 25 µl total volume contained 2.5 µl Red*Taq* PCR buffer, 10X dNTPs 2.5 mM; 20 pM forward and reverse primer, 5 U Red*Taq* DNA Polymerase (Sigma, Germany) and 20-40 ng template DNA. All the amplification were carried out as follows: 1 cycle at 94°C for 2 min; 30 cycles of 94°C for 30 sec, 53°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min. SDW and samples devoid of DNA template were added as negative controls and the DNAs of European stone fruit yellows phytoplasmas (ESFY, 16SrX-B), aster yellows (16SrI-B) and "stolbur" (16SrXII-A) from the EPPO-QBank collection were used as control (Bertaccini *et al.*, 2014). Amplified nested PCR products were analyzed by electrophoresis on 1.2% agarose gel in TAE 1X buffer (Annex 2).

The PCR products (about 300 ng each) were subjected to RFLP analyses using *Tru1*I restriction endonuclease (FastEnzyme Fermentas, Vilnius, Lithuania) at 65°C for 10 min. The number and size of the resulting fragments were analyzed by vertical electrophoresis in 6.7% polyacrylamide gel in TBE buffer (Tris-borate 0.09 M, H_3BO_3 0.09 M). Fragments obtained were compared with those of classified phytoplasmas used as control or to those available in literature.

The same samples tested for phytoplasma were also analyzed for the presence of FMV in order to study the possible etiological role of phytoplasmas in disease symptoms. Accordingly, cDNA synthesis and RT-PCR were performed according to the procedure described above in the part of fig viruses.

2.3.4 Molecular characterization of non-ribosomal genes

Further investigation on non-ribosomal genes of the 16SrXII strain was carried out with fTUF1/ rTUF1 and fTUFAY/ rTUFAY (Schneider and Gibb, 1997) in nested PCR to amplify a fragment of the *tuf* gene, as well as with POSecF1/ POSecR1 and PoSecF3/ PoSecR3 to amplify a portion of *secY* gene (Fialová *et al.*, 2009) (**Table 8**Table 8). PCR reaction mix volume was 25 µl containing 1 µl of DNA, 1× High Fidelity buffer (Invitrogen), 2 mM $MgSO_4$ (Invitrogen), 200 µM dNTPs (Applied Biosystems), 0.02 U/µl Platinum Taq DNA

Polymerase High Fidelity (Invitrogen) and 0.2 µM (secY)/ 0.4 µM (tuf) of each primer. The PCR amplification procedure was performed in a PCR cycler (PCR System 9700 Gene Amp). 1 µl of first PCR product was used as template for nested PCR. The thermocycling conditions of PCR and nested PCR were the same except for annealing temperature as shown in (Table 7).

Table 7: Thermocycling conditions of PCR and nested PCR of non-ribosomal genes.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Number of cycles	Final extension
tuf	3 min at 94°C	30 sec at 94°C	30 sec at 45°C (PCR) 53°C (nested PCR)	1 min at 68°C	35	7 min at 68°C
secY	2 min at 94°C	15 sec at 94°Cs	30 sec at 54°C (PCR) 62°C (nested PCR)	1 min at 68°C		no

For further 16SrI strain characterization, *groEl* gene was analyzed. The primer sets AYgroesF/AYampR and AYgroelF/AYgroelR (Mitrović *et al.*, 2011) were employed to amplify a portion of the *groEl* gene in direct and nested PCR, respectively (Table 8). Dilution (1: 30) of first PCR product was prepared to be used as DNA template for nested PCR. Each 25 µl PCR reaction mix consisted of 1 µl of DNA, 1× High Fidelity buffer (Invitrogen), 2 mM $MgSO_4$ (Invitrogen), 200 µM dNTPs (Applied Biosystems), 0.02 U/µl Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and 0.4 µM (groEl) of each primer. Thirty-five cycles were implemented for both primer pairs under the following PCR cycling conditions: [30 sec (groEl) at 94°C for denaturation, 30 sec at 55°C (groEl) for annealing, 2 min (1.5 min in nested PCR) at 68°C for extension]. In addition *tuf* and *secA* genes were also tested with ftufu/rtufu (Schneider and Gibb, 1997) and secAFor1/secARev3 (Dickinson and Hodgetts, 2013) primers (Table 8). The final reaction volume of PCR mix was 30 µl consisting of 3 µl DNA, 1×GoTaqFlexi Buffer (Promega, Madison, WI, USA) containing, 0.25 mM dNTPs, 5 pMol of each forward and reverse primer and 0.025 U\ µl GoTaq DNA polymerase (Promega, Madison, WI, USA). The thermocycling conditions consisted of initial denaturation at 94°C for 4 min, a total of 40 cycles of [35 sec at 94°C for denaturation, annealing for 30 sec at 45°C (tuf)/ 1 min at 53°C (secA), 60 s (tuf) /1 min at 72°C (secA) for

extension] and 7 min at 72°C for final extension. PCR and/or nested PCR were separated on 1.2% agarose gel stained with ethidium bromide and put under UV light.

Table 8: Sequences of PCR primers targeting 16S rDNA and non-ribosomal genes.

Gene	Method	Primer	Sequence (3′ - 5′)	Length (bp)	References
16S rDNA	PCR	R16F1/R0	TAAAAGACCTAGCAATAGG/CAA TCCGAACTGAGACTGT	1200bp	(Lee *et al.*, 1994)
	PCR/Nested PCR	R16F2n/R2	GAAACGACTGCTAAGACTGG/TGA CGGGCG GTGTGTACAAACCCCG		(Gundersen and Lee, 1996)
	Nested PCR	M1/M2	GTCTTTACTGACGCTGAGGC/ CTTCAGCTACCCTTTGTAAC	509 bp	(Gibb *et al.*, 1995)
	Nested PCR	fU5/rU3	CGGCAATGGAGGAAACT/ TTCAGCTACTCTTTGTAACA	862 bp	(Lorenz *et al.*, 1995)
secY	PCR	PoSecF1/ PoSecR1	TCTGCTTTGCCTTTGCCTTT/ ATTAGTAAACTAGTTCCTCC	998 bp	(Fialová *et al.*, 2009)
	Nested PCR	PoSecF3/ PoSecR3	GGATTGATAGATGCTGCCCC/ GCCCCTATAACGGTGATTTTGA		
tuf	PCR	fTufl/ rTufl	CACATTGACCACGGTAAAAC/ CCACCTTCACGAATAGAGAAC	950 bp	(Schneider and Gibb, 1997)
	Nested PCR	fTufAy/ rTufAy	GCTAAAAGTAGAGCTTATGA /CGTTGTCACCTGGCATTACC		
	PCR	fTufu/ rTufu	CCTGAAGAAAGAGAACGTGG/ CGGAAATAGAATTGAGGACG	840 bp	
groEl	PCR	AYgroesF/AY ampR	ATCAGAAAAAGAAAAATCCT/ GCAACAGCAGCAAATAAAAC	1.4 kbp	(Mitrović *et al.*, 2011)
	Nested PCR	AYgroelF/AY groelR	GGCAAAGAAGCAAGAAAAG/ TTTAAGGGTTGTAAAAGTTG		
secA	PCR	secAFor1/secA Rev3	GARATGAAAACTGGRGAAGG/ GTTTTRGCAGTTCCTGTCATNCC	840 bp	(Dickinson and Hodgetts, 2013)

2.3.5 Sequencing and phylogenetic analysis of 16S rDNA

Ligation: the DNA fragments generated from R16F2n/R2 primers in nested PCR were cloned into StrataClone™ PCR Cloning vector pSC-A (Stratagene, USA), and then introduced in *Escherichia coli* DH5α cells. The ligation process was done by mixing 5 µl of ligation buffer 2X with 1 µl (50 ng/ µl) of pSC-A vector, 1 µl of T4 DNA ligase and 3 µl of PCR product according to manufacturer's instructions.

Preparation of competent cells: all steps in this procedure were conducted in aseptic conditions. A colony of *E. coli* DH5α was incubated into 20 ml of LB liquid medium (Annex 4), and the solution was incubated overnight at 37°C at 250 rpm. Bacteria were harvested by centrifugation at 5,000 rpm for 5 min at 4°C. The pellet was gently resuspended in 7 ml cold 0.1 M $CaCl_2$, and then kept in ice for at least 2 h before transformation.

Transformation of competent cells: ligation mixture (2 µl) was added to 50 µl competent cells and incubated in ice for 20 min. Cells were shocked by heating at 42°C for 45 sec, immediately chilled in ice for 2 min, then incubated at room temperature for 1 min before adding 800 µl of LB medium. The bacteria were incubated at 37°C for 90 min with slow shaking. A final centrifugation was performed for 3 min at 5,000 rpm, the supernatant was eliminated and the bacterial pellet re-suspended in a low quantity of LB media. An aliquot of 35 µl of bacteria was plated on LB, containing 100 mg/ml ampicillin and 20 µl X-Gal (40 µg/µl) (for white/blue selection), with a sterile glass rod. The plates were incubated overnight at 37°C.

Screening of colonies: white colonies, carrying the insert, were selected, re-inoculated and numbered in another plate using sterile toothpicks.

Extraction of DNA plasmid: plasmids were extracted from bacteria cells using the boiling method (Sambrook *et al.*, 1989). Single, well isolated white colonies, likely containing the recombinant plasmids, were inoculated in 2ml in the liquid LB with 100 mg/ml ampicillin and incubated overnight at 37°C, at 250 rpm. Grown bacteria pellets were collected by centrifugation at 13,000 rpm for 1 min, re-suspended in 400 µl STET (Annex 4) and 20 µl lysozyme (20 mg/ml), incubated in boiling water for 45 sec and chilled in ice for 3 min.

After centrifugation at 13,000 rpm for 20 min and elimination of the pellet, 2 µl of RNase (10 µg/µl) were added to the supernatant, which was then left to incubate at 37°C for 30 min. A phenol-chloroform extraction was carried out, followed by a centrifugation at 13,000 rpm for 10 min. 300 µl of plasmid DNA were transferred in fresh tubes containing 200 µl NH_4OAC and 1ml cold absolute ethanol and centrifuged at 13,000 rpm for 15 min.

The pellet was then washed with 500 µl of 70% cold ethanol, centrifuged at 13,000 rpm for 10 min, dried at room temperature and finally re-suspended in 30 µl sterile water.

Enzymatic digestion of plasmid: to verify if extracted plasmids contained the appropriate nested PCR insert, a digestion with the restriction enzyme *EcoR*I was carried out. Three µl of plasmid DNA were incubated with 1 µl 10 X buffer H (Roche), 0.2 µl 10 U/µl *EcoR*I (Promega, Madison, WI, USA), (10 µl, final volume of digestion) at 37°C for 2h. Digestion of products was checked on 1.2% TAE agarose gel (Annex 2). The electrophoresis was carried out for 45 min at 100 V, and the gel was stained by ethidium bromide (10 mg/ml). DNA bands were observed under an UV transilluminator.

DNA clone's purification and sequencing: plasmids chosen for sequencing were further purified by using a commercial kit (Quick Lyse Miniprep Kit, Qiagen, USA). Bacterial cells were transferred to Quick lyse spin columns, placed in Eppendorf tubes and centrifuged at 13,000 rpm for 30 sec. After washing with 400 µl of buffer (QLW with isopropanol) and a new centrifugation, the supernatant was discarded and the pellet retained on the quick lyse spin columns was dried by a rapid centrifugation of 13,000 rpm for 35 sec. The filter columns were transferred to new Eppendorfs and the pellet were re-suspended in 50 µl of sterile water. An incubation at room temperature took place for 2 min followed by the last centrifugation at 13,000 rpm for 1 min. The enzymatic digestion was carried out by mixing 1 µl of plasmid DNA with 0.2 µl *EcoR*I, 1 µl buffer H 10 X and 7.8 µl SDW.

The DNA fragments generated from the use of R16F2n/R2 primers in nested PCR were custom sequenced (Eurofins Genomics, Germany). Four clones from each positive sample were sequenced bidirectionally. Nucleotide sequences were analyzed with DNA Strider 1.1 program (Marck, 1988) and multiple alignments were obtained using the default options of CLUSTALX 1.8 (Pearson and Lipman, 1988). Search for homology with nucleotides was done with BlastX programs (Altschul *et al.*, 1990). Phylogenetic trees using the Neighbor-joining method in MEGA 6 (Tamura *et al.*, 2013) were constructed based on available 16S rDNA sequences of '*Candidatus* Phytoplasmas' retrieved from the NCBI. *Acholeplasma laidlawii* was used as outroot.

2.3.6 Virtual RFLP analysis of 16S rDNA

Virtual RFLP analysis to define the subgroup of the phytoplasmas detected in the infected plants was performed using the online software tool *i*PhyClassifier (Zhao *et al*., 2009b). Each 16S rDNA consensus sequence obtained was subjected to *in silico* digestion with 17 restriction endonucleases. The generated virtual RFLP profiles were compared with available representative of 16SrI and 16SrXII subgroups and the similarity coefficient was calculated.

2.3.7 Detection of phytoplasmas by qPCR

Twenty positive nested PCR samples were further investigated for the presence of phytoplasmas by qPCR employing universal plant assay, universal (Christensen *et al.*, 2004), AY group specific (Angelini *et al.*, 2007; Nikolić *et al.*, 2009), and "stolbur" group specific (Hren *et al.*, 2007) phytoplasma assays (Table 9). Five PCR master mix tubes were prepared and the final reaction volume of 10 µl consisted of 2 µl of ten-fold/100-fold diluted DNA, 2 X TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM of forward primer for universal phytoplasma assay, 900 nM of all primers except forward primer of phytoplasma universal assay and 100 nM of probe for phytoplasma universal and AY specific assay, 250 nM of probe for 16SrXII specific assay. Each dilution was tested in duplicates. Positive and negative quality controls as described by (Dermastia *et al.*, 2017) were included. The qPCR reaction amplification procedure was conducted in Real-time PCR instrument (Applied Biosystems 7900 HT) and the thermocycling conditions were as follows: 95°C for 10 min (AmpliTaq activation) and 45 amplification cycles, consisting of 15 sec at 95°C and 1 min at 60°C. If an exponential amplification curve was observed to be discernible from negative controls, then the sample was considered positive.

Table 9: Sequences of primers and probes used in quantitative PCR.

Gene	Sequences of primers and probes 5'-3'		References
18S rRNA	Forward	GACTACGTCCCTGCCCTTTG	(Christensen *et al.*, 2013)
	probe	FAM-ACACACCGCCCGTCGCTCC-TAMRA	
	Reverse	AACACTTCACCGGACCATTCA	
16S rDNA	Forward	CGTACGCAAGTATGAAACTTAAAGGA	(Christensen *et al.*, 2013)
	Probe	FAM-TGACGGGACTCCGCACAAGCG-TAMRA	
	Reverse	TCTTCGAATTAAACAACATGATCCA	
AY 16S rRNA	AYnib-F	GGGTTAAGTCCCGCAACGA	(Nikolić *et al.*, 2009)
	AYnib-S	FAM-CAACCCTTATTGTTAGTTRCCAG-MGB	
	AYnib-R	TCTTGCTAAAGTCCCCACCATTAC	
AY 16S rRNA	AYan-F	TTGGGTTAAGTCCCGCAAC	(Angelini *et al.*, 2007)
	AYan-S	FAM-CCAGCACGTAATGGTGGGGACTT-TAMRA	
	AYan-R	CCCACCTTCCTCCAATTTATCA	
BNgen	Forward	AAGCAGGTTTAGCGATGGTTGT	(Hren *et al.*, 2007)
	Probe	FAM-TTAATACCACCTTCAGGAAA-NFQ	
	Reverse	TGGTACCGTTGCTTCATCATTT	

2.4 Results

2.4.1 Detection of phytoplasmas

In nested PCR, the 16S rDNA gene sequence was amplified in 20 out of 43 samples tested, showing the phytoplasma presence in the 46.5% of symptomatic fig samples. Nucleotide sequences of the 16S rDNA clones, generated from different infected plants, showed high similarities (99.5%) with sequences of '*Ca*. P. asteris' (16SrI-B) and '*Ca*. P. solani' (16SrXII-A). The sequences of three phytoplasma strains, named 70-3, 70-6 (16SrI) and P1-1-3 (16SrXII-A), respectively, in different combinations, were detected from infected plants analyzed, with no variations among them (Table 10), the CT values averages of all genes analyzed in qPCR are reported in (Table 11). The presence of two diverse phytoplasmas was further verified through group specific qPCR assays, which showed the presence of 16SrI phytoplasma in 20 samples, whereas 16SrXII phytoplasmas were detected in 4 samples in co-infection with 16SrI phytoplasmas (Table 10). No PCR amplifications were obtained from the primers used to amplify *secA*, *secY*, *groEl* and *tuf* genes of 16SrI and 16SrXII strains, although the positive controls were successfully amplified.

2.4.2 Detection of fig mosaic virus

The RT-PCR results showed the presence of FMV in 36 symptomatic fig plants out of the 43 tested, showing an infection rate of 83.7%. Seventeen of the 20 positive phytoplasma infected samples were found co-infected with FMV (Table 10), thus only 3 were FMV-free. These samples showed different types of symptoms varying from leaf mottling to deformation. Four symptomatic samples also resulted negative to all the searched pathogens.

Table 10: Detection of phytoplasmas by nested and qPCR and of FMV by RT-PCR.

Sample code		Nested PCR	**qPCR** (Christensen *et al*., 2004)	**qPCR 16SrI-** (Nikolić *et al.*, 2009)	**qPCR 16SrXII-** (Hren *et al.*, 2007)	**RT-PCR FMV**
1	B60-1	-	nt	nt	Nt	+
2	B60-2	+	+	+	-	+
3	B60-3	-	nt	nt	Nt	-
4	B60-4	-	nt	nt	Nt	-
5	B60-6	+ **(LR584982, LR584983)**	+	+	-	+
6	B60-11	+	+	+	-	+
7	B65-1	+	+	+	-	+
8	B65-2	+	+	+	-	+
9	B65-3	-	nt	nt	Nt	+
10	B65-4	+ **(LR584982, LR584983, LR584984)**	+	+	-	+
11	B65-5	-	nt	nt	Nt	+
12	B65-6	-	nt	nt	Nt	+
13	B65-7	+	+	+	-	+
14	B65-8	+ **(LR584982, LR584983, LR584984)**	+	+	+	+
15	B65-9	+	+	+	-	-
16	B65-10	+	+	+	-	+
17	B65-11	+ **(LR584982, LR584984)**	+	+	+	+
18	B65-12	+	+	+	+	+
19	B65-13	+	+	+	-	-
20	B65-14	+	+	+	-	-
21	B65-15	+	+	+	-	+
22	B65-16	+	+	+	-	+
23	B65-17	+	+	+	-	+
24	B65-18	-	nt	nt	Nt	+
25	B65-19	-	nt	nt	Nt	-
26	B65-20	-	nt	nt	Nt	+
27	B66-64	-	nt	nt	Nt	+
28	B66-65	-	nt	nt	Nt	+
29	B66-66	-	nt	nt	Nt	-
30	B66-67	-	nt	nt	Nt	+
31	B66-68	-	nt	nt	Nt	+
32	B66-69	-	nt	nt	Nt	+
33	B66-70	+ **(LR584982, LR584983, LR584984)**	+	+	+	+
34	B66-71	-	nt	nt	Nt	+
35	B66-72	-	nt	nt	Nt	+
36	B66-73	-	nt	nt	Nt	+
37	B66-74	+	+	+	-	+
38	B66-75	+	+	+	-	+
39	B66-76	-	nt	nt	Nt	+

40	B66-77	-	nt	nt	Nt	+
41	B66-78	-	nt	nt	Nt	+
42	B66-79	-	nt	nt	Nt	+
43	B66-80	-	nt	nt	Nt	+
Total (percentage)		**20 (46.5%)**	**20 (46.5%)**	**20 (46.5%)**	**4 (9.3%)**	**36 (83.7%)**

+: positive; -: negative; nt: not tested; boxes with dark green background: samples infected by phytoplasmas but not by FMV

Table 11: CT values of samples tested in qPCR

Sample	**18S**	(Christensen *et al*., 2004)	**AYnib**	**AYan**	**BNgen**
B60-2	21.68	35.57	37.73	undt	undt
B60-6	15.73	32.14	32.79	undt	undt
B60-11	19.30	33.02	35.22	undt	undt
B65-1	24.28	36.65	36.94	undt	undt
B65-2	20.09	34.43	36.57	undt	undt
B65-4	18.06	33.79	36.05	undt	undt
B65-7	17.22	34.67	33.67	undt	undt
B65-8	18.15	32.61	35.33	undt	38.41
B65-9	19.46	29.67	29.95	30.49	undt
B65-10	19.24	34.01	34.35	undt	undt
B65-11	18.74	33.06	36.03	undt	38.81
B65-12	17.50	31.56	33.93	undt	37.62
B65-13	19.65	32.33	36.36	undt	undt
B65-14	21.10	34.91	35.75	undt	undt
B65-15	19.84	33.07	35.02	undt	undt
B65-16	19.97	31.24	32.59	undt	undt
B65-17	19.55	32.04	32.71	undt	undt
B66-70	15.80	25.16	27.84	27.69	37.89
B66-74	18.44	33.16	33.39	undt	undt
B66-75	18.87	33.55	33.70	undt	38.21
NTC	undt	undt	undt	undt	undt
PC	20.86	28.23	21.76	21.16	32.76

2.4.3 RFLP and phylogenetic analysis

Amplicons of the expected size, generated with primer pairs M1/M2, fU5/rU3 and R16F2n/R2 from the fig sample Bio66-70, were digested with *Tru*I restriction enzyme. Mixed RFLP patterns for aster yellows (16SrI) and "stolbur" (16SrXII-A) phytoplasmas were generated from R16F2n/R2 (Figure 20) and fU5/rU3 amplicons, whereas the restriction profile of M1/M2 amplicon was identical to that of "stolbur" phytoplasma (data not shown).

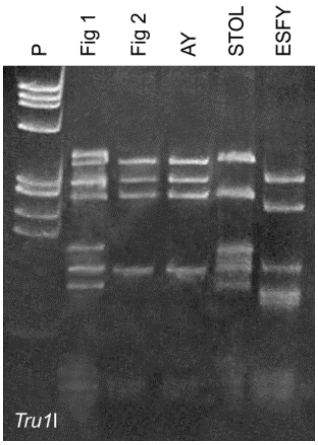


Figure 20*:* Restriction fragment length polymorphism patterns of the two fig phytoplasmas obtained in this study (Fig 1 and Fig 2) compared with those of reference strains maintained in periwinkle: AY, aster yellows (16SrI-B); STOL, "stolbur" (16SrXII-A) and ESFY (European Stone Fruit Yellows) of 16S rDNA amplified in nested-PCR with R16F2n/R2 and digested with *Tru1*I. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

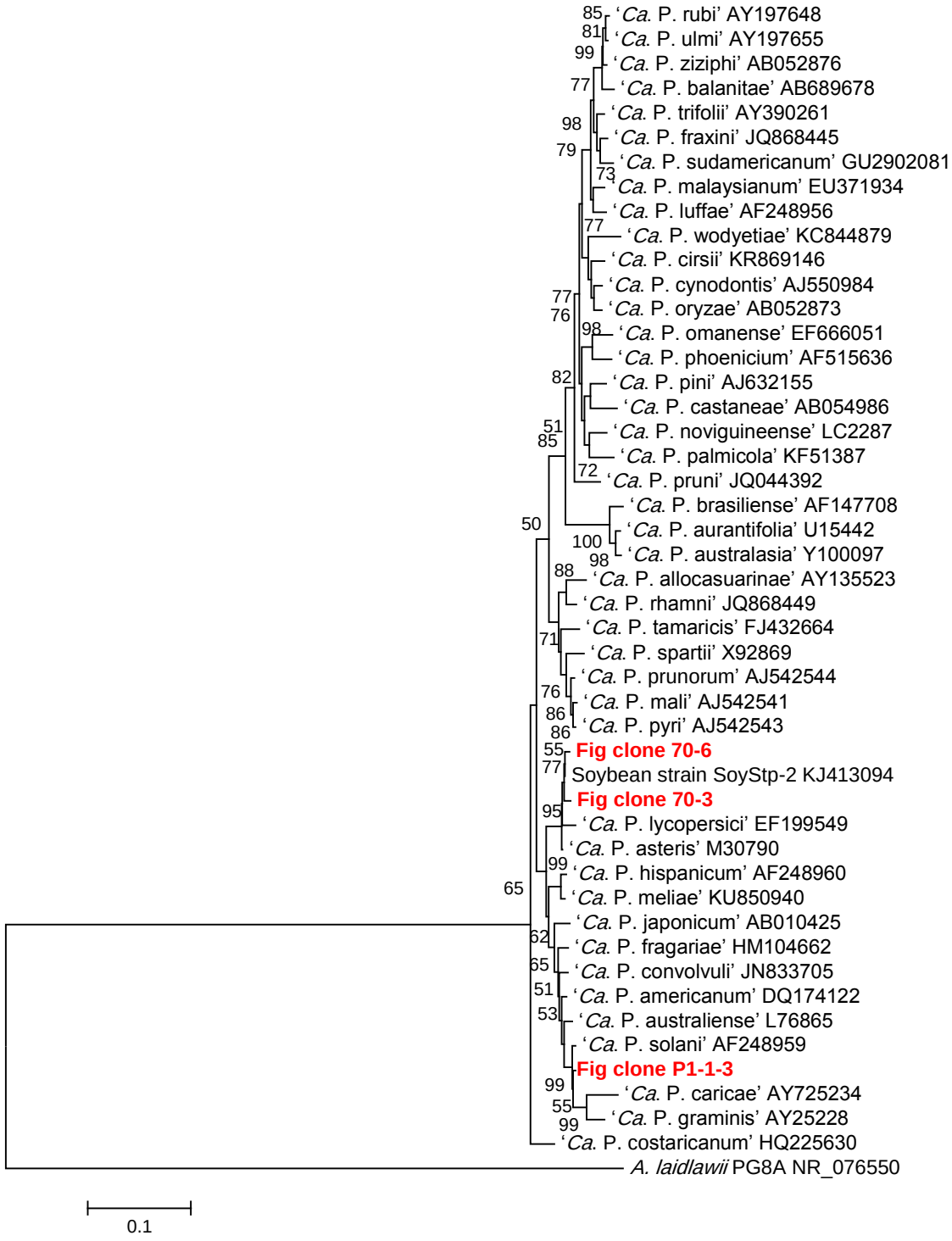
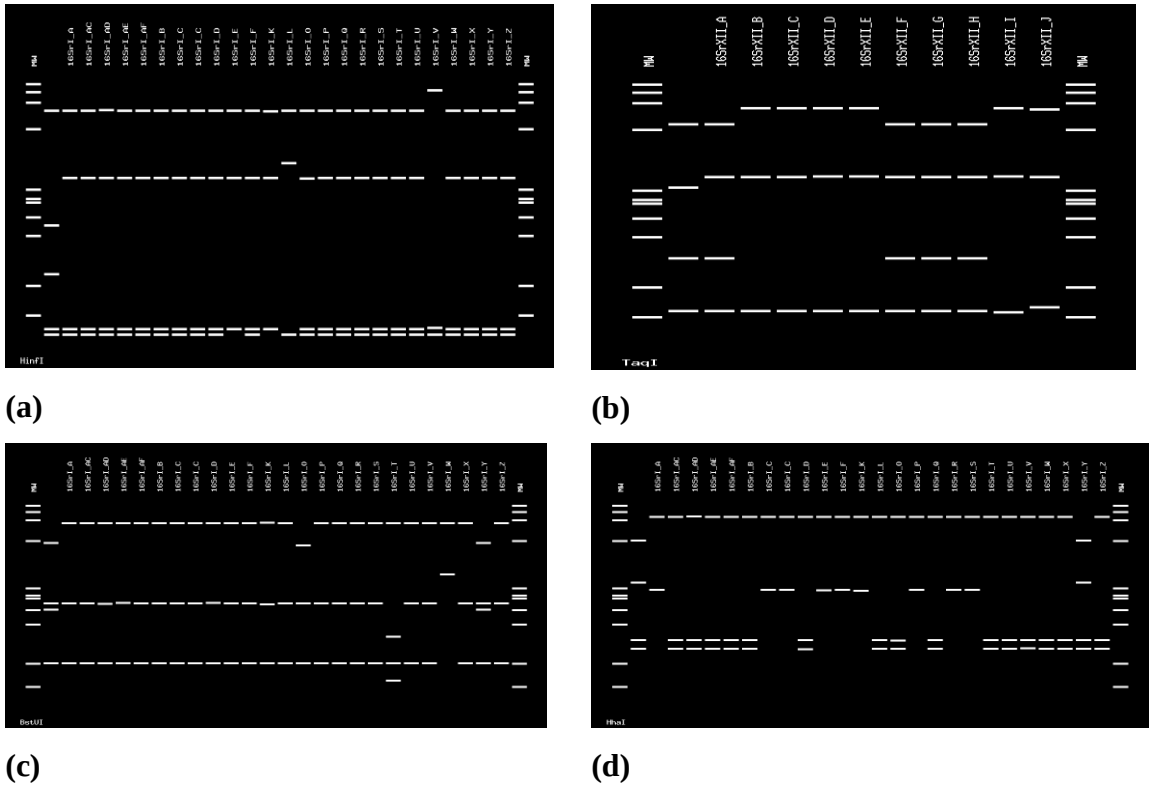


Figure 21: Phylogenetic relationships of phytoplasma clones from fig tree samples with the '*Candidatus* Phytoplasma' taxa available. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The

evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 48 nucleotide sequences.

The phylogenetic tree constructed using the sequences of 16S rDNA gene obtained for each of the three phytoplasma strains (70-3 , 70-6 and P-1-1-3) and submitted to the GenBank (accession numbers: LR584982, LR584983 and LR584984, respectively) allow to cluster the first two with '*Ca.* P. asteris'- related strains (GenBank accession numbers M30790, EF199549 and KJ413094) and the last one with the '*Ca.* P. solani'-related strain (accession number: AF248959) (Figure 21).



(a) **(b)**

(c) **(d)**

Figure 22: RFLP profiles generated after in silico digestion of 16S rDNA fragments from phytoplasma clones from fig (A): 70-3 (LR584982), (B): P1-1-3 (LR584984) and (C-D): 70-6 (LR584983) compared with subgroups in 16SrI and 16SrXII groups with informative enzymes using the online *i*PhyClassifier.

Virtual RFLP analysis confirmed the presence of three phytoplasma strains, two of which were belonging to 16SrI group and one to 16SrXII group. However, two of these strains (clone70-3 and clone P1-1-3), obtained from sample the Bio66-70, showed polymorphisms with HinfI and TaqI endonucleases (Figure 22) to the respective phytoplasma subgroups

present in the *i*PhyClassifier and showed similarity coefficient of 0.94 and 0.98 with strains 16SrI-B and 16SrXII-A, respectively. The third strain. 70-6, showed identity, with similarity coefficient of 1.0, to the subgroup reported as 16SrI-Y in the *i*PhyClassifier (GenBank accession number KJ413094) showing identical virtual RFLP profile with the restriction enzymes that allow its differentiation from the other reported 16SrI subgroups (Figure 22).

2.5 Discussion

Phytoplasma diseases have never been reported in fig plants, therefore plants displaying different types of symptoms have been inspected in the field, sampled and analyzed in the laboratory. Analyses showed that twenty fig plants from different varieties (out of 43 tested) were infected with phytoplasmas sharing the highest identity with members of '*Ca*. P. asteris' and '*Ca*. P. solani'. However, in all the samples but seven the presence of fig mosaic virus (FMV) was also detected, thus rendering difficult to distinguish symptoms related only to the presence of phytoplasma.

The presence of the latter prokaryotes was confirmed by using 16SrI and 16SrXII-A specific primers and probes in Real-time PCR assays and the virtual RFLP analyses indicated that two of the phytoplasma strains detected could represent a novel subgroup (16SrI) and a variant of 16SrXII-A subgroup, respectively. This result could be however influenced by the presence of phytoplasma mixed infection in the samples even if identical sequences were detected in more than one sample tested. Moreover, considering that when a mixed phytoplasma infection is present the detection of both phytoplasmas is very often scattered among samples (Duduk *et al*., 2009), the presence of chimeric sequences cannot completely be discarded. Moreover, all the attempts to amplify *secA*, *secY*, *groEl* and *tuf* genes of 16SrI and 16SrXII strains were unsuccessful, although a clear amplification was obtained from positive controls. This indicated either the presence of sequence variation in the targeted regions or the existence of phytoplasmas in low concentrations.

Both phytoplasmas detected have a wide distribution and infect a large range of plant species. In Europe, 16SrI phytoplasmas were reported in grapevine (Alma *et al.*, 1996; Canik *et al.*, 2011; Landi *et al.*, 2013), blackberry (Reeder *et al*., 2010), *Brassica* spp. (Kaminska *et al*., 2012), and ornamentals. They were also found to be associated with clover phyllody and strawberry green petal and detected in a wide range of vegetable crops worldwide (Kumari *et al.*, 2019). On the other hand the 16SrXII-A phytoplasmas were widely reported in grapevine (Torres *et al.*, 2005; Belli *et al.*, 2010; Pasquini *et al.*, 2010; Quaglino *et al.*, 2016), stone fruits (Quaglino *et al.*, 2013), solanaceous crops (Navrátil *et al.*, 2009), ornamentals,

vegetable crops and in diverse weed plants (Garnier, 2000; Fialová *et al.*, 2009; Ember *et al.*, 2011).

In this study the symptomatology is the puzzling part since the presence of co-infection in 17 fig plants of phytoplasmas and FMV, responsible for a wide range of symptoms on fig, have hindered somehow the real phytoplasma symptom association. It is noteworthy that the three fig plants, with leaf mottling and deformation symptoms, infected with phytoplasma and free from FMV, could suggest a possible involvement of phytoplasma with specific disease symptoms in fig. Further screening including asymptomatic and virus free plants will be necessary to determine if there is an association between specific symptoms and phytoplasma presence. However, the high incidence of phytoplasmas found in the fig collection plot could be attributed to the possible presence of insect-vectors (not investigated in this study) that could have played a decisive role in the spread of reported phytoplasmas.

Conclusions

Fig mosaic disease is widely diffused in fig growing areas worldwide, with a high variability of symptoms induced by this disease. To date, at least 10 viruses belonging to different families have been described to be associated with this disease. Previously, RT-PCR was the only molecular based technique available to detect fig viruses. Since the sanitary status of fig plants is critical according to European Union regulations (Commission Executive Directive 2014/98 and Council Directive 2008/90, that enforce the Conformitas Agraria Communitatis, CAC), fig plant material free of mosaic is highly needed. Several sanitation techniques should be therefore applied to obtain mosaic-free mother stock to produce virus free propagative materials. The high incidence of this disease is correlated to the propagation mode of their viral agents in nature (by cuttings and grafting) and the presence of very efficient virus vectors (eriophyid mites, mealybugs and aphids). Accordingly, a new diagnostic molecular assay was developed to detect single and multiple infections caused by FMD-associated viruses. This newly developed assay is more sensitive and reliable than RT-PCR. It can be applied for high throughput screening and to ease the management of this disease through the limitation of fig viruses spread, thus providing a remarkable contribution to support quarantine and certification programs. Once the healthy material is planted in the field, the rate of reinfection by FMV mediated by viruliferous mites could be very high if in the area is present a high inoculum (infected trees), also in consideration of the vulnerability of commercial fig cultivars to FMV. Furthermore, due to information shortage about the epidemiology of FMD, and knowing that the disease is essentially transmitted by grafting and by plant propagating material, sanitary selection could be adopted as an appropriate control strategy for the establishment of certified fig orchards. Finally, the sanitary selection is a crucial step for virus control but it is also important to take into consideration the epidemiology of the detected viruses which is essential to preserve the selected fig plants. Besides to the use of healthy nursery stocks, chemical treatment to control the vector could help to reduce or delay the spread of FMD in the field.

This work aimed also to study the possible presence of an undescribed bacterial disease in fig plants. The presence of phytoplasma was assessed by PCR, universal and group specific

Real-time PCR assays through the amplification of their 16S rDNA. In addition, RFLP and phylogenetic analysis of 16S rDNA showed the presence of two ribosomal phytoplasma groups (16SrI and 16SrXII). The most important outcome of this study is the first report of phytoplasmas occurrence in fig plants. Further studies are highly recommended at the epidemiological level to verify the identity of insect vectors, their natural reservoirs and distribution as well as the possible role of the infected material in their spread, which all together will contribute in the designing of an effective management strategies.

Theoretically, phytoplasma diseases can be managed either by controlling the vector or eliminating the pathogen from plant material through the use of insecticides or antibiotics, respectively. However, as European policy aims to reduce the insecticides use and the application of antibiotics is not allowed, an alternative approach should be found to replace these methods. Environment friendly strategies could be the best choice to control these bacteria through the identification of resistant varieties and avoid the use of chemicals.

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Annexes

Annex 1. Buffers and solutions of total nucleic acid extraction.

Buffer	Material	Quantity	Note
Grinding buffer pH 5.6-5.8	Guanidine thiocianate. NaOAc, pH 5.2 EDTA. KOAc PVP-40 NaHSO4	4M 0.2 M 25 mM 1.0 M 2.5% 2%	– Adjust pH using CH_3COOH and sterilize by autoclaving – Keep it at 4°C – Add NaHSO4 before using
NaI	Na_2SO_3 NaI (Sigma S8379)	0.75 g 36 g	– Dissolve in 40 ml distilled water – Sterilize by autoclaving – Keep it in dark at 4°C
Silica particles solution pH 2.0	Silica particles (Sigma 12% S5631)	12%	– Add 60 g silica to 500 ml H_2O – Mix and let settle for 24 hours – Discard the upper 470 ml supernatant (90% of the supernatant) – Add H_2O up to 500 ml and mix well – Let settle 5 h – Discard 440 ml (85% of the supernatant) – Adjust the remaining 60 ml slurry to a pH 2.0 with HCl – Autoclave and store in dark at room temperature
Washing buffer 1x	Tris-HCl, pH7.5 EDTA NaCl EtOH	10 mM 0.5 mM 50 mM 50%	– Sterilize by autoclaving before adding EtOH. – Keep it at 4°C
CTAB buffer 2X	Tris-HCl, PH8 NaCl EDTA CTAB PVP40	100 mM 1.4 M 20 mM 2% 2%	– Autoclave and at room temperature

Annex 2. Buffers and gels used for electrophoresis.

Buffer	Material	Quantity	Note
TBE 10x (stock solution) pH 8.3	Tris Boric acid 99.5% EDTA	1M 61.3 g 0.01M	– Dissolve in 1 l distilled water – Sterilize by autoclaving – Keep it at room temperature
TAE 10x (stock solution) PH 7.2	Tris Sodium acetate EDTA	0.4 M 0.4 M 0.02 M	– Dissolve in 1 l distilled water – Sterilize by autoclaving – Keep it at room temperature

Annex 3: PCR universal primers commonly used for the detection of phytoplasmas.

Primer/probe	gene	Reaction	References
P1/ P7	16Sr/23SR	PCR	(Deng and Hiruki, 1991; Smart *et al.*, 1996)
16R758F/16R1232R	16Sr	Nested PCR	(Gibb *et al.*, 1995)
R16F2/R16R2 R16F2n /R6R2	16Sr	PCR	(Lee *et al.*, 1993a; Gundersen and Lee, 1996)
	16Sr	Nested PCR	
F1/B6	16S/23SR	semi-nested PCR	(Davis and Lee, 1993; Padovan *et al.*, 1995)
fU3/fU5	16Sr	Nested PCR	(Lorenz *et al.*, 1995)
SecAfor 1/SecArev 3	secA	PCR	(Hodgetts *et al.*, 2008)
SecAfor 2/SecArev 3	sec A	semi-nested PCR	(Hodgetts *et al.*, 2008)
UPH-F (F)	16Sr	qPCR	(Christensen *et al.*, 2004, 2013)
UPH-R (R)	16Sr	qPCR	(Christensen *et al.*, 2004, 2013)
UPH-Pb (P)	16Sr	qPCR	(Christensen *et al.*, 2004, 2013)
UPH-P (P)	16Sr	qPCR	(Malandraki *et al.*, 2015)
UPHr2 (R)	16Sr	qPCR	(Ito and Suzaki, 2017)
D-UPHr2 (R)	16Sr	qPCR	(Ito and Suzaki, 2017)
JH-F 1 (F)	23S	qPCR	(Hodgetts *et al.*, 2009)
JH-F all (F)	23S	qPCR	(Hodgetts *et al.*, 2009)
JH-R (R)	23S	qPCR	(Hodgetts *et al.*, 2009)
JH-P uni (P)	23S	qPCR	(Hodgetts *et al.*, 2009)

Annex 4. Solution used for the extraction of plasmid DNA.

Solutions	Materials	Quantities	Notes
STET (100 ml)	NaCl Tris-HCl EDTA Saccarose Triton X-100	0.1 M 20 mM 50 mM 8% 0.5%	pH 8.0
Lysozyme		20 mg/ml	Store at -20°C
Lysogeny broth (LB) liquid	Tryptone Yeast extract NaCl	1% 0.5% 0.5%	Sterilized by autoclave
Lysogeny broth (LB) solid	Tryptone Yeast extract NaCl Agar bacteriological	1% 0.5% 0.5% 0.3%	Sterilized by autoclave
Ampicillin	aminobenzylpenicilin	75 µg/ml	
Isopropanol	Isopropyl alcohol		Absolute
Ethanol	EtOH	70% in deionized water	