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STUDIES ON PHYTOPLASMA SEED TRANSMISSION IN DIFFERENT BIOLOGICAL SYSTEMS

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Alle mie radici, con immensa riconoscenza;
per il mio futuro, con piena fiducia.

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Summary

The transmission of phytoplasmas by seed in *Sesamum indicum, Brassica napus, Solanum lycopersicum and Zea mays,* was studied. The seeds, derived from infected mother-plants, were sown in sterile substrates and their germination percentage was evaluated. The seedlings were analyzed for the presence of phytoplasmas in different stages of growth by "nested"-PCR/RFLP analysis. The four species resulted positive for phytoplasmas belonging to different ribosomal groups and the number of positive plants decreased in the later stages of growth. For *S. lycopersicum* samples the presence of phytoplasmas was also analyzed in second generation plants: 7 seedlings out of the 60 tested resulted positive for phytoplasmas. Phytoplasma isolation in artificial medium CB was performed for all samples resulted positive, to verify the viability of the phytoplasmas. From *Z. mays* samples, colonies positive to phytoplasmas belonging to ribosomal groups 16SrI and 16SrXII were obtained. These colonies positive to 16SrI were reproducible for at least three subsequent passages liquid/solid media carried out every 5 days. Some of these samples produced colonies also from broth maintained for seven months at 25°C after isolation. These preliminary results indicate the viability of 16SrI phytoplasmas isolated from corn seedlings and confirm seed transmission of viable phytoplasmas.

A quantitative PCR assay with SYBR Green chemistry, with generic "primers" to detect phytoplasmas belonging to different ribosomal groups was successfully applied both to seedlings and symptomatic field infected plants. Contrasting results were obtained from phytoplasma in liquid media and colonies. This technique demonstrated high sensitivity for phytoplasma at low concentrations and high specificity for the *Mollicutes* that can be differentiated from non-*Mollicutes* by the analysis of melting temperatures.

In carrot samples from Gran Canaria Island (Spain), symptoms of shoot and root malformation were observed. '*Candidatus* Liberibacter solanacearum', haplotype D, and phytoplasmas belonging to the ribosomal group 16SrI were detected.

1. Phytoplasmas

1.1. What phytoplasmas are?

In 1967 Doi and collaborators showed that several plant diseases were associated with prokaryotic organisms similar to mycoplasmas. Because of their morphologic and ultrastructural similarities with the mycoplasmas pathogens of animals and humans, known as agents of pneumonia diseases since the end of 1800 (Nocard and Roux, 1898), these organisms were named MLO, mycoplasma-like organisms. They were then designed with the trivial name of "phytoplasmas" by the Phytoplasma Working Team during the 10th Congress of the International Organization of Mycoplasmology in 1994, and then in 2004 the name '*Candidatus* phytoplasma' was officially adopted for the taxon (IRPCM, 2004) considering their incomplete biologic description mainly due to the lack of their cultivation in media (Murray and Stackebrandt, 1995).

Phytoplasmas are now associated to many different diseases showing different symptoms in plants as stunting, yellows, dwarf, phyllody, witches' brooms, redness and virescence. These bacteria are associated with devastating diseases in diverse crops worldwide with severe economic losses.

The application of molecular technologies made it possible to investigate the phytoplasma phylogeny, and indicated that they form a monophyletic group in the class *Mollicutes* (Sears and Kirkpatrick, 1994; Gundersen *et al*., 1994; Seemüller *et al*., 1994) and are related to each other more closely than with the other groups of prokaryotes of this class as mycoplasmas, ureaplasmas, spiroplasmas and acholeplasmas (Razin *et al*., 1998).

Restriction enzyme-based studies on the *16S rRNA* gene allowed dividing the monophyletic group of phytoplasmas into ribosomal groups and subgroups (Lee *et al*., 1998).

1.2. Morphology

Phytoplasmas are pleomorphic bacteria surrounded by a lipopropteic membrane, but lacking the cell wall and for this reason they are resistant to all the antibiotics that act on the cell wall; they have a very small genome ranging from 350 to 1,350 kb and a low G+C content (Dickinson *et al*., 2013). Their average diameter is ranging from 0.2 to 1 µm and they are phloem-limited mainly located in mature sieve tubes (Hogenhout *et al*., 2008). The distribution of phytoplasmas in the

host plant is variable according with the season and the type of plant infected (Bertaccini and Duduk, 2009). For instance, their concentration in the roots is usually lower than in the stems, and they are more concentrated in leaves than in sink tissues. The phytoplasma ancestor is considered a Gram-positive bacterium as *Clostridium* or *Lactobacillus* spp. (Woese, 1987; Gundersen *et al*., 1994; Lee *et al*., 1998b; Seemuller *et al*., 1994). Extrachromosomal DNAs similar to plasmids were also detected in different phytoplasmas as maize bushy stunt (Davis *et al*., 1990), western aster yellows (WAY) (Kuske *et al*., 1991), tomato big bud (Tran-Nguyen and Gibb, 2006) periwinkle little leaf (Davis *et al*., 1990), chrysanthemum yellows, American aster yellows (Bertaccini *et al*., 1990a), aster yellows witches' broom (AYWB) (Bai *et al*., 2006). These plasmids contain also geminivirus-like sequences, and have the ability to recombine with phytoplasma chromosomes (Bai *et al*., 2006) and between each other (Nishigawa *et al.,* 2002; Liefting *et al.,* 2004), increasing the biological diversity of phytoplasmas. For instance, *'Candidatus* Phytoplasma asteris', onion yellows strain (OY), contains two types of plasmids, each of which possesses a gene encoding a putative transmembrane protein, ORF3, that plays a role in the interactions of OY with the insect host of this phytoplasma (Ishii *et al*., 2009).

1.3. Economic importance

Among the phytoplasma affected species there are many crops of agricultural interest because food reserves, ornamental and forest plant species (Bertaccini, 2007; Bertaccini *et al*., 1992a; Lee *et al*., 2007). The principal diseases associated with phytoplasma presence worldwide are described below: fruit trees decline, citrus witches' broom, grapevine yellows and palm lethal yellowing. Others important diseases are those diseases associated to corn, tomato, *Sesamum indicum* and *Brassica napus* (See chapter 2).

1.3.1. Fruit trees decline

Phytoplasma economically important diseases in Europe affecting apple, pear, apricot, peach and plum trees are apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY). These diseases are associated with 3 different quarantine pathogens: '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum' transmitted by diverse psyllids and having some host range specificity (Seemüller and Schneider, 2004).

AP is present in almost all Europe (Rui *et al*., 1950; Seemüller and Schneider, 2004) and apple is the main host of '*Ca.* P. mali', but it was also detected in wild and ornamental *Malus* species (Seemüller *et al*., 2008), in hazelnut (*Corylus* spp.) (Marcone *et al*., 1996), cherry (*Prunus avium*),

apricot (*P. armeniaca*) and plum (*P. domestica*) (Mehle *et al*., 2007). The psyllids *Cacopsylla picta* (Forster), *C. melanoneura* (Frisinghelli *et al*., 2000; Jarausch *et al*., 2004) and *Fieberiella florii* are the main responsible of the transmission of AP in Italy and Germany.

PD was firstly reported in North American pear orchards affecting more than one million of pear trees (McLarty, 1948; Woodbridge *et al*., 1957). In northern Italy it was known with the name "moria" (Refatti, 1948) and recently was described also in southern Italy (Marcone *et al*., 1996). In North America and UK, the vector is *Cacopsylla pyricola* (Foerster), instead *Cacopsylla pyri* (L.) has been reported in Europe.

ESFY disease it is due to '*Candidatus* Phytoplasma prunorum' presence (Seemüller and Schneider, 2004) and belongs to the ribosomal subgroup 16SrX-B. It is affecting several stone fruit species including apricot (Morvan, 1977), plum (Giunchedi *et al*., 1982), almond (Seemüller *et al*., 1998) and peach (Poggi Pollini *et al*., 2001), but it infects also several other wild and cultivated *Prunus* species. When the conditions are favorable for host-plants and insect vectors, ESFY disease can have a rapid and widespread diffusion. Symptom are variable: foliar yellowing, leaf roll and leaf reddening, reduction or suppression of dormancy with the consequent risk of frost damage, severe and progressive necrosis, decline and mortality (Figure 1.1).

Figure 1.1. Symptoms associated with ESFY phytoplasma in peach in Italy.

1.3.2. Citrus witches' broom disease

Witches' broom disease of lime (WBDL), associated with the presence of '*Ca*. P. aurantifolia', is responsible for major losses of Mexican lime trees (*Citrus aurantifolia* L.) (Mardi *et al*., 2011). The disease was first observed more than 30 years ago, in Oman (Garnier *et al*., 1991; Zreik *et al*., 1995) where the destruction of 98% of Mexican lime trees occurred and it has after spread to the

United Arab Emirates, Iran (Bové *et al*., 2000), and India (Ghosh *et al*., 1999). Infected lime trees produced large number of shoots with small and clustered leaves and branches (Garnier *et al*., 1991; Chung *et al*., 2006). In Brazil in 2007, phytoplasmas belonging 16SrI and 16SrIX were described associated with a devastating citrus disease known as huanglongbing (HLB) mainly attributed to three species of Gram negative bacteria in the '*Candidatus* Liberibacter' genus, highly destructive to citrus production worldwide (Chen *et al*., 2008). Mixed infection of '*Ca.* P. asteris' and '*Ca.* L. asiaticus' was also reported (Teixeira *et al*., 2009).

1.3.3. "Flavescence dorée" and "bois noir"

The main phytoplasma diseases of grapevine in Europe are "flavescence dorée" (FD) and "bois noir" (BN). The first is a quarantine disease associated with phytoplasmas belonging to 16SrV-C and 16SrV-D ribosomal subgroups (Bertaccini *et al*., 1995; Daire *et al*., 1997; Martini *et al*., 1999) and is transmitted by the leafhopper of American origin *Scaphoideus titanus* Ball (Schvester *et al*., 1969); however the transmission by *Dictiofora europea* from *Clematis vitalba* to grapevine under experimental condition was also reported (Filippin *et al*., 2009). *Oncopsis alni* transmits 16SrV-C phytoplasma from alder to grapevine and the disease is known as Palatinate grapevine yellows (Maixner *et al*., 200). Recently another insect species was associated with 16SrV phytoplasmas in vineyards: the *Orientus ishidae* that resulted positive to 16SrV-C and -D in Slovenia, Italy e Switzerland (Mehle *et al*., 2010; 2011; Gaffuri *et al*., 2011; Trivellone *et al*., 2015). The BN disease is associated with phytoplasmas belonging to ribosomal subgroup 16SrXII-A, "stolbur", that are transmitted by the occasional grapevine feeder, the cixiid *Hyalesthes obsoletus* Signoret (Sforza *et al*., 1998) and by *Reptalus panzeri* as recently reported in Serbia (Cvrković *et al*., 2014).

1.3.4. Lethal yellowing

Lethal yellowing (LY) is a severe disease that affects coconut (*Cocos nucifera* L.) and 36 other palm species in the Americas (Harrison and Jones, 2004). The phytoplasmas associated with LY in North America and in the Caribbean are members of group 16SrIV. There is more than one phytoplasmas associated with coconut diseases according with the location, thus the collective name of these diseases is "lethal yellowing-type diseases" (LYTD). The symptoms begin with a premature shedding of most or all fruit. Aborted fruit usually develop a brownish black calyx-end rot and reduced seed viability. Other symptoms are: inflorescence necrosis, leaf discoloration from the oldest to the youngest. Affected leaves can turn brown, desiccate and hang down forming a skirt around the trunk for several days before falling to the ground (Bertaccini *et al*., 2014).

1.4. Symptomatology

Characteristic symptoms of phytoplasma infection are: phyllody which in tomatoes was demonstrated to be associated to deregulation of a gene involved in the formation of the inflorescence (Pracros *et al*., 2006); yellowing of the leaves, due to the presence of phytoplasmas in the phloem that reduce the functionality and can induce changes in the translocation of carbohydrate or, since phytoplasmas affect the physiological processes of photosynthesis, plants infected by these pathogens often show a yellowing caused by the drastic reduction in the concentration of chlorophyll and carotenoids, whose biosynthesis is inhibited (Bertamini and Nedunchezhian, 2001); virescence, development of green flowers because of the change and/or loss of the pigments of the petals cells (Lee *et al*., 2000); witches' broom due to the elimination of apical dominance resulting in proliferation of the secondary side shoots (Lee *et al*., 2000) and reduction of the internode length.

1.5. Phytoplasmas transmission

Phytoplasmas are distributed within the host plant not homogeneously, but subjected to seasonal fluctuation (Bertaccini and Duduk, 2009): their concentration is high in mature leaves and low in sink leaves. Phytoplasmas can be transmitted by insect vector, vegetative propagation as grafting, cuttings, tubers or rhizomes, micropropagation (Bertaccini *et al*., 1992b), and dodder (*Cuscuta* sp.). Another method for phytoplasma transmission is by seed, considered a controversial issue until few years ago (Cordova *et al*., 2003; Khan *et al*., 2002; Botti e Bertaccini, 2006; Calari *et al*., 2011) (See chapter 2).

1.5.1. Transmission by insects

Insect vectors have a stinging-sucking mouthparts and feed in phloem cells, obtaining nutrition from free amino acids and sugars; but only a small number of insects have been confirmed as vectors of phytoplasmas, they mainly belong to the families Cicadellidae, Fulgoromorpha and Psyllidae (Weintraub and Beanland, 2006). The acquisition access period (APP) is the period of feeding necessary to the insect to acquire phytoplasmas, which is very variable. The period of time from initial acquisition to the ability to transmit the phytoplasma is known as the latent period (LP) or incubation period. During this time, the phytoplasmas move through the insect body and replicate. The phytoplasmas enter into the insects through the stylet together with the lymph, they then pass through the intestine, are absorbed and transported by hemolymph until the salivary glands. The phytoplasmas can also spend the winter inside insect vectors or in perennial

host plants; the effect of their presence in the insect can be either an increase or a reduction of their fitness (Christensen *et al*., 2005).

Generally, the insect vector-phytoplasma relationship is very close, and greatly influences the number of insects and / or plant possible hosts. It was identified in phytoplasmas an antigenic protein (amp) that is the main component of the cell membrane protein capable of interacting with the complex of microfilaments of intestinal muscles of insect vectors and therefore is considered very important for the specific transmission of phytoplasmas and their subsequent ability to induce disease (Suzuki *et al*., 2006; Hoshi *et al*., 2007).

1.5.2. Transovarial transmission

For many years phytoplasmas were considered not transmissible vertically to the progeny of infected insects. However, until now the transovarian transmission of phytoplasmas was described for the following insect-phytoplasma combinations: *Scaphoideus titanus* / aster yellows (Danielli *et al*., 1996; Alma *et al*., 1997); *Hishimonoides sellatiformis* / mulberry dwarf (Kawakita *et al*., 2000), *Matsumuratettix hiroglyphicus* (Matsumura) / sugarcane white leaf (Hanboonsong *et al*., 2002), and *Cacopsylla melanoneura* / European stone fruit yellows (Tedeschi *et al*., 2006).

1.6. Phytoplasma detection

Before the possibility to use molecular biology to detect phytoplasmas, the diagnostic methods employed were the observation of the symptomatology (Errampalli *et al*., 1991; Lee and Davis, 1992) and the pathogens transmission by grafting or insect to healthy plants (mainly periwinkle or celery) (Bertaccini and Duduk, 2009). With the advent of the transmission electron microscopy (TEM) phytoplasma presence was described in sieve tubes (Doi *et al*., 1967). Fluorescence microscopy with DAPI staining was developed resulting sufficiently able to detect structures referable to phytoplasmas (Lederer and Seemüller, 1992). To detect phytoplasmas in vital plant tissue, Christensen and collaborators in 2004 developed a confocal laser scanning microscope technique with the use of fluorescent dyes. Serological techniques were also employed for phytoplasma detection since the early ‘80s. Monoclonal and polyclonal antisera were used for the identification of phytoplasma (Lee *et al*., 1993b; Chen *et al*., 1993; 1994), and some of them are still commercially available. By serological tools, different phytoplasmas were successfully identified in the insect vectors by immunoelectron microscopy (Lherminier *et al*., 1990), classical serology (Sinha, 1979; Sinha and Benhamou, 1983), "dot blot" or ELISA (Boudon-Padieu *et al*., 1989), tissue blotting that allows the direct or indirect detection of antigens in the infected tissue

(Lin and Chen, 1985; 1996). Antibodies binding partial sequences of immunodominant proteins of some phytoplasmas were produced (Berg *et al*., 1999; Blomquist *et al*., 2001; Hong *et al*., 2001; Mergenthaler *et al*., 2001) and in some cases, they were successfully expressed *in planta* (Kakizawa *et al*., 2009).

1.6.1. PCR and nested-PCR

Kirkpatrick and collaborators in 1987 succeeded with the first phytoplasma DNA cloning, and then probes, obtained by random cloning phytoplasma nucleic acids, were employed for the detection and differentiation of phytoplasmas in plants and insect vectors (Lee and Davis, 1988; Bertaccini *et al*., 1990a; Bonnet *et al*., 1990; Harrison *et al*., 1992). In the early 1990s, detection based on the *16SrRNA* gene allowed the identification and differentiation of a wide range of phytoplasmas (Lee and Davis, 1988; Bertaccini *et al.*, 1990b; Deng and Hiruki, 1991; Namba *et al*., 1993; Gundersen and Lee, 1996). With the use of the restriction fragment length polymorphism (RFLP) analysis on the amplicons of the 16S rDNA obtained in PCR it was then possible to classify the phytoplasmas in ribosomal groups and subgroups using universal and group-specific primers (Namba *et al*., 1993; Lee *et al*., 1993a; Schneider *et al*., 1993). Universal primers amplifying conserved region (16S rRNA and intergenic spacer region 16S-23S) allowed to identify phytoplasmas associated with plants and insects (Deng and Hiruki, 1991; Ahrens and Seemüller, 1992; Bertaccini *et al*., 1992a; Davis and Lee, 1993; Lorenz *et al*., 1995; Gundersen and Lee, 1996; Schneider *et al*., 1997; Lee et al., 1993a, 1998a; 1998b); primers for the amplification of ribosomal proteins (*rp*), *secY, secA* and *tuf* genes (Gundersen *et al.*, 1996; Schneider *et al.*, 1997; Marcone *et al*., 2000; Martini *et al*., 2002; 2007; Hodgetts *et al*., 2008), membrane protein genes (*imp*, *amp*, *stamp*, *groEL*) (Danet *et al*., 2007; Kakizawa *et al*., 2006; Fabre *et al*., 2011; Mitrović *et al*., 2011a; 2011b; 2015) allow a finer differentiation of phytoplasmas. To have good amplification results in PCR analysis, a clean DNA is required (Firrao *et al*., 2007), and several protocols of DNA extraction were developed. Another issue is represented by the low phytoplasma DNA concentration in plants that is usually less than 1% on the total amount of plant DNA (Bertaccini, 2007). To overcome this problem, the nested-PCR tool was developed: this technique provides a first amplification (direct PCR) using universal primers to amplify a region of the *16SrRNA* gene phytoplasmas in all ribosomal groups and then a second amplification using primers amplifying an inner region; in this second step, universal primers or group-specific primers can be used; using specific primers it is possible to detect also phytoplasmas present in mixed infection in hosts (Lee *et al*., 1994). This system allows increasing

both the sensitivity and the specificity of the assay; however it is also possible false positive detection.

1.6.2. LAMP

A rapid tool for phytoplasma detection is the loop-mediated isothermal amplification (LAMP). It provides phytoplasma detection in an hour and the sensitivity level is reported as comparable to that obtained by nested PCR; moreover, the assay is specific for the phytoplasmas it is designed for (Tomlinson *et al*., 2010). Several studies confirm the application of this methodology for the detection of phytoplasmas belonging to different ribosomal groups (Bekele *et al*., 2011; Hodgetts *et al*., 2011; Kogovšek *et al*., 2015; 2016).

1.6.3. DNA barcoding

DNA barcoding is an identification method based on the comparison of short DNA sequences (barcodes) of unknown species with sequences present in a database and for phytoplasmas allows assigning the sample to a '*Candidatus* Phytoplasma' species (Makarova *et al*., 2012). Two barcodes, based on *tuf* and 16S rRNA genes (Makarova *et al*., 2012) have been developed and one based on *SecA* gene has been proposed (Bertaccini *et al*., 2011; Contaldo *et al*., 2015). Thus, sequences of phytoplasmas infecting plants worldwide were collected in a Q-Bank Phytoplasma database that can be freely used for phytoplasma identification (http://www.q-bank.eu/Phytoplasmas/).

1.6.4. Quantitative PCR (qPCR)

To avoid cross-contamination and time-consuming q-PCR method can also be employed for phytoplasmas. This method allows to amplify, and also quantify the target DNA. The PCR mix contains a fluorescent dye that binding the DNA molecule, after each PCR cycle, emits florescence captured by a detection system. During the exponential phase, there is a threshold cycle (Ct) at which the emission of fluorescence is detected because of the accumulation of the PCR product. The increase in fluorescence is directly proportional to the initial amount of DNA target. The DNA concentration, in term of copy number or relative amount, is calculated building standard curves of serial dilution of the PCR target by plotting the log of the starting quantity against the Ct value obtained in the amplification of each dilution. Different types of chemistry are available: TaqMan, Molecular Beacons and Scorpion are probes labeled with a fluorescent dye and specific for a determined amplicon. The advantages of these probes are high specificity and ability to perform multiplex reactions; disadvantages are the high cost, difficulty in use and the possibility of false negatives when variable target DNAs are used (Anderson *et al*., 2003; Papin *et al*., 2004). Other

products are DNA dyes as SYBR Green I and EvaGreen: they are not sequence-specific, but bind the minor groove of the dsDNA. These probes are cheap and easy to use, but are non-specific and can overestimate the pathogen concentration. To overcome the low-specificity a melting curve analysis is needed to be built after the amplification reaction. The fluorescence decreases when the dsDNA denatures and the negative first derivative of the change in fluorescence is plotted against temperature. In fact, the melting temperature (Tm) is the peak of temperature of the amplicon that can distinguish true from false positives.

1.6.5. Droplet digital PCR

Droplet digital PCR (ddPCR) is a detection method very useful to quantify the nucleic-acid copy number without the construction of standard curves (Hindson *et al*., 2011). It was successfully applied to viruses, bacteria and genetically modified organisms quantification (Henrich *et al*., 2012; Hayden *et al*., 2013; Strain *et al*., 2013; Rothrock *et al*., 2013; Morisset *et al*., 2013; Racki *et al*., 2014). Each sample is fractionated into a large number of droplets, and PCR amplification occurs in each individual droplet containing different copies of the target. The number of copies in the original sample can be calculated from the ratio of positive to total partitions using binomial Poisson statistics (Hindson *et al*., 2011; Pinheiro *et al*., 2012). This method was firstly applied to phytoplasma associated to “flavescence dorée” by Mehle *et al*. (2014) in comparison to qPCR suggesting a good overlap of the two techniques.

1.7. Phytoplasma classification

The RFLP analysis is the main method used for the phytoplasma classification. The amplified 16S rDNA sequence is analyzed with different restriction enzymes to differentiate among phytoplasmas ribosomal groups on the restriction profile obtained (Lee *et al*., 1998a; 1998b; 2000). Nevertheless, in some cases like for ‘*Ca.* P. asteris’ that is associated with different diseases, it is needed to be more precise in the differentiation of closely related strains, thus, analysis of *rp*, *secY* and *tuf* genes have a complementary discrimination role (Lee *et al*., 2007; 2010; Martini *et al*., 2007; Makarova *et al*., 2012).

On the other hand, a new species of '*Candidatus* Phytoplasma' can be defined if the sequence of the *16S rRNA* gene is at least 1.200 nucleotides, and if its homology is less than 97.5% with the others '*Ca*. Phytoplasma' species described. Up today, 40 ‘*Ca*. Phytoplasma’ species, 33 ribosomal groups and 130 ribosomal subgroups have been described (Table 1.1). In some cases, for the high conservation of the *16S rRNA* gene, is not possible to differentiate among different phytoplasmas

and specific biologic properties as host plants, insect vectors, specific antibodies, and other criteria must be included for '*Candidtus* Phytoplasma' speciation (Seemüller and Schneider, 2004).

Table 1.1. Classification of phytoplasmas based on RFLP analyses and/or sequencing of 16S rDNA.

16Sr group/subgroup	Strain (acronym) **'*Candidatus* sp.'**	GenBank number
16SrI: aster yellows (America, Europe, Asia, Africa)		
I-A	Aster yellows witches' broom (AYWB)	NC007716
I-A	Tomato big bud (BB)	L33760
I-B	Onion yellows mild strain (OY-M)	NC005303
I-B	Aster yellows (MAY) **'*Ca.* P. asteris'**	M30790
I-C	Clover phyllody (CPh)	AF222065
I-D	Paulownia witches' broom (PaWB)	AY265206
I-E	Blueberry stunt (BBS3)	AY265213
I-F	Aster yellows apricot - Spain (A-AY)	AY265211
I-I	Strawberry witches' broom (STRAWB1)	U96614
I-K	Strawberry witches' broom (STRAWB2)	U96616
I-L	Aster yellows (AV2192)	AY180957
I-M	Aster yellows (AVUT)	AY265209
I-N	Aster yellows (IoWB)	AY265205
I-O	Soybean purple stem (SPS)	AF268405
I-P	Aster yellows from *Populus* (PopAY)	AF503568
I-Q	Cherry little leaf (ChLL)	AY034089
I-R	Strawberry phylloid fruit (StrawbPhF)	AY102275
I-S	Mexican potato purple top phytoplasma (COAH10)	FJ914654
I-U	Mexican potato purple top phytoplasma (JAL6)	FJ914650
I-V	Mexican potato purple top phytoplasma (SON18)	FJ914642
I-W	Peach rosette-like disease (PRU0382)	HQ450211
I-Y	"Brote grande" of tomato **'*Ca.* P. lycopersici'**	EF199549
16SrII: peanut witches' broom (America, Africa, Europe, Asia, Australia)		
II-A	Peanut witches' broom (PnWB)	L33765
II-B	Lime witches' broom (WBDL) **'*Ca*. P. aurantifolia'**	U15442
II-C	Faba bean phyllody (FBP)	X83432
II-D	Papaya mosaic (PpM) **'*Ca*. P. australasiae'**	Y10096
II-E	Pichris echioides phyllody (PEY)	Y16393
II-F	Cotton phyllody (CoP)	EF186827
16SrIII: X-disease (America, Europe, Asia)		
III-A	Peach X-disease (PX11CT1) **'*Ca*. P. pruni'**	JQ044392/JQ044393
III-B	Clover yellow edge (CYE)	AF173558
III-C	Pecan bunch (PBT)	GU004371
III-D	Goldenrod yellows (GR1)	GU004372
III-E	Spiraea stunt (SP1)	AF190228
III-F	Milkweed yellows (MW1)	AF510724
III-G	Walnut witches' broom (WWB)	AF190226/AF190227
III-H	Poinsettia branch-inducing (PoiBI)	AF190223
III-I	Virginia grapevine yellows (VGYIII)	AF060875
III-J	Chayote witches' broom (ChWBIII)	AF147706

III-K	Strawberry leafy fruit (SLF)	AF274876
III-L	Cassava frog skin disease (CFSD)	EU346761
III-M	Potato purple top (MT117)	FJ226074
III-N	Potato purple top (AKpot6)	GU004365
III-P	Dandelion virescence (DanV)	AF370119/AF370120
III-Q	Black raspberry witches' broom (BRWB7)	AF302841
III-T	Sweet and sour cherry (ChD)	FJ231728
III-U	Cirsium white leaf (CWL)	AF373105/AF373106
III-V	Passion fruit phytoplasma (PassWB-Br4)	GU292082
	16SrIV: coconut lethal yellows (America, Africa)	
IV-A	Coconut lethal yellowing (LYJ-C8)	AF498307
IV-B	Yucatan coconut lethal decline (LDY)	U18753
IV-C	Tanzanian coconut lethal decline (LDT)	X80117
	16SrV: elm yellows (Europe, America, Asia, Africa)	
V-A	Elm yellows (EY) **'*Ca*. P. ulmi'**	AY197655
V-B	Jujube witches' broom (JWB-G1) **'*Ca*. P. ziziphi'**	AB052876
V-C	"Flavescence dorée" (FD-C)	X76560
V-D	"Flavescence dorée" (FD-D) **'**	AJ548787
V-E	Rubus stunt (RuS) **'*Ca*. P. rubi'**	AY197648
V-F	Balanite witches' broom (BltWB) **'*Ca*. P. balanitae'**	AB689678
V-G	Korean jujube witches' broom	AB052897
V-H	Bischofia polycarpa witches' broom	KJ452547
	16SrVI: clover proliferation (Europe, America, Asia)	
VI-A	Clover proliferation (CP) **'*Ca*. P. trifolii'**	AY390261
VI-B	Strawberry multiplier disease (MC)	AF190224
VI-C	Illinois elm yellows (EY-IL1)	AF409069/AF409070
VI-D	Periwinkle little leaf (PLL-Bd)	AF228053
VI-E	*Centaurea solstitialis* virescence (CSVI)	AY270156
VI-F	Catharanthus phyllody phytoplasma (CPS)	EF186819
VI-H	Portulaca little leaf phytoplasma (PLL-Ind)	EF651786
VI-I	Passionfruit (WB-Br3) **'*Ca*. P. sudamericanum'**	GU292081
	16SrVII: ash yellows (America, Europe)	
VII-A	Ash yellows (AshY) **'*Ca*. P. fraxini'**	AF092209
VII-B	Erigeron witches' broom (ErWB)	AY034608
VII-C	Argentinian alfalfa witches' broom (ArAWB)	AY147038
VII-D	Erigeron bonariensis witches' broom (EboWB)	KJ831066
VII-E	Vernonia brasiliana shoot proliferation (VbSP)	KX342018
	16SrVIII: Loofah witches' broom (Asia)	
VIII-A	Loofah witches' broom (LufWB)	AF086621
	16SrIX: pigeon pea witches' broom (Europe, Asia, America)	
IX-A	Pigeon pea witches' broom (PPWB)	AF248957
IX-B	Almond witches' broom (AlWB) **'*Ca*. P. phoenicium'**	AF515636
IX-C	Naxos periwinkle virescence (NAXOS)	HQ589191
IX-D	Almond witches' broom (AlWB)	AF515637
IX-E	*Juniperus* witches' broom	GQ925918
IX-F	Almond and stone fruit witches' broom (N27-2)	HQ407532
IX-G	Almond and stone fruit witches' broom (A1-1)	HQ407514

IX-H	Sarson phyllody and witches' broom (SAR-2)	KU892213
	16SrX: apple proliferation (Europe, America)	
X-A	Apple proliferation (AP) **'*Ca*. P. mali'**	AJ542541
X-B	European stone fruit yellows (ESFY) **'*Ca*. P. prunorum'**	AJ542544
X-C	Pear decline (PD) **'*Ca*. P. pyri'**	AJ542543
X-D	Spartium witches' broom (SpaWB) **'*Ca*. P. spartii'**	X92869
X-E	Black alder witches' broom (BAWB[BWB])	X76431
	16SrXI: rice yellow dwarf (Europe, Asia, Africa)	
XI-A	Rice yellow dwarf (RYD) **'*Ca*. P. oryzae'**	AB052873
XI-B	Sugarcane white leaf (SCWL)	X76432
XI-C	Leafhopper-borne (BVK)	X76429
XI-D	Sugar cane white leaf (SCWL) China	KR020685
XI-E	*Cirsium* yellows and stunting (CirYS) **'*Ca*. P. cirsii'**	KR869146
	16SrXII: "stolbur" (Europe, Asia, America, Africa, Australia)	
XII-A	Stolbur (STOL11) **'*Ca*. P. solani'**	AF248959
XII-B	Australian grapevine yellows **'*Ca*. P. australiense'**	L76865
XII-C	Strawberry lethal yellows (StrawLY)	AJ243045
XII-D	Japanese hydrangea phyllody **'*Ca*. P. japonicum'** (JHp)	AB010425
XII-E	Yellows diseased strawberry (StrawY) **'*Ca*. P. fragariae'**	DQ086423
XII-F	"Bois noir" (BN-Op121)	EU836651
XII-G	"Bois noir" (BN-Fc3)	EU836647
XII-H	Bindweed yellows (BY-S57/11) **'*Ca*. P. convolvuli'**	JN833705
	16SrXIII: Mexican periwinkle virescence (America)	
XIII-A	Mexican periwinkle virescence **'*Ca*. P. hispanicum'**	AF248960
XIII-B	Strawberry green petal (SGP)	U96616
XIII-C	China-tree yellows (CbY1)	AF495882
XIII-D	Potato purple top-PPT/SINPV	FJ914647
XIII-E	Papaya apical curl necrosis (PACN)	JQ792171
XIII-F	Strawberry red leaf (Argentina)	KJ921641
XIII-G	China tree yellows (ChTY) **'*Ca*. P. meliae'**	KU850940
	16SrXIV: Bermudagrass white leaf (Europe)	
XIV-A	Bermudagrass white leaf (BGWL) '***Ca*. P. cynodontis'**	AJ550984
XIV-B	Bermudagrass white leaf Iran	EF444485
	16SrXV: hibiscus witches' broom (America)	
XV-A	Hibiscus witches' broom (HibWB) **'*Ca*. P. brasiliense'**	AF147708
XV-B	Guazuma witches' broom (GWB)	HQ258882
	16SrXVI: sugarcane yellow leaf syndrome (America)	
XVI-A	Sugarcane yellow leaf syndrome **'*Ca*. P. graminis'**	AY725228
	16SrXVII: papaya bunchy top (America)	
XVII-A	Papaya bunchy top **'*Ca*. P. caricae'**	AY725234
	16SrXVIII: American potato purple top wilt (America)	
XVIII-A	American potato purple top wilt **'*Ca*. P. americanum'**	DQ174122
	16SrXIX: Chestnut witches' broom (Asia)	
XIX-A	Chestnut witches' broom **'*Ca*. P. castaneae'**	AB054986
	16SrXX: Rhamnus witches' broom (Europe)	
XX-A	Rhamnus witches' broom **'*Ca*. P. rhamni'**	AJ583009
	16SrXXI: Pinus phytoplasmas (Europe)	

XXI-A	Pinus phytoplasma (PinP) **'*Ca.* P. pini'**	AJ310849
	16SrXXII: Lethal yellowing-type (Africa)	
XXII-A	Coconut lethal yellowing-Nigerian Awka disease (LDN)	Y14175
XXII-A	Mozambique coconut (LYDM-185) **'*Ca.* P. palmicola'**	KF751388
XXII-B	Cape St. Paul wilt (CSPW)	KF419286
	16SrXXIII: -	
XXIII-A	Buckland valley grapevine yellows	AY083605
	16SrXXIV: -	
XXIV-A	Sorghum bunchy shoot	AF509322
	16SrXXV: -	
XXV-A	Weeping tea witches' broom	AF521672
	16SrXXVI: -	
XXVI-A	Sugarcane phytoplasma D3T1	AJ539179
	16SrXXVII: -	
XXVII-A	Sugarcane phytoplasma D3T2	AY539180
	16SrXXVIII: -	
XXVIII-A	Derbid phytoplasma	AY744945
	16SXXIX: Cassia witches' broom (Asia)	
XXIX-A	Cassia witches' broom (CaWB) **'*Ca*. P. omanense'**	EF666051
XXIX-B	Bindweed witches' broom (RBiWB)	KY047493
	16SXXX: Salt cedar witches' broom (Asia)	
XXX-A	Salt cedar witches' broom **'*Ca*. P. tamaricis'**	FJ432664
	16SXXXI: Soybean stunt (America)	
XXXI-A	Soybean stunt (SoyST1c1) **'*Ca*. P. costaricanum'**	HQ225630
	16SXXXII: Malaysian periwinkle virescence and phyllody (Asia)	
XXXII-A	Malaysian p. virescence (MaPV) **'*Ca*. P. malaysianum'**	EU371934
XXXII-B	Malayan yellow dwarf phytoplasma (MYD)	EU498727
XXXII-C	Malayan oil palm phytoplasma (MOP)	EU498728
	16SXXXIII: *Allocasuarina muelleriana* phytoplasma (Australia)	
XXXIII-A	Allocasuarina phytoplasma **'*Ca.* P. allocasuarinae'**	AY135523

- the ribosomal group has no designation since only Genbank sequences were employed for its determination

1.8. Genomics

As members of the *Mollicutes* group, phytoplasmas have a circular double-stranded DNA molecule (Lim and Sears, 1989; 1991a; 1991b; Neimark and Kirkpatrick, 1993). Sequence analysis of 16S rDNA and other housekeeping genes indicate that are closely related to the *Acholeplasma* spp. or to some of the mycoplasmas rather than to the *Spiroplasma* spp., other plant pathogenic Mollicutes (Lim and Sears, 1992). Ancestor of phytoplasmas is considered *Acholeplasma laidlawii*: in this specie the tryptophan is encoded by the triplet UGG, while in the other prokaryotes as mycoplasmas and spiroplasmas it is encoded by UGA. In phytoplasma UGA is a stop-codon. Between the *16Sr* and *23Sr* genes there is a characteristics intergenic spacer region (about 300

bp), encoding for the isoleucine tRNA and part of the sequence for the alanine tRNA, this is one of the regions that allow genetic differentiation between phytoplasmas and mycoplasmas. Phytoplasma genome has a low content in G+C, but several genes are present in two or more copies; they contain two RNA operons. Moreover, there is a huge number of transposons and insertion sequences unique of these microorganims and named variable mosaic (SVM) (Jomantiene and Davis, 2006; Jomantiene *et al*., 2007; Wei *et al*., 2008) or potential mobile units (PMUs) (Bai *et al*., 2006; Toruno *et al*., 2010; Ku *et al*., 2013), all these elements are important for the genetic variability and the phytoplasma adaptation to different environments.

Differences in the chromosomes size among phytoplasmas have also been described; for example, '*Ca*. P. cynodontis' and a strain of "stolbur" have the chromosomal size of respectively 530 and 1,350 kb; phytoplasmas of rape virescence and hydrangea phyllody, both belonging to ribosomal subgroup 16SrI-B, have a chromosome respectively of 1,130 kb and 660 kb (Marcone *et al*., 1999). This variability is usually due to gene duplication and redundancy. In the genome of OY, it has been estimated that the 18% of the genes is the result of repeated copies of only five genes: *uvrD* (ATP-dependent DNA helicase, 3,117 nucleotides, 7 copies), *hflB* (ATP-dependent Zn protease, 1,551 nucleotides, 17 copies), *tmk* (thymidylate kinase, 624 nucleotides, 6 copies), *dam* (DNA methylase, 660 nucleotides, 4 copies) and *ssb* (single-stranded DNA-binding protein, 345 nucleotides, 15 copies). These genes are not always present or they are present usually in a single copy in the other *Mollicutes*. Moreover, 5 genes encoding components of the transport system have multiple copies, probably not all functional (Oshima *et al*., 2004). Multiple copies of sequences similar to insertion elements and functional genes for thymidine kinase are present in the genome of OY and other phytoplasmas (Lee *et al*., 2005; Miyata *et al*., 2003). Phytoplasmas lack several genes for the classic metabolic function, while they have a sec transport pathway (Bai *et al*., 2006) and other genes encoding transporter systems (Oshima *et al*., 2001). Their genome encodes for even less metabolic functions than that of mycoplasmas (Razin, 2007).

The OY genomic size is approximately 861 kb and contains 754 ORF, corresponding to 73% of the coding capacity. As other *Mollicutes*, this phytoplasma lack genes for the biosynthesis of amino acids and fatty acids, the tricarboxylic acid cycle (TCA) and the oxidative phosphorylation (ATP production), while, on the contrary of what occurs in mycoplasmas, OY lacks genes for phosphotransferase system and for the metabolism of UDP-galactose to glucose-1-phosphate, suggesting that this phytoplasma has a unique source of sugars and a specific metabolic system (Oshima *et al*., 2004). In addition, phytoplasmas have not the pentose phosphate pathway and the

subunits for the ATP synthesis, which are believed to be essential for life, suggesting that phytoplasmas imports ATP from the host through a yet unknown mechanism, or that the synthesis of ATP is highly dependent on glycolysis. The absence of these important functions can explain their parasitic life (Oshima *et al*., 2004). Two sets of 5 glycolytic enzymes are present in a tandem duplicated region of '*Ca*. P. asteris' OY-W (Oshima *et al*., 2007) but not in AY-WB (Bai *et al*., 2006) and OY-M that have a glycolytic gene organization more similar. Phytoplasmas also have maltose ABC transporter, but not the enzymes to converts sugars to glucose-6-phosphate (Christensen *et al*., 2005). The '*Ca.* P. asteris' OY-M genome encodes for a number of genes for folate biosynthesis as *folk*, *folP*, *folC*, and *folA*. '*Ca.* P. mali' and '*Ca.* P. australiense' genomes encode only *folA*, while in the '*Ca.* P. asteris' AY-WB strain genome the *folA* gene is complete, but *folk* and *folP* are pseudogenes (Oshima *et al*., 2013). Even if there are only a few metabolic genes in phytoplasma genome, many genes encoding malate, aminoacids and metal-ions transport systems are present; some of these genes are present in multiple copies, suggesting an aggressive import of many metabolites from host cells, which may explain also the expression of disease symptoms. Their genome is also capable of encoding the synthesis of folate, which could allow the phytoplasma to adapt to different environments in plants and insects (Oshima *et al*., 2004). Recently, in phytoplasma genomes six types of ATP-binding cassette (ABC) transporters are observed. Through these ABC transporters, metabolites can cross bacterial membranes, and nutrient and metabolites can be uptaken from the host. A superoxide dismutase enzyme (SOD), was also observed involved in counteracting reactive oxygen species produced by hosts (Miura *et al*., 2012).

1.9. Interaction with hosts

Studies on the translocation of phytoplasmas (Garcia-Chapa *et al*., 2003; Wei *et al*., 2004b) provide evidence that it cannot be explained only by assimilate flow, however active movement seems unlikely, considering the lack of genes coding for cytoskeleton elements or flagella (Christensen *et al*., 2005). Phytoplasmas may overwinter in insect vectors or in perennial plants; their concentration varies greatly from plant to plant, and periwinkle is the species able to accumulate them at the highest concentration (Berges *et al*., 2000). Different strains of the same phytoplasma can reach different concentrations in the same host (Sinclair and Griffiths, 2000). Furthermore, the concentration of phytoplasmas in the tree species can largely differ depending on the season, as in the case of pear decline (Errea *et al*., 2002; Garcia-Chapa *et al*., 2003), European stone fruit yellows (Jarausch *et al*., 1999) and "bois noir" (Škorić *et al*., 1998).

Immunodominant membrane proteins were described for the first time in mycoplasmalike organisms (MLO) by Shen and Lin in 1993. Serological studies demonstrated the localization of these proteins with transmembrane domains. Most of the surface proteins of *Mollicutes* have an important role in host-pathogen recognition and subsequent adherence to the host cells. They are also involved in the expression of pathogenicity and in triggering of host resistance responses (Christensen *et al*., 2005). The presence on the surface of epitopes characteristic for each phytoplasmas group suggests that these proteins may be involved in specific interactions with host cells.

Genes encoding immunodominant membrane proteins have been identified in several phytoplasmas and the corresponding proteins have been classified into four types: (1) immune-dominant membrane protein (Imp) identified in sweet potato witches' broom (SPWB) (Yu *et al*., 1998), apple proliferation (AP) (Berg *et al*., 1999), European stone fruit yellows (ESFY) (Morton *et al*., 2003), pear decline (PD) (Morton *et al*., 2003), and peach yellow leaf roll (PYLR) (Morton *et al*., 2003); (2) immunodominant membrane protein A (IdpA) identified in western X-disease (WX) (Blomquist *et al*., 2001); (3) membrane protein antigen (Amp) detected in aster yellows (AY) (Barbara *et al*., 2001; 2002), clover phyllody (CPh) (Barbara *et al*., 2002), and onion yellows (OY) (Kakizawa *et al*., 2004; and (4) putative membrane protein (vmp1) studied in the "stolbur" phytoplasmas (STOL) (Cimerman *et al.,* 2009). A high level of Amp protein expression was confirmed in phytoplasmas AY, CPh, and OY (Barbara *et al*., 2002; Kakizawa *et al*., 2004) where the protein is transported by the sec system, after the cleavage of the N-terminal signal (Kakizawa *et al*., 2004). Suzuki *et al*., in 2006 showed that Amp in OY phytoplasma form complexes with microfilaments of actin-myosin of insects and this is related to the transmissibility of the phytoplasma by leafhoppers.

The isolation of the genes encoding Imp having identical sequence in different phytoplasmas (Morton *et al*., 2003) has shown that there is no correlation between membrane proteins and the *16S rRNA* gene, thus the variability of these proteins is not linked to evolutionary factors.

Recently, experiments with leafhopper vector fed with monoclonal anti-AMP from '*Candidatus* Phytoplasma asteris' chrysanthemums yellows strain (CY), demonstrated a reduction in the phytoplasma internalization and transmission, implying that anti-Amp reduces attachment of the bacteria in the vector gut (Rashidi *et al*., 2015). Studies regarding other types of immunodominant membrane proteins is still in progress (Kakizawa *et al*., 2009).

1.10. Virulence and pathogenicity

Up today, phytoplasmas remain a poorly characterized group of plant pathogens and understand the energetic metabolism could be important to understand phytoplasmas' biology. Some evidences of disorders in different plant species were observed: Sears *et al*. in 1997 described the alteration of oxygen and carbon dioxide levels in *Oenothera* crops; Lepka *et al*. in 1999 and Maust *et al*. in 2003 observed changes in carbohydrates concentration in phloem, roots and leaves of plants infected by phytoplasmas: this is due to the inhibition of the phloem transport in phytoplasma-infected plants, that leads to accumulation of carbohydrates in source leaves, and reduction of these sugars in sink organs as roots. The accumulation of sugar in source leaves is generally believed to be the result of photosynthesis inhibition and the cause of typical chlorosis (Lepka *et al*., 1999; Bertamini *et al*., 2002; Maust *et al*., 2003). Infected plants present changes in photosynthate translocation, reduced photosynthesis, alteration in stomata conductance and root respiration, altered secondary metabolism and disturbed plant hormone balance (Marcone, 2010). However, due to the wide range of symptoms exhibited by infected plants it seems that also other nutrients are subjected to changes, and this was confirmed by the discovered reduction in the concentration of photosynthetic pigments and of total soluble proteins (Bertamini and Nedunchezhian, 2001; Bertamini *et al*., 2002; Musetti *et al*., 2005), as well as alterations in the hormone balance (Jagoueix-Eveillard *et al*., 2001; Maust *et al*., 2003), amino-acid transport (Lepka *et al*., 1999) and the occurrence of folate and endopetidase gene homologues in phytoplasma genomes (Davis *et al*., 2003). A marked reduction in the phloem translocation, until obstructing the lumen of sieve tubes, can also be assumed in cases of high multiplication rate, although this phenomenon has been observed in plants with a very low level of phytoplasma titer, suggesting an indirect influence on phloematic functions and host metabolism (Siddique *et al*., 1998; Guthrie *et al*., 2001). Recently, in infected *A. thaliana* plants, phytoplasmas were reported as inducing phloem disorganization and impairment (Musetti *et al*., 2013). In fact, the plant responds to phytoplasma infection by the agglutination of phloem protein that change their configuration, and by callose deposition, responses calcium-mediated, in order to occlude the sieve elements (Marco *et al*., 2016).

Phytoplasmas do not have virulence genes, as the *hrp*, present in other phytopathogenic bacteria (Oshima *et al*., 2004) and, the comparative study of the two strains OY-M, causing mild proliferation and yellowing, and OY-W causing severe yellowing, stunting, proliferation and witches' broom, showed that five glycolytic genes were duplicated in the severe strain OY-W.

Oshima *et al*. in 2011 reported that OY-W phytoplasma titter is higher than OY-M. So, the higher consumption of carbon source (due to the duplication of these genes) may affect the growth rate of this phytoplasma and may also directly or indirectly produce more severe symptoms (Oshima *et al*., 2007).

Recently, a single virulence factor, "tengu-su" (TENGU), was discovered inducing witches' broom and dwarfism after cloning and expression in transgenic plants of *Nicotiana benthamiana* and *Arabidopsis thaliana* (Hoshi *et al*., 2009; Sugawara *et al*., 2013). Although the location of phytoplasmas is restricted to the phloem, the TENGU protein has been identified in apical buds suggesting that it was transported from the phloem to other cells. Moreover, the genes responsible for the metabolism of auxin were significantly down-regulated in TENGU-transgenic plants suggesting that it inhibits the pathway of the auxin metabolism (Hoshi *et al*., 2009). Moreover, Minato et al., 2014 demonstrated that TENGU induces both male and female sterility without floral malformations in transgenic *A. thaliana* affecting the expression of flower maturation genes and altering jasmonic acid and auxin syntheses also in flowers.

Phytoplasmas have two secretion systems: the *YidC* system for the integration of membrane proteins and the Sec system for the integration and secretion of proteins into the host cell cytoplasm (Kakizawa *et al*., 2001; Wei *et al*., 2004a; 2004b; Lee *et al*., 2012). In the '*Ca.* P. asteris' AY-WB strain genome more than 56 genes encoding secreted proteins were identified, but the two more studied are SAP11 and SAP54. SAP11 contains a signal required for nuclear targeting in plant cells (Bai *et al*., 2008; Sugio *et al*., 2014). The SAP11-expressing plants exhibit crinkled leaves and produce many stems morphologic changes in transgenic *A. thaliana* flower development and also induce altered root architecture. Recently it was found that transgenic *N. benthamiana* plants expressing SAP11 from '*Ca.* P. mali' exhibit an altered aroma phenotype leading to understand that this phytoplasma effector has the ability to modulate host volatile organic compound emissions (Tan *et al*., 2016). Moreover, SAP11 destabilizes the proliferation cell transcription factors suppressing the jasmonic acid responses in *N. benthamiana* (Sugio *et al*., 2011). SAP54 is an effector protein that alters floral development in *A. thaliana* resulting in the production of leaf-like flowers (Bai *et al*., 2006; MacLean *et al*., 2011; Lu *et al*., 2014). Another effector recently discovered is PHYL1, homologue of SAP54 that was identified in OY–W phytoplasma induces phyllody-like floral abnormalities (Maejima *et al*., 2014). SAP54/PHYL1 interacts and promotes the degradation of floral transcription factors MAD-box proteins, critical for floral development (McLean *et al*., 2014; Maejima *et al*., 2014). Another recently discovered protein is P38, a

conserved *Mollicutes* adhesion motif (MAM) identified in the onion yellows phytoplasma genome that interacts with crude insect extracts and plants extracts but its specific hosts are still unknown (Neriya *et al*., 2014). Moreover, HflB, an ATP- and Zn^{2+}-dependent proteases, is a virulence factor for '*Ca*. P. mali' (Seemüller *et al*., 2013; Wang *et al*., 2014): *hflB* gene is present in phytoplasmas genomes in high copy number that is unusual in other prokaryotes but it is not clear how these proteins are involved in parasitism. The study of variable membrane protein A (VmpA) expressing the protein in *S. citri* showed that VmpA is involves in interaction with insects and provides a new method for studying interactions of phytoplasma surface proteins with host cells (Renaudin *et al*., 2015).

1.11. Control and prevention strategies

The principal management strategy to reduce the phytoplasma spread is acting on the insect vectors, however despite the large use of insecticides, phytoplasma diseases continue to be severe in several areas of the world (Firrao *et al*., 2007) Others methods are thermotherapy and chemotherapy (Bertaccini, 2007). However, these methods are not sufficient and in some cases, they could also be environmentally unsafe. The use of healthy plant material and the reduction of the sources of inoculum are very important prevention tools when the vectors are polyphagous and can feed wild plants as the case of *Hyalesthes obsoletus* that transmit "stolbur" phytoplasma to grapevine from bindweed and nettle (Bertaccini and Duduk, 2009). The use of antibiotics is expensive and forbidden in many countries. The use of kaolin power, reducing the insect mobility and eggs anchorage on the leaf gave good results also at the environmental level (Tedeschi and Alma, 2007). Alternative strategies are based on biocontrol agents (Bextine *et al*., 2004) and the use of RNA interference (RNAi), a regulatory mechanism that causes specific gene silencing (Tedeschi, 2012): the host organism recognizes as foreign a double-stranded RNA (dsRNA) and hydrolyzes it with a ribonuclease. This hydrolysis produces small and specific RNA fragments (21–28 nucleotides) called small interfering RNAs (siRNAs) that combine with constitutive proteins to form the RNA-induced silencing complex (RISC). The RISC diffuses in the cell, and its siRNA hybridizes to the specific messenger RNAs (mRNAs) with sequences complementary to that of the siRNA. The new double-stranded region stimulates the hydrolysis of that mRNA to produce more siRNAs. This process is repeated each time the siRNA hybridizes to its complementary mRNA, effectively destroying and preventing that mRNA from being translated, thus "silencing" the expression of that specific gene (Eamens *et al*., 2008). The RNAi is widely applied in entomologic

field, but there are some problems to overcome as selecting the target sequence (Tedeschi, 2012), the massive production of dsRNA (Tenllado *et al*., 2004) and the choice of a dsRNA delivery system (Turner *et al*., 2006; Whyard *et al*., 2009; Huvenne and Smagghe, 2010).

2. Phytoplasma seed transmission and cultivation

2.1. Introduction

2.1.1. Phytoplasma diseases in tomato and corn

The symptoms of phytoplasma presence in tomato are yellowing and reduction of leaves, sterility or fruit alterations, stunting of the plants (Figure 2.1a and b). Different groups of phytoplasmas were identified in symptomatic tomatoes including aster yellows (16SrI) subgroups -A (Lee *et al*., 1998a) and -B (Marcone and Ragozzino, 1995; Marcone *et al*., 1997; Okuda *et al*., 1997; Arocha *et al*., 2007), peanut witches' broom (16srII) subgroups -A (Xu *et al*., 2013) and -D (Omar and Foissac, 2012; Singh *et al*., 2012), Western-X (16SrIII) (Del Serrone *et al*., 2001; Tapia-Tussell *et al*., 2012; Amaral Mello *et al*., 2006), elm yellows (16SrV) (Del Serrone *et al*., 2001; Gungoosingh-Bunwaree *et al*., 2013), clover proliferation (16SrVI) (Boudon-Padieu *et al*., 1996; Lee *et al*., 1998a; Anfoka *et al*., 2003; Du *et al*., 2013) and "stolbur" (16SrXII-A) in several countries as Italy (Del Serrone *et al*., 2001), Bulgaria (Vibio *et al*., 1996), Turkey (Sertkaya *et al*., 2007), Greece (Alivizatos, 1989; Vellios and Lioliopoulou, 2007), Israel (Zimmermann-Gries and Klein, 1978), Iran (Salehi *et al*., 2014), in USA (Dale and Smith, 1975) and Australia (Samuel *et al*., 1933; Gibb *et al*., 1996).

Diseases associated with Mollicutes in corn are reported worldwide and are maize bushy stunt (MBS) and corn stunt (CS), both present in North, Central, and South America (Ebbert *et al*., 2001; Harrison *et al*., 1996; Lee *et al*., 2004). MBS is associated with a phytoplasma that is a member of the aster yellows 16SrI-B subgroup (Lee *et al*., 2004). CS is a disease caused by *Spiroplasma kunkelii* (Chen and Liao 1975; Williamson and Whitcomb, 1975). These pathogens are both transmitted by the leafhopper *Dalbulus maidis* and by other *Dalbulus* species (Ebbert *et al*., 2001). Corn reddening is another phytoplasma-associated disease of corn, described for the first time in Serbia in 1957 (Marić and Savić, 1965) with reddening symptoms that involve the main leaf midrib, then they spread to the stalk and eventually to the whole plant. Typical symptoms in cobs are: reduction in grain filling and weight, drying of the poor and shrivelled grains. The disease was recently associated to "stolbur" phytoplasma (Duduk and Bertaccini, 2006) reporting, for the first time, "stolbur" phytoplasma in corn (Figures 2.2a and b).

Figure 2.1. a) Symptoms referable to phytoplasma presence in a tomato field; **b)** reduction in seeds production in infected tomato.

Figure 2.2. a) Symptoms of corn reddening in field in Serbia; **b)** cobs with reduction in grain filling and weight.

2.1.2. Phytoplasma diseases in *Sesamum indicum*

Sesamum indicum L. is an economically important species because of its high oil content (50–60%) with a high ratio of unsaturated fatty acids (Uzun *et al*., 2008). The most common phytoplasmas' symptom in sesame is phyllody that appears as stunting of the plants and transformation of the floral parts into leafy structures bearing no capsule nor seeds (Figure 2.3). This disease was described in Turkey, India, Iran, Iraq, Israel, Burma, Sudan, Nigeria, Tanzania, Pakistan, Ethiopia, Thailand, Uganda, Upper Volta, and Mexico (Akhtar *et al*., 2009a; 2009b; Ikten *et al*., 2014; Tseng *et al*., 2014; Pamei and Makandar, 2016).

Figure 2.3. Symptoms referable to phytoplasmas in *S. indicum*.
https://www.apsnet.org/publications/imageresources/Pages/FI00148.aspx

2.1.3. Phytoplasma diseases in *Brassica napus*

Winter oilseed rape (*Brassica napus* L.) is an important oil-yielding crop for industrial processing and human and animal feed, and also for biodiesel production (Maliogka *et al*., 2009). The phytoplasma infected plants show stunted growth, leaf reddening, virescence and flower malformation (Figure 2.4). A few flowers are able to complete the maturation, carrying an extremely limited production and small, shriveled and malformed seeds. In different parts of the world oilseed has proved susceptible to phytoplasma diseases, and phytoplasmas belonging to subgroup 16SrI-B are the main detected ('*Ca.* P. asteris') (Bertaccini *et al*., 1998; Olivier *et al*., 2008; Maliogka *et al*., 2009).

Figure 2.4. Symptoms referable to phytoplasma presence in *B. napus.*

2.1.4. Transmission by seeds

Since the lack of a direct connection between the phloem system and the embryos, the phytoplasma transmission by seed was considered not possible, (Menon and Pandalai, 1960). Moreover, floral abnormalities and fruit malformations led to believe that the seeds originated from infected plants were not viable and germinating (McCoy *et al*., 1989).

However, phytoplasmas are pleomorphic and have a small size that allow them to pass through the pores of the phloem, and be transported by the flow of the assimilates; therefore, they are potentially able to reach organs connected to the phloem. Thus, they were found in different organs of plants as in floral structures (Bertaccini and Marani, 1982; Clark *et al*., 1989), in inflorescences and also in stems, racemes, male and female flowers in palms infected by the "Cape St. Paul wilt disease" (CSPWD) (Nipah *et al*., 2007). European stone fruit yellows (ESFY) phytoplasmas were tedected in apricot in flowers, fruits, but not in pollen samples (Nečas *et al*., 2008). In mulberry trees (*Morus* spp.) infected with mulberry dwarf (MD), phytoplasmas were found in the ovary, filaments, stigmas, sepals and anthers (Jiang *et al*., 2004). The demonstration of LY phytoplasma presence in coconut palm fruits embryos (*Cocos nucifera* L.) using nested PCR (Harrison *et al*., 1994; Harrison and Oropeza, 1997; Nipah *et al*., 2007) and *in situ* PCR (Cordova *et al*., 2003), led to the national and international legislation to prohibit commercial movement of coconuts from areas where lethal yellowing (LY) is epidemic ("Centre for Information on Coconut Lethal Yellowing" http://www.avxl82.dsl.pipex.com/ CICLY/main.html). Phytoplasmas have been identified in seed tegument and kernels of apricot and mulberry infected respectively by ESFY (Nečas *et al.,* 2008) and MD (Jiang *et al*., 2004) and in corn kernels infected by '*Ca*. P. asteris' (aster yellows: AY) (Nipah *et al*., 2007). AY has been detected also in *Brassica rapa* seeds in Canada (Olivier *et al*., 2006).

The phloem is the ideal tissues for phytoplasma propagation and plant colonization. The sieve tubes consist of cells without nuclei with a reduced cytoplasm which offer low resistance to flow of assimilates (van Bel *et al*., 2002); oligopeptides, lipids, proteins and phytoplasmas migrate through the pores of sieve plates (Sugio and Hogenhout, 2012). Phytoplasmas were also found in the companion (Sears and Klomparens, 1989) and parenchyma (Siller *et al*., 1987) cells even if their dimensions are not suitable to pass through the plasmodesmata (Stadler *et al*., 2005). Ultrastructural changes in cytoskeleton were never observed in this type of cells in infected plants (Siller *et al*., 1987; Rudzinska-Langwald *et al*., 1999): viruses, for example, can open passages, dilating plasmodesmata pores with the help of movement proteins (Oparka, 2004), while in the

genomes of phytoplasmas was not identified any gene encoding comparable proteins (Zambryski, 2004). In general, many plant bacteria have the type-III secretion system that injects bacterial proteins (effectors) into the host cytoplasm (Cornelis and van Gijsegem, 2000; Büttner and Bonas, 2003); and some type-III effectors are virulence factors that suppress plant defense responses (Jackson *et al*., 1999; Abramovitch *et al*., 2003; Hauck *et al*., 2003), but up today there are no descriptions of effectors able to modify the cytoskeleton, except for *Xanthomonas campestris*, that produces a protein inducing mesophyll cell swelling (Marois *et al*., 2002), indicating a possible disruption to the plant microtubule cytoskeleton. *Pseudomonas syringae* pv. *tomato* DC3000 contains more than 36 type-III effectors whose function was not defined (Collmer *et al*., 2002), but the plant cytoskeleton could be a target for some of these proteins.

The production of seeds from infected plants is severely compromised by phytoplasma presence in mother plants both in quantity and quality, due to malformations, withering, small size and low weight. A frequent symptom is early germination of the seeds. De La Rue *et al*., in 2002 conducted a two years' study on the effect of stylosanthes little leaf disease on production of *Stylosanthes scabra* seeds and no significant reductions of seed were observed in plants in case of late infections, while a decrease of 98.8% and 56.5% has been observed in plants showing symptoms respectively at 79 and 110 days after planting, indicating that the seed production was closely linked to the precocity of the infection. Other observations in coconut palm suggest that, if the infection occurs in the early stages of development, the flowers became necrotic and unable to ripe the fruits. The time between pollination and maturity of the fruit is around 12 months that is also the time of incubation of LY, thus only the fruits under development before the infection are able to complete the maturation (Cordova *et al*., 2003). Data on the germination percentage are variable and depending on the species and on the precocity of the infection. For instance, apricot seeds infected by ESFY showed a vitality 4.5 times lower (21.6%) and a germination 7 times less (9.4%) of the healthy control (Nečas *et al*., 2008); on the contrary, in coconuts, higher germination values (72.1%) were reported for diseased plants compared to the healthy ones (57.6%) (Nipah *et al*., 2007). Olivier and Galka in 2008 demonstrated the presence of two different types of seeds from plants of *Brassica napus* infected by AY. Malformed seeds, from symptomatic and asymptomatic plants were positive to phytoplasma presence respectively in the 25-80% and in 9-20% of the cases, while normal seeds were positive in the 20-60% and 2-10% of the cases. Malformed seeds, both from symptomatic and asymptomatic plants, had no germination, while normal seeds reached values of 50-90%.

In all these studies phytoplasma DNAs were detected in plants by PCR methodologies, but it was not possible confirm their presence by microscopy, thus the criticism is that only phytoplasma DNA is present in these tissues, and that it cannot be associated with a living organism able to survive the during the plant growth stages. In 2002 Khan and co-author presented the first evidence of symptomatic and phytoplasma positive plants sowing *in vitro* seeds of alfalfa from phytoplasma infected plants. A similar research was shown in 2004 regarding carnation (*Dianthus* L.) (Šeruga Music *et al*., 2004) and in 2006 regarding tomato (*Solanum lycopersicum*) and *Citrus aurantifolia* (Botti and Bertaccini, 2006). The identification of phytoplasmas in pea plants (*Pisum sativum*) germinated and grown in a protected environment and produced from seed derived from "stolbur" infected plants was also reported (Zwolińska *et al*., 2010). One hundred plants of *S. indicum* and the same number of *Cicer aretinum* (chickpea) plants obtained from plants infected by phytoplasmas belonging to the ribosomal group 16SrII-D were kept under observation in an insect-proof greenhouse until maturity without detecting any symptom referable to the presence of phytoplasmas (Akhtar *et al*., 2009a; 2009b). It was also not possible to identify phytoplasmas by nested PCR techniques in plants of *C. aurantifolia* originated from seeds of plants affected by WBDL (witches' broom disease of lime) cultivated for two years in an insect-proof greenhouse. The DNA of phytoplasmas was however detected in seed tegument, but not in embryos, while the analysis carried out every three months, was negative for leaves, stems and roots of these seedlings. No symptoms referable to WBDL have been observed in individuals generated from symptomatic plants. However, the germination percentage of the seeds derived from symptomatic plants was higher when compared with the percentages of seeds resulting from healthy plants (Faghihi *et al*., 2011).

Seeds from plants of *Brassica napus* infected by AY show malformations such as an increase in the number of trichomes, a reduced growth, enlargement of the stem, leaf wilting and a generalized developmental delay. In addition, it was possible to identify DNA of phytoplasmas belonging to the 16SrI-B group in the stem of these young plants, but it was very surprising that, after the fourth leaf stage of these individuals, PCR results previously positive for the presence of phytoplasmas, become negative (Olivier and Galka, 2008). Lebsky *et al*. (2010) observed by scanning electron microscopy (SEM), but not supported by molecular analysis, the presence of structures related to phytoplasmas in different phloem tissues and organs, including flowers, mature seeds and germinated seeds within the fruit in papaya plants (*Carica papaya*) showing symptoms referable to

phytoplasmas. Phytoplasma presence was also identified in seedlings of tomato, corn and oilseed rape derived from infected plants (Calari *et al*., 2011).

In conclusion, phytoplasmas appear able to infect the floral structures, the fruits, the seeds and even the embryos. The seeds produced by infected plants are generally viable and capable of germinating, although the production would result qualitatively and quantitatively affected according with the relationship flowering stage / time of infection.

For a confirmation of phytoplasma transmission by seeds, obtaining progeny in which the bacterium is maintained over the time and induce symptoms, is therefore to be demonstrated together with the ability of seed phytoplasmas to transmit the disease to healthy plants by grafting or insect vectors or their isolation in artificial media.

Furthermore, since phytoplasmas are not yet contemplate in propagation material by the plant protection quarantine protocols, the movement of seeds from infected plants imply the geographic dissemination of the pathogen, and therefore of the associated diseases in still uncontaminated areas.

2.1.5. Phytoplasma cultivation

The term mycoplasma was first used by A.B. Frank in 1889 to define some filamentous microorganisms similar to fungi; then Julien Nowak, in 1929 used the same term to define the contagious bovine pleuropneumonia. In 1955 E. A. Freundt and D. G. Edward used the term mycoplasma to describe organisms similar in colonial morphology and filterability to the agent of the contagious bovine pleuropneumonia (PPLO, pleuropneumonia-like" organisms). The name mycoplasma, because of the similarity in the morphology of this organism with other organisms (MLOs, Mycoplasma-like organisms) became the name including also L-form bacteria (Sabin, 1941; Edward, 1967). These latter were first isolated by Klieneberger in 1935 and are bacteria that may exist in two status: (1) unstable and revert to the original bacterial form or (2) stable which cannot revert. They can be produced in laboratory by inhibiting the wall formation and are not pathogenic. L-form bacteria are morphologically identical to mycoplasma (Dienes and Bullivant, 1968), but they are genetically differentiable (Somerson *et al*., 1967; McGee *et al*., 1967). Originally mycoplasmas were characterized by Sabin in 1941 studying their behavior in culture, but only between 1967 and 1969 they were better morphologically described thanks to the electron microscopy (Edward, 1967; Hayflick, 1969; Razin 1969).

Phytoplasmas were discovered in these same years, but were considered for long time impossible to cultivate in artificial media and this is till today a controversial issue even if same elucidating contributions were provided (Contaldo *et al*., 2012; 2016).

For many decades, the agent causing yellows-type diseases (Kunkel, 1926) was considered a virus but in 1967 Doi and collaborators described after electron microscopy studies many structures resembling mycoplasmas (MLOs) in association with yellows-type and witches' broom symptoms in diverse plant species. The electron microscopy was for long time used as instrument to investigate the MLO morphology by cutting thin section of plants and insects showing that a high accumulation of MLO could be detected in sieve tubes of infected plants. Moreover, from these studies resulted that small and big cells were simultaneous present suggesting a co-presence of different stage of growth (Ploaie *et al*., 1968) or, considering the modern studies, a possible mix infection of phytoplasma belonging to different ribosomal groups (Lee *et al*., 1995). To satisfy the Koch's postulates, attempts to cultivate MLO were carried out.

At the beginning, artificial media similar to those used for mycoplasmas growth supplemented with components used for vertebrate and invertebrates were tried (Jones *et al*., 1977). For instance, Lombardo and Pignatelli in 1970 described the cultivation of the agent present in *Catharanthus roseus* showing symptoms of phyllody in flowers. In 1971 Saglio and collaborators were able to grow in solid and liquid media the agent of the stubborn disease of citrus. In the same year, Gianotti and Vago reported the success of cultivating the MLO of the clover phyllody; while the corn stunt agent was maintained in liquid media for a long period (Chen and Granados, 1970). Other studies, then demonstrated that the agent of the corn stunt is the *Spiroplasma kunkelii* (Chen and Liao 1975; Williamson and Whitcomb, 1975) belonging to genus *Mollicutes*, morphologically different from phytoplasmas. Despite spiroplasmas were successfully cultivated in artificial media, MLO remained uncultured. Many attempts to cultivate MLOs were described (Müller *et al*., 1975; Elmendorf, 1977; Whitcomb and Tully, 1979) but the experiments resulted not repeatable in other laboratories (Maramorosch, 2011). Thus, the claims of the MLOs cultivation were not accepted by the scientific community also because the isolates were not deposited in collections and were not available for comparison with other *Mollicutes* isolated strains according to the rules established by the International Organization of Mycoplasmology (IOM) (Maramorosch and Harris, 1981).

The scientists started asking why it was so difficult to cultivate plant MLOs; their intracellular location in plants and insects, together with their *status* of parasites very well adapted to the host condition were suggested as main difficulties to develop an appropriate artificial medium.

Nevertheless, it is important to note that also some mycoplasmas were not cultivable when discovered, for instance, this is the case of *M. hyopneumoniae*. It is well known that in many laboratories, mycoplasmas grow very easily as contaminant in cellular lines, but they are very difficult to grow in artificial cell-free media. The cause could be that the mycoplasma is dependent on the other cells to grow (Hopps *et al*., 1976). Thus, to explain the difficult in cultivating MLO, two hypotheses were formulated: (1) the media developed are lacking essential elements and (2) the MLO are cell-dependent to grow. However, since using phloem saps as nutrient in the media did not permit their multiplication, the second explanation seems more plausible. A fascinating story about the phytoplasmas cultivation concerns the so named "Olympic Guide to MLO Cultivation" unpublished document written by the "MLO Dream Team" composed by K. Hackett, D. Pollack and F.F. Whitcomb that is a report prepared for the MLO culture session which was held during the 9th IOM congress in Ames (Iowa, USA) in 1992. However, no cultivation was tried again or at least published until very recently, when a preliminary method of phytoplasma cultivation was described by Bertaccini and collaborators during the 18th Congress IOM in Chianciano Terme, Italy (Bertaccini *et al*., 2010). This methodology was eventually implemented and first in 2012 and then in 2016, the phytoplasma cultivation was described in scientific journals from Contaldo and collaborators. The first described methodology of cultivation was reported for some phytoplasma strains from micropropagated periwinkles, however the used media composition PivL® (liquid) and PivS® (solid) that were complimentary provided by the UK company Mycoplasma Experience Ltd, are covered by patent. Only in 2016 a new medium was developed and published (Contaldo *et al*., 2016) comparing three different complex media enclosing Piv®. Grapevine field-collected materials showing symptoms and infected by "flavescence dorée", "bois noir" and aster yellows were used as phytoplasma source. The new developed medium CB, composed by TSB, an Oxoid medium containing 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 the advantage that it is possible to modify its composition increasing the specificity for the diverse phytoplasmas and adapting the medium to the different stages of the phytoplasma growth.

2.2. Materials and methods

2.2.1. Plant material

2.2.1.1. *Sesamum indicum*

Sesame is an annual plant and is one of the oldest and traditional oilseed crops. Phytoplasmas associated with sesame induce phyllody, witches' broom, virescence, yellowing, floral sterility (Akhtar *et al*., 2008). So far diverse phytoplasmas were detected in sesame and classified as: 16SrI-B in Myanmar, 16SrII-A in Thailand and Taiwan, 16SrII-D in Oman and India, 16SrIX-C in Iran, and 16SrVI-A and 16SrIX in Turkey (Catal *et al*., 2013). Sesame phytoplasmas are transmitted by leafhopper (Vasudeva and Sahambi, 1955) and the associated disease causes significant economic losses also because it affects the seeds production (Ikten *et al*., 2014). Some seeds deriving form infected mother plants were sown in sterile soil in order to test the deriving seedlings for phytoplasma presence. In this experiment 200 seeds from 5 genotypes were sown in soil. The plantlets were grouped in batches of 10 for the testing (Table 2.1).

Sample	Number of sown seeds in Murashige and Skoog (MS) medium (1962)	Number of tested seedlings
Sesame 2	20	20
Sesame 6	42	40
Sesame 7	83	80
Sesame 8	30	30
Sesame 14	25	20
Total	200	190

Table 2.1. Sesame seedlings tested to verify the phytoplasma presence.

2.2.1.2. *Brassica napus*

B. napus phytoplasma infected plants show stunted growth, leaf reddening, virescence and floral malformation. Only a few flowers are able to complete the maturation; the seeds production is very limited and they are small, shriveled and malformed. In different parts of the world, these symptoms are associated with phytoplasma presence; in particular, phytoplasmas belonging to ribosomal subgroup 16SrI-B ('*Ca.* P. asteris') were identified in symptomatic samples (Bertaccini *et al*. 1998; Olivier *et al*., 2008; Maliogka *et al.*, 2009). In this experiment, 130 seeds from 3 plants were sown directly in soil. The plantlets were grouped in batches of 10 seedlings and each batch was tested for phytoplasma presence (Table 2.2).

Sample	Number of sown seeds in MS	Number of tested seedlings
Rape 5	42	40
Rape 6	10	10
Rape 7	81	80
Total	133	130

Table 2.2. *B. napus* seedlings tested to verify the phytoplasma presence.

2.2.1.3. *Solanum lycopersicum*

Phytoplasma infections in tomato have been described in different parts of the world and indicate that this plant species is host of numerous phytoplasmas belonging to different ribosomal groups. The symptoms generally consist of stunted growth, leaf yellowing or reddening, proliferation of lateral buds and emission of adventitious roots, hypertrophy of goblets, virescence, phyllody, malformations and abortion of the floral organs. The plant also produces a few berries, of small size and only on older branches. The berries ripen early and have inappropriate texture and flavor. The phytoplasmas most frequently identified in Italy, and more generally in Europe, belong to the ribosomal subgroups 16SrXII-A ("stolbur") and 16SrI-B ("aster yellows"), even if sporadically phytoplasmas belonging to groups 16SrV and 16SrIII were also found (Del Serrone *et al*., 2001). In Italy, the disease is mainly localized in the central and southern regions: Puglia (Martelli et al., 1969; Giuliani *et al*., 2010), Calabria (Albanese *et al*., 1998), Campania (Marcone and Ragozzino, 1995), Basilicata (Marcone *et al*., 1997), Sicily (Polizzi *et al*., 1990) and Sardinia (Lisa *et al*., 1983; Minucci and Boccardo, 1997). Some cases were also reported in northern region as Veneto, Piedmont (Marzachì *et al*., 2000) and Emilia Romagna (Favali *et al*., 2000). “Stolbur” phytoplasma present in tomato crops in the districts of Piacenza, Parma, Ferrara and Ravenna was identified and characterized by Terlizzi *et al*. in 2010 on the *tuf* gene as tuf-type b. Tomato seeds from symptomatic plants were collected from farms in the district of Parma and the same plants were assayed for the presence of phytoplasmas. Two groups of seeds consisting in 40 (group A) and 250 (group B) seeds respectively were sown in soil (Table 2.3). Germination percentage was verified two weeks after the *in vitro* sowing and the seed viability was evaluated one month after the sowing.

Sample	Number of sown seeds in MS	Number of tested seedlings
Tomato group A	68	44
Tomato group B	250	196 (28 batches of 7 plants each)

Table 2.3. Tomato seedlings tested to verify the phytoplasma presence.

2.2.1.4. *Zea mays*

The most well-known and widespread diseases due to *Mollicutes* in maize plants are the “bushy stunt" (MBS) and the "corn stunt" (CS), both present in the American continent. MBS is associated with a phytoplasma belonging to the ribosomal group 16SrI-B ("aster yellows") and is characterized by symptoms as marginal chlorosis of young leaves, gradual reddening of the base and the proliferation of axillary buds, which are also showing chlorosis and redness. In case of early infection, the plant has very short internodes and numerous small cobs with a limited number of seeds (Ebbert *et al*., 2001; Harrison *et al*., 1996; Lee *et al*., 2004). CS is a disease caused by *Spiroplasma kunkelii* and characterized by symptoms similar to that of MBS (Chen and Liao 1975; Williamson and Whitcomb, 1975). Both pathogens are transmitted in America by leafhoppers belonging to the genus *Dalbulus*. In Europe, a disease named redness was reported since 1957 in Serbia (Mari and Savić, 1965). The disease became epidemic in the early 60s and in the late 90s (Šutić *et al*., 2003). During these epidemic stages, symptoms can be present in more than 90% of the plants and these can cause crop losses of more than 50% (Blaženčić, 1982; Starovič *et al*., 2004). In the middle of July, the redness affects the midribs, and then spreads to the entire leaf blade, then to the sword, and finally to the whole plant, reaching the maximum in August and September and culminating in the premature withering of the infected plant. The dimensions of the symptomatic plants are similar to those of asymptomatic, but the kernels are dry and their weight is severely compromised. In particular, the few seeds are malformed and ripen earlier. Duduk and Bertaccini in 2006 detected phytoplasmas belonging to ribosomal subgroup 16SrXII-A (“stolbur”) only in symptomatic samples, and suggested their association with the redness disease. In most cases corn infected fields have the most severely infected plants along the edges suggesting that the infection could come, through insect vectors, from wild or cultivated plants nearby. Some symptomatic plants were sampled in Serbia (Bojan Duduk, Institute of Pesticides and Environmental Protection, Belgrade, Serbia) and tested for phytoplasma presence, resulting positive for “stolbur” phytoplasmas. From these symptomatic and phytoplasma infected plant the whole cobs were collected in order to verify the phytoplasma transmission by seed. In total 109 seeds deriving from 6 cobs from Serbia were sterilized and then placed *in vitro* in groups of 5 in Magenta boxes on MS agar for germination. Because of the microbial load, it was difficult to maintain the plantlets in aseptic conditions, thus some seeds were directly sown in soil in the green-house under insect- proof cages. The corn gemination started at 3 days until one week after sowing. At 10 days from germination, each plant was tested

for the presence of phytoplasmas. From each plant 0.5 g of tissue was sampled for the extraction of nucleic acids without killing the plant. The same sampling was done after 90 days from the germination. The percentage of germination *in vitro* was also determined calculating the average percentages after one week from sowing (Table 2.4).

Sample	Number of sown seeds in MS	Number of tested seedlings
Corn cob H	14	9
Corn cob 1	19	13
Corn cob 2	19	12
Corn cob 3	19	18
Corn cob 4	19	17
Corn cob 5	19	10
Total	109	79

Table 2.4. Corn seedlings tested to analyze the phytoplasma presence.

2.2.2. Seed sterilization

According with the seed tissue two protocols of sterilization were used:

1) Protocol A for small seeds and for thin skins seeds (sesame, tomato and oil seed rape).

- Batches of 5-10 seeds were put in 2 ml sterile Eppendorf tubes;
- the seeds were suspended in a solution of 1.5 ml of NaClO (sodium hypochlorite) at 15% (in sterile distilled water) and 2 drops of Tween 20 under laminar flow hood, in sterile tubes and put in gentle agitation for 15 min in a shaker;
- NaClO solution was discarded and the seeds were washed in sterile distilled water (SDW);
- then the seeds were washed with ethanol at 70% in SDW for 3 min, under gentle agitation;
- discarded the ethanol, the seeds were washed 4 times with SDW dried and stored at 4°C until use;

2) protocol B (corn seeds)

- Batches of 5 seeds were put in 15 ml tubes after washing with soapy water and rinsing in running water for 5-10 min.
- Under the hood, 8 ml of a solution at 80% ethanol were added for 3 minutes, maintaining the tubes under constant agitation.
- Removed the ethanol, a 50% solution of NaClO (in SDW) was added for 20 minutes with the addition of two drops of Tween 20, maintaining the tubes under constant agitation.
- The above two points have been repeated two times.

- The seeds were washed 4 times with SDW, dried maintained in Eppendorf tubes at 8°C until use.

2.2.3. *In vitro* seed sowing

After the sterilization processes the seeds were placed in groups of 5, under sterile laminar flow hood, in Magenta boxes containing approximately 12 ml of agar medium sterilized by autoclaving at 121°C for 20 minutes at a pressure of 1 atmosphere.

The medium, based for macronutrients, micronutrients and vitamins on components and quantities indicated by Murashige and Skoog (1962), was solidified with the addition of 8 g/l of agar and enriched with 20 g/l of sucrose, the pH was adjusted to 5.80 (Table 2.5).

The cultures were maintained in climatic chambers at a temperature of about 24±1°C and subjected to an illumination of 3,000 lux for a photoperiod of 16 hours per day.

Macronutrients		Micronutrients		Vitamins	
KNO_3	1,90 g/l	H_3BO_3	6,20 mg/l	Nicotinic Acid	0,5 mg/l
NH_4NO_3	1,65 g/l	$MnSO_4$ $4H_2O$	22,30 mg/l	Glycine	2 mg/l
KH_2PO_4	0,17 g/l	$ZnSO_4$ $4H_2O$	8,60 mg/l	Pyridoxine	0,5 mg/l
$CaCl_2$ $2H_2O$	0,44 g/l	KJ	0,83 mg/l	Thiamine	0,1 mg/l
$MgSO_4$ $7H_2O$	0,37 g/l	$NaMoO_4$ $2H_2O$	0,25 mg/l		
Fe-EDTA	0,04 g/l	$CuSO_4$ $5H_2O$ $CoCl_2$ $6H_2O$	0,025 mg/l 0,025 mg/l	Myo-Inositol	100 mg/l

Table 2.5. Medium composition for seed sowing (Murashige e Skoog, 1962).

2.2.4. DNA extraction

Total DNA from 0.5 g of plant leaves of *S. indicum*, *B. napus*, *S. lycopersicum* and *Z. mays* seedlings was extracted using a method based on chloroform and final precipitation in isopropanol described by Angelini *et al*., 2001 (Appendix 1). The DNAs extracted were then stored at -20°C until use.

2.2.5. PCR assay

To detect the presence of phytoplasmas, PCR assays with REDtaq DNA polymerase (Sigma-Aldrich, Co., St. Louis, MO, USA), were carried out using universal and specific primers combinations to amplify the phytoplasma 16S rDNA region. In particular, in direct PCR assays P1/P7 or R16F2n/R16R2 or fU5/rU3 primers pairs were employed, followed by nested PCR assays on these amplicons diluted 1:30 using the universal primers R16F2n/R16R2 or fU5/rU3 or 16R758f/16S1232r (=M1/M2) and the group specific primers R16(I)F1/R1. The nested PCR was sometime followed by a second nested PCR on the amplicons of the first reaction diluted 1:30 with the primers M1/M2 (Appendix 2-Table 1A). PCR analysis was performed also on *tuf* gene: two pairs of primer cocktails were used, Tuf340/Tuf890 and Tuf400/Tuf835 in direct and nested PCR assays respectively (Appendix 2). In each amplification assay, sterile distilled water as negative control and DNA of selected reference strains (Bertaccini, 2014) extracted from periwinkles as positive controls were added.

2.2.6. Restriction fragment length polymorphism (RFLP) analysis

The identification of detected phytoplasmas was performed using RFLP analyses that are based on the use of endonucleases able to recognize specific sequences of DNA and generating a specific restriction pattern. The fragments of amplified 16S rDNA region using R16(I)F1/R1, fU5/rU3 and M1/M2 primer pairs were subjected to digestion with *Tru1*I and *Tsp509*I restriction enzymes according to the manufacturer's instructions (Appendix 3).

2.2.7. Sequencing and phylogenetic analyses

Selected amplicons obtained with primer pairs R16(I)F1/R1, fU5/rU3 and M1/M2 were sequenced directly after purification with Nucleospin extract II kit (Macherey-Nagel, Germany) at a private company (Macrogene, NL). The sequences were edited, assembled and analyzed following the methodologies described in the Appendix 4.

2.2.8. Phytoplasma cultivation in artificial media

2.2.8.1. Media

In order to demonstrate that the detected phytoplasmas were viable in the plants generated from seeds deriving from infected mother plant, two artificial media were used:

- PivL®: pH 7.3 ± 0.2 (Contaldo *et al*., 2012) Mycoplasma Experience (composition covered by patent), ready to use.
- CB (Contaldo *et al*., 2016): pH 7.3 ± 0.2, containing TSB (Oxoid, UK; CM 1065), tryptone and soya peptone. This medium was sterilized for 20 min at 121°C and then a supplement

containing 20 ml of sterile porcine serum (Oxoid, SR0035), 25 µg/ml of ampicillin (Sigma, A9393) and 50 µg/ml of nystatin (Sigma, N6261) both 0.22 µm filter sterilized, 10 ml of autoclaved yeast extract (25% w/v) and 0.005% of phenol red for each 80 ml of medium were added.

2.2.8.2. Isolation and growing condition

The isolation trials were performed from 2 midribs per sample (tomato, corn, oilseed rape, sesame). The midribs were surface sterilized for 1 min in 1% NaClO, rinsed in deionized, distilled, sterile water (DDSW), and dried on sterile filter paper disks under sterile hood. After the ends discarding, midribs were moistened in 2.5 ml of each of the 2 media and sliced with sterile scalpels. The slurry was then transferred to an 8 ml Monovette urine tubes (Sigma Aldrich) and incubated at 25±1°C under atmospheric conditions. Uninoculated tubes (UT) and tubes inoculated with midribs from healthy (phytoplasma negative) seedlings were also maintained under the same conditions. The isolation procedure was repeated twice for each sample in each medium at each isolation time.

When colour change from orange-red to orange-yellow occurred, 100 µl of broth cultures were inoculated onto plates containing 8 ml of the corresponding solid media: PivS® and CBs (solid). The last medium contains TSB 30 g/l, NaCl 20 g/l, agar No. 3 12 g/l (Oxoid, LP0013), autoclaved and then added with 0.22 µm filter sterilized ampicillin and nystatin 50 µg/ml. The incubation was carried out in a 2.5 l anaerobic jar (Oxoid, AG0025) in a microaerophilic atmosphere using CampyGen sachets (Oxoid, CN0025).

2.2.8.3. Culture purification by sequential passages liquid/solid media

After 24-48 hours from the agar insemination, distinct single colonies were picked and transferred into the corresponding fresh liquid media, for subsequent purification steps, following a slightly modified procedure from ICSB (1979). After colour change 100 µl of each broth culture was 0.8 µm filtered using syringe filter non-pyrogenic hydrophilic (Sartorius Stedim, Biotech, Germany) and again plated; this procedure was repeated three times per selected colony of each sample in each medium when there was colony formation. After the above procedure one colony was picked, transferred in broth medium and plated after 2 days of incubation using 1: 1,000 and 1: 10,000 dilutions. The procedure was repeated 2 times. The two and five days old colonies were photographed on the plates and also under optical bifocal microscope at 40X magnification. Single colonies were then collected, dissolved in 100 µl DDSW, and subjected to nucleic acid extraction

by DNeasy Plant Minikit (Qiagen, Germany). The agar surface between colonies was also collected and extracted separately as negative control for each of the media that allowed colony growth.

2.2.8.4. Culture purification by differential centrifugations

After colour change, 1 ml of broth culture was centrifuged at 6,000 rpm for 10 min and the supernatants were centrifuged again at 14,000 rpm for 10 min. The DNAs were extracted from the obtained pellets by DNeasy Plant Minikit (Qiagen, Germany). The supernatants obtained from the first centrifugation at 6,000 rpm were plated.

2.2.8.5. Phytoplasma molecular identification from colonies

Phytoplasma detection and identification was carried out from colonies by specific nested PCR/RFLP assays on *16S rRNA* gene. One microliter of extracted nucleic acid was employed as template using primers R16(I)F1/R1 followed by nested PCR with primers M1/M2. In each PCR and nested-PCR two negative control samples represented by DDSW and media not inseminated were used. Each 25 µl of PCR reaction mix contained 12.5 µl of 2 x Red PCR Master Mix (Rovalab, Germany), 10.5 µl of DDSW (Sigma) and 0.4 mM of each primer. Nested-PCR assays were carried out using 1 µl of a 1: 30 dilution of amplicons from direct PCR as template. Six microliters of PCR products were separated in 1% agarose gel, stained with ethidium bromide and visualized under UV transilluminator. Identification of detected phytoplasmas was done using RFLP analyses with *Tru1*I (Fermentas, Vilnius, Lithuania) restriction enzyme. RFLP products were separated in a 6.7% polyacrylamide gel, stained with ethidium bromide and visualized under UV transilluminator as reported above.

Phytoplasma detection was also carried out in corn samples, both in colonies and liquid CB medium by qPCR method with SYBR Green I chemistry, as described in chapter 3.

2.3. Results

2.3.1. Germination percentage

2.3.1.1. *Sesamum indicum*

S. indicum seeds were sown both in MS medium and directly in sterile soil. After the sterilization, according to protocol A, 208 seeds deriving from 9 plants were sown in MS medium. The total germination was 17.3% with 36 germinated seeds. The germination percentage was higher for full and pulpy seeds as samples 5, 6, 7 and 8, but lower for empty seed as samples 1, 2, 3, 4, 9. In total 8 seedlings were tested by PCR/RFLP analysis (Table 2.6).

Samples	Sown seeds in MS	Germinated seeds	Germination percentages	Tested seedlings
Sesame 1	31	0	0%	0
Sesame 2	23	2	8.7%	1
Sesame 3	25	0	0%	0
Sesame 4	14	0	0%	0
Sesame 5	19	4	21.1%	1
Sesame 6	25	9	36%	2
Sesame 7	24	2	8.3%	1
Sesame 8	30	19	63.3%	2
Sesame 9	17	0	0%	1
Total	208	36	17.3%	8

Table 2.6. Germination percentages for *S. indicum* seeds sown in MS medium.

After the sterilization, according to protocol A, 200 seeds deriving from 5 plants were sown in sterile soil (Figure 2.5.). The samples with a high germination percentage in the first sowing were chosen (6 and 8), but also the sample 2 in order to estimate if the cause of the difficult germination was the medium or the seed condition; a new sample (14) was chosen too because it showed slightly malformed seeds. The total germination was 95% with 190 germinated seeds. The germination percentage was again higher for full and pulpy seeds (2, 6, 7 and 8) and slightly lower for empty seed as the sample 14, but the percentage of germinated seeds was increased sowing them directly in sterile soil. In total 190 samples grouped in 10 seedlings per sample were tested by PCR/RFLP analysis (Table 2.7).

Sample	Sown seeds in soil	Germinated seeds	Germination percentages	Tested seedlings (in groups of 10)
Sesame 2	20	20	100%	20
Sesame 6	42	40	95.2%	40
Sesame 7	83	80	96.4%	80
Sesame 8	30	30	100%	30
Sesame 14	25	20	80%	20
Total	200	190	95%	190

Table 2.7. Germination percentage for *S. indicum* seeds sown in sterile soil.

Figure 2.5. *S. indicum* seedlings in the greenhouse.

2.3.1.2. *Brassica napus*

After the sterilization, according to the protocol A, 133 seeds deriving from 3 plants were sown in sterile soil (Figure 2.6). The total germination was 97.7% with 130 germinated seeds grouped in 10 seedlings per sample that were tested by PCR/RFLP analysis (Table 2.8).

Sample	Sown seeds in MS	Germinated seeds	Germination percentage	Tested seedlings (in groups of 10)
Rape 5	42	40	95.2%	40
Rape 6	10	10	100%	10
Rape 7	81	80	98.8%	80
Total	133	130	97.7%	130

Table 2.8. Germination percentage for *B. napus* seeds sown in sterile soil.

Figure 2.6. *B. napus* seedlings in the greenhouse.

2.3.1.3. *Solanum lycopersicum*

After the sterilization, according to the protocol A, 68 seeds deriving from a pool of plants of the same field were sown in MS medium. The total germination was 64.7% with 44 germinated seeds and all were tested by PCR/RFLP analysis (Table 2.9).

Tomato sample group A	Sown seeds in MS	Germinated seeds	Germination percentage	Tested seedlings
Total	68	44	64.7%	44

Table 2.9. Germination percentage for *S. lycopersicum* group A seeds sown in MS medium.

From the *S. lycopersicum* plants resulted positive to phytoplasmas, it was also possible to obtain new seeds that were sterilized according to the protocol A and 85 seeds from 5 plants were sown in MS medium and transplanted in sterile soil (Figure 2.7) producing 58 plants of second generation with a germination percentage of 68.2%; they were all tested (Table 2.10).

Sample	Collected-sown seeds	Germinated seeds	Germination percentages	Tested seedlings
		Positive samples		
Tomato 1a	0	-	-	-
Tomato 1b	0	-	-	-
Tomato 2	34	27	79.4%	27
Tomato 3	0	-	-	-
Tomato 9	0	-	-	-
Tomato 11	7	4	57.1%	4
Tomato 20	23	23	100%	23
Tomato 23	4	4	100%	4
		Negative samples		
Tomato 24	17	0	0%	-
Tomato 26	85	20/20	100%	14
Tomato 31	41	20/20	100%	14

Table 2.10. Germination percentage for *S. lycopersicum* second generation seeds sown in MS medium.

After the sterilization, according to the protocol A, 250 seeds deriving from a pool of plants of the same field were sown in MS medium. The total germination was 78.4% with 196 germinated seeds and all were tested by PCR/RFLP analysis (Table 2.11).

Tomato sample group B	Sown seeds in MS	Germinated seeds	Germination percentage	Tested seedlings
Total	250	196	78.4%	196

Table 2.11. Germination percentage for *S. lycopersicum* group B seeds sown in MS medium.

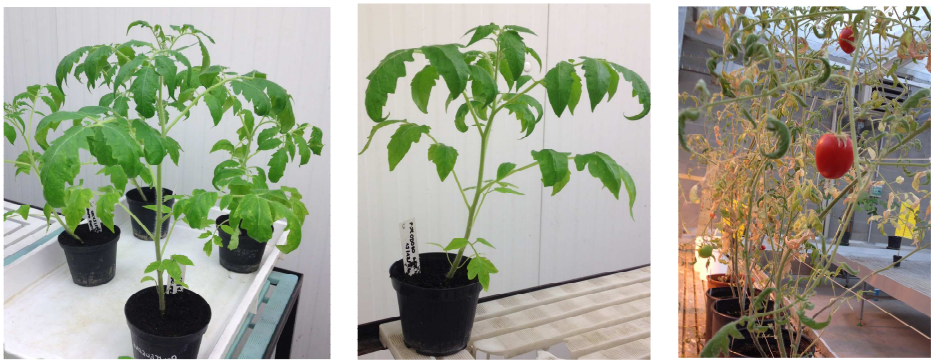

Figure 2.7. *S. lycopersicum* seedlings in greenhouse.

2.3.1.4. *Zea Mays*

Z. mays seeds were sown both in MS medium and directly in sterile soil. After the sterilization, according to the protocol B, 36 seeds deriving from 6 plants were sown in MS medium. The total germination was 66.7% with 24 germinated seeds. The seeds from sample cob 5 were malformed and maybe this is the cause of the low germination observed. In total 10 seedlings were tested (Table 2.12). After the sterilization, according to protocol B, 109 seeds deriving from 6 plants were sown in sterile soil (Figure 2.8). The germination percentage was 82.6 % with 90 germinated seeds. To note that the germination performed better in soil than in MS medium increasing the percentage of almost 20% and 79 seedlings were tested by PCR/RFLP analysis (Table 2.13).

Sample	Sown seeds in MS	Germinated seeds in MS	Germination percentages	Tested seedlings
Corn cob H	6	5	83.3%	3
Corn cob 1	6	5	83.3%	-
Corn cob 2	6	5	83.3%	1
Corn cob 3	6	3	50%	1
Corn cob 4	6	6	100%	5
Corn cob 5	6	0	0%	-
Total	36	24	66.7%	10

Table 2.12. Germination percentage for *Z. mays* seeds sown in MS medium.

Sample	Sown seeds in soil	Germinated seeds	Germination percentages	Tested seedlings
Corn cob H	14	12	85.7%	9
Corn cob 1	19	14	73.7%	13
Corn cob 2	19	12	63.2%	12
Corn cob 3	19	18	94.7%	18
Corn cob 4	19	19	100%	17
Corn cob 5	19	15	78.9%	10
Total	109	90	82.6%	79

Table 2.13. Germination percentage for *Z. mays* seeds sown in sterile soil.

Figure 2.8. *Z. mays* seedlings in greenhouse.

2.3.2. Phytoplasma detection

2.3.2.1. *Sesamum indicum*

The seedlings sown in MS medium were tested at 30 days after germination, the 7 seedlings survived to the transplanting were all negative for phytoplasma presence. The 190 plantlets obtained from the soil germination were grouped in 19 batches: the DNAs were extracted at 30 days after germination and as shown in Table 2.15, 15 samples out of 19 resulted positive for phytoplasmas belonging to ribosomal groups 16SrI, 16SrII and 16SrXII-A.

Sample	Germination percentages	Phytoplasma detected at 30 days
Sesame 2A	100%	16SrXII-A*
Sesame 2B		16SrXII-A
Sesame 6A		16SrXII-A
Sesame 6B	95.2%	16Sr?
Sesame 6C		16SrXII-A**
Sesame 6D		16SrII
Sesame 7A		16SrI
Sesame 7B		16SrI
Sesame 7C		Negative
Sesame 7D		Negative
Sesame 7E	96.4%	16SrI
Sesame 7F		16SrI
Sesame 7G		16SrII
Sesame 7H		Negative
Sesame 8A		16SrI
Sesame 8B	100%	16SrI
Sesame 8C		16SrI
Sesame 14A	80%	Negative
Sesame 14B		16SrI

Table 2.15. Results of phytoplasma detection in *S. indicum* samples (batches). * and **, RFLP profile is different from16SrXII-A, sequence homology is 99%, see Fig. 2.9 and 2.10.

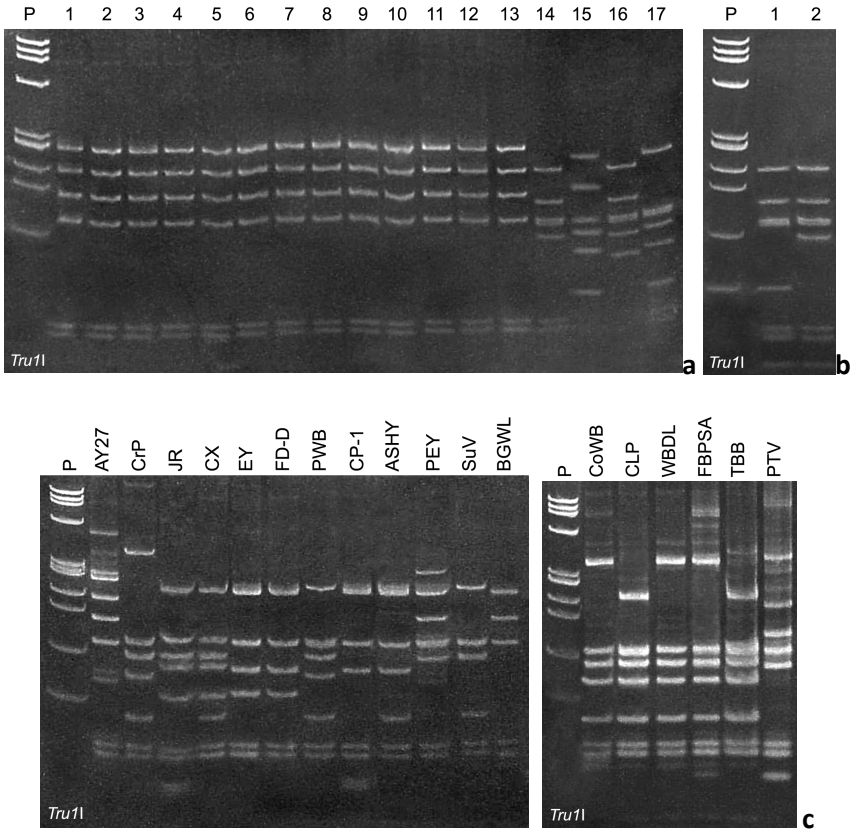

Figure 2.9. RFLP analyses in polyacrylamide gels of sesame samples amplified in nested PCR with primers fU5/rU3 and digested with *Tru1*I restriction enzyme; **a)** 1-13: samples positive to 16SrI; 14: sesame 6A positive to 16SrXII-A; 15: sesame 6B positive to 16Sr?; 16: sesame 6C positive to 16SrXII-A (RFLP difference for a SNP, single nucleotide polymorphism in sequence); 17: sesame 6D positive to 16SrII; **b)** 1: sesame 2A positive to 16SrXII-A (RFLP difference for a SNP in sequence); 2: sesame 2B positive to 16SrXII-A; **c)** reference phytoplasma strains: AY27, 16SrI-B; CrP, 16SrII-C; JR, 16SrIII; CX, 16SrIII-A; EY, 16SrV-A; FD-D, 16SrV-D; PWB, 16SrVI; CP-1, 16SrVI-A; ASHY, 16SrVII-A; PEY, IX-C; SuV, 16SrXV; BGWL, 16SrXIV-A; CoWB, 16SrII-F; CLP, 16SrII-A; WBDL, 16SrII-B; FBPSA, 16SrII-C; TBB, 16SrII-D; PTV, 16SrXII-A; P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

The amplicons were obtained with primers fU5/rU3 (Figure 2.9), R16(I)F1/R1 (Figure 2.10) and M1/M2 (data not shown). RFLP analyses on fU5/rU3 amplicons indicated the presence of phytoplasmas belonging to 16SrII ribosomal group, they were sequenced and showed 99% of identity to the sequence of 16SrII, peanut witches' broom available in GenBank (accession number KX358563). Sample sesame 2A and sesame 6C amplified with primers 16R(I)F1/R1 resulted positive for "stolbur" and showed 99% of identity to the sequence of '*Ca*. P. solani' available in GenBank (accession number KT595210).

The samples sesame 6D and sesame 14B were also individually tested at 80 days from germination. The amplicons were obtained with primers fU5/rU3, R16(I)F1/R1 and M1/M2 (Figure 2.11). Nineteen out of 20 plantlets resulted positive for phytoplasmas in groups 16SrI, 16SrII and 16SrXII-A, in some cases in mixed infection (Table 2.16).

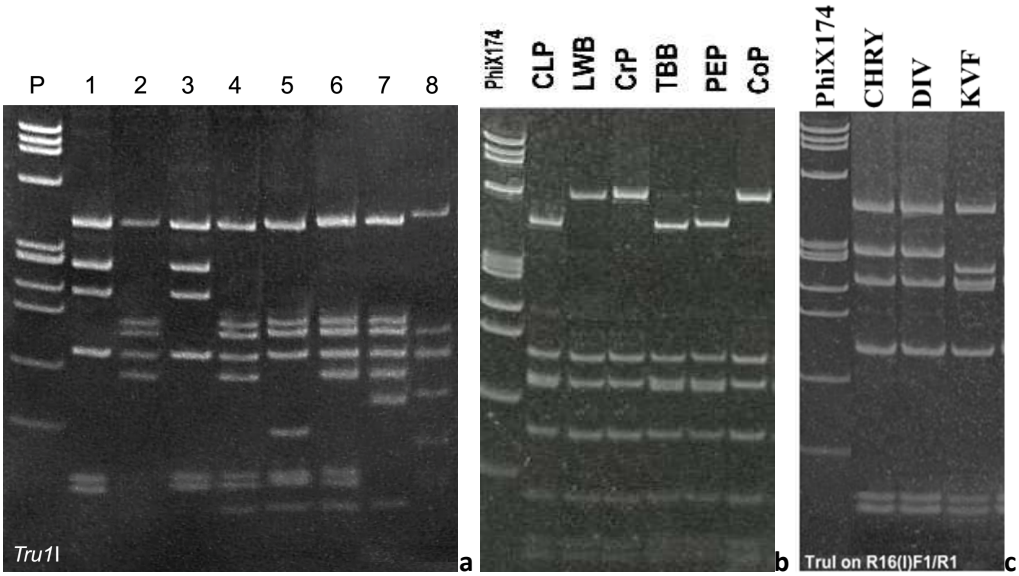

Figure 2.10. a) RFLP analyses in polyacrylamide gels of sesame samples amplified in nested PCR with primers R16(I)F1/R1 and digested with *Tru1*I restriction enzyme; 1, 3: samples positive to 16SrI; 2, 4, 6: sesame positive to 16SrXII-A; 5: sesame 2A positive to 16SrXII-A (RFLP difference for a SNP in sequence); 7: sesame 6C positive to 16SrXII-A (RFLP difference for a SNP in sequence); 8: sesame 6D positive to 16SrII; **b)** reference phytoplasma strains: CLP, 16SrII-A; LWB, 16SrII-B; CrP, 16SrII-C; TBB, 16SrII-D; PEP, 16SrII-E; CoP, 16SrII-F; **c)** CHRY, 16SrI-A; DIV, 16SrI-B; KVF, 16SrI-C; P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

Sample	Germination percentage	Phytoplasma detected in single tested seedlings at 80 days
Sesame 6D1		16SrI
Sesame 6D2		16SrXII-A
Sesame 6D3	95.2%	16SrXII-A
Sesame 6D4		16SrXII-A
Sesame 6D5		16SrXII-A
Sesame 6D6		16SrII + 16SrI
Sesame 6D7		16SrXII-A
Sesame 6D8		Negative
Sesame 6D9		16SrII + 16SrI
Sesame 14B1		16SrI
Sesame 14B2		16SrI
Sesame 14B3		16SrII + 16SrI
Sesame 14B4		16SrXII-A
Sesame 14B5	80%	16SrII + 16SrI
Sesame 14B6		16SrI
Sesame 14B7		16SrII+ 16SrI
Sesame 14B8		16SrI
Sesame 14B9		16SrXII-A
Sesame 14B10		16SrII + 16SrI
Sesame 14B11		16SrII + 16SrI

Table 2.16. Results of phytoplasma detection in *S. indicum* single seedlings.

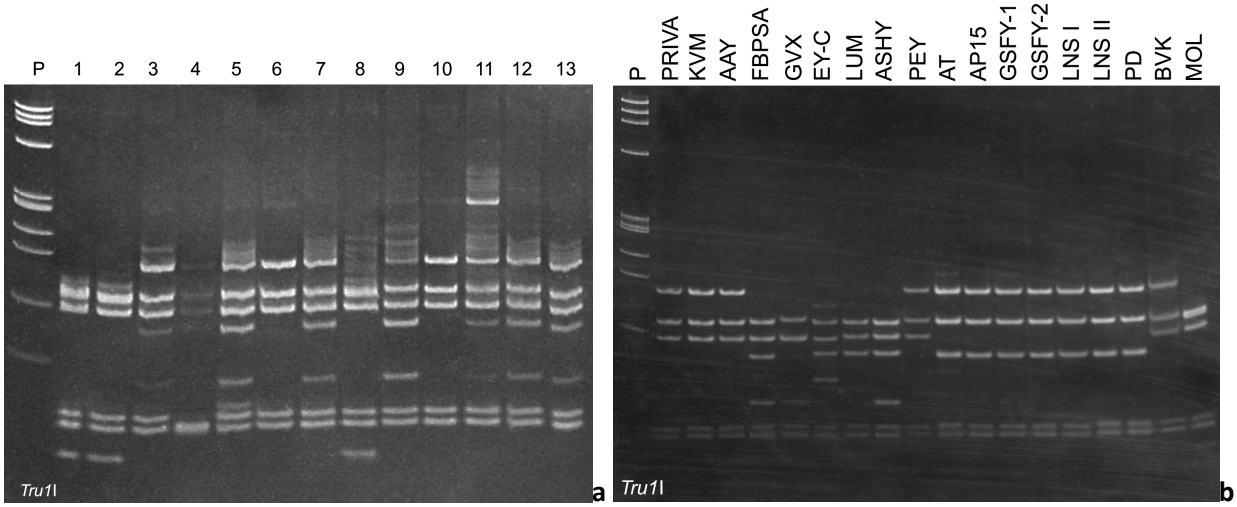

Figure 2.11. RFLP analyses in polyacrylamide gels of sesame samples amplified in nested PCR with primers M1/M2 and digested with *Tru1*I restriction enzyme; **a)** 1, 2, 8: sesame positive to 16SrXII-A; 6, 10: sesame positive to 16SrI; 3, 5, 7, 9, 11-13: sesame positive to 16SrII + 16SrI; b) reference strains: PRIVA, 16SrI-B; KVM, 16SrI-C; AAY, 16SrI-F; FBPSA, 16SrII-C; GVX, 16SrIII-A; EY-C, 16SrV-A; LUM, 16SrVI-A; ASHY, 16SrVII-A; PEY, IX-C; AT, 16SrX-A; AP-15, 16SrX-A; GSFY-1 16SrX-B; GSFY-2, 16SrX-B; LNS I, 16SrX-B; LNS II, 16SrX-B; PD, 16SrX-C; BVK, 16SrXI-C; MOL, 16SrXII-A. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

2.3.2.2. *Brassica napus*

The 130 plantlets grouped to form 13 batches were tested carrying out the DNA extraction at 30 days after germination and performing PCR analysis with primers fU5/rU3, R16(I)F1/R1 and M1/M2. Seven samples out of 13 resulted positive for phytoplasmas in 16SrI and 16SrXII-A groups at 30 days (Table 2.17, Figure 2.12).

Sample	Germination percentages	Phytoplasma detected at 30 days
B. napus 5°		16SrI
B. napus 5B	95.2%	16SrI
B. napus 5C		Negative
B. napus 5D		Negative
B. napus 6	100%	Negative
B. napus 7°		16SrI
B. napus 7B		16SrXII-A
B. napus 7C		16SrI
B. napus 7D	98.8%	16SrXII-A
B. napus 7E		16SrXII-A
B. napus 7F		Negative
B. napus 7G		Negative
B. napus 7H		Negative

Table 2.17. Results of phytoplasma detection in *B. napus* samples (batches).

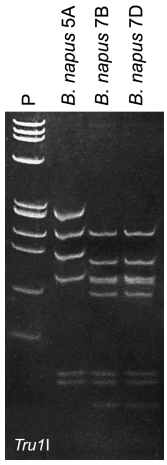

Figure 2.12. RFLP analyses in polyacrylamide gels of *B. napus* samples amplified in nested PCR with primers fU5/rU3 and digested with *Tru1*I restriction enzyme. *B. napus* 5A positive to 16SrI; *B. napus* 7B and 7D are positive to 16SrXII-A. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

The 10 seedlings of samples *B. napus* 5B, 7A and 7E were also singly tested at 80 days from germination and 7 seedlings out of 20 resulted positive as shown in Table 2.18 for phytoplasmas belonging to ribosomal groups 16SrI, 16SrVI and 16SrXII-A (Figure 2.13). Sample 5B1 obtained in amplification with primers fU5/rU3 resulted positive for phytoplasmas belonging to 16SrVI ribosomal group, and its sequence showed 99% of identity to the sequence of 16SrVI, '*Ca*. P. trifolii' available in GenBank (accession number KX092011).

Sample	Germination percentages	Phytoplasma detected in single tested seedlings at 80 days
B. napus 5B1		16SrVI
B. napus 5B2		16SrI
B. napus 5B3	95.2%	16SrI
B. napus 5B4		16SrI
B. napus 5B5		16SrI
B. napus 5B6		Negative
B. napus 5B7		16SrI
B. napus 7A1		16SrXII-A
B. napus 7A2	98.8%	16Sr?
B. napus 7A3		16SrI
B. napus 7A4		Negative
B. napus 7A5		16SrI
B. napus 7A6		Negative
B. napus 7A7		16SrI
B. napus 7E1		16SrI
B. napus 7E2		16Sr?
B. napus 7E3	98.8%	16SrI
B. napus 7E4		16SrI
B. napus 7E5		16SrI
B. napus 7E6		16SrI

Table 2.18. Results of phytoplasma detection in *B. napus* single seedlings.

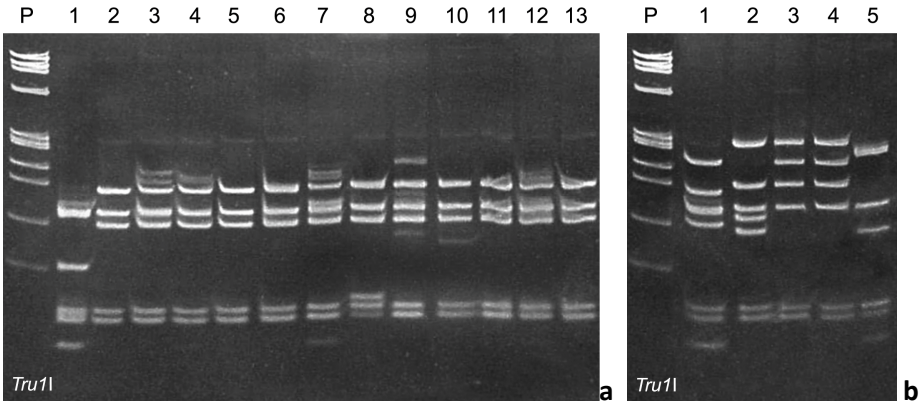

Figure 2.13. a) RFLP analyses in polyacrylamide gels of samples amplified in nested PCR with primers M1/M2 and digested with *Tru1*I restriction enzyme. 1: *B. napus* 7A2 positive to 16Sr?; 2-13: *B. napus* positive to 16SrI; **b)** RFLP analyses of samples amplified in nested PCR with primers fU5/rU3 and digested with *Tru1*I restriction enzyme; 1: *B. napus* 7A1 positive to 16SrXII-A; 2: *B. napus* 7E2 positive to 16Sr?; 3, 4: *B. napus* positive to 16SrI; 5: *B. napus* 5B1 positive to 16SrVI; P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

2.3.2.3. *Solanum lycopersicum*

The 44 seedlings of group A were tested at 30 and 150 days after germination: 8 resulted positive for phytoplasma presence at 30 days and showed the predominant presece of for phytoplasmas in group 16SrI; only one sample resulted belonging to subgroup 16SrXII-A (Figure 2.14a) in nested-PCR with primers R16(I)F1/R1 and M1/M2. The sample tomato 11 resulted 16SrI positive also at 150 days after germination (Table 2.19).

Sample	Germination percentages	Phytoplasma detected at 30 days	Phytoplasma detected at 150 days
Positive samples			
Tomato 1°	64.7%	16SrI	Negative
Tomato 1b		16SrXII-A	Negative
Tomato 2		16SrI	Negative
Tomato 3		16SrI	Negative
Tomato 9		16SrI	Negative
Tomato 11		16SrI	16SrI
Tomato 20		16SrI	Negative
Tomato 23		16SrI	Negative
Negative samples			
Tomato 24	64.7%	Negative	Negative
Tomato 26		Negative	Negative
Tomato 31		Negative	Negative

Table 2.19. Results of phytoplasma detection in *S. lycopersicum* seedlings group A.

Sample	Second generation positive/tested samples	Second generation positive samples	Phytoplasma detected at 30 days
Positive samples			
Tomato 2	3/27	Tomato 2.3	16SrXII-A
		Tomato 2.12	16SrIII
		Tomato 2.19	16SrXII-A
Tomato 11	0/4	-	-
Tomato 20	4/23	Tomato 20.5	16SrXII-A
		Tomato 20.8	16SrI
		Tomato 20.13	16SrI
		Tomato 20.17	16SrI
Tomato 23	0/4	-	-
Negative samples			
Tomato 26	0/14	-	-
Tomato 31	0/14	-	-

Table 2.20. Results of phytoplasma detection in *S. lycopersicum* second generation seedlings obtained both from positive and negative samples.

Fifty-eight second generation seedlings deriving from positive plants and 28 second generation seedlings deriving from negative plants were also tested at 30 days after germination: in nested-PCR with primers R16(I)F1/R1 and M1/M2, 7 seedlings deriving from positive plants resulted positive for 16SrI and 16SrXII-A phytoplasmas and one sample resulted positive for 16SrIII phytoplasmas also with ribosomal group III specific primers (Figure 2.14b). The seedlings deriving from first generation negative plants resulted all negative (Table 2.20).

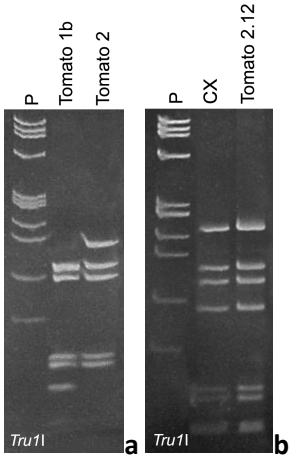

Figure 2.14. a) RFLP analyses in polyacrylamide gels of tomato samples amplified in nested PCR with primers M1/M2 and digested with *Tru1*I restriction enzyme. Tomato 1b positive to 16SrXII-A; tomato 2 positive to 16SrI; **b)** RFLP analyses of samples amplified in nested PCR with primers R16(III)F2/R1 and digested with *Tru1*I restriction enzyme. Tomato 2.12 positive to 16SrIII; CX, reference strain (16SrIII-A); P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

The 196 seedlings of group B were tested grouped in 28 batches (7 seedlings per batch) at 30 days after germination: 15 of them resulted positive for phytoplasma presence at 30 days from the germination showing positivity for phytoplasmas of groups 16SrI, 16SrV and 16SrII with primers fU5/rU3 and M1/M2 (Table 2.21, Figures 2.15, 2.16 and 2.17). The amplicons obtained with primers M1/M2 were sequenced and showed 99% of identity to the sequence of 16SrII, peanut witches' broom present in GenBank (accession number KX358572).

Sample	Germination percentages	Phytoplasma detected at 30 days
Tomato a		16SrI
Tomato b		Negative
Tomato c		Negative
Tomato d		16SrI
Tomato e		Negative
Tomato f		16SrII
Tomato g		16SrI
Tomato h		16SrI
Tomato i	78.4%	Negative
Tomato j		16SrII
Tomato k		Negative
Tomato l		16SrI
Tomato m		Negative
Tomato n		Negative
Tomato o		Negative
Tomato p		Negative
Tomato q		16SrI
Tomato r		16SrV
Tomato s		16SrV
Tomato t		Negative
Tomato u		16SrI
Tomato v		16SrI
Tomato w		16SrI
Tomato x		16SrI
Tomato y		Negative
Tomato z		Negative
Tomato a1		16SrI
Tomato b1		Negative

Table 2.21. Results of phytoplasma detection in *S. lycopersicum* seedlings group B.

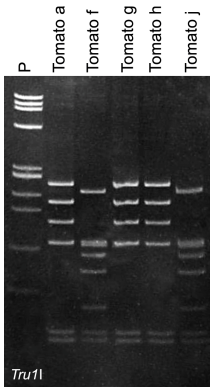

Figure 2.15. RFLP analyses in polyacrylamide gels of tomato samples amplified in nested PCR with primers fU5/rU3 and digested with *Tru1*I restriction enzyme. Tomato a, g and h positive to 16SrI; tomato f and j positive to 16SrII. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

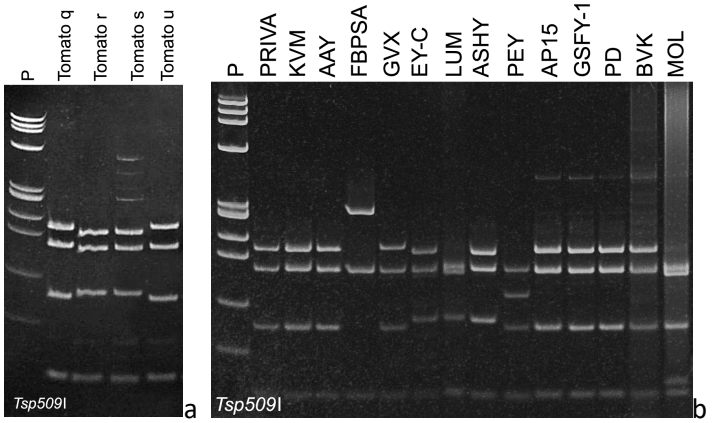

Figure 2.16. RFLP analyses in polyacrylamide gels of samples amplified in nested PCR with primers M1/M2 and digested with *Tsp509*I restriction enzyme; **a)** tomato q and u positive to 16SrI; tomato r and s positive to 16SrV; **b)** reference strains: PRIVA, 16SrI-B; KVM, 16SrI-C; AAY, 16SrI-F; FBPSA, 16SrII-C; GVX, 16SrIII-A; EY-C, 16SrV-A; LUM, 16SrVI-A; ASHY, 16SrVII-A; PEY, 16SrIX-C; AP-15, 16SrX-A; GSFY-1 16SrX-B; PD, 16SrX-C; BVK, 16SrXI-C; MOL, 16SrXII-A. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

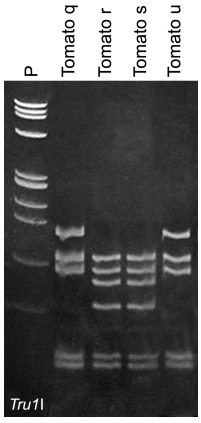

Figure 2.17. RFLP analyses in polyacrylamide gels of tomato samples amplified in nested PCR with primers M1/M2 and digested with *Tru1*I restriction enzyme. Tomato q and u positive to 16SrI; tomato r and s positive to 16SrV. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

2.3.2.4. *Zea mays*

In MS medium 24 seedlings were obtained, but only 9 were tested and, as shows the Table 2.22, 6 were positive for 16SrI phytoplasmas alone or in mixed infection with 16SrXII-A phytoplasmas at 30 days after germination in nested-PCR with primers R16(I)F1/R1 and M1/M2.

Sample	Germination percentages	Phytoplasma detected at 30 days
Corn H		16SrI
Corn HA	83.3%	16SrI+16SrXII-A
Corn HB+HC		16SrI+16SrXII-A
Corn 2	83.3%	Negative
Corn 3	50%	Negative
Corn 4		16SrI
Corn 4A		16SrI
Corn 4B	100%	Negative
Corn 4C		16SrI
Corn 4D		Negative

Table 2.22. Results of phytoplasma detection in *Z. mays* seedlings obtained in MS medium.

From the sowing in sterile soil 79 corn plants were tested, and 17 were positive mostly for 16SrI and 16SrXII-A phytoplasmas at 40 days after germination. Of these plants, 6 were still positive at 90 days after germination in nested-PCR with primers M1/M2, R16(I)F1/R1 and Tuf400/Tuf835 (Table 2.23, Figures 2.18, 2.19 and 2.20).

Sample	Germination percentages	Phytoplasma detected at 40 days	Phytoplasma detected at 90 days
Corn HF	85.7%	16SrI	16SrI
Corn HD		16SrI	Negative
Corn 1.1	73.7%	16SrI	16SrX-A
Corn 1.2		16SrXII-A	Negative
Corn 1.4		16SrI	Negative
Corn 1D		16SrI	16SrI
Corn 3F	94.7%	16SrV	Negative
Corn 3E		16SrI	Negative
Corn 3.1v		16SrI	16SrXII-A
Corn 4A	100%	16SrXII-A	16SrI
Corn 4.5		16SrI	Negative
Corn 4.3v		16SrI	16SrXII-A
Corn 4.1v		16SrXII-A	Negative
Corn 4.4v		16SrI	Negative
Corn 4.1n		16SrI	Negative
Corn 5B	78.9%	16SrI	Negative
Corn 5.4		16SrI	Negative

Table 2.23. Results of phytoplasma detection in *Z. mays* seedlings obtained in sterile soil at 40 and 90 days after germination.

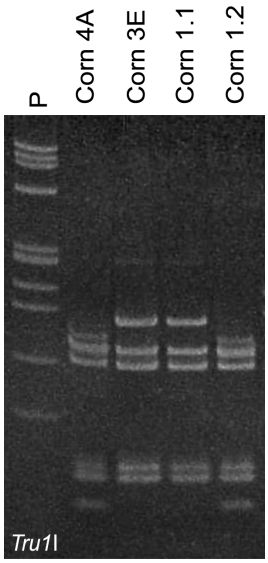

Figure 2.18. RFLP analyses in polyacrylamide gels of corn samples amplified in nested PCR with primers M1/M2 and digested with *Tru1*I restriction enzyme. Corn 4A and 1.2 are positive to 16SrXII-A; corn 3E and 1.1 are positive to 16SrI phytoplasmas. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

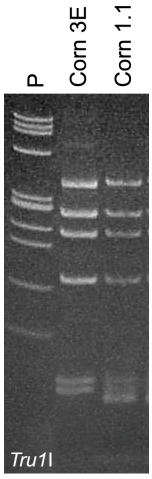

Figure 2.19. RFLP analyses in polyacrylamide gels of corn samples amplified in nested PCR with primers R16(I)F1/ R16(I)R1 and digested with *Tru1*I restriction enzyme. Corn 3E and 1.1 are positive to 16SrI phytoplasmas. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

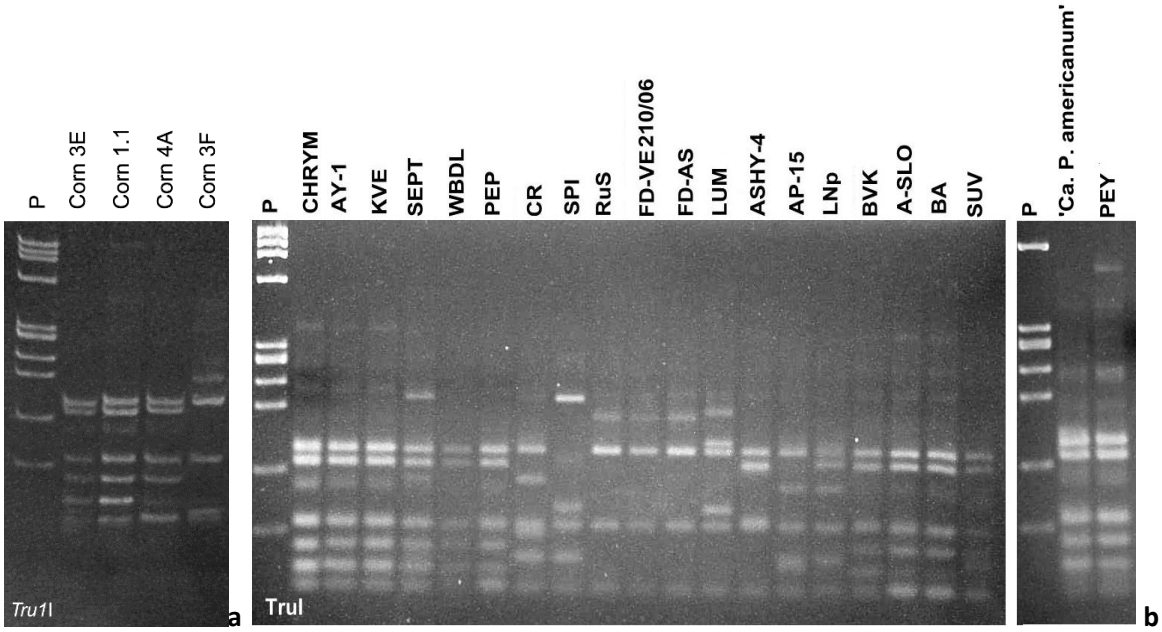

Figure 2.20. RFLP analyses in polyacrylamide gels of corn samples amplified in nested PCR with primers Tuf and digested with *Tru1*I restriction enzyme; **a)** corn 3E and 1.1 are positive to 16SrI; corn 4A is positive to 16SrXII-A and corn 3F is positive to 16SrV phytoplasmas; b) reference strains: CHRYM, 16SrI-A; AY-1, 16SrI-B; KVE, 16SrI-C; SEPT, 16SrI-II-C; WBDL, 16SrII-B; PEP, 16SrII-E; CR, 16SrIII-B; SPI,16SrIII-F; RuS, 16SrV-A; FD-VE, 16SrV-D; FD-AS, 16SrV-C; LUM, 16SrVI-A; ASHY, 16SrVII-A; AP-15, 16SrX-A; LNp, 16SrX-B; BVK, 16SrXI-C; A-SLO, 16SrXII-A; BA, 16SrXII-A; SUV, 16SrXV-A; '*Ca*. P. americanum', 16SrXVIII-A; PEY, 16SrIX-C; P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

2.3.3. Cultivation in artificial media

The samples of *S. indicum*, *B. napus*, *S. lycopersicum* and *Z. mays* obtained from seeds resulted positive for phytoplasma presence, and were also employed for phytoplasmas isolation in artificial media: *S. indicum* and *B. napus* did not show color changing in broth and no colonies where obtained. Some samples of *S. lycopersicum* showed color changing in broth, but the liquid medium resulted negative in PCR/RFLP analysis and no colonies were obtained.

2.3.3.1. *Zea mays*

The Table 2.24 shows the 10 corn seedlings obtained from the sowing in MS medium tested in PCR and used for the isolation trials in medium PivL®. Thirty tubes were tested and three samples resulted positive for 16SrI and 16SrI plus 16SrXII-A phytoplasmas.

Sample	Phytoplasmas detected in seedlings	Inoculated tubes tested by PCR after DNA extraction	Phytoplasma detected in inoculated tubes
Corn H	16SrI	3	Negative
Corn HA	16SrI + 16SrXII-A	3	Negative
Corn HB+HC	16SrI + 16SrXII-A	3	Negative
Corn 2	Negative	3	Negative
Corn 3	Negative	3	16SrI
Corn 4	16SrI	3	16SrI+16SrXII-A
Corn 4A	16SrI	3	Negative
Corn 4B	Negative	3	Negative
Corn 4C	16SrI	3	16SrI+16SrXII-A
Corn 4D	Negative	3	Negative
Total positive	6	30	3

Table 2.24. *Z. mays* samples obtained in MS medium and positive to phytoplasma presence in PivL® medium.

The 17 positive samples at 40 days and the 6 positive samples at 90 days after germination obtained from the sowing in sterile soil were also used for isolation trials in CB liquid medium obtaining after subsequent passages for purification, 52 tubes from the 40 day-old samples and 67 tubes from the 90 day-old samples. Three tubes from both the first and the second group of isolated samples resulted positive for phytoplasma presence after testing by PCR/RFLP analysis (Table 2.25).

Cob sample	40 day-old seedlings used in phytoplasma isolation	Tested tubes inoculated with 40 days-old seedlings	Positive tubes inoculated with 40 days-old seedlings	90 day-old seedlings used in phytoplasma isolation	Tested tubes inoculated with 90 days-old seedlings	Positive tubes inoculated with 90 days-old seedlings
Corn H	2	3	0	1	2	0
Corn 1	4	16	0	2	14	0
Corn 3	3	16	1	1	20	1
Corn 4	6	9	2	2	26	2
Corn 5	2	8	0	0	5	0
Total	17	52	3	6	67	3

Table 2.25. Tubes of CB liquid medium inoculated with corn seedlings and tested for phytoplasmas presence.

Sample	Phytoplasma detected in 40 days-old seedlings	Phytoplasma detected in tubes inoculated with 40 days-old seedlings	Phytoplasma detected in 90 days-old seedlings	Phytoplasma detected in tubes inoculated with 90 days-old seedlings
Corn HD	16SrI	Negative	Negative	Negative
Corn HF	16SrI	Negative	16SrI	Negative
Corn 1.1	16SrI	Negative	16SrX-A	Negative
Corn 1.2	16SrXII-A	Negative	Negative	Negative
Corn 1.4	16SrI	Negative	Negative	Negative
Corn 1D	16SrI	Negative	16SrI	Negative
Corn 3.1	16SrI	Negative	16SrXII-A	Negative
Corn 3E	16SrI	16SrI+16SrVII	Negative	16SrI+16SrIII+16SrXII-A
Corn 3F	16SrV	Negative	Negative	Negative
Corn 4E	16SrXII-A	16SrXII-A	16SrI	16SrXII-A
Corn 4.1	16SrXII-A	Negative	Negative	Negative
Corn 4.2	16SrI	Negative	Negative	Negative
Corn 4.3	16SrI	Negative	16SrXII-A	Negative
Corn 4.4	16SrI	Negative	Negative	Negative
Corn 4.5	16SrI	16SrI	Negative	16XII-A+16SrI+16SrIII
Corn 5B	16SrII	Negative	Negative	Negative
Corn 5.4	16SrI	Negative	Negative	Negative
Total	17	3	6	3

Table 2.26. *Z. mays* samples positive in CB medium with isolation carried out at 40 and 90 days in sterile soil.

Sample	Phytoplasma detected in tubes inoculated with 40 day-old seedlings	Colonies in plates	Phytoplasma detected in tubes inoculated with 90 day-old seedlings	Colonies in plates
Corn HD	Negative	Negative	Negative	Negative
Corn HF	Negative	Negative	Negative	Negative
Corn 1.1	Negative	Negative	Negative	16SrI
Corn 1.2	Negative	Negative	Negative	16SrI
Corn 1.4	Negative	Negative	Negative	Negative
Corn 1D	Negative	Negative	Negative	Bacteria
Corn 3.1	Negative	Negative	Negative	Negative
Corn 3E	16SrI+16SrVII	Negative	16SrI+16SrIII+16SrXII-A	16SrI
Corn 3F	Negative	Negative	Negative	Negative
Corn 4E	16SrXII-A	Negative	16SrXII-A	Bacteria
Corn 4.1	Negative	Negative	Negative	Negative
Corn 4.2	Negative	Negative	Negative	Negative
Corn 4.3	Negative	Negative	Negative	Negative
Corn 4.4	Negative	Negative	Negative	Negative
Corn 4.5	16SrI	Negative	16XII-A+16SrI+16SrIII	Bacteria
Corn 5B	Negative	Negative	Negative	Negative
Corn 5.4	Negative	Negative	Negative	Negative
Total positive	3	0	3	6

Table 2.27. *Z. mays* positive samples isolated from 40 and 90 days old seedlings giving colonies positive for phytoplasma presence in CB medium.

The same 3 samples resulted positive for phytoplasma after 40 and 90 days from germination. Sample corn 3E was positive for mixed infection of phytoplasmas 16SrI and 16SrVII after 40 days and for 16SrI, 16SrIII and 16SrXII-A phytoplasmas after 90 days; sample corn 4E was positive for 16srXII-A; sample corn 4.5 was positive for 16SrI after 40 days and for a mixed infection of 16XII-A, 16SrI and 16SrIII after 90 days (Table 2.26).

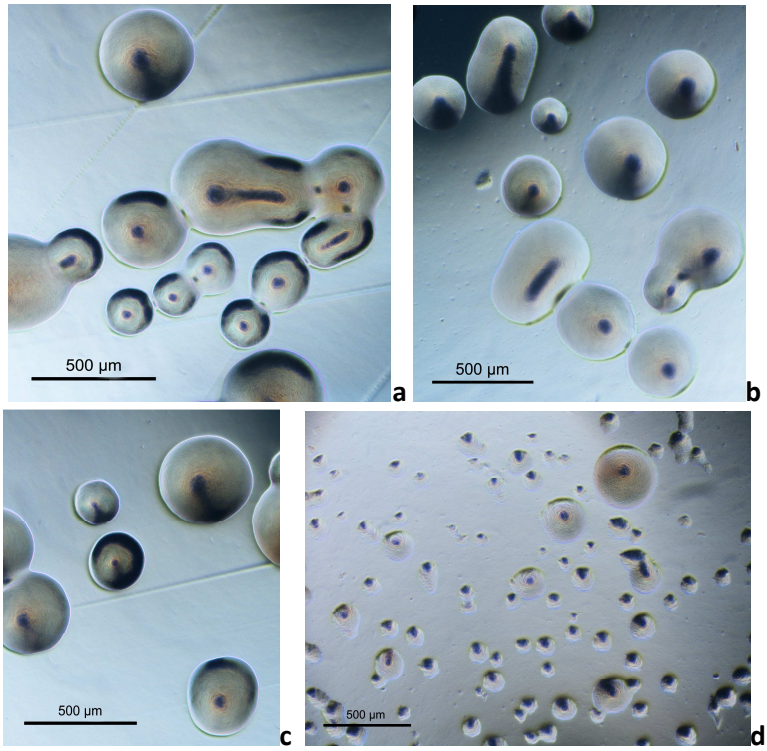

Figure 2.22. Colonies obtained in CB medium from samples corn 1.1 (a), 4.5 (b), 3E (c) and 4E (d).

Using tubes inseminated with plant materials from seedlings at 90 days after germination, it was possible to obtain colonies of different sizes and shapes for the samples corn 3E, 4.5, 4E, 1.1, 1.2 and 1D (Table 2.27). Samples corn 3E, 4.5 and 4E generated colonies also from tubes maintained for seven months at 25°C after initial isolation (Figure 2.22). Single colonies were picked and transferred in broth for purification steps. Small size colonies resulting positive to 16SrI (aster yellows) were obtained from sample corn 3E. (Figure 2.23). These colonies were observed for at least 3 subsequent passages liquid/solid media carried out every 5 days.

Phytoplasma presence in colonies and in CB liquid medium was also tested by qPCR analysis and the results are shown in chapter 3.

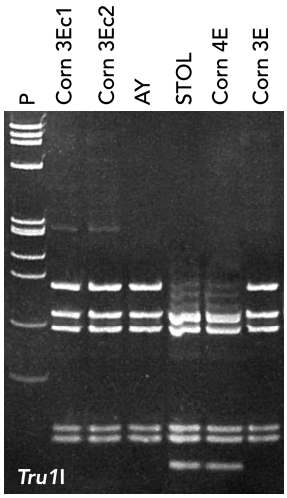

Figure 2.23. RFLP analyses in polyacrylamide gels of samples amplified in nested PCR with primers M1/M2 and digested with *Tru1*I of two single colonies of samples corn 3E positive to 16SrI (corn 3Ec1, c2) and samples corn 4E and 3E from broth positive respectively to 16SrXII-A and 16SrI; P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

2.3.3.2. Differential centrifugations

The CB broth cultures corresponding to different subsequent passages of the sample corn 3E that gave small colonies positive to 16SrI phytoplasmas were employed for differential centrifugations, DNA extraction and PCR/RFLP analysis. The analysis of the pellet obtained after a first centrifugation at 6,000 rpm gave negative results for phytoplasma presence, while the pellet obtained after a father centrifugation at 14,000 rpm resulted positive for 16SrI and 16SrXII-A phytoplasmas (Table 2.28). The supernatants obtained from the first centrifugation at 6,000 rpm were plated and small single colonies positive to 16SrI and 16SrXII-A phytoplasmas were obtained.

Sample	1st pellet	2nd pellet	3rd pellet	Colonies from 1st supernatant
Corn 3Ea	Negative	16SrXII	-	16SrI
Corn 3Eb	Negative	16SrXII	16SrI	16SrXII
Corn 3Ec	Negative	16SrI	16SrI	16SrI

Table 2.28. Analysis by PCR/RFLP of sample corn 3E pellets obtained from CB broth after differential centrifugations and analysis of the colonies obtained from first centrifugation supernatant plated.

2.4. Discussion

Generally, phytoplasmas spreading is explained by propagation of plant material and accidental or feeding movement of insect vectors. However, the sudden epidemic events associated with the

presence of phytoplasmas molecularly indistinguishable, in very distant geographical areas, on the same herbaceous species, seem to indicate the transmission of these prokaryotes also by seeds.

In this study the germination percentages detected depend on the species and on the sowing substrate: when the seeds were sown in sterile soil the chance of obtain seeglings was higer then in sterile substarte, indicating that there is not a decreasing in germination percentage in seeds deriving from phytoplasma-infected mother-plants. Previous studies reported contrasting data on the germination percentage, for instance, Nečas *et al*. (2008) demonstrated that apricot seeds infected by ESFY showed viability and germination lower than the healthy ones, on the contrary, Nipah *et al*. (2007) reported higher germination values for seeds from diseased plants than from healthy plants in coconuts, indicating that the germination is variable and depending on the species and possibly also on the precocity of the infection.

There are some studies that described the presence of phytoplasmas in seeds (Harrison e Oropeza, 1997; Harrison *et al*., 1994; Nipah *et al*., 2007; Nečas *et al*., 2008; Olivier e Galka, 2008; Calari *et al*., 2011) even if is still not clear how they can pass to the seed from the mother-plant because of structural, physical and morphological conformation of the seed. However, there are studies reporting the detection of phytoplasmas in seedlings from a few days after germination (Khan *et al*., 2002; Šeruga Music *et al*., 2004; Botti e Bertaccini, 2006; Zwolinska *et al*., 2010; Calari *et al*., 2011).

In this study, the phytoplasma passage from an infected mother-plant to the first generation seedling through the seed was investigated: the transmission was confirmed by the detection of phytoplasmas in the first generation seedlings. In the case of *S. lycopersicum*, phytoplasmas presence was also detected until the second generation seedlings: this could be considered an evidence of the pathogens transmission because it seems unlikely to be able to detect only phytoplasma DNA after two generations.

Moreover, the seedlings positive to phytoplasmas at about 30-40 days after germination were tested again at about 80-90 (150 for *S. lycopersicum*) days after germination: some samples were still positive even if a lower number of positive samples were detected respect the first testing.

If the phytoplasma detected in both seeds and seedlings, were only in the form of DNA of the pathogen or were a viable and capable of self-replication organism was the subsequent aim of this research. In order to answer this question, isolation in artificial media from the seedlings was performed, obtaining, in the case of some samples of *Z. mays*, colonies positive for phytoplasmas.

These preliminary results indicate the viability of 16SrI (aster yellows) phytoplasmas isolated from corn seedlings and obtained from phytoplasma-infected mother plants and confirm the transmission of viable phytoplasmas trough the seeds.

In all the analyzed species, the phytoplasmas detected in seedlings belong to different ribosomal groups (*S. indicum*: 16SrI, 16SrII and 16SrXII-A, in some cases in mixed infection; *B. napus*: 16SrI, 16SrVI and 16SrXII-A; *S. lycopersicum* group A: 16SrI, 16SrIII, and 16SrXII-A; group B: 16SrI, 16SrII and 16SrV; *Z. mays*: 16SrI, 16srV and 16SrXII-A) indicating a mixed infection of different phytoplasmas, and thus supporting the always present issue of their difficult detection. However, in some *Z. mays* samples, the mixed infection of 16SrI and 16SrXII-A (aster yellows and "stolbur" respectively) was detected in the same seedlings.

This range of phytoplasmas was detected employing different system and combination of primers in the amplification assays. For instance, the use of specific primers sometimes does not allow detecting some phytoplasma groups, but it was useful to confirm the presence of specific phytoplasmas. The use of phytoplasma generic primers instead allows to amplify phytoplasma groups sometimes present at lower concentration in mixed infection with, for example aster yellows, that is the more common phytoplasma detected in the seedlings of the species studied.

To control the spread by seeds of phytoplasma related diseases is of relevant importance mostly about *Z. mays* that, in some areas of the word as their subtropics, undergoes two consecutive sowing in the same field and seeds deriving from the first growing cycle possibly infected by phytoplasmas could remain as disease source allowing the phytoplasmas spread through the insect vectors (*D. maydis*) to the second sowing cycle plants.

3. General phytoplasma detection by a q-PCR method using mycoplasma primers

3.1. Introduction

Phytoplasmas and mycoplasmas are pathogenic bacteria belonging to the class *Mollicutes* (trivial name mollicutes) because they lack a rigid cell wall (Lee *et al*., 1998a; Razin *et al*., 1998). They are the living prokaryotes with the smaller genome size having also a low G+C content, capable of self-replication but with relatively limited metabolic capacities (Razin and Freundt, 1994; Oshima *et al*., 2004; Kube *et al*., 2012). Both bacteria have economic and clinical importance. Despite their phylogenetic relatedness, phytoplasmas and mycoplasmas differ in several aspects, and while phytoplasmas are plant pathogens, mycoplasmas are vertebrate parasites (Chen *et al*., 2012). Phytoplasmas are phloem limited bacteria and cause devastating diseases in many agricultural important crops; they are transmitted by insects, grafting, dodder or seeds (Lee *et al*., 2000; Calari *et al*., 2011; Bertaccini *et al*., 2014). Considering that phytoplasmas pure axenic culture is still a problematic issue (Contaldo *et al*., 2016), the availability of fast and reliable molecular methods able to increase the sensitivity of their detection is very important. The nested-PCR method, using primers based on conserved genes, is the most used and sensitive methodology for phytoplasma detection (Lee *et al*., 1995), but this method may encounter contamination problems and reduced sensitivity due to the presence of inhibitors in plant extracts (Heinrich *et al*., 2001). A quantitative PCR (qPCR) approach could help to overcome these issues, considering the low levels of manual operation obtained also by system automatization that is reducing the contamination possibility and does not need gel electrophoresis to verify results reducing therefore the testing time. Several qPCR assays were developed to detect group specific phytoplasmas (Angelini *et al*., 2007; Torres *et al*., 2005; Baric *et al*., 2006; Aldaghi *et al*., 2009; Nejat *et al*., 2010; Monti *et al*., 2013; Mehle *et al*., 2013a). However, only some qPCR assays were developed to detect all phytoplasmas based on both *16S rRNA* (Christensen *et al*., 2004; Galetto *et al*., 2005) and *23S rRNA* genes (Hodgetts *et al*., 2009); these studies or considered only a limited number of ribosomal groups or they are expensive because based on TaqMan chemistry. A general qPCR assay able to verify phytoplasma presence at very low concentrations, such as in plant propagation materials (dormant cuttings, seedlings) or in cultures is of great practical relevance. Vega-Orellana *et al*. (unpublished)

optimised a SYBR Green-based real time PCR assays from a conventional PCR previously described by Botes *et al*. (2005) being able to detect mycoplasmas, acholeplasmas, mesoplasmas, hemoplasmas, spiroplasmas and ureaplasmas in a highly sensitive and specific manner with the combination of fluorescence reading and melting peak temperature analysis. In this study a qPCR analysis with these universal mycoplasmas primers pair targeting the 16S *rRNA* gene (Botes *et al*., 2005) was tested to detect phytoplasmas belonging to several ribosomal groups.

3.2. Materials and methods

3.2.1. DNA samples

Catharanthus roseus healthy and phytoplasma infected micropropagated shoots were used as source of phytoplasma DNA (Bertaccini, 2014). The following phytoplasma strains were used: '*Candidatus* Phytoplasma asteris' (primula virescence, PRIVA, 16SrI-B), '*Ca*. P. australasia' (tomato big bud, TBB, 16SrII-D), faba bean phyllody (FBPSA, 16SrII-C), '*Ca*. P. pruni' (peach X-disease, CX, 16SrIII-A), '*Ca*. P. ulmi' (elm yellows, EY, 16SrV-A), lucerne virescence (LUM, 16SrVI), '*Ca*. P. faxini' (ash yellows, ASHY, 16SrVII-A), *Pichris echioides* yellows (PEY, 16SrIX-C), '*Ca*. P. prunorum' (European stone fruit yellows, ESFY, 16SrX-B), '*Ca*. P. mali' (apple proliferation, AP-15, 16SrX-A), '*Ca*. P. pyri' (pear decline, PD, 16SrX-C), leafhopper-borne phytoplasma (BVK, 16SrXI-C), '*Ca*. P. solani' ("stolbur", STOL, 16SrXII-A), Suriname virescence (SuV, 16SrXV). Field infected samples were also tested: peach (*Prunus persica*), plum (*Prunus domestica*) and apricot (*Prunus armeniaca*) showing symptoms of leptonecrosis (plum), chlorotic leafroll, (apricot) and yellows (peach). Moreover, tomato (*Solanum licopersicum*) and corn (*Zea mays*) plantlets deriving from seeds produced by phytoplasma-infected mother-plants were analysed (Table 3.1 and 3.2). Total DNA was extracted grinding with liquid nitrogen 1 g of tissue from each sample with pestles in sterile porcelain mortars. A healthy periwinkle shoot was prepared in the same way and employed as negative control. The DNA was then extracted with a phenol/chloroform method (Prince *et al*., 1993) (Appendix 1) and re-suspended in 1X TE buffer, quantified by spectrophotometer at 260 nm and diluted until 20 ng/µl. Some DNAs extracted from both phytoplasma liquid CB medium (Contaldo *et al*., 2016) and from colonies derived from these liquid cultures were also analysed: Corn 3E, Corn 4.5, Corn 4E and Corn 1.1 (Table 3.3). For these samples the DNA was extracted by DNaesy Plant Minikit, QIAGEN (Appendix 1).

3.2.2. Phytoplasma detection by qualitative analyses

The presence of phytoplasmas in the above materials was verified by conventional PCR and nested-PCR using 16Sr DNA universal primers. In particular, for the phytoplasma infected micropropagated shoots, P1 (5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3') as forward (Deng and Hiruki, 1991) and P7 (5'-CGT CCT TCA TCG GCT CTT-3') as reverse (Schneider *et al*., 1995) generic primers for phytoplasma detection, were used. In nested PCR, primer pairs R16F2n (5'-GAAACGACTGCTAAGACTGG-3') (Gundersen and Lee, 1996) /R16R2 (5'-TGACGGGCGGTGTGTACAAACCCCG-3') (Lee *et al*., 1993a) and 16R758f (=M1) (5'-GTCTTTACTGACGCTGAGGC-3')/16R1232r (=M2) (5'-CTTCAGCTACCCTTT GTAAC-3') (Gibb *et al*., 1995) were used for the field-collected plants and seedlings produced by infected mother-plants. For the DNA samples from liquid CB medium and colonies, primers pair R16F2n /R16R2 in direct PCR and M1/M2 in nested PCR were employed for the qualitative analysis.

Further PCR using GPO3F as forward (5'-TGGGGAGCAAACAGGATTAGATACC-3') and MGSO as reverse (5'-TGCACCATCTGTCACTCTGTTAACCTC-3') (Botes *et al*., 2005), generic primers originally designed for mycoplasma conventional PCR detection, were used for all samples. The PCR protocol described in Appendix 2 was done using the Cabru Red *Taq* DNA polymerase, Rovalab. In order to confirm phytoplasma identity, RFLP analysis of P1/P7, M1/M2 and GPO3F/MGSO PCR products was performed with *Tru1*I (Fermentas, Vilnius, Lithuania) restriction enzyme according with the instruction of the manufacturer (Appendix 3).

3.2.3. q-PCR conditions

The same DNAs were then tested by qPCR using the primer pair GPO3F/MGSO in an ICycler-IQ5 (Bio-Rad). The reaction mixture was prepared in a final volume of 25 µl, including 12.5 µl of 2X SYBR Green I (Bio-Rad), 1 µl of GPO3F/MGSO primers, and 2.5 µl of diluted DNA (20 ng/µl). Amplifications were performed in 96-well Hard-shell PCR plates (Bio-Rad). The following thermal cycling conditions were used: one cycle at 95°C for 3 min followed by 35 cycles at 95°C for 1 min, 61°C for 1 min, and 72°C for 1 min. To verify the specificity of the product obtained, a melting curve was performed at the end of the PCR reaction with an increase of the temperature specificity of 0.5°C/s, from 60 to 97.5°C. The assays were carried out in duplicate, each experiment was repeated three times and the mean comparisons were evaluated. An additional samples containing water instead of DNA, were added to each plate in triplicate as DNA-free negative controls.

3.2.4. qPCR specificity evaluation

In order to determine the assay specificity, DNAs of bacteria were used as controls in qPCR assays: '*Ca.* L. solanacearum' from carrot (Satta *et al*., 2016) and *X. fastidiosa* strain CoDiRO-SC from olive from Apulia, Salento (Italy) (Contaldo *et al*., unpublished), *Ralstonia solanacearum* (strain IPV-BO 5836) and *Clavibacter michiganensis* (strain IPV-BO 7695) (Biondi *et al*., 2014; Blasioli *et al*., 2014) from pure cultures. These latter were extracted by DNeasy Plant Mini Kit- QIAGEN and suspended in 1X TE buffer. All these DNAs were quantified by spectrophotometer at 260 nm and diluted until 20 ng/µl. They were positive for specific pathogen presence with the specific primer pairs (Liefting *et al.,* 2009; Jagoueix *et al.,* 1996; Munyaneza *et al.,* 2009; Minsavage *et al*., 1994; Seal *et al*., 1993; Pastrik, 2000) respectively.

3.2.5. Establishment of standard curves for DNA quantification

For phytoplasma quantification two standard curves were produced. The PCR products obtained from the phytoplasma strains ESFY (16SrX-B) and STOL (16SrXII-A) with the primer pair GPO3F/MGSO were extracted from the electrophoresis gel by Promega Wizard Gel and PCR Clean-Up System kit, ligated into pGEM-T Easy Vector plasmid and transformed into competent *Escherichia coli* strain JM109 (Promega). The DNA fragments were then excised from plasmid and sequenced to verify the cloned fragment sequences. The DNA extracted was suspended in 1X TE buffer, quantified by using NanoQuant Infinite M200 PRO (Tecan), and stored at -20°C until use.

3.2.5.1. Insert isolation and plasmid ligation

The ESFY and STOL amplicons obtained with primer pair GPO3F/MGSO were run in agarose gel 1% in 1X TBE buffer, stained in ethidium bromide and visualized by UV. The corresponding bands were recovered from gel in Eppendorf tubes and purified by Wizard® SV Gel a PCR Clean-Up System (Promega): 10 µl per mg of membrane binding solution was added to each sample. The mixtures were vortexed for a few seconds and then incubated at 65°C in a thermo-block; every 2-3 minutes, the Eppendorf tubes were vortexed to faster dissolve the gel. Subsequently, the content of the tube was transferred to the SV minicolumn placed in a 2 ml collection tube and incubated at room temperature for 1 minute. The tube was then centrifuged for 1 minute 11,000 rpm to link only DNA to the membrane of the column. The eluate was eliminated and the column was put back in the collection tube; 700 µl of Membrane Wash solution were added. The tube was centrifuged and the eluate was again eliminated. The washing was repeated with 500 µl of Membrane Wash Solution, followed by a centrifugation at 11,000 rpm for 5 minutes. The contents of the collection

tube were eliminated again and the column replaced on the tube and centrifuged for 1 min. The column was transferred to a 1.5 ml Eppendorf tube and 50 µl of nuclease free water were added in the centre of the column. After a centrifugation for 1 min at 11,000 rpm, the eluate was recovered and stored at -20°C until use. The recovered amplicons were then ligated in the plasmid pGEM-T Easy Vector (Promega) following the cloning manufacturer instructions. The reaction mixtures were prepared in a final volume of 10 µl containing 1 µl of plasmid pGEM-T, 1 µl of T4 DNA Ligase, 5 µl of Buffer 2X and 3 µl of insert. The ligation reaction was set up at 4°C overnight.

3.2.5.2. Transformation and cloning in competent cells

Three µl of ligase mixture were added to 100 µl of competent cells JM109 (Promega) and maintained for 30 min in ice. After that, the cells were put at 42°C for 90 seconds and in ice for 2 min in order to obtain the plasmid uptake through the open membrane pores of the cells. After the addition of 900 µl of SOC medium (Fluka SOC broth), the cells were incubated, in agitation, at 37°C for 2 hours. The cells were then plated (200 and 400 µl) in LB agar medium (Sigma) containing ampicillin (100 µg/ml), IPTG (isopropil-β-D-tiogalattopyranoside; 0.5 mM; Promega) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; 80 µg/ml; Promega). The plates were incubated al 37°C overnight and the day after the white colonies were counted. PCR with universal primer pair M13f (5'-TGTAAAACGACGGCCAGT-3') and M13r (5'-CAGGAAACAGCTATGACC-3') was carried out to confirm successful transformation. Some isolated colonies were transferred with sterile loop on another plate of LB agar medium (Sigma) containing IPTG, X-Gal and Ampicillin, on which a numbered grid was drawn. The plate was incubated at 37°C overnight and then stored at 4°C. Each sterile loop was put in PCR tube containing SDW and washed. Of this SDW, 5 µl were used for PCR that was visualized in agarose gel 1% in 1X TBE buffer.

3.2.5.3. Purification and quantification of the plasmid

The DNA of the transformed cells was extracted: from 10 ml of bacterial culture, the pellet was collected by centrifugation for 5 min at 10,000 rpm, and the supernatant was eliminated; 250 µl of resuspeding solution from Wizard Plus SV Minipreps kit were added and the pellet was re-suspended by vortexing. Then 250 µl of cell lysis buffer were added and mixed by inverting the tube four times; the tubes were incubated at room temperature for 1-5 min. After that, 10 µl of alkaline protease solution were added and mixed. Subsequently, 350 µl of neutralization solution were added and mixed. The bacterial lysate was centrifuged for 10 min at 14,000 rpm at room temperature. Approximately 850 µl of lysate were transferred with a micropipette in a "Wizard Plus SV Minipreps spin column" contained in a 2 ml collection tube. The samples were centrifuged

for 1 min at 14,000 rpm at room temperature and subsequently, the flow-through was eliminated. The column was washed adding 750 µl of washing solution previously diluted with 95% ethanol. After centrifugation for 1 min at 14,000 rpm at room temperature, the flow-through was eliminated. This step was repeated adding 250 µl of washing solution, again centrifuged for 2 min at 14,000 rpm at room temperature. The "Wizard Plus SV Minipreps spin column" was transferred to a clean Eppendorf tube and the DNA Plasmid was eluted by adding 100 µl of nuclease free water. It was centrifuged for 1 min at 14,000 rpm at room temperature. Finally, the column was removed from the tube and discarded; the sample obtained was stored at -20°C until use.

The plasmid DNA was then digested with the restriction enzyme *Eco*RI. The mix was composed by 1 µl buffer 10X, *Eco*RI (12 U) 0.5 µl, DNA 1 µl and SDW 7.5 µl. The samples were incubated for 3 hours at 37°C and digestion products were examined after electrophoresis in 0.8% agarose gel.

The ligated amplicons were used in qPCR to perform the standard curves that were obtained using 10-fold serial dilutions ranging from 2 ng $\times 10^{-3}$ to 2 ng $\times 10^{-8}$ of plasmid with insert amplicon GPO3F/MGSO of pESFY and pSTOL. The standard curves were constructed by the interpolation of the log10 of the copy number of each standard with their respective Ct values. The concentration was converted in copy number of insert per sample, obtaining dilutions ranging from 6.86 $\times 10^{6}$ to 6.86 $\times 10^{2}$ copy numbers/µl. The number of copies of template (phytoplasma *16S rRNA* gene) in the sample was calculated by the formula: number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650). The tool used for the calculation is available at URI Genomics & Sequencing Center, http://cels.uri.edu/gsc/cndna.html.

3.2.6. qPCR sensitivity, accuracy and repeatability evaluation

To test the sensitivity of the qPCR, a comparison with the conventional nested PCR approach was carried out: 10-fold serial dilutions (from 2 ng $\times 10^{-3}$ to 2 ng $\times 10^{-8}$) of pESFY and pSTOL DNAs were tested in both methods with primers pair GPO3F/MGSO. According to Vaerman *et al*., 2004, accuracy was taken in care by a careful design, and calibration of the DNA standard curves. Repeatability of the assay was evaluated by running three replicates for each sample with a standard curve in each run (Bustin *et al*., 2009).

3.2.7. qPCR data interpretation

Analysis of the Ct value was performed using the machine supplied qPCR software (Real-Time Detection System software version 3.0. Bio-Rad, for Windows). About the melt-curve analysis, the

iCycler iQ5 system allows to record the fluorescence generated by the SYBR Green I that binds to the double-strand DNA and converts this florescence in temperature changes. A melt curve is a plot of the first derivative of the change in fluorescence intensity as a function of temperature. The -dF/dT on the temperature gives the peaks and the Tm for a sample is the temperature at which the peak is higher. A sample was considered positive for phytoplasma DNA if it had a Tm of 82.5 ± 0.5°C. The results are reported as means ± SE.

The software allows to calculate the fluorescence acquisition and the Ct. The slope (k) of the linear regression line between logarithmic values of relative DNA concentrations (y-axis) and Ct values (x-axis) was used to calculate the amplification efficiency, E =(10[-1/k])-1, where a value of one corresponds to 100% amplification efficiency. The squared regression coefficient after the linear regression (R^2) was determined. The dynamic range, that is the range of concentrations for which Ct values were in linear relationship with logarithms of concentrations and range of detection, was also determined. Relative quantification of phytoplasma samples was estimated from the extrapolation of the values from a 10-fold dilution standard curve.

3.3. Results

3.3.1. Qualitative PCR assay

Tables 3.1 and 3.2 show the list of phytoplasma DNAs extracted from periwinkles, from field infected plants and seedlings and the results of their testing in conventional nested PCR with primer pairs P1/P7 or M1/M2 and GPO3F/MGSO. The melting temperature of the phytoplasma DNAs varied between 82-83ºC. The DNAs from seedlings were positive in direct PCR with primers GPO3F/MGSO and in second nested PCR with the system P1/P7, R16F2n/R16R2, 16R758/16R1232r. The phytoplasma groups characterization by RFLP analysis on GPO3F/MGSO amplicons shows different patterns in agreement with the available classification (Lee *et al*., 1998a; 1998b) (Figure 3.1). The field infected plants resulted positive in direct PCR with primers P1/P7 and were classified as ESFY-infected after appropriate RFLP analyses (data not shown). The seedlings were positive for 16SrI and 16SrXII phytoplasma ribosomal group in nested PCR/RFLP analyses (data not shown).

Phytoplasma strain and acronym	Subgroup determined by conventional PCR /RFLP	Tm (°C)	Ct values (°) (±SE)
Primula virescence - PRIVA	16SrI-B	83.0	26.22 (±0.34)
Tomato big bud - TBB	16SrII-D	82.5	23.84 (±0.23)
Faba bean phyllody - FBPSA	16SrII-C	82.5	27.26 (±0.40)
Peach X-disease - CX	16SrIII-A	-	-
Elm yellows - EY	16SrV-A	82.5	24.72 (±0.38)
Lucerne virescence - LUM	16SrVI	82.5	20.91 (±0.13)
Ash yellows - ASHY	16SrVII-A	82.5	26.07 (±0.29)
Pichris echioides yellows - PEY	16SrIX-C	-	-
European stone fruit yellows - ESFY	16SrX-B	82.5	24.52 (±0.18)
Apple proliferation - AP-15	16SrX-A	82.5	26.68 (±0.21)
Pear decline - PD	16SrX-C	82.5	24.83 (±0.01)
Leafhopper-borne - BVK	16SrXI-C	83.0	23.37 (±0.08)
"Stolbur" - STOL	16SrXII-A	82.5	22.22 (±0.01)
Suriname virescence - SuV	16SrXV	83.0	25.35 (±0.43)
Other bacteria			
Ralstonia solanacearum	n.a.	-	-
Clavibacter michiganensis subsp. *sepedonicus*	n.a.	-	-
'*Ca*. Liberibacter solanacearum'	n.a.	85	27.52 (±0.01)
Xylella fastidiosa subsp. *pauca* strain CoDIRO-SC	n.a.	-	-

Table 3.1. Phytoplasmas and other bacteria used in conventional PCR/RFLP analysis and qPCR amplified with primer pair GPO3F/MGSO (Botes *et al*., 2005); n.a., not applicable; -, not obtained.

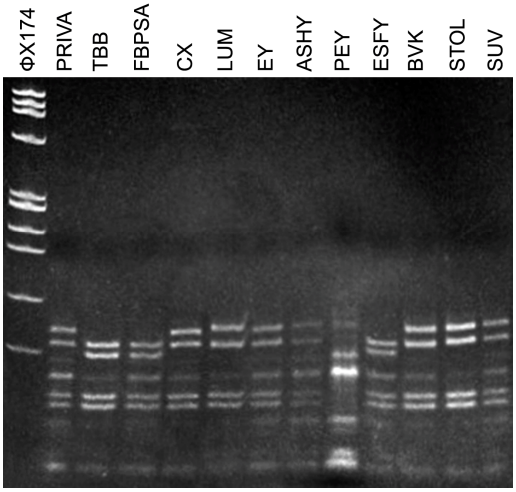

Figure. 3.1. RFLP analyses in polyacrylamide 6.7% gels of samples amplified in PCR with primers GPO3F/MGSO and digested with *Tru1*I. From the left: marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72. Phytoplasma strains: PRIVA, primula virescence (16SrI-B); TBB, tomato big bud (16SrII-D), FBPSA, faba bean phyllody (16SrII-C) CX, peach X disease (16SrIII-A); LUM, lucerne virescence (16SrVI); EY, elm yellows (16SrV-A); ASHY, ash yellows (16SrVII-A); PEY, *Pichris echioides* yellows (16SrIX-C). ESFY, European stone fruit yellows (16SrX-B); BVK, leafhopper-borne (16SrXI-C); STOL, "stolbur" (16SrXII-A); SuV, Suriname virescence (16SrXV-A).

Sample	Conventional PCR/RFLP	Tm (°C)	Ct values (°) (±SE)
Corn 1.1	16SrI/X-A	-	-
Corn 1.2	16SrXII-A	82.0	28.02 (±0.41)
Corn 4.1v	16SrXII-A	-	-
Corn 4.1n	16SrI	-	-
Corn 3.1v	16SrI/XII-A	83.0	30.79 (±0.15)
Corn 4.4v	16SrI	82.0	25.86 (±0.39)
Corn 4.3v	16SrI/XII-A	-	-
Corn 4.5	16SrI	-	-
Tomato 9	16SrI	-	-
Tomato 11	16SrI	82.5	27.44 (±0.24)
Tomato 23	16SrI	82.5	30.01 (±0.26)
Apricot 1C2	16SrX-B	82.5	24.55 (±0.39)
Plum Rome 4	16SrX-B	82.5	24.69 (±0.37)
Apricot 1A7	16SrX-B	-	-
Plum Rome 7	16SrX-B	-	-
Peach Verona 8	16SrX-B	-	-
Peach Verona 5	16SrX-B	82.5	26.24 (±0.37)

Table 3.2. Conventional PCR/RFLP analysis and qPCR results from field infected and seedling samples amplified with primer pair GPO3F/MGSO (Botes *et al*., 2005); -, not obtained.

Sample	Subgroup determined by conventional PCR /RFLP	GPO3F/MGSO Tm (°C) /Ct values (°) (±SE)
Corn 3E (liquid)	16SrI, -VII, -III, -XII	Double peak: 80.5-83.0/30.56 (± 0.15)
Corn 3E (colonies)	16SrI	-
Corn 4.5 (liquid)	16SrI, -XII	-
Corn 4.5 (colonies)	Bacteria	Double peak: 77.0-81.0/ 30.19 (± 0.47)
Corn 4E (liquid)	16SrXII, -I, -III	Double peak: 77.0-82.5/ 27.61 (± 0.05)
Corn 1.1 (colonies)	16SrI	-

Table 3.3. Results of qPCR on phytoplasmas in liquid media and colonies. -, not obtained.

The table 3.3 shows the result of the qPCR on DNAs from colonies and CB liquid medium: in the samples Corn 3E from CB liquid medium a double peak was generated indicating the presence of phytoplasma DNA detected by the melting at 83.0°C but also the presence of another organism because of the other peak at 80.5°C. The figure 3.2 shows the double peak. The same for the sample Corn 4E (liquid). Different is the double peak of the sample Corn 4.5 (DNA from colonies) where no phytoplasma was detected in fact, the two peaks have a Tm different from 82.5 ± 0.5 °C that is the one indicating phytoplasma presence.

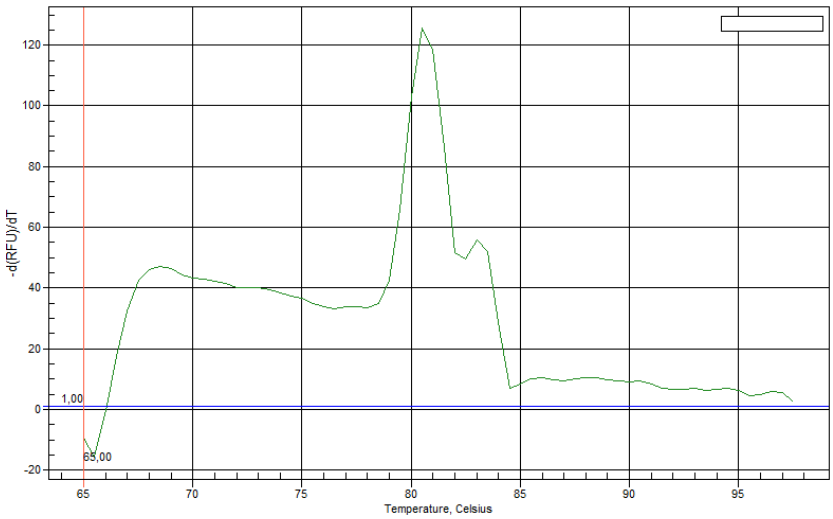

Figure 3.2. Sample corn 3E liquid CB medium amplified in qPCR with primers GPO3F/MGSO. Two peaks were obtained indicating the presence of phytoplasmas, (Tm peak at 83.0°C), and another bacterium for the other peak (Tm peak at 80.5°C).

3.3.2. q-PCR assay specificity

The q-PCR assay using primers GPO3F/MGSO detected a wide range of phytoplasma groups (Table 3.1), but no amplification curves were observed with the tested bacteria: *R. solanacearum*, *C. michiganensis* subsp. *sepedonicus*, and *X. fastidiosa subps. pauca.* '*Ca.* L. solanacearum' instead was amplified but with a different Tm (85°C) and a Ct of 27.52 (±0.01). In fact, the analysis of the Tm allowed discriminating the results obtained from phytoplasmas to those of non-mollicutes. All the dissociation curves from phytoplasma DNAs showed a single peak at 82.5°C (±0.5) and amplification curves at Ct values from 21 and 27. The Tm of the field infected apricot, peach and plum samples resulted 82.5°C with Cts of 24.55, 26.24 and 24.69 respectively, in the same range of the phytoplasma control Ct values. Moreover, also tomato and corn seedlings positive for the presence of phytoplasmas belonging to the ribosomal groups 16SrI or 16SrXII resulted positive in qPCR with a Tm of 82.5 ± 0.5 and Ct values between 25.9 and 30.8 respectively.

3.3.3. Evaluation of standard curves efficiency and phytoplasma quantification

The melting temperature analysis of the standard curves showed a single peak at Tm 82.5°C (±0.5) and non-specific products were not detected. To simplify the quantification analysis, the pESFY and pSTOL curves were overlapped showing a perfect match in term of efficiency, thus the pESFY curve was selected as reference to quantify the phytoplasma titer in the samples. A mean slope of -3.228 and a $R^2 > 0.995$ demonstrated an efficiency of 104.1% (Figure 3.3). Samples with Ct higher than the one of the last standard (standard 10^{-7}), which corresponds to Ct 31.56, were considered

negative. Phytoplasma titer and copy number were evaluated as shown in table 3.4: phytoplasma copy number ranged from 1.75×10^{6} ($\pm$ 1.62×10^{5}) to 1.15×10^{3} ($\pm$ 4.54×10^{2}) depending on sample.

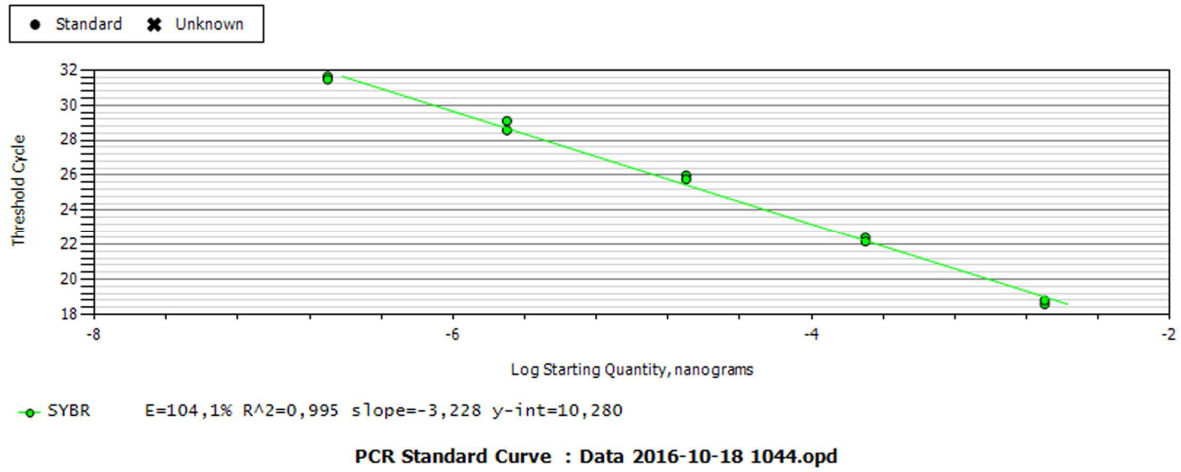

Figure 3.3. CT values obtained with 10-fold serial dilutions ranging from 2 ng $\times10^{-3}$ to 2 ng $\times10^{-7}$ of plasmid with insert amplicon GPO3F/MGSO ESFY phytoplasma DNA.

Sample	Ct Mean (±SE)	Quantity/ ng (±SE)	Quantity/ copy number (±SE)
ESFY 2×10^{-3}	18.68 (± 0.15)	2.00×10^{-3} (-)	6.86×10^{6} (-)
ESFY 2×10^{-4}	22.29 (± 0.16)	2.00×10^{-4} (-)	6.86×10^{5} (-)
ESFY 2×10^{-5}	25.88 (± 0.15)	2.00×10^{-5} (-)	6.86×10^{4} (-)
ESFY 2×10^{-6}	28.84 (± 0.37)	2.00×10^{-6} (-)	6.86×10^{3} (-)
ESFY 2×10^{-7}	31.56 (± 0.12)	2.00×10^{-7} (-)	6.86×10^{2} (-)
PRIVA	26.22 (± 0.33)	1.15×10^{-5} ($\pm$ 2.74×10^{-6})	3.95×10^{4} ($\pm$ 9.40×10^{3})
ASHY	26.07 (± 0.28)	1.81×10^{-5} ($\pm$ 2.60×10^{-6})	4.41×10^{4} ($\pm$ 8.91×10^{3})
TBB	23.84 (± 0.22)	6.30×10^{-5} ($\pm$ 9.85×10^{-6})	2.16×10^{5} ($\pm$ 3.38×10^{4})
FBPSA	27.26 (± 0.40)	5.49×10^{-6} ($\pm$ 1.56×10^{-6})	1.89×10^{5} ($\pm$ 5.37×10^{3})
ESFY	24.52 (± 0.21)	3.88×10^{-5} ($\pm$ 5.92×10^{-6})	1.33×10^{5} ($\pm$ 2.03×10^{4})
AP	26.68 (±0.21)	8.34×10^{-6} ($\pm$ 1.31×10^{-6})	2.86×10^{4} ($\pm$ 4.48×10^{3})
PD	24.83 (±0.01)	3.15×10^{-5} ($\pm$ 6.36×10^{-7})	1.08×10^{5} ($\pm$ 2.18×10^{3})
BVK	23.37 (± 0.08)	8.82×10^{-5} ($\pm$ 4.70×10^{-6})	3.03×10^{5} ($\pm$ 1.61×10^{4})
LUM	20.91 (± 0.13)	5.09×10^{-4} ($\pm$ 4.72×10^{-5})	1.75×10^{6} ($\pm$ 1.62×10^{5})
STOL	22.22 (± 0.01)	1.99×10^{-4} ($\pm$ 5.16×10^{-7})	6.84×10^{5} ($\pm$ 1.77×10^{3})
EY	24.72 (± 0.39)	3.36×10^{-5} ($\pm$ 9.31×10^{-6})	1.15×10^{5} ($\pm$ 3.19×10^{4})
SuV	25.35 (± 0.45)	2.14×10^{-5} ($\pm$ 6.85×10^{-6})	7.34×10^{4} ($\pm$ 2.35×10^{4})
Apricot 1C2	24.39 (± 0.42)	4.35×10^{-5} ($\pm$ 1.30×10^{-5})	1.49×10^{5} ($\pm$ 4.45×10^{4})
Plum Rome 4	24.51 (± 0.40)	3.98×10^{-5} ($\pm$ 1.11×10^{-5})	1.37×10^{5} ($\pm$ 3.81×10^{4})
Peach Verona 5	26.07 (± 0.45)	1.30×10^{-5} ($\pm$ 3.25×10^{-6})	4.47×10^{4} ($\pm$ 1.12×10^{4})
Corn 1.2	28.02 (± 0.41)	3.36×10^{-6} ($\pm$ 2.37×10^{-7})	1.15×10^{4} ($\pm$ 8.14×10^{2})
Corn 3.1v	30.79 (± 0.15)	3.36×10^{-7} ($\pm$ 1.32×10^{-7})	1.15×10^{3} ($\pm$ 4.54×10^{2})
Corn 4.4v	25.86 (± 0.39)	2.01×10^{-5} ($\pm$ 8.65×10^{-6})	6.90×10^{4} ($\pm$ 2.97×10^{4})
Tomato 11	27.44 (± 0.24)	5.00×10^{-6} ($\pm$ 2.53×10^{-7})	1.72×10^{4} ($\pm$ 8.67×10^{2})
Tomato 23	30.01 (± 0.26)	8.78×10^{-7} ($\pm$ 1.60×10^{-7})	3.01×10^{3} ($\pm$ 5.49×10^{2})

Table 3.4. qPCR quantification results for standard curves, phytoplasma controls and phytoplasma infected samples in terms of Ct values and quantity in ng and copy number.

3.3.4. q-PCR assay sensitivity

To test the sensitivity of the qPCR assay developed, the serial dilutions of the cloned fragment GPO3F/MGSO from ESFY phytoplasma DNA were compared with the same serial dilutions obtained in conventional PCR.

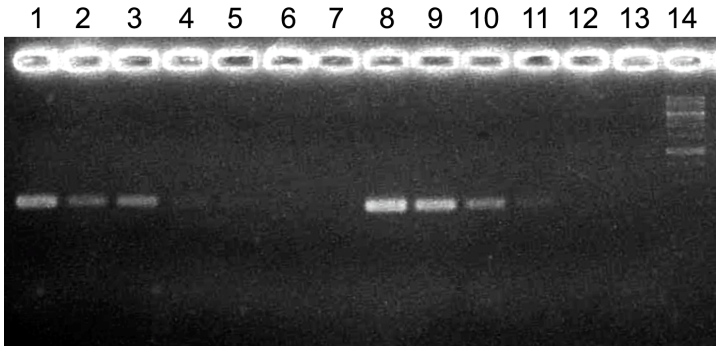

Figure 3.4. Sensitivity of conventional PCR in 1% agarose gel of samples amplified with primers GPO3F/MGSO. From the left: standard dilutions of ESFY plasmid from 2 $\times 10^{-3}$ (1) to 2 $\times 10^{-8}$ (6); negative control, water (7); standard dilutions of STOL plasmid from 2 $\times 10^{-3}$ (8) to 2 $\times 10^{-8}$ (13); marker 1Kb (14), fragment sizes in base pairs from top to bottom of 10,000; 8,000; 6,000; 5,000; 4,000; 3,500; 3,000; 2,500; 2,000; 1,500; 1,000; 750; 500 and 250.

The last dilution observed in conventional PCR was 10^{-6}, while the last dilution detected by this qPCR method was 10^{-7} corresponding to Ct 31.56 indicating a better performance of the qPCR; in Figure 3.4 it is shown the sensitivity of the qualitative PCR with primers GPO3F/MGSO where differences in band intensity of the different sample dilutions from 2 ng $\times 10^{-3}$ to 2 ng $\times 10^{-8}$ are present. The last dilution in which the DNA was detected is 2×10^{-6}.

3.4. Discussion

Several qPCR protocols for phytoplasma specific detection and quantification were developed, however they mostly concern the detection of specific phytoplasma ribosomal groups. For example, many studies regard the detection of '*Ca*. P. mali', '*Ca*. P. prunorum' and '*Ca*. P. pyri' representing respectively the subgroups -A, -B and -C of the ribosomal group 16SrX (Torres *et al*., 2005; Nikolic *et al*., 2010; Yvon *et al*., 2009; Monti *et al*., 2013; Mehle *et al*., 2013a). Similar studies concern the detection of '*Ca.* P. phoenicium' (Jawhari *et al*., 2015) in stone fruit, "flavescence dorée" (FD) and "bois noir" (BN) phytoplasmas in grapevine (Mehle *et al*., 2013b; Galetto *et al*., 2005). However only a few qPCR universal phytoplasma assays were developed (Galetto *et al*., 2005; Hodgetts *et al*., 2009). These studies mentioned above were obtained utilizing different chemistry, from SYBR Green and EvaGreen to TaqMan probes. Moreover, the primer pairs used in

previous studies of universal qPCR produced 500-600 bp amplicons (Christensen *et al*., 2004; Hodgetts *et al*., 2009) while the primer pair GPO3F/MGSO are 270 bp long, allowing to achieve better sensitivity. On the other hand, amplicon shorter than GPO3F/MGSO were also employed (Galetto *et al*., 2005) but they were tested only for phytoplasmas belonging to ribosomal groups 16SrV -XII and -X.

The aim of this study was to develop a quick very sensitive assay for the detection of a wide range of phytoplasmas and not just a phytoplasma-group specific assay. Among the different chemistry available for qPCR analysis as mentioned before SYBR Green I was chosen. SYBR green I is a generic dye that binds in the minor groove of the double-stranded DNA, it exhibits minimal fluorescence when free in solution and it is very easy to use with minimal cost. SYBR Green I has however the disadvantage that non-specific double stranded reaction products could be detected, but this fact could be overcome with a precise experimental design, such as assay specificity, standard curve establishment as reference, and a very careful melting peak temperature evaluation. With the use of SYBR Green I a melt curve of each product is required and the data are analysed at the end of each run.

The Tm was already demonstrated as a valid parameter to discriminate among different viruses (Nicolas *et al*., 2002a; 2002b; Mouillesseaux *et al.*, 2003; Varga and James, 2005; 2006), protists (Robinson *et al*., 2006) and bacteria (Tseng *et al*., 2003; Winder *et al*., 2011) because it is nucleotide sequence specific, and depends on the length of the amplicon and on its GC/AT content.

The Tm of the field infected plants resulted in the same range of the phytoplasma controls Tm values. These results indicate that the qPCR assay developed in this study is able to detect phytoplasma presence in symptomatic field-collected plants. This confirms the data reported by Vega-Orellana *et al*. (2016) indicating that the mollicutes have a Tm different from the one of the non-mollicutes species such as mycoplasmas. However, to achieve comparable results it is important to be consistent with the use of qPCR machine and qPCR master-mix because the Tm can change up to 2°C depending on these parameters (Ramírez *et al*., 2016).

In this study the assay specificity was clearly demonstrated: in facts, no detection or, in the case of '*Ca*. Liberibacter solanacearum', a different melting peak temperature (Tm 85°C) was observed when bacteria were tested, demonstrating that with primers GPO3F/MGSO the qPCR assay, could discriminate non-mollicutes also from phytoplasmas.

Since phytoplasmas can be present at low concentration in infected plants (Lee *et al*., 2000), it is useful to have a high-sensitive assay to detect them. An universal assay is very often less sensitive than a specific assay because of the mismatches present in the primers for widening their detection ability, nevertheless this study showed a high sensitivity, maybe due to the short size of the amplicons obtained. In fact, the last dilution observed in conventional PCR was 10^{-6}, while the last dilution detected by this qPCR method was 10^{-7}. Furthermore, in qualitative PCR, the phytoplasma sample DNAs were amplified in direct PCR with primers GPO3F/MGSO, instead with other phytoplasma primer pairs at least a nested PCR was needed for this.

In order to quantify the phytoplasma titer in the samples, two standards curves were built using serial dilutions of the two plasmids pESFY and pSTOL containing GPO3F/MGSO amplicon. Several tests to set the qPCR conditions and standards curves were performed, and it was observed that the curves of the two plasmids were overlapped; therefore the use of pESFY curve simplify the quantification of the samples from field (plum, apricot and peach) in agreement with several reported qPCR experiments (Torres *et al.*, 2005; Pignatta *et al.*, 2008; Yvon *et al*., 2009; Nikolić *et al*., 2010).

With this assay a wide range of phytoplasmas belonging to different ribosomal groups can be detected and quantified based on a single standard curve. This work could contribute to implement phytoplasma detection in plant materials, as seedlings which have very low phytoplasma titer, until 1.15×10^{3} ($\pm$ 4.54×10^{2}) copy number.

Up today the phytoplasma cultivation in artificial media is still problematic, even if good results were recently obtained (Contaldo *et al*., 2016). In this study, the DNAs extracted from colonies and CB liquid medium are sometimes the results of a co-presence of phytoplasma and other bacteria, because selective media for phytoplasmas are not yet developed. The application of the same protocol used for DNAs from plants, to DNAs from colonies and from CB liquid medium showed a partial gap in the ability of the primer pair GPO3F/MGSO to detect phytoplasmas: the presence of double peaks indicates detection of two organisms. A good deal is represented by possibility to detect phytoplasmas by the Tm value that must be 82 ± 0.5 °C. The setting of a different qPCR assay with other primers specific only for phytoplasmas has to be considered for the future in order to detect and quantify the phytoplasma titre and verify effectiveness of different developed media in supporting specific phytoplasma growth.

4. Simultaneous detection of mixed '*Candidatus* Phytoplasma asteris' and '*Ca*. Liberibacter solanacearum' infection in carrot

(From Satta *et al*., Phytopathologia Mediterranea (2016) 55, 3, 401−409
with permit of the senior editor)

4.1. Introduction

'Candidatus Liberibacter' and '*Candidatus* Phytoplasma' are phloem limited and insect-transmitted bacteria associated with economically important plant diseases. During surveys on carrot (*Daucus carota* L.) carried out in the municipality of Guía, on the North Atlantic island of Gran Canaria (Spain), symptoms of leaf curling with yellow, bronze, and purple discoloration, twisting of petioles, stunted growth of shoots and roots, and proliferation of secondary roots were observed. Nine fields showing symptoms possibly related to *'Candidatus* Liberibacter' and '*Candidatus* Phytoplasma' presence were observed. The percentage of symptomatic plants ranged from approx. 5 to 35% per field and no psyllids were detected in any of the fields at the time of the survey.

The symptom observed (Figure 4.1) resembled those reported by Duduk *et al.,* (2009) and Munyaneza (2010a), associated with the presence of phloem-inhabiting bacteria, in particular *'Candidatus* Liberibacter' and '*Candidatus* Phytoplasma' species. The first group are prokaryotes related to the α-*Proteobacteria*, which are Gram negative bacteria with thin cell walls (Munyaneza, 2012), and are associated with economically important diseases such as citrus huanglongbing (HLB) (Bové, 2006) and zebra chip (ZC) of potato (*Solanum tuberosum*) in America (Crosslin *et al.,* 2010; Secor *et al.,* 2009; Wen *et al.,* 2009). Yellows in tomato (*Solanum lycopersicum*) associated with the presence of potato psyllids (EPPO, 2013; Munyaneza *et al.,* 2010b) and vegetative disorders in celery (*Apium graveolens*) evidenced by abnormal numbers of shoots, curling of stems, and yellowing (Teresani *et al.,* 2014), were linked with the presence of these prokaryotes and in particular of '*Candidatus* Liberibacter solanacearum' haplotypes. Phytoplasmas are also phloem limited and insect-transmitted bacteria that lack cell walls and are associated with severe diseases in many important crops worldwide (Bertaccini *et al.,* 2014). Aster

yellows (16SrI-B) phytoplasmas have previously been detected in carrots in mainland Spain, while "stolbur" (16SrXII-A) phytoplasmas were detected in the Canary Islands, Spain (Font *et al.,* 1999).

Figure 4.1. Two fields showing disease symptoms in carrot (variety Cordoba), in Guía, Gran Canaria Island (Spain).

4.2. Materials and methods

4.2.1. Field sampling

Samples from carrot (variety Cordoba) collected in 2015 and 2016 from, respectively, 26 and eight symptomatic plants were randomly selected in two fields located in the North of Gran Canaria Island. Samples were also collected from two asymptomatic plants in each year and each field as negative controls.

4.2.2. Total DNA extraction

Total DNA was extracted from 1 g of leaf tissue samples ground in mortars with liquid nitrogen, using a reported method based on cetyl-trimethyl-ammonium-bromide (CTAB) (Angelini *et al.,* 2001) (Appendix 1).

4.2.3. Amplification protocols

The extracted DNA samples were tested by PCR using the primer pairs reported in Table 4.1 to verify the presence of '*Ca*. L. solanacearum' and '*Ca*. Phytoplasma' spp. DNA samples from phytoplasma strains maintained in collection (Bertaccini, 2014) were also employed as positive controls for phytoplasma detection: in particular, ash yellows (ASHY, (16SrVII-A); European stone fruit yellows (ESFY, 16SrX-B); "stolbur" (STOL, 16SrXII-A); aster yellows (AY, 16SrI-B); faba bean phyllody (FBPSA, 16SrII-C); peach X disease (CX, 16SrIII-A) and *Picris echioides* yellows (PEY, 16SrIX-

C) were used. DNA samples from asymptomatic carrots and samples containing sterile distilled water as template were used as negative controls.

Name	Sequence 5'-3'	Literature	Amplicon length (bp)	Gene(s)
Reported target '*Ca*. L. solanacearum'				
Clipo-F (f) O12c (r)	TACGCCCTGAGAAGGGGAAAGATT GCCTCGCGACTTCGCAACCCAT	Secor *et al.,* 2009 Jagoueix *et al.,* 1996	1,070	16S rRNA
OA2 (f) O12c (r)	GCGCTTATTTTTAATAGGAGCGGCA GCCTCGCGACTTCGCAACCCAT	Liefting *et al.,* 2009 Jagoueix *et al.,* 1996	1,168	16S rRNA
CL514F CL514R	CTCTAAGATTTCGGTTGGTT TATATCTATCGTTGCACCAG	Munyaneza *et al.,* 2009	669	ribosomal protein *rplJ/rplL*
Reported target '*Ca*. Phytoplasma' spp.				
P1 P7	AAGAGTTTGATCCTGGCTCAGGATT CGTCCTTCATCGGCTCTT	Deng & Hiruki, 1991 Schneider *et al.,* 1995	1,784	16S rRNA + spacer + beginning of 23S rRNA
R16F2n R16R2	GAAACGACTGCTAAGACTGG TGACGGGCGGTGTGTACAAACCCCG	Gundersen & Lee, 1996 Lee *et al.,* 1993a	1,248	16S rRNA
R16(I)F1 R16(I)R1	TAA AAG ACC TAG CAA TAG G CAA TCC GAA CTA AGA CTC T	Lee *et al.*,, 1994	1,095	16S rRNA
16R758f (=M1) 16S1232r (=M2)	GTCTTTACTGACGCTGAGGC CTTCAGCTACCCTTTGTAAC	Gibb *et al.,* 1995	509	16S rRNA

Table 4.1. The table describes the PCR systems used to detect '*Ca*. L. solanacearum' presence in carrot DNAs using primer pairs specific for its 16S rRNA (Clipo-F /O12c and OA2/O12c) and ribosomal protein *rplJ/rplL* (CL514F/CL514R). To detect '*Ca*. Phytoplasma' spp. The phytoplasma universal (P1/P7, R16F2n/R16R2 and M1/M2) and group 16SrI- specific primers (R16(I)F1/R16(I)R1) were employed.

To detect phytoplasmas, the direct PCRs were carried out with primer pair P1/P7 and the first nested amplification was performed in two systems: system I, with the primer pair R16F2n/R16R2; and system II, with primer pair R16(I)F1/R16(I)R1 (Table 4.1). A second nested PCR was carried out with the primer pair 16R758f/16S1232r (=M1/M2) in both systems (Table 4.1). For the PCR protocols followed, Promega *Taq* was used as described in Appendix 2. Identification of detected phytoplasmas was performed using RFLP analyses with *Tru1*I (Fermentas) (Appendix 3). Virtual RFLP analyses were also carried out on 967 bp of the 16S ribosomal gene from the obtained '*Ca*. L.

solanacearum' sequences and reference sequences for the reported haplotypes (A, B, C, D and E) available in Genbank, using pDRAW32 (http://www.acaclone.com/).

4.2.4. Phylogenetic analyses

Amplified products of selected DNA samples obtained from all the primer pairs employed were purified and directly sequenced in both directions with the primers used in amplification by Macrogene (the Netherlands). The sequences were aligned using CromasPro 2.4 software. They were then compared with nucleotide sequences in the GenBank database using BLAST at the National Center for Biotechnology Information (NCBI) website (http://ncbi.nlm.nih.gov/BLAST).

Phylogenetic analyses were carried out using the obtained 16S rDNA sequences, sequences from ribosomal protein gene *rplJ/rplL* and sequences of '*Ca.* L. solanacearum' haplotypes described in the literature. '*Ca.* P. asteris', carrot 2 sequence and '*Ca*. L. asiaticus' were used as outgroups according with the sequence employed. The evolutionary history in both cases was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and were in the units of the number of base substitutions per site. The ME trees were searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial trees. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

4.3. Results

Positive results were obtained in PCR and nested PCR with all the primer pairs and primers combinations used. The negative controls as well as four of the symptomatic samples resulted negative with all the primers and systems employed. In particular, in 2015 and 2016 respectively, the results were: 16 of 26 (61.5%) and four of eight (50%) positive samples with primer pair Clipo-F/O12c (998 bp), 22 of 26 (84.6%) and eight of eight (100%) with primers OA2/O12c (1,168 bp), and 22 (84.6) and six (75%) with primers CL514F/R (669 bp). All the amplicons were of the expected length (data not shown). Twenty-six positive samples were obtained with primer pairs M1/M2 in nested amplification: 16 (61.5%) in 2015 and six (75%) in 2016 were obtained by system I, and four (15.4%) in 2015 by system II (Table 4.2).

Primers and primer combinations	**2015**		**2016**
	'*Ca*. P. asteris'	**'*Ca*. L. solanacearum'**	**'*Ca*. L. solanacearum'**
16S rRNA gene primer combinations			
ClipoF/O12c	-	61.5%	50.0%
OA2/O12c	-	84.6%	100%
System I (P1/P7 + R16F2n/R2 + M1/M2)	-	61.5%	75.0%
System II (P1/P7 + R16(I)F1/R1 + M1/M2)	15.4%*	-	-
Ribosomal protein gene *rplJ/rplL* primer			
CL514 F/R	-	84.6%	75.0%

Table 4.2. Results of *'Ca*. L. solanacearum' and '*Ca*. P. asteris' detection in carrot with the different primer combinations. *'Ca*. L. solanacearum' was detected with the primers ClipoF/O12c, OA2/O12c and CL514 F/R but also with the System I (P1/P7 + R16F2n/R2 + M1/M2). '*Ca*. P. asteris' was detected with the System II (P1/P7 + R16(I)F1/R1 + M1/M2); *Mixed infection with '*Ca*. L. solanacearum'; -, not tested.

RFLP analyses carried out on the M1/M2 amplicons using Tru1I restriction enzyme showed the presence of diverse profiles. In particular, 22 positive samples, amplified with nested PCR system I, showed RFLP profiles identical to each other that matched with the virtual profile of the same amplicon from carrot 1 (Figure 4.2a). The remaining four amplicons obtained with nested PCR system II showed profiles referable to those of phytoplasmas belonging to the ribosomal group 16SrI (Figure 4.2b).

The CL514F/R sequenced amplicons showed 100% identity to each other and to the homologous sequence of a '*Ca*. L. solanacearum' strain from Morocco [GenBank accession number (AC) KJ754507]; the 526 bp sequence of sample carrot 1 was deposited in GenBank under AC KX181862. The direct sequencing and alignment of selected ClipoF/O12c and OA2/O12c amplicons showed 100% of sequence homology to each other and to the '*Ca*. L. solanacearum' strain found in carrots in Morocco (AC KJ740160). The aligned sequence of 967 bp from OA2/O12c amplicon of the same carrot 1 strain was deposited in GenBank under AC KX163276.

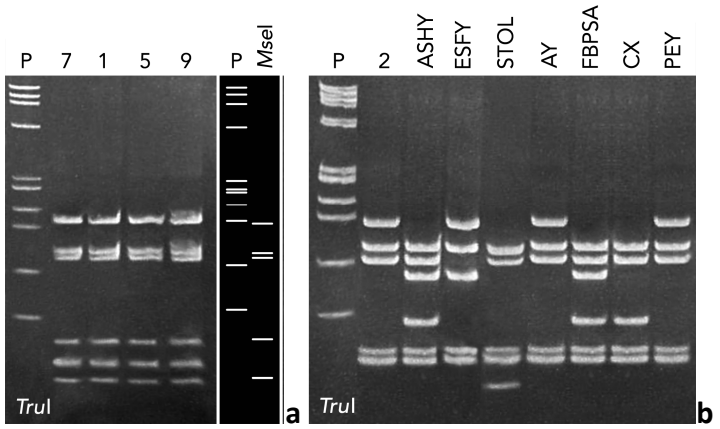

Figure 4.2. RFLP analyses in polyacrylamide gels of samples amplified in nested PCR with primers M1/M2 and digested with *Tru*I (= *Tru1*I). In **a)**, carrot samples 7, 1, 5, 9 amplified with system I and the virtual RFLP profile of the strain carrot 1 (AC KX163276) sequence cut at the same length as the M1/M2 amplicons and digested with the same enzyme (*Mse*I and *Tru1*I are isoschizomers). The middle bands present in the real RFLP (on the left) represents non-specific amplification (primer dimers). In **b)** carrot sample 2 amplified with system II and reference phytoplasma strains: ash yellows (ASHY, 16SrVII-A); European stone fruit yellows (ESFY, 16SrX-B); "stolbur" (STOL, 16SrXII-A); aster yellows (AY, 16SrI-B); faba bean phyllody (FBPSA, 16SrII-C); CX, peach X disease (16SrIII-A); *Picris echioides* yellows (PEY, 16SrIX-C). P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

The sequences obtained from M1/M2 amplicons in nested PCR system I (510 bp) were identical to each other and showed 99% of identity to '*Ca*. L. solanacearum' strain detected in carrots in Finland (AC GU373048); in particular they showed one single nucleotide polymorphism (SNP) at position 116 nt where a T was substituted with a C. One or two further SNPs were also present to other '*Ca*. L. solanacearum' strains detected in *Bactericera cockerelli*, in pepper, potato and tomato in the USA, Mexico and Thailand (AC KF776424, KF776423, KF776422 and KC771216). The carrot sequence from sample 1 was deposited in Genbank under AC KX163277. The sequenced strains amplified with nested PCR system II were 100% identical to each other, and the 498 bp sequence of strain carrot 2 was deposited in GenBank (AC KX163275). These sequences showed 99% identity to aster yellows phytoplasma strains available in GenBank, with one SNP at position 269 nt where a C substitution of a T was present.

These sequencing results verify that the profile obtained in RFLP analyses in all the samples amplified in nested PCR with system I was referable to the '*Ca*. L. solanacearum' profile: (Figure 4.2a). On the other hand, the four positive samples obtained in amplification system II showed RFLP profiles referable to phytoplasmas (Figure 4.2b), in particular to aster yellows (16SrI) as confirmed by the sequencing results. Phytoplasma presence was therefore detected in only four samples and in mixed infection with '*Ca*. L. solanacearum'. The primer pair M1/M2, known as

universal for phytoplasmas, detected '*Ca*. L. solanacearum' when used in nested PCR system I and aster yellows phytoplasmas in nested PCR system II (Table 4.2).

The detected SNPs in the 16S rRNA, 16S/23S ISR and rplJ/rplL ribosomal protein sequences of 'Ca. L. solanacearum' strains from Gran Canaria agreed with those present in the haplotype D (Nelson *et al.,* 2012). The same results were obtained using *the virtual RFLP analyses on* OA2/O12c amplicon sequences. These further confirm the assignment of the studied '*Ca*. L. solanacearum' strains to the haplotype D, and allow differentiation of all the reported haplotypes (Figure. 4.3). In particular, *Stu*I (Figure 4.3A) differentiates haplotype B from all the others; *Mbo*I, *BsmA*I, *Set*I (Figures 4.3B, C and D) and *Bsa*I (data not shown) differentiate haplotype E from all the others; *Set*I (Figure 4.3D) differentiates haplotypes C and D from haplotypes A and B, *BstAP*I (Figure 4.3E) and *Mwo*I (data not shown) differentiate haplotypes C and D from all the others; while *Xba*I (Figure 4.3F) differentiate the haplotype C from all the others.

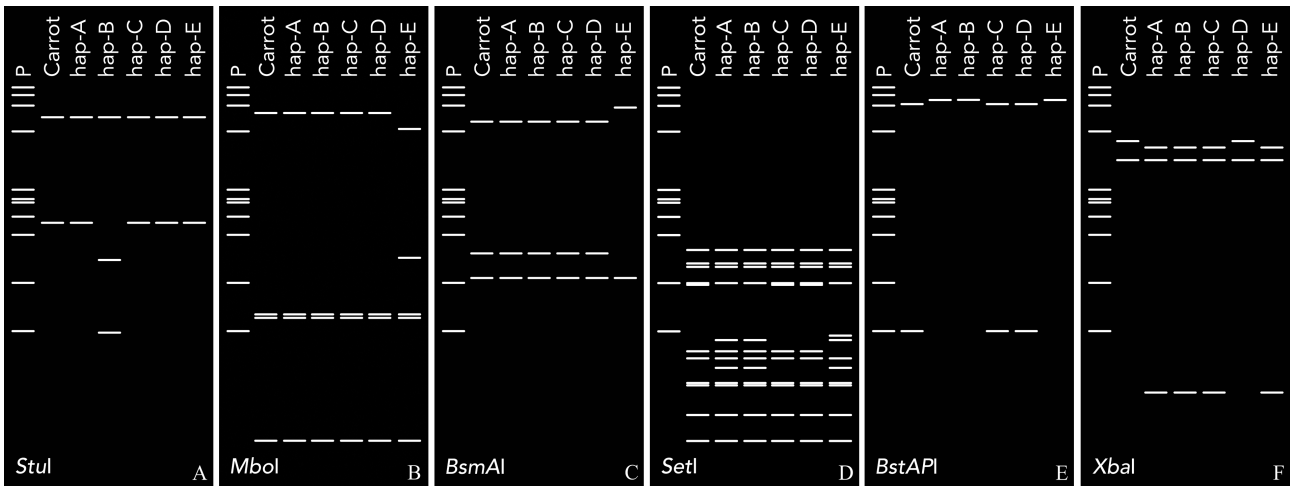

Figure 4.3. Virtual RFLP gel DNA with selected restriction enzymes showing the polymorphisms among '*Ca*. L. solanacearum' detected in carrot and the diverse haplotypes reported on full length OA2/O12c sequences, obtained in this work (Carrot) and retrieved from Genbank. Enzymes used are reported at the bottom of each gel. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

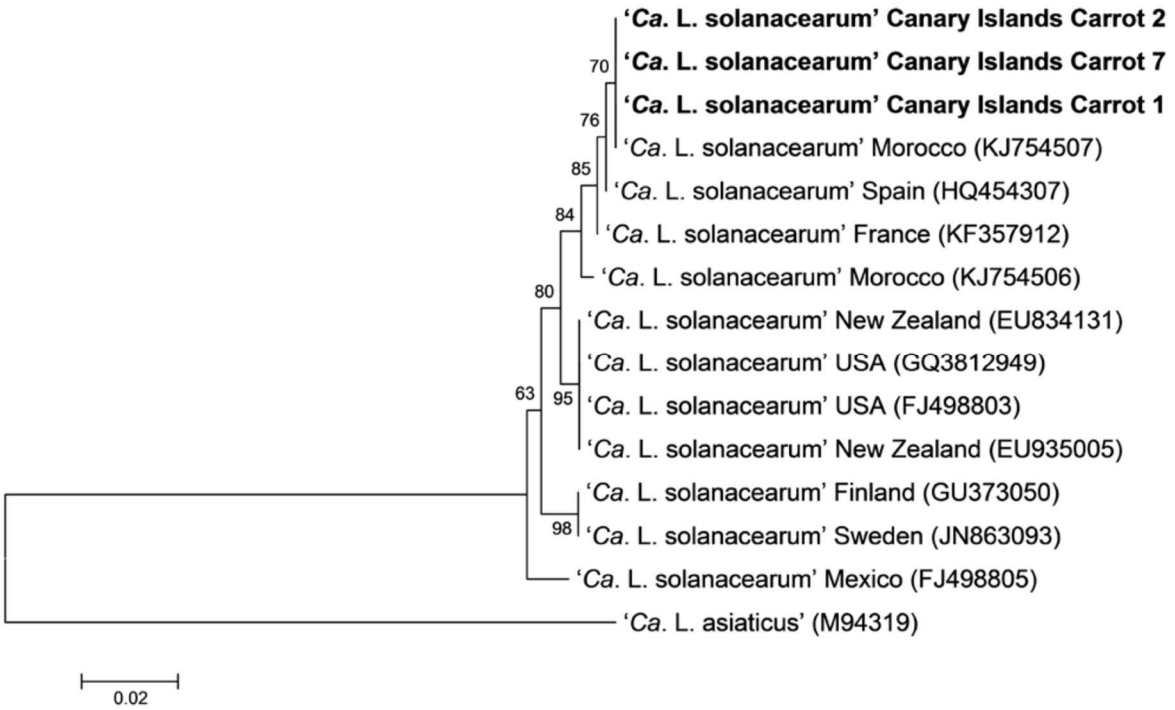

Figure 4.4. Phylogenetic tree showing the evolutionary history using the Minimum Evolution method enclosing ribosomal protein gene rplJ/rplL sequences from '*Ca*. L. solanacearum' from different geographic areas; '*Ca*. L. asiaticus' was used as outgroup. In bold sequences of strains obtained from carrots 1, 2 & 7 from Guia, Gran Canaria, Canary Islands, Spain; on the right geographic distribution and in parenthesis GenBank accession numbers. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein, 1985). The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 524 positions in the final dataset.

Phylogenetic analyses on CL514F/R sequences confirmed the clustering of the obtained sequences with those of '*Ca*. L. solanacearum, in particular with haplotype D sequences (GenBank AC HQ454302). The CL514F/R sequences showed 100% identity with '*Ca*. L. solanacearum' strains haplotype D (Figure 4.4).

4.4. Discussion

The results obtained in this study indicate the presence of '*Ca*. L. solanacearum' and phytoplasmas belonging to the ribosomal group 16SrI in mixed infections in samples of carrots from Gran Canaria Island (Spain). Virtual RFLP analyses were applicable for '*Ca*. L. solanacearum' as an alternative tool for haplotype discrimination. It could be easier than SNPs detection on two genes as required now since only one gene and no sequencing can provide the same result. The nested PCR system(s) reported also allowed detection and RFLP differentiation of the two detected prokaryotes, as confirmed by the sequencing results.

'*Ca.* L. solanacearum' was described in 2008 (Hansen *et al.,* 2008; Liefting *et al.,* 2008) and it was shown to be associated with zebra chip disease of potato tubers, an economically important pathogen for solanaceous crops in New Zealand and the USA. In Europe, *Ca.* L. solanacearum' has only been previously detected in carrot and celery (Alfaro-Fernández *et al.,* 2012a; Hansen *et al.,* 2008; Liefting *et al.,* 2009; Secor *et al.,* 2009; Munyaneza *et al*., 2010a; Munyaneza, 2012; Munyaneza *et al.,* 2010b; Buchman *et al.,* 2011; EPPO, 2012). In addition, the bacterium causes serious damage in pepper (*Capsicum annuum*) in Mexico, aubergine (*Solanum melongena*) in Honduras, tamarillo (*Solanum betaceum)* and tomatillo (*Physalis peruviana*) in New Zealand, and tobacco (*Nicotiana tabacum*) in Honduras. It also infects weeds in the family Solanaceae (Munyaneza, 2012; EPPO 2013). Five haplotypes of '*Ca.* L. solanacearum' have been described that are discriminated by the presence of specific single SNPs in the 16S rRNA, 16S/23S ISR and 50S rplJ and rplL ribosomal protein genes (Nelson *et al.,* 2011). Two haplotypes (LsoA and LsoB) are transmitted by the psyllid *Bactericera cockerelli*. A third (LsoC) was detected in carrots in Finland, Sweden and Norway, and is transmitted by the carrot psyllid *Trioza apicalis* (Nissinen *et al*., 2007; Nelson *et al.,* 2011). Haplotypes D and E were associated with carrots and celery in Spain and in the Canary Islands and are vectored by the psyllid *Bactericera trigonica* (Alfaro-Fernández *et al.,* 2012b; Teresani *et al.,* 2014). The same psyllid was previously demonstrated to vector the disease from carrot to carrot, when only phytoplasmas were reported to be associated with the disease (Font *et al.,* 1999).

Further research is in progress to confirm the transmission of both prokaryotes by seeds (Bertolini *et al.,* 2015; Calari *et al.,* 2011) and/or by insect vectors, considering that psyllids were not detected in the present survey and that the disease is present in Gran Canaria at epidemic levels similar to those reported in other affected areas.

5. Appendix

Appendix 1: DNA extraction methods

- Angelini *et al*., 2001

This method of extraction was employed for the purification of nucleic acids from 0.5 g of plant tissue through the use of chloroform and final precipitation in isopropanol.

The samples were grinded in sterile porcelain mortars through the use of liquid nitrogen and sterile pestles; 7 ml of 3% CTAB (cetyltrimethylammonium bromide) buffer per gram of material (with addition of 2 µl of 0.2% 2-mercaptoethanol per ml of buffer) were used for the homogenization of the tissue. Then 1 ml of each sample was transferred in 2 ml Eppendorf tubes and incubated at 65°C for 20 minutes; 1 ml of chloroform was added to each sample; the samples were subjected to centrifugation at 11,000 rpm for 10 minutes in a microcentrifuge. The supernatant obtained was mixed with 1 ml of isopropanol and incubated at -20°C for 5 min. After centrifugation at 11,000 rpm for 15 minutes and elimination of the supernatant, the pellet was washed with 1 ml of ethanol 70% and centrifuged at 11,000 rpm for 5 min, ethanol is discarded and the samples are air-dried at room temperature. The pellet was re-suspended in 100 µl of TE (Tris-EDTA: ethylenediaminetetraacetic acid; 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) buffer.

- Prince *et al*., 1993

Total nucleic acid was extracted from 1 g of tissue of each sample (frozen until use) and ground in liquid nitrogen using a sterilized mortar and pestle with the addition of 8 ml of grinding buffer [K_2HPO_4 $3H_2O$, 21.7 g/l; KH_2PO_4, 4.1 g/l; sucrose, 100 g/l; BSA (Fraction V), 1.5 g/l; PVP-10, 20 g/l; L-ascorbic acid, 0.53 g and adjust pH to 7.6 with 2N NaOH]. The tissue was transferred in plastic tubes and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was discarded, and 4 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 250 mM NaCl) and 80 µl of proteinase K (5 mg/ml in Sterile Distilled Water-SDW) were added. The pellet was gently re-suspended, adding 440 µl of 10% sarkosyl and incubating 1-2 hours at 55°C. The samples were centrifuged for 10 min at 8,000 rpm at 4°C, the supernatant was then saved and 0.6 volumes of isopropanol (2.5 ml) were added and mixed gently. The tubes were placed at –20°C for 30 min. After centrifugation at 8,000 rpm for 15 min the pellet was re-suspended in 3 ml Tris-EDTA (10

mM Tris-HCl, pH 8.0 and 1 mM EDTA) buffer, adding 75 µl 20% SDS and 60 µl of proteinase K, mixed gently and incubated 1 hour at 37°C. Afterward, 525 µl 5 M NaCl and 420 µl CTAB/NaCl (10% CTAB in 0.7 M NaCl) solutions were added, mix thoroughly and incubated 10 min at 65°C. Then 2 ml of chloroform/isoamylic alcohol and 2 ml of phenol were added, mixed thoroughly and centrifuged at 8,000 rpm for 10 min. The supernatant was transferred to a new tube and an equal volume of chloroform was added, centrifugation at 8,000 rpm for 10 min was then performed. The supernatant was transferred to a Corex tube and added with 2.5 ml of isopropanol alcohol then placed at 4°C overnight. Next day, tubes were centrifuged at 11,000 rpm for 30 min at 4°C. The supernatant was eliminated and the pellet washed in 1 ml of cold ethanol 70% and then centrifuged 11,000 rpm for 10 min at 4°C. Pellet was dried and suspended in 50-100 µl of TE buffer and maintained at 8°C for one month or at –20°C for longer periods.

- DNaesy Plant Minikit, QIAGEN

Total nucleic acid was extracted from 250 µl of liquid medium or from single colonies added to 250 µl of SDW. Firstly, 400 µl of lysis buffer AP1 were added and the samples were vortexed. The tubes placed at 65°C for 10 min were shacked 2-3 times during incubation; 130 µl Buffer AP2 (containing acetic acid) were added to precipitate proteins, polysaccharides and detergent, the tubes were put 5 min on ice and centrifuged at 13,000 rpm for 5 min. The lysate was then transferred, taking care to not disturb the pellet in a column with a membrane, placed on a 2 ml tube and subjected to centrifugation for 2 min at 13,000 rpm. This membrane retains the majority of the precipitates and cell fragments. Subsequently, 1.5 volumes of Buffer AP3 (containing guanidine hydrochloride) were added. The obtained mixtures were transferred to a new column containing a membrane, placed on a 2 ml tube, and subjected to centrifugation for 1 min at 8,000 rpm. In this step the impurities are removed, thanks to the silica gel membrane that retains the DNA. Placing the column into a new 2 ml collection tube, the membrane was washed through the addition of 1,000 µl in two times of Buffer AW and centrifuging for 1 min at 8,000 rpm and for 2 min at 13,000 rpm. The DNA bounded to the membrane was eluted in a new 1.5 ml tube, adding for 2 times 25 µl of Buffer AE, incubated for 5 min at room temperature and centrifuged for 1 min at 8,000 rpm.

Appendix 2: *16Sr* gene and elongation factor (*tuf*) gene amplification (PCR and nested-PCR)

- REDtaqDNA polymerase, Sigma-Aldrich, Co., St. Louis, MO, USA

Direct PCR was performed in a 25 µl reaction (Schaff *et al*., 1992) prepared by mixing, in 0.5-ml tubes, 17.5 µl SDW, 10X REDtaq PCR reaction buffer, 0.2 mM dNTPs (Invitrogen Life Technologies, Carlsbad, CA, USA), 0.4 µM of each primer, 0.05 U/µl REDtaqDNA polymerase (Sigma-Aldrich, Co., St. Louis, MO, USA). As template, 1 µl of DNA extracted by Angelini *et al*., 2001 method and diluted 1: 30, was used. The reactions were performed in a Thermal Cycler BIOMETRA (Germany) without hot lid, with the following parameters for 16Sr gene: 94°C for 10 min, 35 cycle of 94°C for 1 min, 55°C (or 50°C according to the primers annealing temperature) (Table 1A) for 2 min, 72°C for 3 min and a final extension for 10 min at 72°C. PCR and nested-PCR reactions were carried out as described above. For tuf gene, two pairs of primer cocktails were used (Table 1A). Each primer cocktail consisted of several variants of the same primer mixed in equimolar proportions to the final concentration of 10 µM. PCR thermal conditions for direct and nested-PCR were 94°C for 3 min followed by 35 cycles of 94°C for 15 sec, 54°C for 30 sec and 72°C for 1 min and a final extension step of 72°C for 7 min. Some drops of mineral oil were added to each tube to prevent evaporation of the mixture. The first nested PCR was performed using as template 1 µl of a 1: 30 dilution in SDW of each amplicon obtained from the direct PCR and to increase sensibility and specificity, a second nested PCR was also implemented using as template 1 µl of a 1: 30 dilution in SDW of each amplicon obtained from the first nested PCR. PCR products were visualized in 1% agarose gels (Agarose 1%, TAE buffer 1X) stained with ethidium bromide 1% for 10 min then moved in distilled water to wash away the excess of bromide and documented with a bench top UV transilluminator at 312 nm. The gel results were recorded by a digital camera Kodak Edas 290.

- Promega GoTaq G2 DNA polymerase, Promega

A 25 µl reaction was prepared by mixing 5 µl PCR 5× Buffer with $MgCl_2$ (Promega), 2 µl of 10 mM dNTPs, 0.5 µl (20 µM) forward and reverse primer, 0.5 µl (20 µM), 0.16 µl (1.25 units) Promega GoTaq G2 DNA polymerase (Promega), and 15.4 µl nuclease-free molecular biology H_2O (Sigma) (Schaff *et al*., 1992). To detect '*Ca*. Liberibacter', 1 µl of DNA template, extracted by Angelini *et al*., 2001 method, was added directly in the PCR mix; while to detect phytoplasmas presence, 1 µl of the same DNA template diluted 1:30 was employed. Reactions were performed in a Thermal

Cycler BIOMETRA (Germany) with the following parameters: i) for primer pair ClipoF/O12c: 94°C for 2 min, 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min, and a final extension cycle of 72°C for 10 min; ii) for primer pair OA2/O12c: 94°C 3min, 35 cycles of 94°C 45 s, 66°C 45 s, 72°C for 1 min and 10 min at 72°C; and iii) for primer pair CL514F/R: 94°C 3 min, 35 cycle of 94°C 45 s, 54°C 45 s, 72° 1 min and 10 min at 72°C. To detect phytoplasma, primers P1/P7, R16F2n/R16R2, R16(I)F1/R16(I)R1 and M1/M2 were used and the reactions were performed in a Thermal Cycler BIOMETRA (Germany) without hot lid, with the following parameters: 94°C for 10 min, 35 cycle of 94°C for 1 min, 55°C (or 50°C according to the primers annealing temperature) (Table 1A) for 2 min, 72°C for 3 min and a final extension for 10 min at 72°C. Some drops of mineral oil were added to each tube to prevent evaporation of the mixture. The first nested PCR was performed using as template 1 µl of a 1:30 dilution in SDW of each amplicon obtained from the direct PCR and to increase sensibility and specificity, a second nested PCR was also implemented using as template 1 µl of a 1:30 dilution in SDW of each amplicon obtained from the first nested PCR. PCR products were visualized in 1% agarose gels stained with ethidium bromide 1% for 10 min and then moved in distilled water to wash away the excess of bromide and documented with a bench top UV transilluminator at 312 nm. The gel results were recorded by a digital camera Kodak Edas 290.

- Rovalab, Teltow, Germany

A 25 µl reaction was prepared by mixing 2.5 µl PCR 10X Buffer, 1 µl of $MgCl_2$ (Rovalab, Teltow, Germany), 2 µl of 10 mM dNTPs, 0.5 µl (20 µM) forward and reverse primer, 0.5 µl (20 µM), 1 µl (100 U) Cabru Red Taq DNA polymerase (Rovalab) and 16.5 µl nuclease free water for molecular biology (Sigma). To detect phytoplasma presence in DNA from plants, 1 µl of DNA diluted at 20 ng/µl, extracted by Prince *et al*., 1993 method was added to the PCR mix. To detect phytoplasma from colonies and liquid medium, 1 µl of DNA, extracted by DNaesy Plant Minikit, QIAGEN, was used as template. Reactions were performed in a Thermal Cycler BIOMETRA (Germany) with the following parameters: for primer pair P1/P7, R16F2n/R16R2 and M1/M2: 95°C 2 min, 35 cycles 94°C 45 s, 55°C (or 50°C according to the primers annealing temperature) (Table 1A) 55 s, 72°C 2 min and 72°C 10 min; for primer GPO3F/MGSO 94°C 4 min, 35 cycles 94°C 45 s, 61°C 45 s, 72°C 1 min 30 s and 72°C 7 min.

PCR	Primers	Sequence 5'-3'	Annealing temperature °C	References	Amplification length (bp)	Target
Direct	P1 P7	AAGAGTTTGATCCTGGCTCAGGATT CGTCCTTCATCGGCTCTT	55	Deng & Hiruki, 1991 Schneider *et al.*, 1995	1,784	16S rDNA + spacer + beginning of 23S rDNA
Direct/I nested	R16F2n R16R2	GAAACGACTGCTAAGACTGG TGACGGGCGGTGTGTACAAACCCCG	55	Gundersen & Lee, 1996 Lee *et al.*, 1993	1,248	16S rDNA
Direct /I/II	fU5 rU3	CGGCAATGGAGGAAACT TTCAGCTACTCTTTGTAACA	50	Lorenz *et al.*, 1995	862	16S rDNA
I/II nested	R16(I)F1 R16(I)R1	TAAAAGACCTAGCAATAGG CAATCCGAACTAAGACTCT	50	Lee *et al.*, 1994	1,095	16S rDNA
I/II nested	16R758f (=M1) 16S1232r (=M2)	GTCTTTACTGACGCTGAGGC CTTCAGCTACCCTTTGTAAC	50	Gibb *et al.*, 1995	509	16S rDNA
I nested	R16(III)F2 R16(III)R1	AAGAGTGGAAAAACTCCC TCCGAACTGAGATTGA	50	Lee *et al.*, 1994	800	16S rDNA
I nested	Tuf340a Tuf340b Tuf890a Tuf890b Tuf890c	GCTCCTGAAGAAARAGAACGTGG ACTAAAGAAGAAAAAGAACGTGG ACTTGDCCTCTTTCKACTCTACCAGT ATTTGTCCTCTTTCWACACGTCCTGT ACCATTCCTCTTTCAACACGTCCAGT	54	Makarova *et al*., 2012	531-553	Elongation factor Tu
II nested	Tuf400aM13 Tuf400bM13 Tuf400cM13 Tuf400dM13 Tuf400eM13 Tuf835aT7 Tuf835bT7 Tuf835cT7	GTAAAACGACGGCCAGTGAAACAGAAAAACGTCAYTATGCTCA GTAAAACGACGGCCAGTGAAACTTCTAAAAGACATTACGCTCA GTAAAACGACGGCCAGTGAAACATCAAAAAGACAYTATGCTCA GTAAAACGACGGCCAGTGAAACAGAAAAAAGACAYTATGCTCA GTAAAACGACGGCCAGTCAAACAGATAAAAGACATTATYCTCA TAATACGACTCACTATAGGGAACATCTTCWACHGGCATTAAGAAAGG TAATACGACTCACTATAGGGAACACCTTCAATAGGCATTAAAAAWGG TAATACGACTCACTATAGGGAACATCTTCTATAGGTAATAAAAAAGG	54	Makarova *et al*., 2012	420-444	Elongation factor Tu

Table 1A. Primer pairs employed for the amplification of phytoplasmas DNAs.

Appendix 3: Restriction Fragment Length Polymorphism (RFLP) analyses

After PCR analysis, RFLP was carried out with commercial fast restrictions enzymes (New England, BioLabs Ipswich, MA, USA; and Fermentas, Vilnius, Lithuania), following the manufacturer's instructions. Usually, 100-200 ng of DNA (generally 3 µl of the amplicons) obtained from the nested PCR were digested with 3 U of the restriction enzymes, according to the brightness of the bands obtained in agarose gel. This quantity is added to a mix of 9.75 µl of SDW, 2 µl of buffer and 0.25 µl of enzyme, and incubated for 10 min at 37 or 65°C depending on the enzyme used. The *Tru1*I, *Tsp509*I and *Rsa*I (New England, BioLabs Ipswich, MA, USA; and Fermentas, Vilnius, Lithuania) enzymes were used. The analysis was carried out in a 6.7% polyacrylamide gel (Table 2A), stained with ethidium bromide 1% for 10 min, visualized under UV transilluminator at 312 nm and recorded by a digital camera Kodak Edas 290.

Reagent	Quantity
Distilled H_2O	18 ml
TBE 10X	2.5 ml
Acrylamide	4.2 ml
Ammonium persulfate 0.1%	310 µl
TEMED	16 µl

Table 2A. Polyacrylamide gel composition.

Appendix 4: Sequences edition and assembling

Sequences were assembled and edited using ChromasPro v1.7.5 (Technelysium Pty Ltd, Tewantin, QLD, Australia) software, aligned using ClustalW as implemented in MEGA v5.1 (Tamura *et al*., 2011) and adjusted manually. They were then compared with selected nucleotide sequences in the NCBI GenBank database (National Center for Biotechnology Information, Bethesda, MD) using BLAST program (version BLASTN 2.2.18).

6. References

Abramovitch R.B., Kim Y.J., Chen S., Dickman M.B., Martin G.B. 2003. Pseudomonas type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. The EMBO Journal, 22: 60–69.

Ahrens U., Seemüller E. 1992. Detection of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplify a sequence of the 16S rRNA gene. Phytopathology, 82: 828-832.

Akhtar K.P., Dickinson M., Sarwar G., Jamil F.F., Haq M.A. 2008. First report on the association of a 16SrII phytoplasma with sesame phyllody in Pakistan. Plant Pathology, 57: 771.

Akhtar K.P., Sarwar G., Dickinson M., Ahmad M., Haq M.A., Hameed S., Iqbal M.J. 2009a. Sesame phyllody disease: its symptomatology, etiology, and transmission in Pakistan. Turkish Journal of Agriculture and Forestry, 33: 477-486.

Akhtar K.P., Shah T.M., Atta B.M., Dickinson M., Hodgetts J., Khan R.A., Haq M.A., Hameed S. 2009b. Symptomatology, etiology and transmission of chickpea phyllody disease in Pakistan. Journal of Plant Pathology, 91 (3): 649-653.

Albanese G., Polizzi G., Grimaldi V., Collodoro S., Tessitori M., Davis R.E. 1998. Detection and molecular identification of a phytoplasma in diseased tomato plants in Calabria (Italy). Phytopathologia Mediterranea, 37: 83–87.

Aldaghi M., Massart S., Dutrecq O., Bertaccini A., Jijakli M.H., Lepoivre P. 2009. A simple and rapid protocol of crude DNA extraction from apple trees for PCR and real-time PCR detection of 'Candidatus Phytoplasma mali'. Journal of Virological Methods, 156: 96–101.

Alfaro-Fernández A., Cebrián M.C., Villaescusa F.J., Hermoso de Mendoza A., Ferrándiz J.C., Sanjuán S., Font M.I. 2012a. First report of '*Candidatus* Liberibacter solanacearum' in carrot in mainland Spain. Plant Disease, 96 (4): 582.

Alfaro-Fernández A., Siverio F., Cebrián M.C., Villaescusa F.J., Font M.I. 2012b. '*Candidatus* Liberibacter solanacearum' associated with *Bactericera trigonica* affected carrots in the Canary Islands. Plant Disease, 96 (4): 581.

Alivizatos A.S., 1989. Occurrence and distribution of tomato stolbur in Greece. Proceedings of the 7th Conference on Plant Pathogenic Bacteria, Budapest, Hungary: 945–950.

Alma A., Bosco D., Danielli A., Bertaccini A., Vibio M., Arzone A. 1997. Identification of phytoplasmas in eggs, nymphs and adults of *Scaphoideus titanus* Ball reared on healthy plants. Insect Molecular Biology, 6: 115-121.

Amaral Mello A.P.O., Flôres D., Bedendo I.P. 2006. Identificação do fitoplasma associado ao enfezamento dorepolho, através de análise de RFLP. Fitopatologia Brasileira, 31: 239.

Anderson T.P., Werno A.M., Beynon K.A., Murdoch D.R. 2003. Failure to genotype herpes simplex virus by real-time PCR assay and melting curve analysis due to sequence variation within probe binding sites. Journal of Clinical Microbiology, 41 (5): 2135-2137.

Anfoka G.H.A., Khalil A.B., Fattash I. 2003. Detection an molecular characterization of phytoplasma associated with big bud disease of tomatoes in Jordan. Journal of Phytopathology, 151: 223-227.

Angelini E., Clair D., Borgo M., Bertaccini A., Boudon-Padieu E. 2001. "Flavescence dorée" in France and Italy- Occurrence of closely related phytoplasma isolates and their near relationship to Palatine grapevine yellows and an alder phytoplasma. Vitis, 40: 79-86.

Angelini E., Bianchi G., Filippin L., Morassutti C., Borgo M. 2007. A new TaqMan method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. Journal of Microbiological Methods, 68 (3): 613-22.

Arocha Y., Antesana O., Montellano E., Franco P., Plata G., Jones P. 2007. '*Candidatus* Phytoplasma lycopersici', a phytoplasma associated with "hoja de perejil" disease in Bolivia. Intermational Journal of Systematic and Evolutionary Microbiology, 57 (8): 1704-1710.

Bai X., Zhang J., Ewing A., Miller S.A., Radek A.J., Shevchenko D.V., Tsukerman K., Walunas T., Lapidus A., Campbell J.W., Hogenhout S.A. 2006. Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. Journal of Bacteriology, 188: 3682–3696.

Bai X., Correa V.R., Toruño T.Y., Ammar E.-D., Kamoun S., Hogenhout S.A. 2008. AY-WB phytoplasma secretes a protein that targets plant cell nuclei. Molecular Plant-Microbe Interactions, 22: 18–30.

Barbara D.J., Morton A., Clark M.F., Davies D.L. 2001. Molecular variation in immunodominant membrane proteins from phytoplasmas. Acta Horticulturae, 550: 405-408.

Barbara D.J., Morton A., Clark M.F., Davies D.L. 2002. Immunodominant membrane proteins from two phytoplasmas in the aster yellows clade (chlorante aster yellows and clover phyllody) are highly divergent in the major hydrophilic region. Microbiology, 148: 157–167.

Baric, S., Kerschbamer, C., Dalla Via, J., 2006. TaqMan real-time PCR versus four conventional PCR assays for detection of apple proliferation pyhtoplasma. Plant Molecular Biology Reporter, 24: 169–184.

Bekele B., Hodgetts J., Tomlinson J., Boonham N., Nikolic P., Swarbrick P., Dickinson M. 2011. Use of a real-time LAMP isothermal assay for detecting 16SrII and XII phytoplasmas in fruit and weeds of the Ethiopian Rift Valley. Plant Pathology, 60: 345-355.

Berg M., Davies D.L., Clark M.F., Vetten J., Maier G., Seemüller E. 1999. Isolation of a gene encoding an immunodominant memebrane protein gene in the apple proliferation phytoplasma and expression and characterization of the gene product. Microbiology, 145: 1937-1943.

Berges R., Rott M., Seemüller E. 2000. Range of phytoplasma concentration in variousplant hosts as determined by competitive polymerase chain reaction. Phytopathology, 90: 1145-1152.

Bertaccini A. 2007. Phytoplasmas: diversity, taxonomy, and epidemiology. Frontieres in Bioscience, 12: 673-689.

Bertaccini A. 2014. http://www.ipwgnet.org/collection (last accessed April 27, 2016).

Bertaccini A., Marani F. 1982. Electron microscopy of two viruses and mycoplasma-like organisms in lilies with deformed flowers. Phytopathologia mediterranea, 21: 8-14.

Bertaccini A., Davis R.E., Lee I-M., Conti M., Dally E.L., Douglas S.M. 1990a. Detection of chrysanthemum yellows mycoplasmalike organism (MLO) by dot-hybridization and Southern blot analysis. Plant Disease, 74: 40-43.

Bertaccini A., Davis R.E., Lee I.-M. 1990b. Distinctions among mycoplasmalike organisms (MLOs) in *Gladiolus*, *Ranunculus*, *Brassica* and *Hydrangea* through detection with non-radioactive cloned DNA probes. Phytopathologia mediterranea, 29: 107-113.

Bertaccini A., Davis R.E., Hammond R.W., Bellardi M.G., Vibio M., Lee I-M. 1992a. Sensitive detection of mycoplasmalike organisms in field collected and *in vitro* propagated plants of *Brassica*, *Hydrangea* and *Chrysanthemum* by polymerase chain reaction. Annals of applied Biology, 121: 593-599.

Bertaccini A., Davis R.E., Lee I-M. 1992b. *In vitro* micropropagation for maintenance of mycoplasmalike organisms in infected plant tissues. Horticultural Science, 27: 1041-1043.

Bertaccini A., Vibio M., E. Stefani. 1995. Detection and molecular characterization of phytoplasmas infecting grapevine in Liguria (Italy). Phytopathologia Mediterranea, 34: 137-141.

Bertaccini A., Vorácková Z., Vibio M, Fránová J., Navrátil M., Špak J., Nebesárová J. 1998. Comparison of phytoplasmas infecting winter oilseed rape in the Czech Republic with Italian Brassica phytoplasmas and their relationship to the aster yellows group. Plant Pathology, 47 (3): 317–324.

Bertaccini A., Duduk B. 2009. Phytoplasma and phytoplasma diseases: a review of recent research. Phytopathologia Mediterranea, 48: 355-378.

Bertaccini A., Contaldo N., Calari A., Paltrinieri S., Windsor H.M., Windsor D. 2010. Preliminary results of axenic growth of phytoplasmas from micropropagated infected periwinkle shoots. 18th Congress IOM, Chianciano Terme, Italy, Abstract P-147, p. 153.

Bertaccini A., Paltrinieri S., Makarova O., Contaldo N., Nicolaisen M. 2011. Sharing information and collections on phytoplasmas: from QBOL to QBANK. Bulletin of Insectology, 64 (Supplement): S289-S290.

Bertaccini A., Duduk B., Paltrinieri S., Contaldo N. 2014. Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. American Journal of Plant Sciences, 5: 1763-1788.

Bertamini M., Nedunchezhian N. 2001. Effect of phytoplasma, “stolbur”-subgroup (“bois noir”-BN)] of photosynthetic pigments, saccarides, ribulose-1,5-bisphosphate carboxylase, nitrate and nitrite reductases and photosynthetic activities in field-grow grapevine (*Vitis vinifera* L. cv Chardonnay) leaves. Photosynthetica, 39 (1): 119-122.

Bertamini M., Nedunchezhian N., Tomasi F., Grando M.S. 2002. Phytoplasma [“stolbur”-subgroup (“bois noir”-BN)] infection inhibitis photosynthetic pigments, ribulose-1,5-biphosphate carboxylase and photosynthetic activities in field grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves. Physiological and Molecular Plant Pathology, 61 (6): 357-366.

Bertolini E., Teresani G.R., Loiseau M., Tanaka F.A.O., Barbé S., Martìnez C., Gentit P., Lòpez M.M., Cambra M. 2015. Transmission of ‘*Candidatus* Liberibacter solanacearum’ in carrot seeds. Plant Pathology, 64: 276–285.

Bextine B., Lauzon C., Potter S., Lampe D., Miller T.A. 2004. Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. Current Microbiology, 48: 327–331.

Biondi E., Blasioli S., Galeone A., Spinelli F., Cellini A., Lucchese C., Braschi I. 2014. Detection of potato brown rot and ring rot by electronic nose: from laboratory to real scale. Talanta, 129: 422–430.

Blasioli S., Biondi E., Samudrala D., Spinelli F., Cellini A., Bertaccini A., Cristescu S.M., Braschi I. 2014. Identification of volatile markers in potato brown rot and ring rot by combined GC-MS and PTR-MS techniques: study on *in vitro* and *in vivo* samples. Journal of Agricultural and Food Chemistry, 62 (2): 337–347.

Blaženčić J. 1982. Some anatomical characteristics of leaf and stem of maize with symptoms of redness. Bullettin Institute Jardin Botaniques Université Beograd (XIII) XV: 1-3.

Blomquist C.L., Barbara D.J., Davies D.L., Clark M.F., Kirkpatrick B.C. 2001. An immunodominant membrane protein gene from the western X-disease phytoplasma is distinct from those of other phytoplasmas. Microbiology, 147: 571-580.

Bonnet F., Saillard C., Kollar A., Seemüller E., Bové J.M. 1990. Detection and differentiation of the mycoplasmalike organism associated with apple proliferation disease using cloned DNA probes. Molecular Plant Microbe Interactions, 3: 438-443.

Botes A., Peyrot B.M., Olivier A.J., Burger W.P., Bellstedt D.U. 2005. Identification of three novel mycoplasma species from ostriches in South Africa. Veterinary Microbiology, 111: 159–169.

Botti S., Bertaccini A. 2006. Phytoplasma infection trough seed transmission: further observations. Book of Abstracts of the 16th International Congress of the IOM, Cambridge, UK, 9-14 July, 76: 113.

Boudon-Padieu E., Larrue J., Caudwell A. 1989. ELISA and dot blot detection of "flavescence dorée" MLO in individual leafhopper vector during latency and inoculative state. Current Microbiology, 19: 357-364.

Boudon-Padieu E., Cousin M.T., Daire X., Hiruki, C. Kuszala C., Roux J. 1996. Molecular and serological characterization of phytoplasma isolates inducing tomato "stolbur" symptoms on tomato. International Organisation for Mycoplasmology Letters, 4: 167–168.

Bové J.M., Danet J.L, Bananej K., Hassanzadeh N., Taghizadeh M., Salehi M., Garnier M. 2000. Witches' broom disease of lime in Iran. Proceedings of the 14th Conference of IOCV, pp 207–212. IOCV, Riverside (USA).

Bové J.M. 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. Journal of Plant Pathology, 88 (1): 7-37.

Buchman J.L., Heilman B.E., Munyaneza J.E. 2011. Effects of liberibacter-infective *Bactericera cockerelli* (Hemiptera: Triozidae) density on zebra chip potato disease incidence, potato yield, and tuber processing quality. Journal of Economic Entomology, 104: 1783–1792.

Bustin S.A., Benes V., Garson J.A., Hellemans J., Huggett J., Kubista M., Mueller R., Nolan T., Pfaffl M.W., Shipley G.L., Vandesompele J., Wittwer C.T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry, 55 (4): 611-22.

Büttner D., Bonas U. 2003. Common infection startegies of plant and animal pathogenic bacteria. Current Opinion in Plant Biology, 6: 312-319.

Calari A., Paltrinieri S., Contaldo N., Sakalieva D., Mori N., Duduk B., Bertaccini A. 2011. Molecular evidence of phytoplasmas in winter oilseed rape, tomato and corn seedlings. Bulletin of Insectology, 64 (Supplement): S157-S158.

Catal M., Ikten C., Yol E., Ustun R., Uzun B. 2013. First report of a 16SrIX group (pigeon pea witches' broom) phytoplasma associated with sesame phyllody in Turkey. Plant Disease, 97: 835.

Chen J., Pu X., Deng X., Liu S., Li H., Civerolo E. 2008. A phytoplasma closely related to the pigeon pea witches' broom phytoplasma (16SrIX) is associated with citrus huanglongbing symptoms in the state of São Paulo, Brazil. Phytopathology, 98: 977-984.

Chen K.H., Guo J.R., Wu X.J., Loi N., Carraro L., Guo H.J., Chen Y.D., Osler R, Pearson R. Chen T.A. 1993. Comparison of monoclonal antibodies, DNA probes, and PCR for detection of the grapevine yellows disease agent. Phytopathology, 83: 915-922.

Chen K.H., Credi R., Loi N., Maixner M., Chen T.A. 1994. Identification and grouping of mycoplasmalike organisms associated with grapevine yellows and clover phyllody diseases based on immunological and molecular analyses. Applied and Environmental Microbiology, 60: 1905-1913.

Chen T.A., Granados R.R. 1970. Plant-pathogenic mycoplasma-like organisms. Maintenance in vitro and transmission to Zea mays L.. Science, 167: 1633-1636.

Chen T.A., Liao C.H. 1975. Corn stunt spiroplasma: isolation cultivation and proof of pathogenicity. Science, 188: 1015-1017.

Chen L-L., Chung W-C., Lin C-P., Kuo C-H. 2012. Comparative analysis of gene content evolution in phytoplasmas and mycoplasmas. PloS One, 7(3): e34407.

Christensen N.M., Nicolaisen M., Hansen M., Schultz A. 2004. Distribution of phytoplasmas in infected plants as revealed by real time PCR and bioimaging. Molecular Plant–Microbe Interactions, 17: 1175-1184.

Christensen N.M., Axelsen K.B., Nicolaisen M., Schultz A. 2005. Phytoplasmas and their interactions with their hosts. Trend in Plant Science, 10: 526-535.

Chung K.R., Khan I.A., Brlansky R.H. 2006. Citrus diseases exotic to Florida: witches' broom disease of lime (WBDL). EDIS Publications, University of Florida, Gainesville, pp 228:1-228:3.

Cimerman A., Pacifico D., Salar P., Marzachì C., Foissac X. 2009. Striking diversity of vmp1, a variable gene encoding a putative membrane protein of the "stolbur" phytoplasma. Applied and Environmental Microbiology, 75: 2951–2957.

Clark M.F., Morton A., Buss S.L. 1989. Preparation of mycoplasma immunogens from plants and a comparison of polyclonal and monoclonal antibodies against primula yellows. Annals of Applied Biology, 114: 111-124.

Collmer A., Lindeberg M., Petnicki-Ocwieja T., Schneider D.J., Alfano J.R. 2002. Genomic mining type III secretion system effectors in *Pseudomonas syringae* yields new picks for all TTSS prospectors. Trends in Microbiology, 10(10): 462-9.

Contaldo N., Bertaccini A., Paltrinieri S., Windosor H.M. 2012. Axenic culture of plant pathogenic phytoplasmas. Phytopathologia Mediterranea, 51: 607-617.

Contaldo N., Paltrinieri S., Makarova O., Bertaccini A., Nicolaisen M. 2015. Q-bank phytoplasma: a DNA bar-coding tool for phytoplasma identification. Chapter 10 In: C. Lacomme editor. Plant Pathology, Techniques and Protocols, Methods in Molecular Biology, Springer, 1302: 123-135.

Contaldo N., Satta E., Zambon Y., Paltrinieri S., Bertaccini A. 2016. Development and evaluation of different complex media for phytoplasma isolation and growth. Journal of Microbiological Methods, 127: 105-110.

Cordova I., Jones P., Harrison N.A., Oropeza C. 2003. *In situ* detection of phytoplasma DNA in embryos from coconut palms with lethal yellowing disease. Molecular Plant Pathology, 4: 99-108.

Cornelis G.R., Van Gijsegem F. 2000. Assembly and function of type III secretory systems. Annual Review of Microbiology, 54: 735–774.

Crosslin J.M., Munyaneza J.E., Brown J.K., Liefting L.W. 2010. Potato zebra chip disease: a phytopathological tale. Plant Health Progress. https://www.plantmanagementnetwork.org/pub/php/review/2010/zebra/

Cvrković T., Jović J., Mitrović M., Krsti O., Toševski I. 2014. Experimental and molecular evidence of Reptalus panzeri as a natural vector of bois noir. Plant Pathology, 63: 42–53.

Daire X., Clair D., Reinert W., Boudon-Padieu E. 1997. Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the "stolbur" subgroup by PCR amplification of nonribosomal DNA. European Journal of Plant Pathology, 103: 507-514.

Dale J.L., Smith L.D. 1975. Mycoplasmalike bodies observed in tomato plants with big bud in Arkansas. Plant Disease Reporter, 59: 455-458.

Danet J.L., Bonnet P., Jarausch W., Carraro L., Skoric D., Labonne G., Foissac X. 2007. Imp and secY, two new markers for MLST (multilocus sequence typing) in the 16SrX phytoplasma taxonomic group. Bullettin of Insectology, 60: 339-340.

Danielli A., Bertaccini A., Alma A., Bosco D., Vibio M., Arzone A. 1996. May evidence of 16SrI-group-related phytoplasmas in eggs, nymphs and adults of *Scaphoideus titanus* Ball suggest their transovarial transmission? IOM Letters, 4: 190-191.

Davis R.E., Lee I.-M. 1993. Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. Phytopathology, 83: 1008-1011.

Davis R.E., Lee I-M., Douglas S.M., Dally E.L. 1990. Molecular cloning and detection of chromosomal and extrachromosomal DNA of the mycoplasmalike organisms (MLO) associated with little leaf disease in periwinkle (*Catharanthus roseus*). Phytopathology, 80: 789-793.

Davis R.E., Jomantiene R., Zhao Y., Dally E.L. 2003. Folate biosynthesis pseudogenes, ψ fol P and ψ fol K, and an O-Sialoglycoprotein endopeptidase gene homolog in the phytoplasma genome. DNA and Cell Biology, 22: 697-706.

De La Rue S.J., Hopkinson R., Gibb K.S. 2002. Assessment of *Stylosanthes* seed yield reduction caused by phytoplasma-associated diseases. Australian Journal of Experimental Agriculture, 42 (8): 1053-1056.

Del Serrone P., Marzachì C., Bragaloni M., Galeffi P. 2001. Phytoplasma infection of tomato in central Italy. Phytopathoogia Mediterranea, 40: 137–142.

Deng S., Hiruki C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. Journal of Microbiology Methods, 14: 53-61.

Dickinson M., Tuffen M., Hodgetts J. 2013. The phytoplasmas: an introduction. In: Phytoplasmas: methods and protocols Dickinson M. and Hodgetts J., (Eds.), 938: 1-14.

Dienes L., Bullivant S. 1968. Morphology and reproductive precesses of the L-forms of bacteria. II Comparative study of L-forms and mycoplasma with the electron microscope. Journal of Bacteriology, 95: 672-687.

Doi Y.T.M., Yora K., Asuyama H. 1967. Mycoplasma or PLTgroup-like organisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows or paulownia witches broom. Annals of the Phytopathological Society of Japan, 33: 259–266.

Du Y., Mou H., Shi B., Xu X., Xiang B. 2013. Molecular detection and identification of a 16SrVI group phytoplasma associated with big bud disease in Xinjiang, China. Journal of Phytopathology, 161: 870-873.

Duduk B., Calari A., Paltrinieri S., Duduk N., Bertaccini A. 2009. Multigene analysis for differentiation of aster yellows phytoplasmas infecting carrots in Serbia. Annals of Applied Biology, 154 (2): 219-229.

Duduk B., Bertaccini A. 2006. Corn with symptoms of reddening: new host of "stolbur" phytoplasma. Plant Disease, 90: 1313-1319.

Eamens A., Wang M.B., Smith N.A., Waterhouse P.M. 2008. RNA silencing in plants: yesterday, today, and tomorrow. Plant Physiology, 147: 456-68.

Ebbert M.A., Jeffers D.P., Harrison N.A., Nault L.R. 2001. Lack of specificity in the interaction between two maize stunting pathogens and field collected *Dalbulus* leafhoppers. Entomologia Experimentalis et Applicata, 101: 49-57.

Edward D.G. 1967. Problems and classification – an introduction. Annals of the New York Academy of Science, 143: 7-8.

Elmendorf E. 1977. Newly discovered prokaryotic plant pathogens - a bibliography. Research Bulletin, College of Agricultural and Life Sciences, University of Wisconsin, Madison, 1-85 (R 2901).

EPPO, 2012. First report of '*Candidatus* Liberibacter solanacearum' on carrots and celery in Spain, in association with Bactericera trigonica. EPPO Reporting Service – Pests and Diseases, 6: 4–5.

EPPO, 2013. Data sheets on pests recommended for regulation. '*Candidatus* Liberibacter solanacearum'. EPPO Bulletin, 43: 197–201.

Errampalli D., Fletcher J., Claypool P.L. 1991. Incidence of yellows in carrot and lettuce and characterization of mycoplasmalike organism isolates in Oklahoma. Plant Disease, 75: 579-584.

Errea P., Aguelo V., Hormaza J. 2002, Seasonal variations in detection and transmission of pear decline phytoplasma. Journal of Phytopathology, 150: 439-443.

Fabre A., Danet J.L., Foissac X., 2011. The "stolbur" phytoplasma antigenic membrane protein gene STAMP is submitted to diversifying positive selection. Gene, 472: 37-41.

Faghihi M.M., Bagheri A.N., Bahrami H.R., Hasanzadeh H., Rezazadeh R. 2011. Witches' broom disease of lime affects seed germination and seedling growth but is not seed transmissible. Plant Disease, 95 (4): 419–422.

Favali M.A., Musetti R., Fossati F., Vighi C. 2000. Association of "stolbur" phytoplasma with diseased tomatoes in Italy. EPPO Bulletin, 30: 347–350.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39: 783-791.

Filippin L., Jovic J., Cvrkovic T., Forte, V. Clair D., Tosevski I., Boudon-Padieu E., Borgo M., Angelini E. 2009. Molecular characteristics of phytoplasmas associated with "flavescence dorée" in clematis and grapevine and preliminary results on the role of *Dictyophara europaea* as a vector. Plant Pathology, 58(5): 826-837.

Firrao G., Garcia Chapa M., Marzachì C. 2007. Phytoplasmas: genetics, diagnosis and relationships with the plant and insect host. Frontiers Bioscience, 12: 1352-1375.

Font I., Abad P., Albinana M., Espino A.I., Dally E.L., Davis R.E., Jorda C. 1999. Amarilleos y enrojecimientos en zanahoria: una enfermedad a diagnostico. Boletín de Sanidad Vegetal Plagas, 25: 405–415.

Frisinghelli C., Delaiti L., Grando M. S., Forti D., Vindimian M.E. 2000. *Cacopsylla costalis* (Flor, 1861), as a vector of apple proliferation in Trentino. Journal of Phytopathology, 148: 425-431.

Gaffuri F., Sacchi S., Cavagna B. 2011. First detection of the mosaic leafhopper, *Orientus ishidae*, in northern Italy infected by the "flavescence dorée" phytoplasma. New Disease Report, 24: 22.

Galetto L., Bosco D., Marzachì C. 2005. Universal and group-specific real-time PCR diagnosis of "flavescence dorée" (16Sr-V), "bois noir" (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant hosts and insect vectors. Annals of Applied Biology, 147: 191-201.

Garcia-Chapa M., Medina V., Viruel M.A., Lavina A., Battle A. 2003. Seasonal detection of pear decline phytoplasma by nested-PCR in different pear cultivars. Plant Pathology, 52: 513–520.

Garnier M., Zreik L., Bové J.M. 1991. Witches' broom, a lethal mycoplasmal disease of lime trees in the Sultanate of Oman and the United Arab Emirates. Plant Disease, 75: 546-551.

Ghosh D.K., Das A.K., Singh S., Singh S.J., Ahlawat Y.S. 1999. Occurrence of witches' broom, a new phytoplasma disease of acid lime (*Citrus aurantifolia*) in India. Plant Disease, 83 (3): 302.

Gianotti J., Vago C. 1971. Role des mycoplasmes dans l'étiologie de la phylloidie du trèfle: culture et transmission expèrimentale de la maladie. Physiologie Végétale, 9: 541-553.

Gibb K.S., Padovan A.C., Mogen B.D. 1995. Studies on sweet potato little-leaf phytoplasma detected in sweet potato and other plant species growing in Northern Australia. Phytopathology, 85: 169-174.

Gibb K.S., Davis R., Constable F. 1996. Tomato big bud phytoplasma. A widespread and "successful" phytoplasma in Australia. International Organisation for Mycoplasmology Letters, 4: 280.

Giuliani M.M., Contaldo N., Nardella E., Quitadamo M., Vocale E., Bertaccini A. 2010. Il giallume dell'astro colpisce i pomodori da industria pugliesi. L'Informatore Agrario, 11: 75-78.

Giunchedi L., Poggi Pollini C., Credi R. 1982. Susceptibility of stone fruit trees to the Japanese plum tree decline causal agent. Acta Horticulturae, 130: 285–290.

Gundersen D.E, Lee I-M., Rehner S.A., Davis R.E, Kingsbury D.T. 1994. Phylogeny of mycoplasmalike organisms (phytoplasmas): a basis for their classification. Journal of Bacteriology, 176: 5244-5254.

Gundersen D.E., Lee I-M. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea, 35: 144-151.

Gundersen D.E., Lee I.-M., Schaff D.A., Harrison N.A., Chang C.J., Davis R.E., Kinsbury D.T. 1996. Genomic diversity among phytoplasma strains in 16S rRNA Group I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). International Journal of Systematic Bacteriology, 46: 64-75.

Gungoosingh-Bunwaree A., Contaldo N., Mejia J.F., Paltrinieri S., Beni Madhu P., Bertaccini A. 2013. Phytoplasma and virus diseases on tomato in Mauritius. Australasian Plant Pathology, 42: 659.

Guthrie J.N., White D.T., Walsh K.B., Scott P.T., Rasmussent T.S. 2001. The phytopathology of Australian papaya dieback: a proposed role for the phytoplasma. Physiological Molecular Plant Pathology, 58: 23-30.

Hanboonsong Y., Choosai C., Panyim S. Damak S. 2002. Transovarial transmission of sugarcane white leaf phytoplasma in the insect vector *Matsumuratettix hiroglyphicus* (Matsumura). Insect Molecular Biology, 11: 97-103.

Hansen A.K., Trumble J.T., Stouthamer R., Paine T.D. 2008. A new Huanglongbing species, '*Candidatus* Liberibacter psyllaurous', found to infect tomato and potato, is vectored by the psyllid *Bactericera cockerelli* (Sulc). Applied and Environmental Microbiology, 74: 5862–5865.

Harrison N.A., Oropeza C. 1997. Recent studies on detection of lethal yellowing disease phytoplasmas in the Americas. In Proceedings of an International Workshop on Lethal Yellowing-Like Diseases of Coconut, Elmina Ghana, November 1995 (Eden-Green, S.J. and Ofori, F., eds), pp. 221–234. Chatham, UK: Natural Resources Institute.

Harrison N.A., Bourne C.M., Cox R.L., Tsai J.H., Richardson P.A. 1992. DNA probes for detection of mycoplasmalike organisms associated with lethal yellowing disease of palms in Florida. Phytopathology, 82: 216-224.

Harrison N.A., Richardson P.A., Kramer J.B., Tsai J.H. 1994. Detection of the mycoplasma-like organism associated with lethal yellowing disease of palms in Florida by polymerase chain reaction. Plant Pathology, 43: 998-1008.

Harrison N.A., Richardson P.A., Tsai J.H., Ebbert M.A., Kramer J.B. 1996. PCR assay for detection of the phytoplasma associated with maize bushy stunt disease. Plant Disease, 80: 263-269.

Harrison N.A., Jones P. 2004. Lethal yellowing. In: Elliott, M.L., Broschat, T.K., Uchida, J.Y. and Simone, G.W., Eds., Compendium of Ornamental Palm Diseases and Disorders, APS Press, St. Paul, 39-41.

Hauck P., Thilmony R., He S.Y. 2003. A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. Proceedings of the National Academy of Sciences USA, 100: 8577–8582.

Hayden R.T., Gu Z., Ingersoll J., Abdulali D., Shi L., Pounds S., Caliendo A.M. 2013. Comparison of droplet digital PCR to realtime PCR for quantitative detection of cytomegalovirus. Journal of Clinical Microbiology, 51: 540-546.

Hayflick L. 1969. Fundamental biology of the class *Mollicutes* order *Mycoplasmatales*. In The *Mycoplasmatales* and the L-phase of bacteria. Ed. L. Hayflick, Amsterdam, North Holland Publ. Co.

Heinrich M., Botti S., Caprara L., Arthofe W., Strommer, S., Hanzer V., Katinger,H., Bertaccini A., Laimer da Câmara Machado M. 2001. Improved detection methods for fruit tree phytoplasmas. Plant Molecular Biology Reporter, 19: 169-179.

Henrich T.J., Gallien S., Li J.Z., Pereyra F., Kuritzkes D.R. 2012. Low-level detection and quabtification of cellular HIV-1 DNA and 2-LTR circles using droplet digital PCR. Journal of Virological Methods, 186: 68-72.

Hindson B.J., Ness K.D., Masquelier D.A., Belgrader P., Heredia N.J., Makarewicz A.J., Bright I.J., Lucero M.Y., Hiddessen A.L., Legler T.C., Kitano T.K., Hodel M.R., Petersen J.F., Wyatt P.W.,

Steenblock E.R., Shah P.H., Bousse L.J., Troup C.B., Mellen J.C., Wittmann D.K., Erndt N.G., Cauley T.H., Koehler R.T., So A.P., Dube S., Rose K.A., Montesclaros L., Wang S., Stumbo D.P., Hodges S.P., Romine S., Milanovich F.P., White H.E., Regan J.F., Karlin-Neumann G.A., Hindson C.M., Saxonov S., Colston B.W. 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical Chemistry, 83: 8604-8610.

Hodgetts J., Boonham N., Mumford R., Harrison N., Dickinson M. 2008. Phytoplasma phylogenetics based on analysis of secA and 23S rRNA gene sequences for improved resolution of candidate species of '*Candidatus* Phytoplasma'. International Journal of Systematic and Evolutionary Microbiology, 58: 1826–1837.

Hodgetts J., Boonham N., Mumford R., Dickinson M. 2009. Panel of 23S rRNA gene-based real-time PCR assays for improved universal and group-specific detection of phytoplasmas. Applied Environmental Microbiology, 75: 2945–2950.

Hodgetts J., Tomlinson J., Boonham N., González-Martín I., Nikolić P., Swarbrick P., Yankey E.N., Dickinson M. 2011. Development of rapid in-field loop-mediated isothermal amplification (LAMP) assays for phytoplasmas. Bulletin of Insectology, 64 (Supplement): S41-S42.

Hogenhout S.A., Oshima K., Ammar E.-D., Kakizawa S., Kingdom H.N., Namba S. 2008. Phytoplasmas: bacteria that manipulate plants and insects. Molecular Plant Pathology, 9: 403–423.

Hong Y., Davies D.L., Wezel R.V., Ellerker B.E., Morton A., Barbara D. 2001. Expression of the immunodominant membrane protein of chlorantie-aster yellows phytoplasma in *Nicotiana benthamiana* from a potato virus X-based vector. Acta Horticulturae, 550: 409-415.

Hopps H.E., Del Giudice R.A., Barile M.F. 1976. Current status of "non-cultivable" mycoplasmas. Proceedings of the Society for General Microbiology, 3: 143.

Hoshi A., Ishii Y., Kakizawa S., Oshima K., Namba S. 2007. Host-parasite interaction of phytoplasmas from a molecular biological perspective. Bulletin of Insectology, 60 (2): 105-107.

Hoshi A., Oshima K., Kakizawa S., Ishii Y., Ozeki J., Hashimoto M., Komatsu K., Kagiwada S., Yamaji Y., Namba S. 2009. A unique virulence factor for proliferation and dwarfism in plants identified from a phytopathogenic bacterium. Proceedings of the National Academy of Sciences, 106: 6416-6421.

Huvenne H., Smagghe G. 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. Journal of Insect Physiology, 56(3): 227-235.

ICSB, Subcommittee on the Taxonomy of Mollicutes. 1979. Proposal of minimal standards for description of new species of the class *Mollicutes*. International Journal of Systematic and Bacteriology, 29: 172-180.

Ikten C., Catal M., Yol E., Ustun R., Furat S., Toker C., Uzun B. 2014. Molecular identification, characterization and transmission of phytoplasmas associated with sesame phyllody in Turkey. European Journal of Plant Pathology, 139: 217–229.

IRPCM 2004. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. International Journal of Systematic and Evolutionary Microbiology, 54 (4): 1243–1255.

Ishii Y., Oshima K., Kakizawa S., Hoshi A., Maejima K., Kagiwada S., Yamaji Y., Namba S. 2009. Process of reductive evolution during 10 years in plasmids of a non-insect-transmissible phytoplasma. Gene, 446 (2): 51–57.

Jackson R.W., Athanassopoulos E., Tsiamis G., Mansfield J.W., Sesma A., Arnold D.L., Gibbon M.J., Murillo J., Taylor J.D., Vivian A. 1999. Microbiology Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar *phaseolicola* (plant disease resistance and

hypersensitive reaction signal transduction). Proceedings of the National Academy of Sciences USA, 96: 10875–10880.

Jagoueix S., Bové J.M., Garnier M. 1996. PCR detection of the two '*Candidatus* liberobacter' species associated with greening disease of citrus. Molecular and Cellular Probes, 10: 43–50.

Jagoueix-Eveillard S., Tarendau F., Guolter K., Danet J-L., Bové J.M., Garnier M. 2001. *Catharanthus roseus* genes regulated differentially by mollicute infections. Molecular Plant-Microbe Interactions, 14: 225-233.

Jarausch W., Lansac M., Dosba F. 1999. Seasonal colonization pattern of European stone fruit yellows phytoplasma in different *Prunus* species detected by specific PCR. Journal of Phytopathology, 147: 47–54.

Jarausch B., Schwind N., Jarausch W., Krczal G. 2004. Overwintering adults and springtime generation of *Cacopsylla picta* (synonym *C. costalis*) can transmit apple proliferation phytoplasmas. Acta Horticolturae, 657: 409-413.

Jawhari M., Abrahamian P., Sater A.A., Sobh H., Tawidian P., Abou-Jawdah Y. 2015. Specific PCR and real-time PCR assays for detection and quantitation of '*Candidatus* Phytoplasma phoenicium'. Molecular and Cellular Probes, 29 (1): 63-70.

Jiang H., Wei W., Saiki T., Kawakita H., Watanabe K., Sato M. 2004. Distribution patterns of mulberry dwarf phytoplasma in reproductive organs, winter buds, and roots of mulberry trees. Journal of General Plant Pathology, 70: 168–173.

Jomantiene R., Davis R.E. 2006. Clusters of diverse genes existing as multiple, sequence-variable mosaics in a phytoplasma genome. FEMS Microbiology Letters, 255 (1): 59-65.

Jomantiene R., Zhao Y., Davis R.E. 2007. Sequence-variable mosaics: composites of recurrent transposition characterizing the genomes of phylogenetically diverse phytoplasmas. DNA Cell Biology, 26 (8): 557-564. Erratum 26 (9): 695.

Jones A.L., Whitcomb R.F., Williamson D.L., Coan M.E. 1977. Comparative growth and primary isolation of spiroplasmas in media based on isect tissue culture formulations. Phytopathology, 67: 738-746.

Kakizawa S., Oshima K., Kuboyama T., Nishigawa H., Jung H.Y., Sawayanagi T., Tsuchizaki T., Miyata S., Ugaki M., Namba S. 2001. Cloning and expression analysis of phytoplasma protein translocation genes. Molecular Plant Microbe Interactions, 14: 1043–1050.

Kakizawa S., Oshima K., Nishigawa H., Jung H.Y., Wei W., Suzuki S., Tanaka M., Miyata S., Ugaki M., Namba S. 2004. Secretion oimmunodominant membrane protein from onion yellows phytoplasma through the Sec protein-translocation system in Escherichia coli. Microbiology, 150: 135–142.

Kakizawa S., Oshima K., Namba S. 2006. Diversity and functional importance of phytoplasma membrane proteins. Trends Microbiology, 14: 254–256.

Kakizawa S., Oshima K., Ishii Y., Hoshi A., Maejima K., Jung H.Y., Yamaji Y., Namba S. 2009. Cloning of immunodominant membrane protein genes of phytoplasmas and their *in planta* expression. FEMS Microbiology Letters, 293 (1): 91-101.

Kawakita H., Saiki T., Wei W., Mitsuhasi W., Watanabe K., Sato M. 2000. Identification of mulberry dwarf phytoplasmas in genital organs and eggs of the leafhopper *Hishimonoides sellatiformis*. Phytopathology, 90: 909-914.

Khan A.J., Botti S., Paltrinieri S., Al-Subhi A.M., Bertaccini A. 2002. Phytoplasmas in alfalfa seedlings: infected or contaminated seeds? Book of Abstracts of the 14th International Congress of the IOM, Vienna, July 07-12 2002: 6.

Kirkpatrick B.C., Stenger B.C., Morris T.J., Purcell A.H. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. Science, 238: 197-200.

Klineberger E. 1935. The natural occurrence of pleuropneumonia-like organisms in apparent symbiosis with *Streptobacillus moniliformis* and other bacteria. The Journal of Bacteriology and Pathology, 40: 93.

Kogovšek P., Hodgetts J., Hall J., Prezelj N., Nikolić P., Mehle N., Lenarčič R., Rotter A., Dickinson M., Boonham N., Dermastia M., Ravnikara M. 2015. LAMP assay and rapid sample preparation method for on-site detection of "flavescence dorée" phytoplasma in grapevine. Plant Pathology, 64 (2): 286–296.

Kogovšek P., Mehle N., Pugelj A., Jakomin T., Schroers H-J., Ravnikar M., Dermastia M. 2016. Rapid loop-mediated isothermal amplification assays for grapevine yellows phytoplasmas on crude leaf-vein homogenate has the same performance as qPCR. European Journal of Plant Pathology, doi:10.1007/s10658-016-1070-z.

Ku C., Lo W-S., Kuo C-H. 2013. Horizontal transfer of potential mobile units in phytoplasmas. Mobile Genetic Elements, 3 (5): e26145.

Kube M., Mitrovic J., Duduk B., Rabus R., Seemüller E. 2012. Current view on phytoplasma genomes and encoded metabolism. The Scientific World Journal, http://dx.doi.org/10.1100/2012/185942

Kuske C.R., Kirkpatrick B.C., Davis M.J., Seemüller E. 1991. DNA hybridization between western aster yellows mycoplasmalike organism plasmid and extracromosomal DNA from other plant pathogenic mycoplasmalike organisms. Molecular Plant-Microbe Interactions, 4: 75-80.

Lebsky V., Poghosyan A, Silva-Rosales L. 2010. Application of scanning electron microscopy for diagnosing phytoplasmas in single and mixed (virus-phytoplasma) infection in papaya. Julius-Kühn-Archiv, 427: 70-78.

Lederer W., Seemüller E. 1992. Detection of mycoplasmas in *Prunus* species in Germany. Journal of Phytopathology, 134: 89-96.

Lee I-M., Davis R.E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organism by using cloned DNA and RNA probes. Plant-Microbe Interactions, 1: 303-310.

Lee I-M., Davis R.E. 1992. Mycoplasmas which infect plants and insects. In: Maniloff J., McElhaney R.N., Finch L.R., Baseman J.B. (eds.). Mycoplasmas - molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C. pp. 379-390.

Lee I-M., Hammond R.W., Davis R.E., Gundersen D.E. 1993a. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology, 83: 834-842.

Lee I-M., Davis R.E., Hsu H-T. 1993b. Differentiation of strains in the aster yellows mycoplasmalike organisms strain cluster by serological assay with monoclonal antibodies. Plant Disease, 77: 815-817.

Lee I-M., Gundersen D.E., Hammond R.W., Davis R.E. 1994. Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology, 84: 559-566.

Lee I-M., Bertaccini A., Vibio M., Gundersen D.E. 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology 85: 728-735.

Lee I-M., Gundersen-Rindal D.E., Davis R.E., Bartoszyk I.M. 1998a. Revised classification scheme of phytoplasmas based on RFLP analysis of 16S rRNA and ribosomal protein gene sequences. International Journal of Systematic Bacteriology, 48: 1153–1169.

Lee I-M., Gundersen-Rindal D.E., Bertaccini A. 1998b. Phytoplasma: ecology and genomic diversity. Phytopathology, 88: 1359-1366.

Lee I-M., Davis R.E., Gundersen-Rindal D.E. 2000. Phytoplasmas: phytopathogenic mollicutes. Annual Review of Microbiology, 56: 1593-1597.

Lee I-M., Gundersen-Rindal D.E., Davis R.E., Bottner K.D., Marcone C., Seemüller E. 2004. '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. International Journal of Systematic and Evolutionary Microbiology, 54: 1037-1048.

Lee I-M., Zhao Y., Bottner K.D. 2005. Novel insertion sequence-like elements in phytoplasma strains of the aster yellows group are putative new members of the IS3 family. FEMS Microbiology Letters, 242 (2): 353-360.

Lee I-M., Zhao Y., Davis R.E., Wei W., Martini M. 2007. Prospects of DNA-based systems for differentiation and classification of phytoplasmas. Bulletin of Insectology, 60 (2): 239.

Lee I M., Zhao Y., Davis R.E., Weintraub P.G., Jones P. 2010. Prospects of multiple gene-based systems for differentiation and classification of phytoplasmas. Phytoplasmas: genomes, plant hosts and vectors, 51-63. Edited by P. G. Weintraub & P. Jones. Wallingford: CAB International.

Lee I-M., Bottner-Parker K.D., Zhao Y., Bertaccini A., Davis R.E. 2012. Differentiation and classification of phytoplasmas in the pigeon pea witches' broom group (16SrIX): an update based on multiple gene sequence analysis. International Journal of Systematic and Evolutionary Microbiology, 62: 2279-2285.

Lepka P., Stitt M., Moll E., Seemüller E. 1999. Effect of phytoplasmal infection on concentration and translocation of carbohydrates and amino acids in periwinkle and tobacco. Physiological Molecular Plant Pathology, 55: 59–68.

Lherminier J., Prensier G., Boudon-Padieu E., Caudwell A. 1990. Immunolabeling of grapevine "flavescence dorée" MLO in salivary glands of *Euscelidius variegatus*: a light and electron microscopy study. Journal of Histochemistry and Cytochemistry, 38: 79-85.

Liefting L.W., Shaw M.E., Kirkpatrick B.C. 2004. Sequence analysis of two plasmids from the phytoplasma beet leafhopper-transmitted virescence agent. Microbiology, 150 (6): 1809-1817.

Liefting L.W., Egusquiza Z.C., Clover G.R.G., Anderson J.A.D. 2008. A new '*Candidatus* Liberibacter' species in *Solanum tuberosum* in New Zealand. Plant Disease, 92: 1474.

Liefting L.W., Beir B.S., Pennycook S.R., Clover G.R.G. 2009. '*Candidatus* Liberibacter solanacearum', associated with plants in the family Solanaceae. International Journal of Systematic and Evolutionary Microbiology, 59: 2274–2276.

Lim P.O., Sears B.B. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasmalike organisms are evolutionarily distinct from animal mycoplasmas. Journal of Bacteriology, 171: 5901-5906.

Lim P.O., Sears B.B. 1991a. DNA sequence of the ribosomal protein genes rp12 and rps19 from a plant-pathogenic mycoplasma-like organism. FEMS Microbiology Letters, 84: 71-74.

Lim P.O., Sears B.B. 1991b. The genome size of a plant-pathogenic mycoplasma-like organism resembles those of animal mycoplasmas. Journal of Bacteriology, 173: 2128-2830.

Lim, P.O., Sears, B.B. 1992. Evolutionary relationships of a plant pathogenic mycoplasma-like organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. Journal of Bacteriology, 174: 2606–2611.

Lin C.P., Chen T.A. 1985. Monoclonal antibodies against the aster yellows agent. Science, 227: 1233-1235.

Lin C.P., Chen T.A. 1996. *In vitro* and *in vivo* immunization techniques for the production of monoclonal antibodies against the aster yellows phytoplasma. Plant Pathology Bulletin, 5: 28-32.

Lisa V., Lovisolo O., D'Agostino G., Loche P., Fresu B. 1983. Malattie associate a micoplasma ed a virus in pomodoro in Sardegna. L'Informatore Agrario 39 (28): 26693–26696.

Lombardo G., Pignatelli P. 1970. Cultivation in a cell-free medium of a mycoplasma-like organism from *Vinca rosea* with phyllody symptoms of the flowers. Annals of Microbiology, 20: 83-88.

Lorenz K.H., Schneider B., Ahrens U., Seemüller E. 1995. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology, 85: 771-776.

Lu Y-T., Li M-Y., Cheng K-T., Tan C.M., Su L-W., Lin W-Y., Shih H-T., Chiou T-J., Yang J-Y. 2014. Transgenic plants that express the phytoplasma effector SAP11 show altered phosphate starvation and defense responses. Plant Physiology, 164: 1456–1469.

MacLean A.M., Sugio A., Makarova O.V., Findlay K.C., Grieve V.M., Toth R., Nicolaisen M, Hogenhout S. 2011. Phytoplasma effector SAP54 induces indeterminate leaf-like flower development in *Arabidopsis* plants. Plant Physiology, 157: 831– 841.

MacLean A.M., Orlovskis Z., Kowitwanich K., Zdziarska A.M., Angenent G.C., Immink R.G.H., Hogenhout S.A. 2014. Phytoplasma effector SAP54 hijacks plant reproduction by degrading MADS-box proteins and promotes insect colonization in a RAD23-dependent manner. PLoS Biology, 2: e1001835.

Maejima K., Iwai R., Himeno M., Komatsu K., Kitazawa Y., Fujita N., Ishikawa K., Fukuoka M., Minato N., Yamaji Y., Oshima K. Namba S. 2014. Recognition of floral homeotic MADS domain transcription factors by a phytoplasmal effector, phyllogen, induces phyllody. The Plant Journal, 78: 541–554.

Makarova O.V., Contaldo N., Paltrinieri S., Kawube G., Bertaccini A., Nicolaisen M. 2012. DNA barcoding for universal identification of '*Candidatus* Phytoplasmas' using a fragment of the elongation factor Tu gene. PLoS One, 7(12): e52092.

Maliogka V.I., Tsialtas J.T., Papantoniou A., Efthimiou K., Katis N.I. 2009. First report of a phytoplasma associated with an oilseed rape disease in Greece. Plant Pathology, 88: 792.

Maramorosch K, 2011. Historical reminiscences of phytoplasma discovery. Bulletin of Insectology, 64 (Suppl.): S5–S8.

Maramorosch K., Harris K.F. eds. 1981. Plant diseases and vectors. Ecology and Epidemiology. Academic press, New York/London, 368 pp.

Marco F.D., Pagliari L., Degola F., Buxa S.V., Loschi A., Dinant S., Hir R.L., Morin H., Santi S., Musetti R. 2016. Combined microscopy and molecular analyses show phloem occlusions and cell wall modifications in tomato leaves in response to '*Candidatus* Phytoplasma solani'. Journal of Microscopy, 263(2): 212-225.

Marcone C. 2010. Movement of phytoplasmas and the development of disease in the plant. In: P.G. Weintraub, P. Jones Phytoplasmas - Genomes, Plant Hosts and Vectors. Pp. 114-131 Wallingford, Oxfordshire, CAB International.

Marcone C., Ragozzino A. 1995. Tomato big bud disease in southern Italy and characterisation of its causal agent by RFLP analysis. Petria, 5: 195–205.

Marcone C., Ragozzino A., Seemüller E. 1996. Association of phytoplasmas with the decline of European hazel in southern Italy. Plant Pathology, 45: 857–63.

Marcone C., Ragozzino A., Seemüller E. 1997. Detection and identification of phytoplasmas infecting vegetable, ornamental and forage crops in southern Italy. Journal of Plant Pathology, 79: 211–217.

Marcone C., Neimark H., Ragozzino A., Lauer U., Seemüller E. 1999. Chromosome sizes of phytoplasmas composing major phylogenetic groups and subgroups. Phytopathology, 89 (9): 805-810.

Marcone C., Lee I-M., Davis R.E., Ragozzino A., Seemüller E. 2000. Classification of aster yellows-group phytoplasmas based on combined analyses of rRNA and tuf gene sequences. International Journal of Systematic and Evolutionary Microbiology, 50 (5): 1703-1713.

Mardi M., Khayam Nekouei S.M., Farsad L.K., Ehya F., Shabani M., Shafiee M., Tabatabaei M., Safarnejad M.R., Salehi Jouzani G., Hosseini Salekdeh G. 2011. Witches' broom disease of Mexican lime trees: disaster to be addressed before it will be too late. Bulletin of Insectology, 64 (Supplement): S205-S206.

Marić A., Savić R. 1965. Dosadašnji rezultati istraživanja crvenila kukuruza. Dokumentacija za tehnologiju i tehniku u poljoprivredi: 8.

Marois E., van den Ackerveken G., Bonas U. 2002. The *Xanthomonas* type III effector protein AvrBs3 modulates plant gene expression and induces cell hypertrophy in the susceptible host. Molecular Plant–Microbe Interactions, 15: 637–646.

Martelli G.P., Russo M., Cirulli M. 1969. Brevi note sullo "stolbur" del pomodoro in Puglia. Phytopathologia Mediterranea, 8: 150–154.

Martini M., Murari E., Mori N. Bertaccini A. 1999. Identification and epidemic distribution of two "flavescence dorée"-related phytoplasmas in Veneto (Italy). Plant Disease, 83: 925-930.

Martini M., Botti S., Marcone C., Marzachì C., Casati P., Bianco P.A., Benedetti R., Bertaccini A. 2002. Genetic variability among "flavescence dorée" phytoplasmas from different origins in Italy and France. Molecular and Cellular probes, 16 (3): 197-208.

Martini M., Lee I-M., Bottner K.D., Zhao Y., Botti S., Bertaccini A., Harrison N.A., Carraro L., Marcone C., Khan J., Osler R. 2007. Ribosomal protein gene-based filogeny for finer differentiation and classification of phytoplasmas. International Journal of Systematic and Evolutionary Microbiology, 57: 2037-2051.

Marzachì, Veratri F., d'Aquilio M., Vischi A., Conti M., Boccardo G. 2000. Molecular hybridization and PCR amplification of non-ribosomal DNA to detect and differentiate "stolbur" phytoplasma isolates from Italy. Journal of Plant Pathology, 82: 201–212.

Maust B.E., Espadas F., Talavera C., Aguilar M., Santamaría J.M., Oropeza C. 2003. Changes in carbohydrate metabolism in coconut palms infected with the lethal yellowing phytoplasma. Phytopathology, 93 (8): 976-981.

McCoy R.E., Caudwell A., Chang C.J., Chen T.A., Chiykowskyi L.N., Cousin M.T., Dale de Leeuw G.T.N., Golino D.A., Hackett K.J., Kirkptrick B.C., Marwitz R., Petzold H., Shina R.H., Sugiura M., Whitcomb R.F., Yang I.L., Zhu B.M., Seemüller E. 1989. Plant diseases associated with mycoplasmalike organisms. In: The Mycoplasmas, vol. 5. (eds. Whitcomb R.F. e Tully J.G.). Academic Press, New York. Pp. 545-640.

McGee Z.A., Rogul M., Wittler R.G. 1967. Molecular genetic studies of relationships among mycoplasma, L-forms and bacteria. Annals of the New York Academy of Science, 143: 21-30.

McLarty H.R. 1948. Killing of pear trees. Annual report of the Canadian plant disease survey, 28: 77.

Mehle N., Brzin J., Boben J., Hren M., Frank J., Petrovič N., Gruden K., Dreo T., ¸Žežlina I., Seljak G. Ravnikar M. 2007. First report of '*Candidatus* Phytoplasma mali' in *Prunus avium*, *P. armeniaca* and *P. domestica*. Plant Pathology, 56: 721.

Mehle N., Seljak G., Rupar M., Ravnikar M., Dermastia M. 2010. The first detection of a phytoplasma from 16SrV (elm yellows) group in the mosaic leafhopper *Orientus ishidae*. New Disease Reports, 22: 11.

Mehle N., Rupar M., Seljak G., Ravnikar M., Dermastia M. 2011. Molecular diversity of "flavescence dorée" phytoplasma strains in Slovenia. Bulletin of Insectology, 64 (Supplement): S29- S30.

Mehle N., Nikolić P., Gruden K., Ravnikar M., Dermastia M. 2013a. Real-time PCR for specific detection of three phytoplasmas from the apple proliferation group. Methods in Molecular Biology, 938, 269-81.

Mehle, N., Prezelj, N., Hren, M., Boben, J., Gruden, K., Ravnikar, M., Dermastia, M., 2013b. A real-time PCR detection system for the “bois noir” and “flavescence dorée” phytoplasmas and quantification of the target DNA. Methods in Molecular Biology, 938: 253-268.

Mehle N., Dreo T., Ravnikar M. 2014. Quantitative analysis of “flavescence doreé” phytoplasma with droplet digital PCR. Phytopathogenic Mollicutes, 4 (1): 9-15.

Menon K.P.V., Pandalai K.M. 1960. The coconut palm, a Monograph. Indian Central Coconut Committee. Ernakulam, India.

Mergenthaler E., Viczian O., Fodor M., Sule S. 2001. Isolation and expression of an immunodominant membrane protein gene of the ESFY phytoplasma for antiserum production. Acta Horticulturae, 550: 355-360.

Minato N., Himeno M., Hoshi A., Maejima K., Komatsu K., Takebayashi Y., Kasahara H., Yusa A., Yamaji Y., Oshima K., Kamiya Y., Namba S. 2014. The phytoplasma virulence factor TENGU causes plant sterility by downregulating of the jasmonic acid and auxin pathways. Scienfic Reports, 4: 7399.

Minsavage G.V., Thompson C.M., Hopkins D.L., Leite R.M.V.B.C., Stall R.E. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology, 84: 456–461.

Minucci C., Boccardo G. 1997. Genetic diversity in the “stolbur” phytoplasma group. Phytopathologia Mediterranea, 36: 45–49.

Mitrović J., Contaldo N., Paltrinieri S., Meja J. F., Mori N., Bertaccini A., Duduk B. 2011a. The use of *groEL* gene in characterisation of aster yellows phytoplasmas in field collected samples. Bulletin of. Insectology, 64 (Supplement): S17-S18.

Mitrović J., Kakizawa S., Duduk B., Oshima K., Namba S., Bertaccini A., 2011b. The *groEL* gene as an additional marker for finer differentiation of '*Candidatus* Phytoplasma asteris'-related strains. Annals of Applied Biology, 154(2): 219-229.

Mitrović J., Smiljković M., Seemüller E., Bertaccini A., Kube M., Duduk B. 2015. Differentiation of ‘*Candidatus* Phytoplasma cynodontis’ based on *16S rRNA* and *groEL* genes and identification of a new subgroup, 16SrXIV-C. Plant Disease, 99 (11): 1578-1583.

Miura C., Sugawara K., Neriya Y., Minato N., Keima T., Himeno M., Maejima K., Komatsu K., Yamaji Y., Oshima K., Namba S. 2012. Functional characterization and gene expression profiling of superoxide dismutase from plant pathogenic phytoplasma. Gene, 510(2): 107-12.

Miyata S., Oshima K., Kakizawa S., Nishigawa H., Jung H.-Y, Kuboyama T., Ugaki M., Namba S. 2003. Two different thymidylate kinase gene homologues, including one that has catalytic activity, are encoded in the onion yellows phytoplasma genome. Microbiology, 149: 2243-2250.

Monti M., Martini M., Tedeschi R. 2013. EvaGreen real-time PCR protocol for specific ‘*Candidatus* Phytoplasma mali’ detection and quantification in insects. Molecular and Cellurar Probes, 27: 129–136.

Morisset D., Štebih D., Milavec M., Gruden K., Žel J. 2013. Quantitative analysis of food and feed samples with droplet digital PCR. PLoS ONE 8: e62583.

Morton A., Davies D.L., Blomquist C.L., Barbara D.J. 2003. Characterization of homologues of the apple proliferation immunodominant membrane protein gene from three related phytoplasmas. Molecular Plant Pathology, 4: 109–114.

Morvan G. 1977. Apricot chlorotic leaf roll. EPPO Bulletin, 7: 37–55.

Mouillesseaux K.P., Klimpel K.R., Dhar A.K. 2003. Improvement in the specificity and sensitivity of detection for the Taura syndrome virus and yellow head virus of penaeid shrimp by increasing the amplicon size in SYBR Green real-time RT-PCR. Journal of Virological Methods, 111: 121–127.

Müller H.M., Kleinhempel H., Rabitschuch A.W., Spaar D., Müller J.H. 1975. Mykoplasmen in der Phytopathologie. Bibliographisches Verzeichnis der Literatur für die Jahre Inst. für Landwirtschaftliche Information und Dokumentation, Berlin, 1967-1974.

Munyaneza J.E. 2012. Zebra chip disease of potato: biology, epidemiology, and management. American Journal of Potato Research, 89: 329–350.

Munyaneza J.E., Sengoda V.G., Crosslin J.M., De la Rosa-Lozano G., Sanchez A. 2009. First report of '*Candidatus* Liberibacter psyllaurous' in potato tubers with zebra chip disease in Mexico. Plant Disease, 93: 552.

Munyaneza J.E., Fisher T.W., Sengoda V.G., Garczynski S.F., Nissinen A., Lemmetty A. 2010a. First report of '*Candidatus* Liberibacter solanacearum' in carrots in Europe. Plant Disease, 94: 639.

Munyaneza J.E., Fisher T.W., Sengoda V.G., Garczynski S.F., Nissinen A., Lemmetty A. 2010b. Association of '*Candidatus* Liberibacter solanacearum' with the psyllid *Trioza apicalis* (Hemiptera: Triozidae) in Europe. Journal of Economic Entomology, 103: 1060-1070.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology, 15: 473-497.

Murray R.G., Stackebrandt E. 1995. Taxonomic note: implementation of the provisional status '*Candidatus*' for incompletely described procaryotes. International Journal of Systematic Bacteriology, 45: 186–187.

Musetti R., Sanità Di Toppi L., Martini M., Ferrini F., Loschi A., Favali M.A., Osler R. 2005. Hydrogen peroxide localization and antioxidant status in the recovery of apricot plants from European Stone Fruit Yellows. European Journal of Plant Pathology, 112: 53-61.

Musetti R., Buxa S.V., De Marco F., Loschi A., Polizzotto R., Kogel K.H., van Bel A.J.E. 2013. Phytoplasma-triggered Ca2+ influx is involved in sieve-tube blockage. Molecular Plant-Microbe Interactions, 26: 379–386.

Namba S., Kato S., Iwanami S., Oyaizu H., Shiozawa H., Tsuchizaki T. 1993. Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. Phytopathology, 83: 786-791.

Nečas T., Mašková V., Krška B. 2008. The possibility of ESFY phytoplasma transmission through flowers and seeds. Acta Horticulturae, 781: 443-448.

Nei M., Kumar S. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York, 352 pp.

Neimark H.C., Kirkpatrick B.C. 1993. Isolation and characterization of full-length chromosomes from non-culturable plant-pathogenic mycoplasma-like organisms. Molecular Microbiology, 7: 21-28.

Nejat N., Sijam K., Abdullah S.N.A., Vadamalai G., Sidek Z., Dickinson M. 2010. Development of a TaqMan realtime PCR for sensitive detection of the novel phytoplasma associated with coconut yellow decline in Malaysia. Jornal of Plant Pathology, 92: 769–773.

Nelson W.R., Fisher T.W., Munyaneza J.E. 2011. Haplotypes of '*Candidatus* Liberibacter solanacearum' suggest long-standing separation. European Journal of Plant Pathology, 130: 5–12.

Nelson W.R., Sengoda V.G., Alfaro-Fernandez A.O., Font M.I., Crosslin J.M., Munyaneza J.E. 2012. A new haplotype of '*Candidatus* Liberibacter solanacearum' identified in the Mediterranean region. European Journal of Plant Pathology, 135: 633-639.

Neriya Y., Maejima K., Nijo T., Tomomitsu T., Yusa A., Himeno M., Netsu O., Hamamoto Hi., Oshima K., Namba S. 2014. Onion yellow phytoplasma P38 protein plays a role in adhesion to the hosts. FEMS Microbiology Letters, 361: 115–122.

Nicolas L., Milon G., Prina E. 2002a. Rapid differentiation of old world *Leishmania* species by lightcycler polymerase chain reaction and melting curve analysis. Journal of Microbiological Methods, 51: 295–299.

Nicolas L., Prina E., Lang T., Milon G. 2002b. Real-time PCR for detection and quantitation of *Leishmania* in mouse tissues. Journal of Clinical Microbiology, 40: 1666–1669.

Nikolić P., Mehle N., Gruden K., Ravnikar M., Dermastia M. 2010. A panel of real-time PCR assays for specific detection of three phytoplasmas from the apple proliferation group. Molecular and Cellular Probes, 24 (5): 303-309.

Nipah J.O., Jones P., Hodgetts J., Dickinson M. 2007. Detection of phytoplasma DNA in embryos from coconut palms in Ghana, and kernels from maize in Peru. Bulletin of Insectology 60 (2): 385-386.

Nishigawa H., Oshima K., Kakizawa S., Jung H., Kuboyama T., Miyata S., Ugaki M., Namba S. 2002. Evidence of intermolecular recombination between extrachromosomal DNAs in phytoplasma: a trigger for the biological diversity of phytoplasma. Microbiology, 148: 1389-1396.

Nissinen A., Vanhala P., Holopainen J.K., Tiilikkala K. 2007. Short feeding period of carrot psyllid (*Trioza apicalis*) females at early growth stages of carrot reduces yield and causes leaf discoloration. Entomologia Experimentalis et Applicata, 125: 277-283.

Nocard E.I.E., Roux E. 1898. Le microbe de la peripneumonie. Annales de l'Institut Pasteur, 12: 240-262.

Nowak J. 1929. Morphologie, nature et cycle èvolutif du microbe de la pèripneumonie des bovidès. Annales de l'Institut Pasteur, 43: 1330-1352.

Okuda S., Prince J. P., Davis R. E., Dally E. L., Lee I-M., Margone B. Kato S. 1997. Two groups of phytoplasmas from Japan distinguished on the basis of amplification and restriction analysis of 16S rDNA. Plant Disease, 81: 301-305.

Olivier C.Y., Séguin-Swartz G., Hegedus D. 2006. First report of '*Candidatus* Phytoplasma asteris'-related strains in *Brassica rapa* in Saskatchewan, Canada. Plant Disease, 90 (6): 832.

Olivier C., Galka B. 2008. Consequences of phytoplasma infection on canola crop production in the Canadian prairies. Proceedings of Endure International Conference, Diversifying crop protection, 12-15 October 2008 La Grande-Motte, France, O-47: 1-4.

Olivier C.Y., Galka B., Rott M., Johnson R. 2008. First report of molecular detection of '*Candidatus* Phytoplasma asteris'-related strains in seeds of *Brassica napus* in Saskatchewan, Canada. Cruciferae Newsletter, 27: 22–23.

Omar A.F., Foissac X. 2012. Occurrence and incidence of phytoplasmas of the 16SrII-D subgroup on solanaceous and cucurbit crops in Egypt. European Journal of Plant Pathology, 133: 353–360.

Oparka K.J. 2004. Getting the message across: how do plant cells exchange macromolecular complexes? Trends in Plant Science, 9: 33–41.

Oshima K., Kakizawa S., Nishigawa H., Kuboyama T., Miyata S.I., Ugaki M., Namba S. 2001. A plasmid of phytoplasma encodes a unique replication protein having both plasmid- and virus-like domains: clue to viral ancestry or result of virus/plasmid recombination. Virology, 285: 270-277.

Oshima K., Kakizawa S., Nishigawa H., Jung H-Y., Wei W., Suzuki S., Arashida R., Nakata D., Miyata S., Ugaki M., Namba S. 2004. Reductive evolution suggested from the complete genome sequence of a plant-pathogenic phytoplasma. Nature Genetics, 36: 27-29.

Oshima K., Kakizawa S., Arashida R., Ishii Y., Hoshi A., Hayashi Y., Kawagida S., Namba S. 2007. Presence of two glycolityc gene clusters in a severe pathogenic line of '*Candidatus* Phytoplasma asteris'. Molecular Plant Pathology, 8: 481-489.

Oshima K., Ishii, Y., Kakizawa S., Sugawara K., Neriya Y., Himeno M., Minato N., Miura C., Shiraishi T., Yamaji Y., Namba S. 2011. Dramatic transcriptional changes in an intracellular parasite enable host switching between plant and insect. PLoS One, 6: e23242.

Oshima K., Maejima K. Namba, S. 2013. Genomic and evolutionary aspects of phytoplasmas. Frontiers in Microbiology, 4: 230, doi: 10.3389/fmicb.2013.00230.

Pamei I., Makandar R. 2016. Association of 16SrII-D phytoplasma with phyllody disease in sesame (*Sesamum indicum*) in Telangana from Southern India. Plant Disease, 100 (8): 1774.

Papin J.F., Vahrson W., Dittmer D. 2004. SYBR Green-based real-time quantitative PCR assay for detection of West Nile virus circumvents false-negative results due to strain variability. Journal of Clinical Microbiology, 42 (4): 1511-1518.

Pastrik K.H. 2000. Detection of *Clavibacter michiganensis* subsp*. sepedonicus* in potato tubers by multiplex PCR with coamplification of host DNA. European Journal Plant Pathology, 106: 155–165.

Pignatta D., Poggi Pollini C., Giunchedi L., Ratti C., Reggiani N., Gobber M., Miorelli P., Forno F., Mattedi L., Ropelato E. 2008. A real-time PCR assay for the detection of European stone fruit yellows phytoplasma (ESFYP) in plant propagation material. Acta Horticolturae, 781: 499-503.

Pinheiro L.B., Coleman V.A., Hindson C.M., Herrmann J., Hindson B.J., Bhat S., Emslie K.R. 2012. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Analytical Chemistry, 84 (2): 1003-1011.

Ploaie P.G., Granados R.R., Maramorosch K. 1968. Mycoplasma-like structures in periwinkle plants with Crimean yellows, European clover dwarf, “stolbur” and “parastolbur”. Phytopathology, 58: 1063.

Poggi Pollini C., Bissani R., Giunchedi L. 2001. Occurrence of European stone fruit yellows phytoplasma (ESFYP) infection in peach orchards in Northern-Central Italy. Journal of Phytopathology, 149: 725-730.

Polizzi G., Grimaldi V., Catara A., Davino M. 1990. La “virescenza ipertrofica” del pomodoro in Sicilia. Istituto di Patologia Vegetale, Università di Catania, Italy, 10 pp.

Pracros P., Renaudin J., Eveillard S., Mouras A., Hernould M. 2006. Tomato flower abnormalities induced by “stolbur” phytoplasma infection are associated with changes of expression of floral development genes. Molecular Plant-Microbe Interactions, 19 (1): 62-68.

Prince J.P., Dams R.E., Wolf T.K., Lee I-M., Mogen B.D., Dally E.L., Bertaccini A., Credi R., Barba M., 1993. Molecular detection of diverse mycoplasmalike organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease and elm yellows MLOs. Phytopathology, 83: 1130-1137.

Racki N., Morisset D., Gutierrez-Aguirre T., Ravnikar M. 2014. One-step RT-droplet digital PCR: a breakthrough in the quantification of waterborne RNA viruses. Analytical and Bioanalytical Chemistry, 406: 661-667.

Ramírez A.S., Dare C., Naylor C., Timofte D., Bradbury J.M. 2016. Comparison of six master mixes in the adaptation of a *Mycoplasma iowae* real time PCR. 21st Congress of the International Organization for Mycoplasmology. Brisbane, Australia, 112, p. 89.

Rashidi M., Galetto L., Bosco D., Bulgarelli A., Vallino M., Veratti F., Marzachì C. 2015. Role of the major antigenic membrane protein in phytoplasma transmission by two insect vector species. BMC Microbiology, 15: 193.

Razin S. 1969. Structure and function in mycoplasma. Annual Review of Microbiology, 23: 317-356.

Razin S. 2007. Molecular biology and genomics of Mollicutes. Bulletin of Insectology, 60 (2): 101-103.

Razin S.V., Freundt E.A. 1994. The Mycoplasmas. In Bergey's Manual of Systematic Bacteriology Volume 1. Edited by: Krieg NR, Holt JG. Baltimore: William & Wilkkins. Pp. 740-793.

Razin S., Yogev D., Naot Y. 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiology and Molecular Biology Reviews, 62: 1094–1156.

Refatti E., 1948. Su di una grave malattia dei peri nelle province di Trento e di Bolzano. La Ricerca Scientifica, 18: 856-860.

Renaudin J., Beven L., Batailler B., Duret S., Desque D., Arricau-Bouvery N., Malembic-Maher S., Foissac X. 2015. Heterologous expression and processing of the "flavescence dorée" phytoplasma variable membrane protein VmpA in *Spiroplasma citri*. BMC Microbiology, 15: 82.

Robinson B.S., Monis P.T., Dobson P.J. 2006. Rapid, sensitive, and discriminating identification of *Naegleria* spp. by real-time PCR and melting-curve analysis. Applied Environmental Microbiology, 72(9): 5857-5863.

Rothrock M.J., Hiett K.L., Kiepper B.H., Ingram K., Hinton A. 2013. Quantification of zoonotic bacterial pathogens within commercial poultry processing water samples using droplet digital PCR. Advances in Microbiology, 3: 403-411.

Rudzinska-Langwald A., Kaminska M. 1999. Cytopathological evidence for transport of phytoplasma in infected plants. Acta Societatis Botanicorum, 68: 261–266.

Rui D., Ciferri R., Refatti E. 1950. La virosi degli ''scopazzi del melo'' nel Veronese. Rivista di Patologia Vegetale, 13: 7–11.

Rzhetsky A., Nei M., 1992. A simple method for estimating and testing minimum evolution trees. Molecular Biology and Evolution, 9: 945-967.

Sabin A.B. 1941. The filterable microorganisms of the *Pleuropneumonia* group. Bacteriological Reviews, 5: 1-65.

Saglio P., Laflèche D., Bonissol C., Bové J.M. 1971. Culture *in vitro* des mycoplasmes, associés au "stubborn" des agrumes et leur observation au microscope électronique. Comptes rendus hebdomadaires des séances de l'Académie des sciences Ser. D, 272: 1387-1390.

Saitou N., Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425.

Salehi E., Salehi M., Taghavi S.M., Izadpanah K. 2014. A 16SrII-D phytoplasma strain associated with tomato witches' broom in Bushehr province, Iran. Journal of Crop Protection, 3 (3): 377-388.

Samuel G., Bald J.G., Eardly C.M. 1933. "Big bud", a virus disease of the tomato. Phytopathology, 23: 641–653.

Satta E., Ramírez A.S., Paltrinieri S., Contaldo N., Benito P., Poveda J.B., Bertaccini A. 2016. Simultaneous detection of mixed '*Candidatus* Phytoplasma asteris' and '*Ca*. Liberibacter solanacearum' infection in carrot. Phytopathologia Mediterranea, 55 (3): 401−409.

Schaff D.A., Lee I-M., Davis R.E. 1992. Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reactions. Biochemical and Biophysical Research Communications, 186: 1503-1509.

Schneider B., Ahrens U., Kirkpatrick B.C., Seemüller E. 1993. Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. Journal of General Microbiology, 139: 519-527.

Schneider B., Seemüller E., Smart C.D., Kirkpatrick B.C. 1995. Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In: Molecular and Diagnostic Procedures in Mycoplasmology, pp. 369-380. Ed. S. Razin and J.G. Tully. San Diego, CA: Academic press.

Schneider B., Gibb K.S., Seeümller E. 1997. Sequence and RFLP analysis of the elongation factor *Tu* gene used in differentiation and classification of phytoplasmas. Microbiology, 143: 3381-3389.

Schvester D., Carl P., Moutous G. 1969. Nouvelles données sur la transmission de la flavescence dorée de la vigne par *Scaphoideus littoralis* Ball. Annales de Zoologie Ecologie Animale, 1: 445–465.

Seal S.E., Jackson L.A., Young J.P.W., Daniels M.J. 1993. Differentiation of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii* and blood disease bacterium by partial 16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. Journal of General Microbiology, 139: 1587–1594.

Sears B.B., Klomparens K.L. 1989. Leaf tip cultures of the evening primrose allow stable, aseptic culture of mycoplasma-like organism. Canadian Journal of Plant Pathology, 11: 343–348.

Sears B.B., Kirkpatrick B.C. 1994. Unveiling the evolutionary relationship of plant-pathogenic micoplasma-like organisms. ASM News, 60 (6): 307-312.

Sears B.B., Klomparens K.L., Wood J.I., Schewe G. 1997. Effect of altered levels of oxygen and carbon dioxide on phytoplasma abundance in *Oenothera* leaftip cultures. Physiological and Molecular Plant Pathology, 50: 275-287.

Secor G.A., Rivera V.V., Abad J.A., Lee I-M., Clover G.R.G., Liefting L.W., Li X., de Boer S.H. 2009. Association of '*Candidatus* Liberibacter solanacearum' with zebra chip disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. Plant Disease, 93: 574–583.

Seemüller E., Schneider B., Maurer R., Ahrens U., Daire X., Kison H., Lorenz K., Firrao G., Avinent L., Sears B.B., Stackebrandt E. 1994. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. International Journal of Systematic Bacteriology, 44: 440-446.

Seemüller E., Stolz H., Kison H. 1998. Persistence of the European stone fruit yellows phytoplasma in aerial parts of *Prunus* taxa during the dormant season. Journal of Phytopathology, 146: 407-410.

Seemüller E., Schneider B. 2004. '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. International Journal of Systematic and Evolutionary Microbiology, 54: 1217-1226.

Seemüller E., Moll E., Schneider B. 2008. Apple proliferation resistance of *Malus sieboldii*-based rootstocks in comparison to rootstocks derived from other *Malus* species. European Journal of Plant Pathology, 121: 109-119.

Seemüller E., Sule S., Kube M., Jelkmann W., Schneider B. 2013. The AAA+ ATPases and HflB/FtsH proteases of '*Candidatus* Phytoplasma mali': phylogenetic diversity, membrane topology, and relationship to strain virulence. Molecular Plant-Microbe Interactions, 26(3): 367-376.

Sertkaya G., Martini M., Musetti R. Osler R. 2007. Detection and molecular characterization of phytoplasmas infecting sesame and solanaceous crops in Turkey. Bulletin of Insectology, 60: 141-142.

Šeruga M., Vrek I., Koriae D. 2004. *Dianthus croaticus* Borb –a new host for phytoplasma from ribosomal groups 16SrI and 16SrIII. Book of Abstracts of the 15th International Congress of the IOM, Athens, USA, 9-14 July, 76: 122-123.

Sforza R., Clair D., Daire X., Larrue J., Boudon-Padieu E. 1998. The role of *Hyalesthes obsoletus* (Hemiptera: Cixiidae) in the occurrence of "bois noir" of grapevine in France. Journal of Phytopathology, 146: 549- 556.

Shen W.C., Lin C.P. 1993. Production of monoclonal antibodies against a mycoplasmalike organism associated with sweet potato witches' broom. Phytopathology, 83: 671-675.

Siddique A.B.M., Guthrie J.N., Walsh K.B., White D.T., Scott P.T. 1998. Histopathology and within-plant distribution of the phytoplasma associated with Australian papaya dieback. Plant Disease, 82: 1112-1120.

Siller W., Kuhbandner B., Marwitz R., Petzold H., Seemüller E. 1987. Occurrence of mycoplasma-like organisms in parenchyma cells of *Cuscuta odorata* (Ruiz et Pav.). Journal of Phytopathology, 119: 147–159.

Sinclair W.A., Griffiths H.M. 2000. Variation in aggressiveness of ash yellows phytoplasmas. Plant Disease, 84: 282-288.

Singh J., Rani A., Kumar P., Baranwal V. K., Saroj P. L., Sirohi A. 2012. First report of a 16SrII-D phytoplasma '*Candidatus* Phytoplasma australasia' associated with a tomato disease in India. New Disease Reports, 26: 14.

Sinha R.C. 1979. Purification and serology of mycoplasmalike organisms antigens from aster yellows-diseased plants by two serological procedures. Canadian Journal of Plant Pathology, 1: 65-70.

Sinha R.C., Benhamou N. 1983. Detection of mycoplasma-like organisms antigens from aster yellows-diseased plants by two serological procedures. Phytopathology, 73: 1199-1202.

Škorić D., Šarić A., Vibio M., Murari E., Krajačić M., Bertaccini A. 1998. Molecular identification and seasonal monitoring of phytoplasmas infecting Croatian grapevines. Vitis, 37: 171-175.

Somerson N.L., Reich P.R., Chanock R.M., Weissman S.M. 1967. Genetic differentiation by nucleic acid homology. III. Relationships among mycoplasma, L-forms and bacteria. Annals of the New York Academy of Science, 143: 9-20.

Stadler R., Wright K.M., Lauterbach C., Amon G., Gahrtz M., Feuerstein A., Oparka K.J., Sauer N. 2005. Expression of GFP-fusions in *Arabidopsis* companion cells reveals non-specific protein trafficking into sieve elements and identifies a novel post-phloem domain in roots. The Plant Journal, 41 (2): 319–331.

Starović M., Tošić M., Tomić T., Živković S., Pavlović S. 2004. Etiological study of redness on maize. V Congress of Plant Protection, Zlatibor, November 22-26, Book of Abstracts: 156-157.

Strain M.C., Lada S.M., Loung T., Rought S.E., Gianella S., Terry V.H., Spina C.A., Woelk C.H., Richman D.D. 2013. Hogjlt precise measurement of HIV DNA by droplet digital PCR. Plos One, 8: e55943.

Sugawara K., Honma Y., Komatsu K., Himeno M., Oshima K., Namba S. 2013. The alteration of plant morphology by small peptides released from the proteolytic processing of the bacterial peptide TENGU. Plant Physiology, 162 (4): 2005-2014.

Sugio A., Hogenhout S.A. 2012. The genome biology of phytoplasma: modulators of plants and insects. Current Opinion Microbiology, 15: 247–254.

Sugio A., Kingdom H.N., MacLean A.M., Grieve V.M., Hogenhout S.A. 2011. Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. Proceedings of the National Academy of Sciences, USA, 108: E1254–E1263.

Sugio A. MacLean A.M., Hogenhout S.A. 2014. The small phytoplasma virulence effector SAP11 contains distinct domains required for nuclear targeting and CIN-TCP binding and destabilization. New Phytologist, 202: 838–848.

Šutić D., Starović M., Tošić M., Stojanović S., Kuzmanović S. 2003. Novija proučavanja crvenila kukuruza u nas. VI savetovanje o zaštiti bilja, Zlatibor, November 24-28, 2003. Book of Abstracts: 42.

Suzuki S., Oshima K., Kakizawa S., Arashida R., Jung H.Y., Yamaji Y., Nishigawa H., Ugaki M., Namba S. 2006. Interaction between the membrane protein of a pathogen and insect microfilament

complex determines insect-vector specificity. Proceedings of National Academy of Sciences, USA, 103 (11): 4252–4257.

Tamura K., Nei M., Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA), 101: 11030-11035.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution, 30: 2725-2729.

Tan C.M., Li C-H., Tsao N-W., Su L-W., Lu Y-T., Chang S.H., Lin Y.Y., Liou J-C., Hsieh L-C., Yu J-Z., Sheue C-R., Wang S-Y., Lee C-F., Yang J-Y. 2016. Phytoplasma SAP11 alters 3-isobutyl-2-methoxypyrazine biosynthesis in *Nicotiana benthamiana* by suppressing NbOMT1. Journal of Experimental Botany, 67 (14): 4415-4425.

Tapia-Tussell R., Suaste-Dzul A., Cortes-Velazquez A., Torres-Calzada C., Quijano-Ramayo A., Martin-Mex R., Nexticapan-Garcez A., Perez-Brito D. 2012. Molecular characterization of Yucatan tomato phytoplasma (group 16SrIII). African Journal of Biotechnology, 11: 2169-2177.

Tedeschi R. 2012. DNA Sequencing and crop protection. In: DNA Sequencing - Methods an Applications, Dr. Anjana Munshi (Ed.), chapter 4, pp. 61-84.

Tedeschi R., Ferrato V., Rossi J., Alma A. 2006. Possible phytoplasma transovarial transmission in the psyllids *Cacopsylla melanoneura* and *Cacopsylla pruni*. Plant Pathology, 55: 18–24.

Tedeschi R., Alma A. 2007. '*Candidatus* Phytoplasma mali': the current situation of insect vectors in northwestern Italy. Bulletin of Insectology, 60 (2): 187-188.

Teixeira D.C., Wulff N.A., Martins E.C., Kitajima E.W., Bassanezi R., Ayres A.J., Eveillard S., Saillard C., Bové J.M. 2009. A phytoplasma related to '*Candidatus* Phytoplasma asteris' detected in citrus showing huanglongbing (yellow shoot disease) symptoms in Guangdong, P. R. China. Phytopathology, 99: 236-242.

Tenllado F., Llave C., Diaz-Ruiz J.R. 2004. RNA interference as a new biotechnological tool for the control of virus diseases in plants. Virus Research, 102 (2): 85–96.

Teresani G.R., Bertolini E., Alfaro-Fernandez A., Martínez C., Tanaka F.A., Kitajima E.W., Roselló M., Sanjuán S., Ferrándiz J.C., López M.M., Cambra M., Font M.A. 2014. Association of '*Candidatus* Liberibacter solanacearum' with a vegetative disorder of celery in Spain and development of a real-time PCR method for its detection. Phytopathology, 104 (8): 804-811.

Terlizzi F., Delvago C., Zambini R., Testi V., Saccò S., Credi R. 2010. "Stolbur" su pomodoro: identificazione e caratterizzazione molecolare del fitoplasma in Emilia-Romagna. Petria, 20 (3): 699-701.

Tomlinson J.A., Boonham N., Dickinson M. 2010. Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. Plant Pathology, 59 (3): 465-471.

Torres E., Bertolini E., Cambra M., Montón C., Martín M.P. 2005 Real-time PCR for simultaneous and quantitative detection of quarantine phytoplasmas from apple proliferation (16SrX) group. Moleculra and Cellular Probes, 19: 334–340.

Toruno T.Y., Music M.S., Simi S., Nicolaisen M., Hogenhout S.A. 2010. Phytoplasma PMU1 exists as linear chromosomal and circular extrachromosomal elements and has enhanced expression in insect vectors compared with plant hosts. Molecular Microbiology, 77: 1406–1415.

Tran-Nguyen L.T., Gibb K.S. 2006. Extrachromosomal DNA isolated from tomato big bud and '*Candidatus* Phytoplasma australiense' phytoplasma strains. Plasmid, 56 (3): 153-66.

Trivellone V., Filippin L., Jermini M., Angelini E. 2015. Molecular characterization of phytoplasma strains in leafhoppers inhabiting the vineyard agroecosystem in Southern Switzerland. Phytopathogenic Mollicutes, 5: 45-46.

Tseng Y-W., Deng W-L., Chang C-J., Huang J-W., Jan F-J. 2014. First report on the association of a 16SrII-A phytoplasma with sesame (*Sesamum indicum*) exhibiting abnormal stem curling and phyllody in Taiwan. Plant Disease, 9 (7): 990.

Tseng C-P., Cheng J-C., Tseng C-C., Wang C., Chen Y-L., Chiu D.T-Y., Liao H.C., Chang S-S. 2003. Broad-range ribosomal RNA real-time PCR after removal of DNA from reagents: melting profiles for clinically important bacteria. Clinical Chemistry, 49 (2): 306–309.

Turner C.T., Davy M.W., MacDiarmid R.M., Plummer K.M., Birch N.P., Newcomb R.D. 2006. RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. Insect Molecular Biology, 15 (3): 383-391.

Uzun B., Arslan C., Furat S. 2008. Variation in fatty acid compositions, oil content and oil yield in a germplasm collection of sesame (*Sesamum indicum* L.). Journal of the American Oil Chemists' Society, 85: 1135–1142.

Vaerman J.L., Saussoy P., Ingargiola I. 2004. Evaluation of real-time PCR data. Journal of Biological Regulators and Homeostatis Agents, 18: 212-214.

van Bel A.J.E., Ehlers K., Knoblauch M. 2002. Sieve elements caught in the act. Trends in Plant Science, 7 (3): 126-132.

Varga A., James D. 2005. Detection and differentiation of *Plum pox virus* using real-time multiplex PCR with SYBR Green and melting curve analysis: a rapid method for strain typing. Journal of Virological Methods, 123: 213–220.

Varga A., James D. 2006. Real-time RT-PCR and SYBR Green I melting curve analysis for the identification of Plum pox virus strains C, EA, and W: effect of amplicon size, melt rate, and dye translocation. Journal of Virological Methods, 132: 146–153.

Vasudeva R.S., Sahambi H.S. 1955. Phyllody in sesamum (*Sesamum orientale* L.). Indian Phytopathology, 8: 124-129.

Vega-Orellana O.M., Poveda J.B., Rosales R.S., Bradbury J.M., Poveda C.G., Mederos-Iriarte L.E., Tavío M.M., Ramírez A.S. 2016. Interpretation of the results of a conventional PCR in real time PCR in the detection of Mollicutes. 21st Congress of the International Organization for Mycoplasmology. Brisbane, Australia, 113, pp. 89-90.

Vellios E., Lioliopoulou F., 2007. Detection and characterization of phytoplasmas infecting tomato plants in Greece. Bulletin of Insectology, 60 (2): 157-158.

Vibio M., Bertaccini A., Lee I-M., Davis R.E., Clark M.F. 1996. Differentiation and classification of aster yellows and related European phytoplasmas. Phytopathologia Mediterranea, 35: 33-42.

Wang Q., Guo Y., Wang N., Li Y., Chen W., Chen W., Yunfeng W. 2014. Identification of a conserved core genome with group-specific genes from comparative genomics of ten different '*Candidatus* Phytoplasma' strains. Journal of Phytopathology, 162: 650–659.

Wei W., Kakizawa S., Jung H.Y., Suzuki S., Tanaka M., Nishigawa H., Miyata S., Oshima K., Ugaki M., Hibi T., Namba S. 2004a. An antibody against the SecA membrane protein of one phytoplasma reacts with those of phylogenetically different phytoplasmas. Phytopathology, 94 (7): 683-686.

Wei W., Kakizawa S., Suzuki S., Jung H.Y., Nishigawa H., Miyata S., Oshima K., Ugaki M., Hibi T., Namba S. 2004b. *In planta* dynamic analysis of onion yellows phytoplasma using localized inoculation by insect transmission. Phytopathology, 94 (3): 244-250.

Wei W., Davis R.E., Jomantiene R., Zhao Y. 2008. Ancient, recurrent phage attacks and recombination shaped dynamic sequence-variable mosaics at the root of phytoplasma genome evolution. Proceedings of National Academy of Science USA, 105 (33): 11827-11832.

Weintraub P.G., Beanland L.A. 2006. Insect vectors of phytoplasmas. Annual Review of Entomology, 51: 91-111.

Wen A., Mallik I., Alvardado V.Y., Pasche J.S., Wang X., Li W., Levy L., Lin H., Scholthof H.B, Mirkov T.E., Rush C.M., Gudmestad N.C. 2009. Detection, distribution, and genetic variability of '*Candidatus* Liberibacter' species associated with zebra complex disease of potato in North America. Plant Disease, 93: 1102–1115.

Whitcomb R.F., Tully J.G. 1979. The Mycoplasmas, Vol. 3. Academic Press, New York.

Whyard S., Singh A.D., Wong S. 2009. Ingested double-stranded RNAs can act as species-specific insecticides. Insect Biochemistry and Molecular Biology, 39 (11): 824-832.

Williamson D.L., Whitcomb R.F. 1975. Plant mycoplasmas: a cultivable spiroplasma causes corn stunt disease. Science, 188: 1018-1020.

Winder L., Phillips C., Richards N., Ochoa-Corona F., Hardwick S., Vink C.J., Goldson S. 2011. Evaluation of DNA melting analysis as a tool for species identification. Methods in Ecology and Evolution, 2: 312–320.

Woese C. R. 1987. Bacterial evolution. Microbiological Reviews, 51: 221–271.

Woodbridge C.G., Blodgett E.C., Diener T.O. 1957. Pear decline in the Pacific Northwest. Plant Disease Reports, 41: 569–572.

Xu X., Mou H-Q., Zhu S.F., Liao, X.L. Zhao W.J. 2013. Detection and characterization of phytoplasma associated with big bud disease of tomato in China. Journal of Plant Pathology, 161: 430-433.

Yu Y., Yeh K., Lin C. 1998. An antigenic protein gene of a phytoplasma associated with sweet potato witches' broom. Microbiology, 144: 1257-1262.

Yvon M., Thébaud G., Alary R., Labonne G. 2009. Specific detection and quantification of the phytopathogenic agent '*Candidatus* Phytoplasma prunorum'. Molecular and Cellular Probes, 23: 227–234.

Zambryski P. 2004. Cell-to-cell transport of proteins and fluorescent tracers via plasmodesmata during plant development. Journal of Cell Biology, 164: 165–168.

Zimmermann-Gries S., Klein M. 1978. A tomato big bud-like disease of tomatoes in Israel and its association with mycoplasma-like organisms. Plant Disease Reporter, 62: 590–594.

Zreik L., Carle P., Bové J.M., Garnier M. 1995. Characterization of the mycoplasmalike organism associated with witches' broom disease of lime and proposition of a '*Candidatus*' taxon for the organism, '*Candidatus* Phytoplasma aurantifolia'. International Journal of Systematic Bacteriology, 45: 449-453.

Zwolinska A., Krawczyk K., Pospieszny H. 2010. First report of "stolbur" phytoplasma infecting pea plants. Book of Abstracts of the 18th International Congress of the IOM, Chianciano Terme, Italy, 11-16 July 145: 152.

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