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FACULTY OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES
MSc IN AGRICULTURAL BIOTECHNOLOGY

COMPARATIVE ANALYSIS OF TRADITIONAL AND MODERN PHYTOPLASMA DIAGNOSTIC METHODS ON APPLE TREES

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Gödöllő
2018

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List of Abbreviations

16S rDNA	16S ribosomal deoxyribonucleic acid
ABC transporters	ATP-binding cassette transporters
Amp	Ampicillin resistance gene
AP	Apple proliferation
ApE	A Plasmid Editor
AT	Adenine Thymine
ATP	Adenosine Tri phosphate
AY-WB	Aster Yellows Witches' broom
BlastN	Nucleotide Basic Local Alignment Search Tool
Bp	Base pair
Bst	Bacillus stearothermophilus DNA polymerase
DAPI	DNA-specific 6-diamidino-2-phenylindole
DFP	Direct Fluorescent Detection
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide
EPPO	European and Mediterranean Plant Protection Organization
ESFY	European stone fruit yellows
EtBr	Ethidium bromide
EU	European Union
GC	Guanine Cytosine
IgG	Immunoglobulin G
Imp	Immunodominant membrane protein
Kb	Kilo bases
LAMP	Loop mediated isothermal amplification
LB	Luria Bertani
MLOs	Mycoplasma like organisms

ORF	Open reading frame
OY-M	Onion Yellows phytoplasma (strain OY-M)
PCR	Polymerase chain reaction
PD	Pear decline
PMUs	Potential mobile units (clusters of repeated gene sequences)
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal ribonucleic acid
SAP	Stress-associated protein 11
SecY	Protein translocase subunit SecY
SOC	Super Optimal Catabolite repression
TBE	Tris base, boric acid and EDTA
TEM	Transmission electron microscopy
tRNA	transfer ribonucleic acid
Tuf	Elongation Factor Tu gene
UV	Ultraviolet

1. INTRODUCTION

Fruit production in Europe is a significant sector in the agriculture. Cultivated fruit trees, apple, pear, apricot, peach, orange, and other fruits, are maintained on 1,29 million hectares, according to Eurostat, 2012 data. The most prominent production of fruit trees cultivated in the European Union is apple tree with 35% of the total orchard area; corresponding to 450 000 ha, followed by 21% of oranges and 15% of peaches. Hungary is one of the six major apple producing countries with 6% of the total EU apple tree area. It was reported in 2016, that the production of apples was 485 900 tons in Hungary. Apple trees cultivation is mainly for consumption of fresh table apples, exportation, processing and production of apple juice, wine/brandy, applesauce, apple chips, preserved foods, and part of baking products (Krautgartner, 2011).

Apple trees are susceptible to fungal, viral and bacterial, including phytoplasmal, diseases which affect the development of these plants. '*Candidatus* Phytoplasma mali' is considered one of the most important vector-borne diseases of apple trees in Europe (Carraro, 2004). Phytoplasmas are plant-pathogenic, unculturable, bacteria that inhabit the plant phloem sieve tubes and are transmitted by insects. The three most economically critical phytoplasmal diseases are apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY) (Seemüller, 2004).

Pear decline, Apple proliferation and European Stone fruit yellows are diseases associated with phytoplasma that produces a reduction in production and quality of fresh fruit in Europe (Bertaccini, 2009). *C*. phytoplasma mali is one of the most severe apple diseases since it affects the quantity and quality of the fruits produced. *C*. P. mali is present mainly in most of the pome fruit production areas in southern and central Europe (Fialová, 2003). Plants that are infected by phytoplasmas exhibit some common symptoms such as virescence, phyllody, sterility of flowers, witches broom (proliferation of axillary buds), stunting, yellowish color of the leaves, etc. These symptoms suggest that the infected plant exhibits a profound disturbance in the balance of growth regulators (Bertaccini, 2009).

The symptomatic detection of phytoplasma-infected trees is unreliable due to the presence of asymptomatic infected plants and also because of the variability of symptoms and distribution of the pathogen among the plant that is affected by seasonal changes (Minguzzi, 2016). Diagnosis and characterization of phytoplasmas are possible by using molecular methods, mostly based on the study of the polymorphisms present in the 16S rDNA gene (Bertaccini, 2009). The molecular

diversity of this pathogens can also be studied using the analysis of genes that codes for non-ribosomal proteins like S3, tuf, SecY, amp and imp. The development of a sensitive and reliable tool to detect the presence of phytoplasma pathogens it's essential for the study of mechanisms of control and management of this diseases. Control of phytoplasma diseases depends on prevention rather than cure (Constable, 2010).

Phytoplasma detection is carried out mostly by polymerase chain reaction (PCR) both from plant host or insect vector samples. Diagnostic technics are vital since they help to prevent the spread of phytoplasmal diseases and to reduce the economic impact of the possible outbreaks. PCR with RFLP (Restriction Fragment Length Polymorphism), the analysis offers an accurate identification of different strains of phytoplasma. The quantity of phytoplasma DNA is 1% compared to the total genomic DNA and also shown spatial variation. That is why it is required an enrichment step like a first DNA amplification followed by a second amplification step of nested PCR, to increase the sensitivity and specificity of the detection of phytoplasma. This technique first uses a preliminary amplification with universal primers, followed by a second amplification with group-specific primers. During the last decade loop-mediated isothermal amplification (LAMP) is acquiring importance in the diagnosis and for phytoplasma could be a widely reliable diagnostic tool (Jonghe, 2016).

The purpose of this study was to determine the presence of '*Candidatus* Phytoplasma mali' from apple samples using molecular techniques such as nested PCR and Loop-mediated isothermal amplification techniques.

1.1 Objectives

1.1.1 Main objectives:

1. To diagnose the presence of '*Candidatus* Phytoplasma mali' in apple trees in Hungary.
2. To optimize specific and sensitive methods using molecular techniques: nested PCR and Lamp, for detection and identification of '*Candidatus* Phytoplasma mali.'

1.1.2 Specific Objectives

1. To isolate DNA from apple plants collected in Olcsvaapáti, Hungary.
2. To detect phytoplasma infection by polymerase chain reaction with reported and newly designed primers.

3. To prove the usefulness of newly designed primers (cloning and sequencing of the amplified part of '*Candidatus* Phytoplasma mali' 16S rDNA).
4. To detect phytoplasma infection by LAMP with reported and newly designed primers.

2. LITERATURE REVIEW

2.1 Phytoplasmas in general

The name ‘phytoplasmas' is used to name wall-less Gram-positive bacteria, which inhabit plant phloem and insect vectors (leafhoppers, planthoppers, and psyllids). They were formerly known until 1993 as mycoplasma-like organisms (MLOs), because of their morphological likeness with mycoplasma organisms. By phylogenetic analyses of conserved genes, it was discovered that these bacteria belong to a distinct monophyletic clade within the Mollicutes class. The name, ‘*Candidatus Phytoplasma*’ came from the Greek *phytos*, for plant and *plasma*, for thing moulded (Firrao, 2004). However, the first evidence of plant diseases associated with prokaryotes that colonize the phloem of vascular plants, which resemble the mycoplasmas was described by (Doi, 1967).

Phytoplasmas are related to significant damages caused on different types of plants, including economically important crops, ornamental plants, and fruit trees, all over the world. Despite their economic importance, they remain as the most poorly characterized phytopathogens. Phytoplasma diseases, at first was attributed to viruses due to the virus-like symptoms and the way of transmissibility by insects (Maejima, 2014).

Agreeing with adopted taxonomic rules, uncultured organisms should be noted by the *Candidatus* designation. The Phytoplasma Working team at the 10^{th} Congress of the International Organization in 1994 named as ‘phytoplasmas’ these types of bacteria. So far it is described 40 species of Phytoplasmas worldwide (Maejima, 2014) (Firrao, 2004).

2.2 Morphology

Phytoplasmas are phloem-limited small bacterial (200 – 800 nm in diameter) surrounded by a single cell membrane, pleomorphic in shape, so that the environment can alter their shape and size. These bacteria have a reduced genome compared with other related Gram-positive bacteria (Firrao, 2004). The first visual observation of phytoplasma was made in 1967 by transmission electronic microscopy (TEM). By TEM is possible to diagnose and determine the morphology, size and concentration of phytoplasma in plant tissues (Figure 2.1).

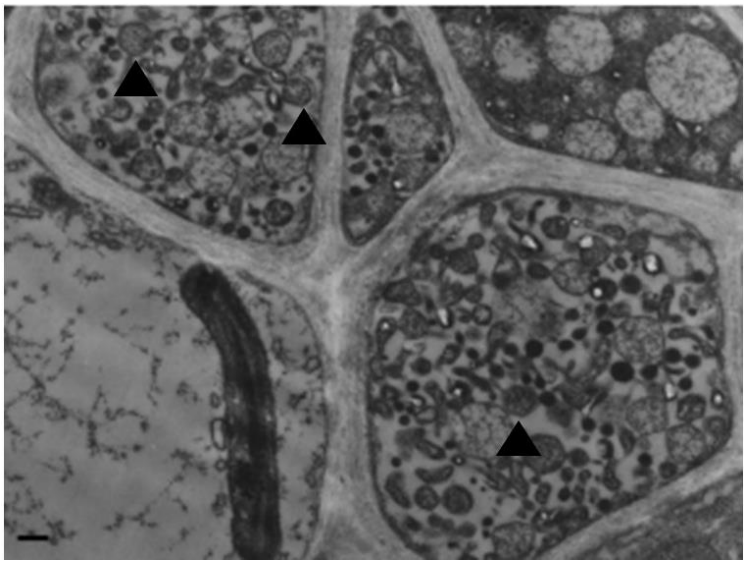


Figure 2. 1 Electron micrograph of cross-sectioned sieve tubes infected with phytoplasma cells (indicated by black arrows) *(Bertaccini, 2009)*.

2.3 Habitat

These pathogens are transmitted by insects that are causing devastating losses in crops worldwide. Phytoplasmas in nature, require plant and insects to survive. It's believed that they undergo rapid genome evolution since they can survive in a broad range of environments like the phloem sieve elements in their plant hosts, and the gut lumen, hemolymph, saliva and endocellular niches in various organs of their sap-sucking insect hosts (Bertaccini, 2007).

2.4 Symptoms

Plants infected by phytoplasma frequently present changes in their development pattern and have a less crop yield. The expression of the phytoplasma symptoms is influenced by species, cultivar, rootstock and environmental factors. The results of many researchers indicate that phytoplasmas alter the levels of endogenous phytohormones in the infected plant, causing particular symptoms like virescence, phyllody, and auxiliary shoots; contrary, other studies do not support the direct effect on plant hormones production. The consumption of the plant nutrients, growth regulators, and metabolites by these pathogens could be directly associated with the virulence (Lee, 2000).

In poinsettia *Euphorbia pulcherrima* phytoplasma causes a desired bushy form, since there is a subtle interaction between phytoplasma and this plant, resulting in dwarfing and moderate branching that is a desired trait for poinsettia growers (Lee, 1997).

These bacteria cause symptoms in their plant hosts (Fig 2.2) like:

- Stunting: Generalized growth reduction
- Virescence: the greening of floral organs
- Phyllody: developing of leaf-like structures instead of flowers
- Sterility of flowers, premature fruit drop
- Rosettes: shortened internodes with many leaves
- Enlarged stipules
- Rolling of leaves
- The proliferation of auxiliary and axillary shoots
- Abnormal elongation of internodes
- Witches broom: the unusual number of tiny shoot branches with small leaves
- Yellowing of leaves, etc. (Lee, 2000) (Maejima, 2014).

Phytoplasmas have uneven distribution among the plant organs that is why a correct sampling procedure is required and crucial to obtain reliable and reproducible results. Phytoplasmas are sensitive to antibiotics of the tetracycline group (Firrao, 2007). Also, the mechanism of movement used by phytoplasmas in different plants is still unknown (Garcia-Chapa, 2003).

The damaging effect of phytoplasma can differ in severity according to the plant species and also depend on the time of its occurrence during plant development. It is suggested that the severity and symptom expression may be related to the concentration of phytoplasma and also with its distribution within the plant (Constanzo, 2012).

Phytoplasmas can act as pathogens to their insect vectors, but commonly it doesn't affect the aptness of the vectors; actually, these bacteria can work increasing the fecundity and persistence of the insect (Hogenhout, 2008).

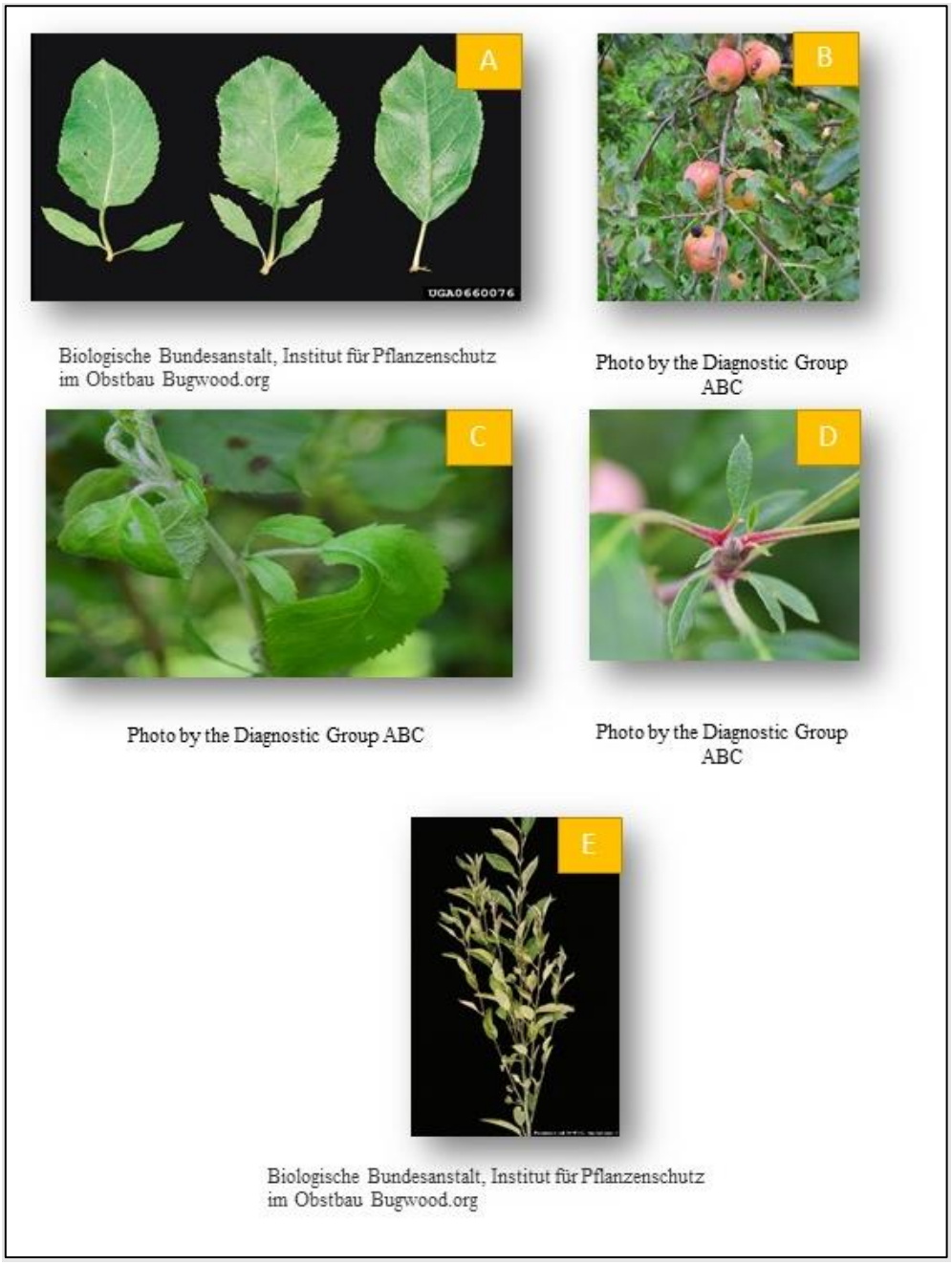


Figure 2.2 Phytoplasma symptoms **A.** Enlarged Stipules. **B.** Reduced fruit size. **C.** Rolling of leaves. **D.** Stipules and **E.** Witches Broom.

2.5 Genome composition of Phytoplasmas

Sequencing of phytoplasma genome provides detailed information about the molecular pathways of these bacteria in their parasitic lives and will give clues for it's in vitro culture because at the present phytoplasmas are not cultivable in cell-free media. So far, four phytoplasma genomes have

been completely sequenced, allowing to understand of their interactions with its vectors and host plants at a molecular level (Bertaccini, 2007).

The completely sequenced genomes of phytoplasmas belong to Onion Yellows M (OY-M), Aster yellows witches-broom (AY-WB), a strain of '*Ca*. Phytoplasma australiense', and a strain of '*Ca*. Phytoplasma mali'. Molecular research in phytoplasma has some difficulties in obtaining high-quality DNA from infected plants, and the instability of DNA rich in AT in large insert genome libraries; that is the reason why just four genomes have been completely sequenced so far (Kube, 2008). All four genomes are composed of a single chromosome, what can be circular or linear and completed with one or more plasmids. Phytoplasma genome is rich in AT; the GC content ranges from 21,4% in '*Ca*. Phytoplasma mali' to 27,7% for '*Ca*. Phytoplasma asteris'. The genomes contain genes for DNA replication, transcription, translation, and protein translocation but their genomes are significantly reduced in the number of genes belonging to essential metabolic pathways. Phytoplasmas lack genes for pentose phosphate cycle, fatty acid biosynthesis, tricarboxylic acid cycle, salvage pathway for recovery of nucleotides, urea cycle and metabolic pathways for phenylalanine, D-glutamine, D-glutamate, D-arginine, D-ornithine, D-alanine and D-glutathione synthesis (Tran-Nguyen, 2008). Contrary to mycoplasma, phytoplasmas lack genes for ATP-synthase subunits (Oshima, 2004).

The reduction of the genome and the lack of genes for critical metabolic pathways explains why phytoplasmas are highly dependent on the metabolic compounds produced by their hosts (Maejima, 2014).

2.6 Classification

The provisional taxonomic classification of phytoplasmas is summarized in Table 2.1.

Table 1. Classification of phytoplasmas *(Constanzo, 2012)*.

Domain	Bacteria
Phylum	Firmicutes
Class	Mollicutes
Order	Acholeplasmatales
Family	Acholeplasmataceae
Genus	'*Candidatus* Phytoplasma'

For purposes of classification, phytoplasmas are grouped in the base of their distinct fragment length polymorphism (RFLP) pattern of PCR amplicons generated from 16S rDNA conserved sequences, subjected to enzymatic digestion (Firrao, 2004).

'*Ca*. Phytoplasma' includes 30 clades according to with the 16S rRNA gene analyses. The taxonomic classification of phytoplasmas was made in a base of a direct comparison of the nucleotide sequence of 16S rRNA genes. 16S rRNA genes exist in all prokaryotes and contain both conserved and variable regions which are appropriate phylogenetic classification (Lee, 1993).

The Phytoplasma/Spiroplasma Working Team IRPCM Team-Phytoplasma Taxonomy Group determined that '*Candidatus* Phytoplasma' species description and classification is based on a single gene 16S rRNA sequence (>1200 bp). A new strain can be recognized if its 16S rRNA sequence has <97,5% similarity comparing with the previously described phytoplasma species (Firrao, 2004).

2.7 Lifecycle

Phytoplasmas can be transmitted by grafting of infected plants, by phloem-feeding insects, and in few cases by natural transfer by root fusion. Phytoplasmas can live as parasites of plants and insects (Fig 2.3). Insects in adult state or as nymphs acquire phytoplasmas when they feed on sap from infected plants, and then phytoplasmas move to the hemolymph and colonize the salivary glands where they are released during feeding (Tedeschi, 2004). During winter time phytoplasmas declines in the sieves of plant phloem due to tube sieve degeneration. Bacteria get concentrated in roots, and during April and May, they invade the stem again, reaching a peak during summer and early April. Phytoplasma distribution is dependent on the temperature (Constanzo, 2012).

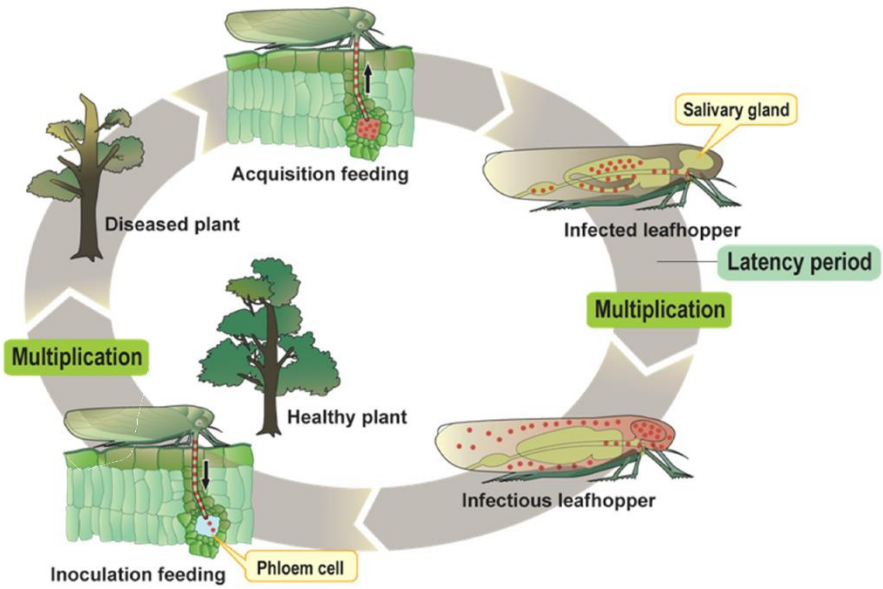


Figure 2.3 Phytoplasma life cycle *(Oshima, 2011).*

2.8 Virulence factors involved in the expression of symptoms

Since phytoplasmas have no cell wall and reside within the host cells, it is believed that the proteins secreted by them have a direct action in the host plants or insects (Maejima, 2014). The presence of functional Sec pathway, identified in phytoplasmas, can deliver proteins to the surroundings without the need of a specialized injection system. Moreover, phytoplasmas secrete proteins that target the nuclei of the host cell and manipulate the host plant processes (Bai, 2009).

There are three genes *secA*, *secY*, and *secE*, present in *Escherichia coli* that have the function of protein translocation. *SecA* was also identified as expressed in the plant host during phytoplasma infection, and since these genes are also found in the genome of phytoplasmas, it is believed that the Sec system is conserved in phytoplasmas (Bai, 2009). The spread of phytoplasmas relies on the phloem-feeding insects. The spreading and reproduction success of phytoplasmas is by making the plants more attractive to the insects by giving them suitable leaves for feeding and oviposition by symptoms such as witches broom, phyllody, and virescence. Some proteins like SAP11 effector can modify endogenous host processes, by targeting the nuclei and affecting the expression of particular genes (Bai, 2009).

Other genes related to the virulence that are present in other bacteria are not found in phytoplasmas genomes. That is why proteins secreted by the Sec system, could be candidates of virulence factors

which produce symptoms in the plant. According to reports of (Hoshi, 2009), a secreted protein called TENGU was identified first in OY-M phytoplasma, as a virulence factor and was associated with witches' broom and dwarfism which are typical symptoms of phytoplasma infection. Microarray analyses of TENGU-transgenic Arabidopsis plants showed that this protein inhibits the pathway related to auxin that is why the development of the plant is altered.

Additionally, to the proteins, secreted by phytoplasmas, the consumption of the plant nutrients and metabolites by these pathogens could be directly associated with their virulence.

2.9 Control

Phytoplasmas are transmitted from one plant to another by phloem/feeding homopterous insects such as leafhoppers, planthoppers, and psyllids (Kube, 2008).

So far, there is no an efficient way of control this pathogen bacteria, currently the strategy trust in its early detection and removal of the diseased plants to prevent the spread (Maejima, 2014). Breeding projects focused on the development of less susceptible or phytoplasma resistant plants, as well as plants capable of detecting and reacting to vector feeding may be the most economical and efficient way of control (Constanzo, 2012).

It was reported by (Mannini, 2007) that in grape hot water treatment of dormant wood could be useful to eliminate phytoplasmas from propagative material and also can be a proper treatment against leafhoppers overwintering eggs.

The frequent inspection and removal of infected plants combined with insecticide spraying may be a way to reduce the spreading of phytoplasma. Antibiotic therapy with tetracycline and oxytetracycline is administered by high-pressure injection or by gravity infusion into the trunk of infected plants. This treatment is a costly and non-curative method that reduce the severity but in a temporary way (Constanzo, 2012).

Phytoplasma control strategies are mostly made using insecticide treatments to minimize the vector population, but there are significant environmental concerns such as their affection non-targeted insects. Some woody species infected with phytoplasma show decreased symptom expression an even recovery. The presence and concentration of phytoplasmas should be considered to develop strategies to manage phytoplasma diseases (Phylis, 2010). Due to the long incubation periods of phytoplasmas in plants and insect's outbreaks are detected too late (Hogenhout, 2008).

2.10 Diagnostics techniques

The first technique applied in the diagnostics of phytoplasma was the transmission electron microscopy (TEM). Because of the high cost of the equipment and the difficulty to prepare ultrathin sections of the tissue, was replaced with simple techniques like DFP or DAPI. While direct fluorescence detection (DFD), detects auto-fluorescence of necrotic cells, DNA-specific 6-diamidino-2-phenylindole (DAPI) staining, more sensitive than TEM, detects phytoplasmal DNA, both techniques utilize fluorescence microscopy. Using DAPI technique a bluish fluorescence in the sieve indicates the likely presence of phytoplasmas (Maejima, 2014).

Different groups of phytoplasmas cannot be morphologically or ultra-structurally distinguished by using TEM, that is why the distinction of different groups of phytoplasma is made by sequence comparison of sequences of 16S rRNA genes (Lee, 1993).

Serological assays are available to detect 'C. P. mali' are composed of monoclonal antibodies (mAbs) to AP phytoplasma obtained from *Catharanthus roseus*. Polyclonal antibodies are also available (pABs) and were prepared against the expression product in *E. coli* of the immunodominant membrane protein (*imp*) (Constanzo, 2012).

Enzyme-linked Immunosorbent assay is commonly used to diagnose viral diseases, but it was rarely used to detect phytoplasma because of the difficulty to prepare the specific antibody and to purify phytoplasmal cells (Maejima, 2014).

The IgG antibody system, to detect apple proliferation (AP) phytoplasma, is commercially available but it is not suitable for the diagnostic of all the classes of AP (Tomlinson, 2010).

Since 1990 direct detection of phytoplasmal DNA by DNA-DNA hybridization and by PCR reaction have been applied. The amplification of the 16S rRNA genes of phytoplasma is the most common and used strategy to diagnose phytoplasmal diseases. PCR also is used to examine the localization and dynamics of the pathogen in its host plant (Deng, 1991) (Nakashima, 1995).

Nested PCR is the commonly used technique for phytoplasma diagnostics; using two PCR amplifications, the first one with a set of universal primers followed by a second amplification with group specific primers. This technique is necessary because of its increased specificity since a single PCR reaction is unable to detect low amounts of phytoplasma (Demeuse, 2016).

Real-Time PCR assays were developed to detect generic or specific phytoplasmas with the advantage of being less labour intensive and automated compared with endpoint PCR (Hodgetts, 2011).

Isothermal amplification of nucleic acids, like loop-mediated isothermal amplification (LAMP), is more rapid and sensitive than PCR. LAMP uses a set of four to six primers and a *Bst* polymerase with strand displacement activity. This technique does not require DNA purification and amplifies the DNA in less than one hour. The results of the amplification can be visualized either by gel electrophoresis or by colorimetric methods which can be used in the field. Since there is a significant amount of DNA obtained in LAMP reaction, a white precipitate of magnesium pyrophosphate can be detected with the naked eye or by real-time turbidimeter. In the presence of SYBR GREEN I, amplification generates a color change that can be determined by the naked eye or by using a UV lamp, having this method the potential to be used in the field (Mumford, 2006). The goal of LAMP assays is to develop a rapid way of diagnostic that is reliable and can be used in the field; in combination with fast DNA extraction.

For the improvement of diagnostic procedures; reliable sampling protocols are required, but due to the irregular distribution of phytoplasmas in the phloem of the host plants, to obtain valid results, it's still a challenge (Phylis, 2010).

Reliable results can be obtained using leaf midribs or stems sampled from late spring to the end of summer. Leaf samples should be collected randomly from all the plant since the normal uneven distribution of phytoplasma cells in the foliage.

2.11 16S rDNA Group X

Candidatus Phytoplasma prunorum, *Candidatus* Phytoplasma mali, and *Candidatus* Phytoplasma pyri belong to the group X based on the classification of phytoplasmas according to the 16S rRNA sequence. P. mali and P. pyri are included in the EPPO A2 List of quarantine pests (EUPHRESCO FRUITPHYTOINTERLAB GROUP, 2011).

Among the whole group of phytoplasmas, P. mali, P. pyri, and P. prunorum are the only ones with a linear chromosome; these types of chromosomes are rare in bacteria. Linear chromosomes are characterized by the presence of large TIRs and covalently closed hairpin terminations. The reason why P. mali and related phytoplasmas have linear chromosomes is unknown (Kube, 2008).

Comparison of nucleotide sequences of 16S rRNA of the three members of the group X revealed differences of 1 and 1,5%; to be assigned to another specie group they need at least 2,5 %. The species distinction was provided by molecular markers like 16S-23S rRNA spacer region and ribosomal protein, also considering the vector transmission host specificity and serological comparisons related on the recognition of imp (immunodominant membrane protein) (Constanzo, 2012).

2.12 *Candidatus* Phytoplasma mali

Candidatus Phytoplasma mali is the causative agent of apple proliferation disease. P. mali is one of the most economically important pathogens in southern and central Europe (Kube, 2008). In 2001, a phytoplasma outbreak affecting apple trees was reported in Germany and Italy that causes losses of €25 and €100 million respectively (Straus, 2009) (Maejima, 2014). AP affects the tree vigor resulting in smaller fruit size, with poor taste (Constanzo, 2012).

Table 2. Scientific and common names of '*Ca*. P. mali.'

Scientific name	**16S rDNA Group-Subgroup**	**Synonym**	**Common names**
'*Candidatus* Phytoplasma mali' (Seemüller and Scheneider, 2004)	16SrX-A	"*Candidatus* Phytoplasma mali"; Phytoplasma AP-MLO; *Phytoplasma mali*; Apple proliferation phytoplasma; Phytoplasma mali (Candidatus); AP, proliferation of apple (EPPO, 2018).	Apple proliferation, witches' broom, 16SrX (apple proliferation group)

AP is one of the most critical phytoplasma diseases of apple causing the reduction in size, weight, and quality of the fruit. The apple plant reduces its tree vigor increasing the susceptibility to other diseases like powdery mildew (*Podosphaera leucotricha*) and silver leaf fungus (*Chondrostereum purpureum*). Once an apple tree becomes infected by AP, it will remain infected throughout its life. Phytoplasma may disappear from the canopy but will stay in the roots and could be transmitted to other apple trees (Constanzo, 2012).

'Candidatus Phytoplasma mali' is transmitted by two primary species of psyllids in Europe: *Cacopsylla melanoneura* and *C. picta. C. picta* is monophagous on *Malus* spp, and *C. melanoneura* is oligophagous on Rosacea family. A third vector reported is the leafhopper *Fieberilla florii* (Tedeschi, 2004).

‘*Ca*. P. mali’ is an uncommon organism that differs in many characteristics comparing with ‘*Ca*. P. asteris and also comparing with all other mycoplasmas (Kube, 2008).

Symptoms of P. mali infection are unevenly distributed on apple trees, based on the symptoms phytoplasma strains can be classified from avirulent to mildly, moderate, or highly virulent; apple trees can be simultaneously infected by more than one strain of AP phytoplasma (Seemuller, 2010).

2.13 Distribution

AP phytoplasma has been reported to occur throughout Europe, specifically in Albania, Austria, Belgium, Bulgaria, Croatia, Czech Republic, France, Germany, Hungary, Italy, Moldova, Norway, Poland, Romania, Serbia and Montenegro, Slovakia, Slovenia, Spain, Switzerland and Ukraine; the areas located in the Figure 2.4. This bacterial presence was also reported in Turkey, Denmark, and Netherlands (Constanzo, 2012).

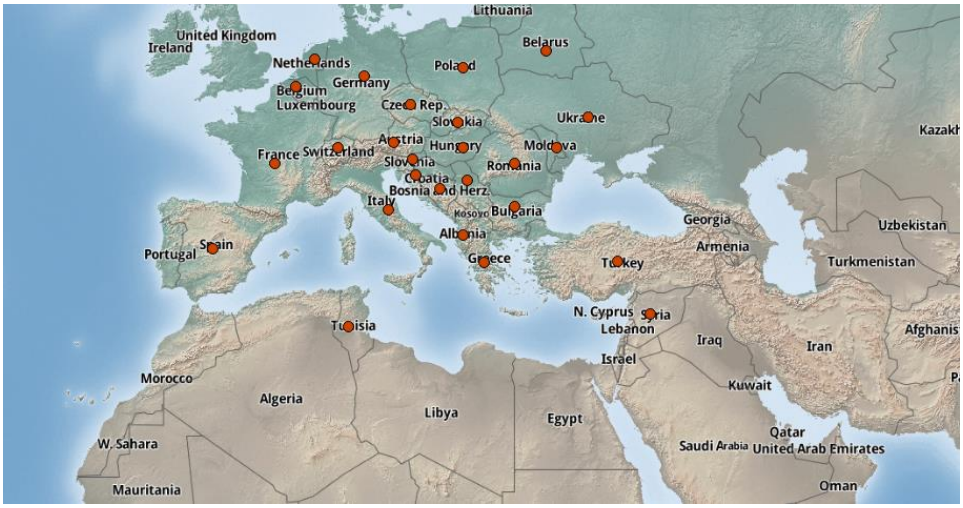


Figure 2. 4 Apple proliferation reported distribution. Source: CABI/EPPO, 2013.

2.14 ‘*Candidatus* Phytoplasma mali’ genome characteristics

An analysis of protein coding genes of P. mali showed that they have an incomplete glycolysis pathway, contrary to ‘*Ca*. P. asteris’. Because of the lack of critical metabolic pathways, it is proposed that as a carbon and energy sources, these bacteria use maltose and malate. Comparing with the genome of P. asteris, P. mali has a smaller genome, lower GC content, a lower number of paralogous genes, and a limited number of ABC transporters for amino acids. Contrary, P. mali has more genes for recombination, SOS response and excision repair compared with P. asteris (Kube, 2008).

AP phytoplasma has a large number of *hflB* genes, so it is believed that they may function as virulence factors since they act in the degradation of host proteins to uptake the essential compounds and to degrade the proteins produced by the defense reaction of the host (Kube, 2008).

Table 3. General features of Phytoplasma mali genome *(Kube, 2008)*.

Strain **'*Ca*. Phytoplasma' species** **16S rDNA group** **Cluster**	**AT** **Mali** **X** **II**
Chromosome size (bp)	601,943
Chromosome composition	Linear
G+C content (%)	21.4
Protein-coding regions (%)	79
Coding sequences	536
Genes encoding proteins	497
Protein coding genes with assigned functions	338
(Conserved) hypothetical proteins	159
Single copy proteins	408
Multiple-copy proteins	89
Multiple-copy proteins in PMUs	4
Transposase similar to tra5	1
Fragmented genes	16
Avg ORF size (bp)	955
tRNA genes	32
rRNA operons	2
Extrachromosomal DNAs	0

*PMUs – Potential mobile units (clusters of repeated gene sequences)

3. MATERIALS AND METHODS

3.1 Sample collection

The research presented here was carried out with samples of different varieties of apple trees collected in Olcsvaapáti at summer 2017, the laboratory work was made between May 2017 and March 2018.

Table 4. Apple sample varieties sampled for this research.

Apple sample varieties
Rebella
Remo
Reglindis
Relinda
Reanda

3.2 DNA extraction

Young, symptomatic and asymptomatic leaves samples were used for total DNA extraction by using the NucleoSpin® Plant II (MACHEREY-NAGEL) kit. The homogenization procedure started with grinding approximately 100 mg of leaves samples using a mortar and a pestle in the presence of 400 µl lysis buffer PL1 and 10µl of RNaseA solution of the NucleoSpin® Plant II (MACHEREY – NAGEL) kit. It was followed by an incubation for 10 min at 65°C.

The process of filtration and clarification of crude lysate was done by loading the lysate into a NucleoSpin®Filter column and then centrifuged for 2 min at 11000 g. Then we collected the clear flow-through and add 450 µl of Buffer PC and mix thoroughly by pipetting. In the next step we loaded 700 µl of the sample in a NucleoSpin Plant II® column and centrifuged it for 1 min at 11 000 g. Washing was made using 400 µl of Buffer PW1 and centrifugation for 1 min at 11 000 g and the flow through was discarded. The second wash was made with 700 µl of Buffer PW2 and centrifugation of 1min at 11 000 g, and again the flow-through was discarded.

The final step was the elution of DNA using 50 µl of Buffer PE, pre-heated to 65°C and incubation for 5 minutes was followed by centrifugation for 1min at 11 000 g.

3.3 Crude DNA extract preparation

Approximately 100-150 mg frozen apple leaves samples were homogenized in a mortar with 500 µl lysis buffer (Plant material DNA extraction kit-Optigene). 10 µl prepared plant material was transferred into a new 1,5 ml microcentrifuge tube, which contains 50 µl dilution buffer (Optigene). It was stored at -20 °C until use.

Another type of crude extract was also prepared by macerating the apple tissue in Milli-Q water.

3.4 Primer design

The nucleotide sequences of *Candidatus* Phytoplasma mali were obtained from a Gene Sequence repository (Genebank) in Fasta format (GenBank Accession No.: AJ542541).

All the primers engaged in this research were designed using the primer design web page Primer3. Once primers were generated, they were analyzed with mFOLD software, which helps in the analysis of secondary structures and thermodynamic features of primers.

Finally, the primers generated were copied to BLASTn (NCBI) to assessed for specificity.

LAMP primers were designed to the target genomic sequence of *'Candidatus.* P. mali' (Accession No. GenBank: CU469464.1). The primer sequences were generated using PrimerExplorer V4 software (Eiken Chemical Co. Ltd., Tokyo, Japan, http://primerexplorer.jp/e/), and then checked in Genebank databases by BLASTn for specificity.

Table 5. Oligonucleotides designed and used for amplification of a segment of 16S rRNA of *C*.P.mali by PCR

Name	Primer	Product size	Sequence 5'-3'
Pmali_16S_542F	Forward	168 bp	TATTGGGCGTAAAGGGTGT
Pmali_16S_709R	Reverse		CTTCGCTACTGGTGTTCCTC
Pmali_16S_1F	Forward	120 bp	GTAGGCGGTTTAATAAGTCTATGGT
Pmali_16S_120R	Reverse		ACGCATTTTACCGCTACACA
Pmali_F3	Forward	204 bp	CGTGCCTAATACATGCAAG
Pmali_B3	Reverse		CTAACTAATGTGCCGCAAG
Pmali_FIP	Forward Inner		CGTCTAAGAGGCAGGTTACTTACG-CGAACGGAAACTTTTAGTTTCAG
Pmali_BIP	Reverse Inner		TTGGAAACGACTGCTAAGACTG-TCTCTTAGCATACCCTTGC

3.5 Gradient PCR

This technique was used to determine the optimal annealing temperature of the primers. Using the gradient function of the universal block, a range between 45 to 65°C was set. The following test parameters were selected: denaturation 98°C, 40 s, annealing 45°C - 65°C, 10 s, elongation 72°C, 20 s.

The tested annealing temperatures were (45, 51.2, 56.2 61.4 and 65°C) to optimize the PCR reaction. For the PCR it was used 1 µl of DNA template, 1 µl of forward and reverse primers (Pmali_16S_542F and Pmali_16S_709R), 3µl of 5x Phire Green Reaction Buffer (ThermoFisher), 0,3 µl of dNTPs, 0,3 µl of Phire Hot Start II DNA Polymerase and 8,4 µl of Milli-Q water. The amount of these components of PCR were the same for each apple sample.

3.6 PCR

PCR amplification was performed in a 15 µl reaction volume with 1 µl of DNA template, 1 µl of forward and 1 µl of reverse primers, 3µl of 5x Phire Green Reaction Buffer (ThermoFisher), 0,3 µl of dNTPs, 0,3 µl of Phire Hot Start II DNA Polymerase and 8,4 µl of Milli-Q water. For the nested PCR the conditions used were the same.

Table 6. PCR conditions.

PCR steps	**Temperature**	**Duration**
Initial denaturation	98 °C	30s
Denaturation	98°C	10s
Annealing	60°C	10s
Elongation	72°C	20s
Extension	72°C	1min
No. of cycles	40 cycles	

3.7 Electrophoresis

To analyze DNA fragments amplified by PCR and detecting the presence of the investigated PCR product, agarose gel electrophoresis was used to separate the DNA products and enable a clear visualization of the results.

For the agarose gel preparation, d 3,6 g of agarose measure and dissolved in 300 ml of 1xTBE (which is a buffer solution containing a mixture of Tris base, boric acid and EDTA) in an Erlenmeyer flask to make a gel of 1,2% of agarose concentration. Then the solution was

microwaved for until the agarose was dissolved entirely. The agarose solution was let it cool down to 50 °C. 1µl of ethidium bromide (EtBr) was added to 25-30 ml agarose gel. The agarose was poured into a gel glass, and then the wells comb was placed. It took around 10 minutes until the gel is completely solidified.

The agarose gel was placed into a gel electrophoresis tank unit previously filled with 1xTBE and added more until the gel is covered. The molecular weight ladder was loaded always in the first well of the gel.

Loading dye was added to the samples, and all the samples were loaded into the wells of the gel.

The separation was done using 80 - 150 V until the dye line is approximately reached the bottom of the gel.

3.8 Visualization by UV light

The agarose gel was visualized using a long wavelength ultraviolet transilluminator (300 nm). Thanks for the intercalating EtBr stain DNA should appear as bright orange bands on a dark background. The gel was removed carefully from the transilluminator after documented the results.

3.9 RT fluorescent LAMP assay

Total DNA and crude extract apple samples were used as template for Loop mediated isothermal amplification reaction. The DNA template and primer sets were mixed with the Optigene LAMP reaction mix to a total volume of 25µL, which contained the components listed in the Table 4. The reaction mixture was incubated at 65°C for 40 min. Amplifications were carried out on a GenieIII instrument. The reaction was terminated by heating the mixture at 80°C for 10 min. Milli-Q water was used as negative control, while DNA and crude extract from diseased apple samples.

Table 7. Master mix for an individual LAMP reaction.

	Individual reaction
Total volume	25 µl
LAMP Master mix	15µl
FIP-BIP primers	3µl - 3µl
F3-B3 primers	0.5 µl- 0.5 µl
Template (0.1ng/µl)	4 µl

3.10 Purification of a gel fragment

For this procedure the Thermo Scientific GeneJET Gel Extraction Kit was used. The DNA fragment of 167bp was excised with a sterile scalpel from an agarose gel trying to cut very close to the DNA band to avoid an excess of agarose gel, then the piece of gel was placed in a 1,5ml microcentrifuge tube previously weighted. The Eppendorf tube with the gel sliced was weighted, and 1:1 volume of binding buffer to the slice was added to dissolve the agarose, denature proteins and promote DNA binding to the column. An incubation at 60°C for 10 min took place until the gel slice is completely dissolved. The solubilized gel solution was transferred to the GeneJET purification column, centrifuged for 10 min and the flow-through was discarded. 100 µl of Binding buffer was added to the GeneJET purification column and centrifuged for 1 min and again the flow-through was discarded. 700µl of Wash Buffer was added to the column and centrifuged for 1 min and discarded the flow-through. The GeneJET column was further centrifuged for 1min to remove the residual wash buffer and in a new Eppendorf tube the column was placed and 25 µl of elution buffer was added to the center of the column membrane and centrifuged for 1 min. The eluted DNA was stored at -20°C.

3.11 Cloning

The cloning process was made using the Thermo Scientific CloneJET PCR Cloning Kit. The linearized cloning vector (The pJET1.2/blunt) accepts inserts from 6 bp to 10 kb. In a 1,5 ml Eppendorf tube a ligation mixture was made using 7,5 µl 2X Reaction Buffer, 0,75µl of the pJET1.2/blunt Cloning Vector (50 ng/µl), 0,5µl of T4 DNA ligase, 1µl of water nuclease-free and 5 µl of the DNA fragment.

The ligation mix was incubated at room temperature for 5 min and passed that time the ligation is ready for transformation.

3.12 Transformation

Competent DH5α E. coli cells stored at -70°C was put on ice for 10 to 15 minutes until they defrost. Empty transformation tubes were placed on ice and 150-200 µl competent cells were carefully pipetted into them, and subsequently 5µl of the ligation mixture was added. The mixture was stored on ice for 20 minutes.

The tubes were transferred to 42°C water bath heat shock for 30 seconds and were transferred back to the ice. 500 µl of SOC medium without antibiotics was added to the tube what was transferred

to a shaking incubator at 37°C. The cultures were incubated for 40 min to allow the bacteria to recover and to express the antibiotic resistance marker encoded in the plasmid.

After this time, on an LB solid medium, containing ampicillin, 250 µl of the transformed competent cells were transferred and spread carefully. The Petri plates were inverted and incubated at 37°C overnight. The transformed colonies were expected to appear in 12-16 hours.

3.13 Inoculation of liquid culture

In an Erlenmeyer flask containing 250 ml of LB medium, 250 µl of ampicillin was added. 3 ml of LB medium + ampicillin was transferred into each inoculation flasks. Of the petri plate incubated overnight we picked 4 colonies, each one was collected with a toothpick and in a new Petri plate with solid LB medium previously labelled we drew a line and the toothpick we placed into the small flask containing the 3ml of medium. The procedure was repeated with each chosen colony. The cultures were placed to 37°C overnight to a shaking thermostat.

3.14 Plasmid purification

The Plasmid DNA purification was made using the NucleoSpin® Plasmid kit (MACHEREY-NAGEL). 1 ml of the transformed *E. coli* cells were transferred into a 1,5 ml Eppendorf tube, centrifuged for 3 min at 8000 rpm to sediment the cells. The supernatant was discarded as much as possible, we kept the pellet and then the remaining cell culture was transferred to this tube the centrifugation procedure was repeated. The remaining pelleted bacterial cells were re-suspended with 250 µl of Buffer A1. The bacteria were completely re-suspended by pipetting up and down. 250 µl of Lysis Buffer A2 was added and mix thoroughly by inverting the tube gently 6 times and incubated for 5 min at room temperature, until the lysate appears clear. 300 µl of Precipitation Buffer A3 was added and mixed immediately by inverting the tube until the mixture was homogeneous, the solution was centrifuged for 5 min at 11 000 g at room temperature. The NucleoSpin® Plasmid column was placed in a collection tube and 750 µl of the supernatant were pipetted onto the column and centrifuged 1 min at 11 000 g. The flow-through was discarded and placed the column back into the collection tube.

600 µl of Buffer A4 were added to the column and centrifuged 1min at 11 000g. The empty column was placed back into the collection tube and centrifuged 2 min for 11 000 g to dry the silica membrane. The NucleoSpin® Plasmid column was placed in 1,5ml micro-centrifuge tube and 30 µl of Buffer TAE was added, incubated for 1 min at room temperature and centrifuged for 1 min at 11 000 g.

3.15 Plasmid Digestion

To check that the plasmid contains the fragment of DNA that we inserted we did the digestion of the plasmid pJET with two restriction enzymes Xho1 and Xba1 (Thermo Fischer Scientific). A mixture of 2 µl 10X Tango Yellow Buffer, 0,2 µl of Xho1, 0,4 µl Xba1 enzymes and 4,4 µl of Milli-Q water and 3µl of the purified plasmid was prepared and incubated for 1 hour at 37°C.

The purified undigested and the digested plasmid were separated on agarose gel by electrophoresis. The plasmids containing the inserted fragment were sent for sequencing.

4. RESULTS

EPPO Global database reported that Phytoplasma mali is widespread in Hungary. In 1998 was also detected in pear, in mixed infection with pear decline phytoplasma. These two phytoplasmas were first identified by molecular methods in pear trees in Hungary by Del Serrone (Del Serrone, 1998). For controlling the spread of phytoplasma disease a specific, sensitive, reproducible and rapid diagnosis tool would be extremely important.

4.1 DNA extraction

Our experiments were carried out on apple samples originating from five different varieties of the same orchard. DNA was extracted from leaf samples using the NucleoSpin® Plant II (MACHEREY-NAGEL) kit. To examine the quality of DNA samples obtained from the DNA extraction we scanned the total DNA samples from 220 to 400 nm on Nanodrop NP-1000. The concentrations obtained are presented in Table 8.

Further test of the quality of the samples was done by gel electrophoresis of the total DNA on 1,2% agarose gel as shown in Figure 4.1. The results of the gel electrophoresis show that in all the samples a high-molecular-weight bands, corresponding to the total genomic DNA, was present. So, we obtained pure genomic DNA, rapidly from plant samples with a high yield and quantity using the NucleoSpin® kit, as we can see in Table 8 and Figure 4.1.

Table 8. DNA concentration of the samples determined with Nanodrop.

Sample name	Concentration (ng/µl)
E5/1	98
E5/2	162.5
E5/3	97.6
E5/4	96.9
E5/5	115.6
E5/6	104.9
E5/7	104.7
E5/8	111.6
E5/9	86.8
E5/10	117.6
E5/11	131.3
E5/12	119.3

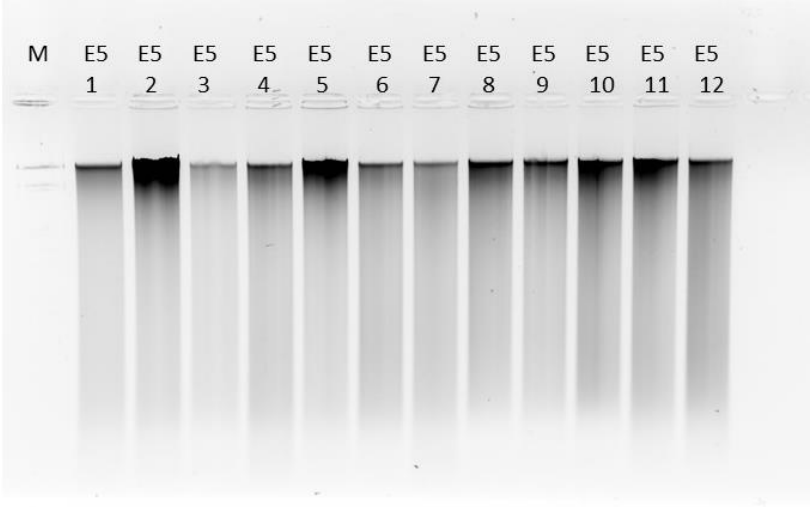


Figure 4. 1 Agarose gel (1,2% w/v) electrophoresis of total DNA prepared from apple leaves from REBELLA and REMO varieties. M, DNA size marker (1 kb DNA ladder). Lanes 1-11, REBELLA; lane 12 REMO.

Phytoplasmas are difficult to detect, because of the low concentration, especially in the case of woody plants. Also, the diverse distribution among the sieve tubes of the infected plant made their detection complicated. According to Bertaccini's work, in general, the total amount of phytoplasma DNA in the plant samples is less than 1% of the total extracted DNA; that is why for their investigation a technique including amplification step was applied. To detect phytoplasma DNA and reduce enzyme-inhibitory molecules like polyphenols and polysaccharides nested PCR was employed to increase sensitivity and specificity. Nested PCR is done using universal primers (P1/P7) at the first PCR reaction what is followed by a second amplification by PCR with a group-specific primer pair to identify the presence of phytoplasma in samples with mixed infections (Bertaccini, 2007).

4.2 Primer design

I have designed new sets of primers for detection of *C.* phytoplasma mali by nested PCR and LAMP reactions using a program available on the web (Primer3 and Primer Explorer (LAMP primers)). Sequences and properties of the newly designed primers are listed in Table 9. Sequences of published primers used for phytoplasma group 16SrX detection are listed in Table 10.

For oligonucleotide design I used published sequence of 16S rDNA of *C.* p. mali (GenBank accession number: AJ542541) by both techniques.

Table 9. Oligonucleotide primers designed for Nested PCR and LAMP.

Name	Primer	Product size	Sequence 5'-3'	TM	GC%
P_16S_542F	Forward	168 bp	TATTGGGCGTAAAGGGTGT	57.96	47.37
P_16S_709R	Reverse		CTTCGCTACTGGTGTTCCTC	57.54	55.00
P_16S_1F	Forward	120 bp	GTAGGCGGTTTAATAAGTCTATGGT	58.28	40.00
P_16S_120R	Reverse		ACGCATTTTACCGCTACACA	59.25	45.00
P_F3	Forward	204 bp	CGTGCCTAATACATGCAAG	55.59	47.90
P_B3	Reverse		CTAACTAATGTGCCGCAAG	55.51	47.00
P_FIP	Forward Inner		CGTCTAAGAGGCAGGTTACTTACG-CGAACGGAAACTTTTAGTTTCAG	77	42.50
P_BIP	Reverse Inner		TTGGAAACGACTGCTAAGACTG-TCTCTTAGCATACCCTTGC	77	46.00

Table 10. Reported primers for Phytoplasma GroupX detection.

Name	Primer	Product size	Sequence 5'-3'	TM	GC%	Phytoplasma isolates	Reference
P1	Forward	1830 bp	AAGAGTTTGATCCTGGCTCAGGATT	62	44	16S-23S rRNA Phytoplasma	(Smart, 1996)
P7	Reverse		CGTCCTTCATCGGCTCTT	57	55		
fO1	Forward	1200 bp	CGGAAACTTTTAGTTTCAGT	52	35	AP group (AP, PD, ESFY, PYLR)	(Lorenz et al. 1995)
rO1	Reverse		AAGTGCCCAACTAAATGAT	52	36.8		
F3	External forward		CCTGCCTCTTAGACGAGGAT	58	55	16s rRNA group X phytoplasma	(Jonghe, 2017)
B3	External reverse		CAATGTGGCCGTTCAACCT	58	52.6		
FIP	Hybrid inner forward		AGCATACCCTTGCGGGTCTTTTT TTTACAGTTGGAAACGACTGCTA	-	-		
BIP	Hybrid inner reverse		AAGAGATGGGCTTGCGGCACTT TTCTCAGTCCAGCTACACATCA	-	-		

Target sites in the sequence of Phytoplasma mali, where the designed primers anneal are shown in Figure 4.2.

Figure 4. 2 Position of the primers P_16S_542F and P_16S_709R, P_16S_1F and P_16S_120R designed for nested PCR in the sequence of 16S rDNA gene.

For LAMP primer design six distinct genomic regions were designated on the target gene 16S rRNA. FIP and BIP are hybrid primers of two regions. Hybridization of the four oligos to the sequence is critical in loop-mediated isothermal amplification, consequently, the melting temperature of the outer primers is lower than the temperature for the inner primers FIP and BIP, to guarantee that the DNA synthesis starts earlier from the inner primers (Obura, 2011). The annealing sites of the designed LAMP primers are displayed in Figure 4.3.

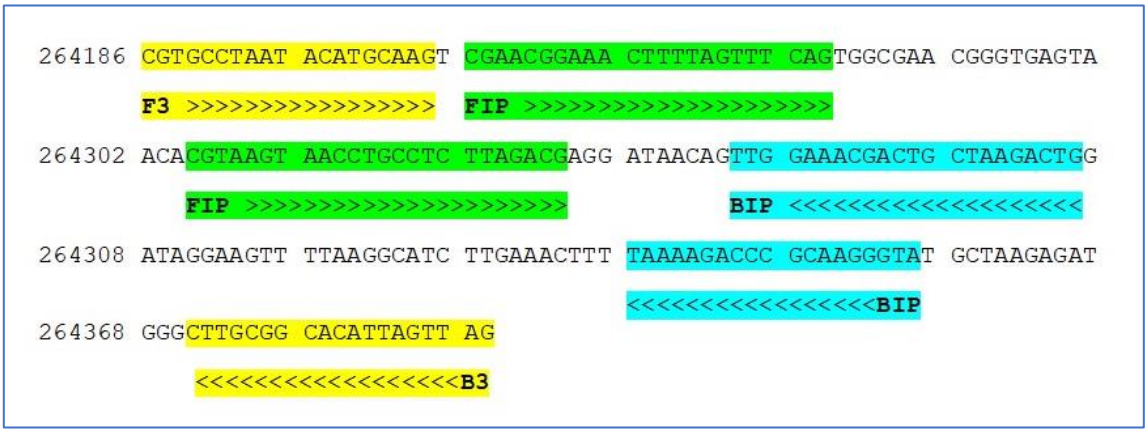
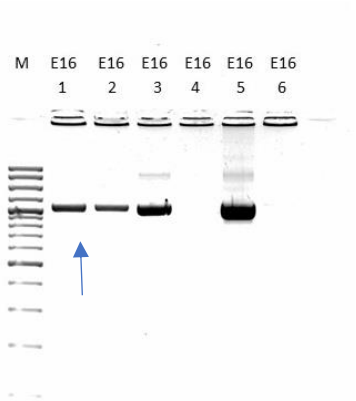


Figure 4. 3 A 204-bp 16S rRNA gene segment. Region amplified by loop-mediated isothermal amplification of DNA (LAMP). Annealing sites for oligonucleotides are indicated.

4.3 Detection of phytoplasma in apple

Different samples from DNA extraction from apple, crude extract from apple, DNA extracted from apricot and the positive and negative controls were amplified with the group-specific primers fO1/rO1, to test the presence of Phytoplasma mali by nested PCR as showed in Figure 4.4. From

the results obtained we concluded that apple samples were infected by a phytoplasma, and E16/1 sample was selected for further assays. A nested PCR with a first reaction using universal primer set followed by a group specific set of primers allows the detection of phytoplasma in a sample containing more than one strain of phytoplasma (Costanzo, 2012) belonging to the 16S RNA group X.



Sample name	Description
M	1 kb DNA ladder
E16/1	Apple DNA
E16/2	Apple crude extract
E16/3	Apricot DNA
E16/4	Negative control apple DNA
E16/5	Positive control Apple crude extract
E16/6	Negative Apricot DNA

Figure 4. 4 1.1 kb 16S rDNA fragments amplified using the primers fO1-rO1. The arrow points show the sample chosen as a template for the next PCR reaction.

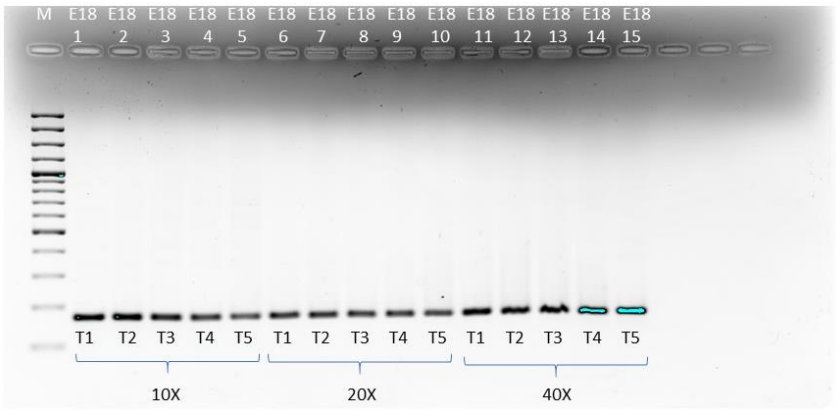
4.4 Gradient PCR

Nested PCR is the leading method for phytoplasma amplification, and it has been reported for diagnosis purposes in a great variety of plant samples where phytoplasmas occur in low titer or where there are inhibitors present in the DNA sample. The diagnosis of phytoplasmas has been usually based on phytoplasma universal primers and group specific primers. The primers used are designed for the amplification of the 16S ribosomal rRNA gene sequences (Marzachi, 2004).

To optimize the nested PCR conditions of the designed primers, we tested 10x, 20x and 40x dilutions of the PCR product obtained from amplification of phytoplasma with the group X specific set of primers fO1-rO1 (Lorenz, 1995). We tested five different annealing temperatures to determine the optimal one.

Optimal annealing temperature of the amplification must be set in order to keep it high enough to decrease the number of nonspecific products but not reduce the yield of the desired product. The gradient feature of the thermocycler allows us to test a range of temperatures in a single experiment.

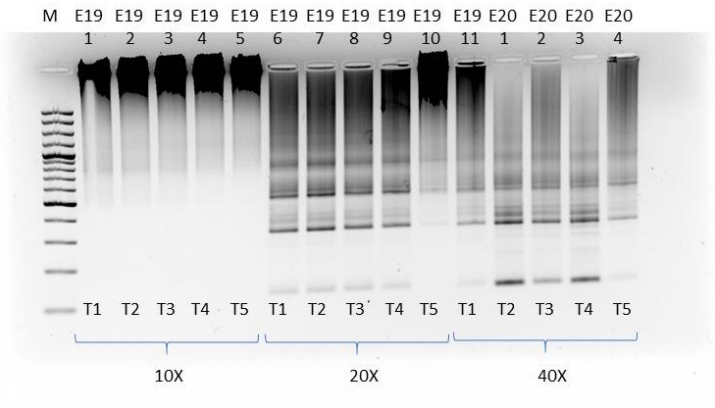
When we checked the optimal annealing temperature for Pmali_16S_542F and Pmali_16S_709R, we obtained the desired PCR product of 168 bp, in all the tested temperatures and we got only specific amplification (Figure 4.5). There is no presence of nonspecific bands. We obtained the best results at an annealing temperature of 51,2°C and with a 10X dilution of the PCR product used as a template.



T1	45°C
T2	512°C
T3	56.2 °C
T4	61.4°C
T5	65.0°C

Figure 4. 5 Optimization of PCR assay for using the primers Pmali_16S_542F and Pmali_16S_709R.

On Figure 4.6. gel electrophoresis results of the experiments for optimization of annealing temperature for P_16S_1F and P_16S_120R primers are shown. In this case, we expected a PCR product of 120 bp. We could observe the expected fragments on the 20X and 40X dilutions of the PCR product. The best result was obtained from the 40X dilution and 51,2°C. In this gel electrophoresis, we can see that unspecific products are also present.

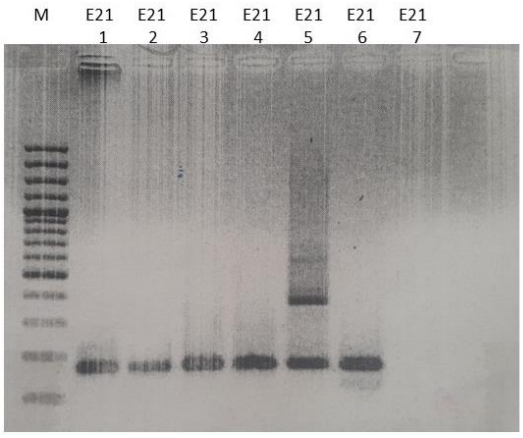


T1	45°C
T2	51.2°C
T3	56.2 °C
T4	61.4°C
T5	65.0°C

Figure 4. 6 Optimization of PCR assay using the primers P_16S_1F and P_16S_120R.

The use of 16SrX/group specific primers fO1/rO1 (Lorenz, 1995) can be used in combination with restriction enzyme digestion (*SspI* and *SfeI*) for the distinction of P.mali, P. pyri, and P. prunorum.

Since we obtained the desired product with the set of primers P_16S_542/P_16S_709, we tested these primers with PCR products obtained with the primers P1/P7 and fO1/rO1 and with samples of '*Candidatus* Phytoplasma mali' and '*Candidatus* phytoplasma prunorum to check the amplification of the 16S rDNA gene in two members of the group 16SrX (Figure 4.7).



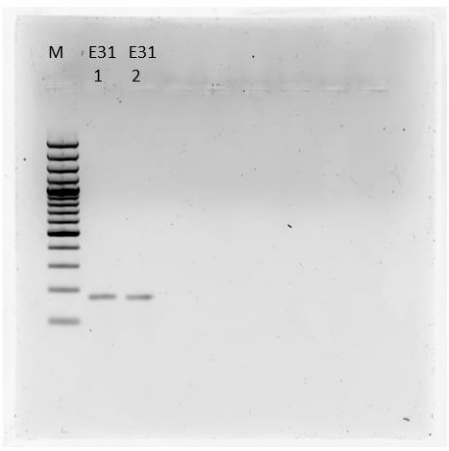
Sample name	Description
E21/1	P.mali PCR product P1/P7
E21/2	P.mali PCR product fO1/rO1
E21/3	P. prunorum PCR product P1/P7
E21/4	P. prunorum PCR product fO1/rO1
E21/5	P. mali Control +
E21/6	P. mali Control +
E21/7	Control -

Figure 4. 7 Nested PCR products with the primers P_16S-542F and P_16S-709R with P. mali and P. prunorum samples previously amplified with the primers P1/P7 and fO1/rO1. In all the wells we obtained a desired 168 bp band product, this can indicate that the primers P_16S-542F and P_16S-709R can be used as a group-specific set of primers to diagnose the presence of 16SrX phytoplasma members.

Good results were obtained using nested PCR with primer combinations P1/P7 – P_16S_542F/P_16S_709R and fO1/rO1 - P_16S_542F/P_16S_709R. The samples tested showed good results using these two combinations of primers for nested PCR, but also showed that they can only be used as group specific primers and not as Phytoplasma mali specific primers.

4.5 Molecular cloning

After Nested PCR amplification, to prove that our 168 bp product amplified by the primers P_16S_542F and P_16S_709 R is the fragment of interest (part of Phytoplasma mali 16srRNA gene); we cloned it into a vector, purified and sent for Sanger sequencing. The PCR products obtained with the primers P_16S_542F/ P_16S_709R were purified from the agarose gel (Figure 4.8) and ligated into the pJET1.2/blunt and transformed into competent *E. coli* cells as described in the materials and Methods section (Figure 4.9).



Sample name	Description
M	1Kb DNA ladder
E31/1 (DNA kit)	E17/2: DNA product P_16S_542F / P_16S_709R
E31/2 (Crude extract)	E21/2: DNA product P_16S_542F / P_16S_709R

Figure 4. 8 Agarose Gel electrophoresis PCR 168 bp product obtained with the primers P_16S_542F / P_16S_709R

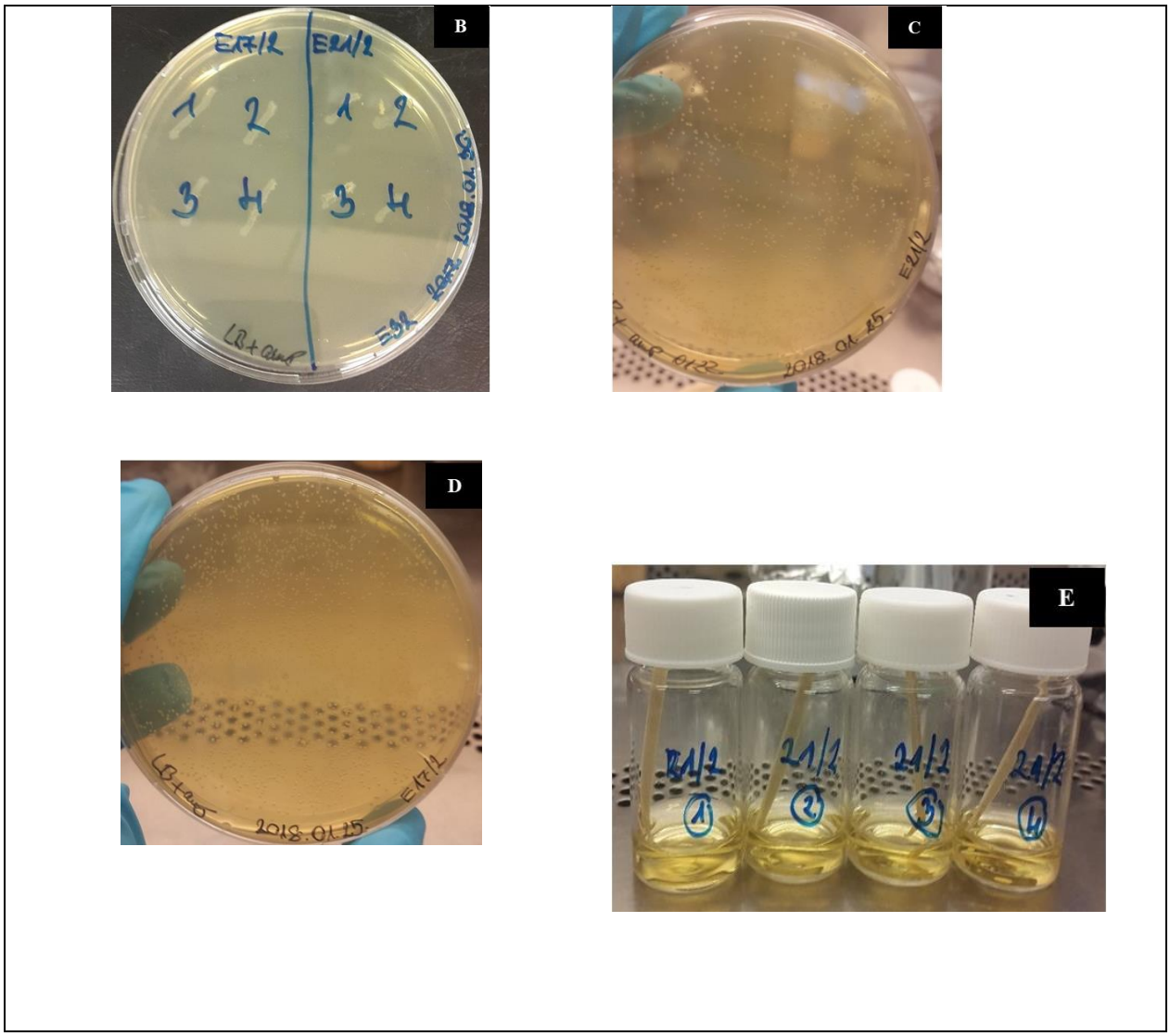
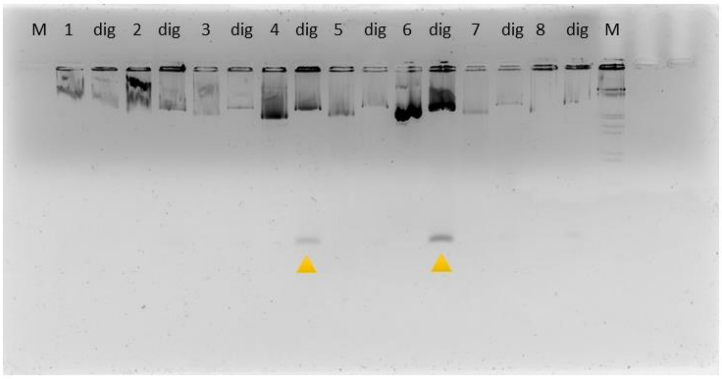


Figure 4. 9 Molecular cloning procedure. **A**. *E.coli* colonies selection. **B.** Master plate of the selected *E. coli* colonies. **C** and **D**. Petri plates showing the transformant *E. coli* colonies. **E.** Liquid culture for plasmid isolation.

Plasmids purified from eight colonies were digested with the restriction enzymes XhoI-XbaI to check the presence of the cloned fragment. In Figure 4.10 two samples pointed with an arrow showed to contain the fragment of 168bp. These two isolated plasmids were sent for sequencing.



Sample name	Description
1	Plasmid DNA
	Digestion
2	Plasmid DNA
	Digestion
3	Plasmid DNA
	Digestion
4	Plasmid DNA
	Digestion
5	Plasmid DNA
	Digestion
6	Plasmid DNA
	Digestion
7	Plasmid DNA
	Digestion
8	Plasmid DNA
	Digestion
M	λHind III EcoRI

Figure 4. 10 The result of restriction enzyme digestion of the purified plasmids. The arrows indicate the cloned 168bp fragments. The digestion was done with the restriction enzymes XbaI and XhoI.

4.6 Sequence analysis

Sequenced part of the plasmid was compared for identification with the available sequences of Phytoplasma mali present in GenBank. Sequence analysis of the plasmids verified and identified the pathogen-derived product in the two clones. The sequences obtained after Sanger sequencing are presented in Figure 4.11. We determined the sequence that belonged to the primers P_16S_542 and P_16S_709R, and it is highlighted in yellow. The sites of cut and recognition of the restriction enzymes are highlighted in green, while the cloned part of Phytoplasma mali 16s rRNA gene is highlighted by red.

Figure 4. 11 Nucleotide sequences obtained after sequencing and location of the oligonucleotides P_16S_542 and P_16S_709R.

Using the ApE A plasmid editor software, we determined the sites of digestion of the restriction enzymes XhoI and XbaI (present in the cloning region of the plasmid pJET). On Figure 4.12 schematic representation of the location of these restriction sites are shown in the sequenced plasmid's sequence.

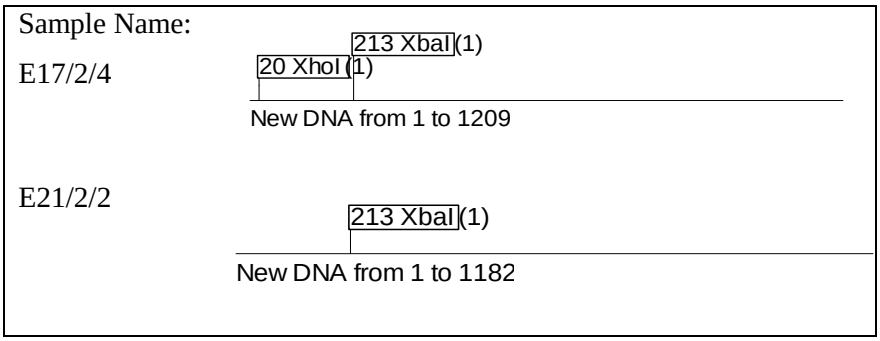


Figure 4. 12 Location of the XbaI and XhoI restriction sites of the enzymes in the sequences.

Results of the evaluation of the plasmid sequences with the Genbank Phytoplasma mali reference sequences using BLASTn is presented in Figure 4.13. Comparison of the sequence obtained for the sample E17/2/4 we have found a mismatch with the reference sequence CU469464.1. The mismatch is highlighted. Nucleotide sequence analysis revealed a 99% homology of the sequence of the sample E17/2/4 compared with the sequence of *Candidatus* Phytoplasma mali strain AT.

Sample E17/2/4

Candidatus Phytoplasma mali strain AT complete chromosome
Sequence ID: CU469464.1Length: 601943Number of Matches: 4

Score	Expect	Identities	Gaps	Strand
295 bits(326)	1e-81	166/168(99%)	0/168(0%)	Plus/Plus

```
Query  1       TATTGGGCGTAAAGGGTGTGTAGGCGGTTAAATAAGTCTATGGTATAAGTTCAACGCTTA       60
               ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  264692  TATTGGGCGTAAAGGGTGTGTAGGCGGTTTAATAAGTCTATGGTATAAGTTCAACGCTTA  264751

Query  61      ACGTTGTGATGCTATAGAAACTGTTTGACTAGAGTTGGATAGAGGCAAGTGGAATTCCAT  120
               ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264752  ACGTTGTGATGCTATGGAAACTGTTTGACTAGAGTTGGATAGAGGCAAGTGGAATTCCAT  264811

Query  121     GTGTAGCGGTAAAATGCGTAAATATATGGAGGAACACCAGTAGCGAAG  168
               ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264812  GTGTAGCGGTAAAATGCGTAAATATATGGAGGAACACCAGTAGCGAAG  264859
```

Sample E21/2/2

Candidatus Phytoplasma mali strain AT complete chromosome
Sequence ID: CU469464.1Length: 601943Number of Matches: 2

Score	Expect	Identities	Gaps	Strand
304 bits(336)	2e-84	168/168(100%)	0/168(0%)	Plus/Plus

```
Query  1       TATTGGGCGTAAAGGGTGTGTAGGCGGTTTAATAAGTCTATGGTATAAGTTCAACGCTTA  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264692  TATTGGGCGTAAAGGGTGTGTAGGCGGTTTAATAAGTCTATGGTATAAGTTCAACGCTTA  264751

Query  61      ACGTTGTGATGCTATGGAAACTGTTTGACTAGAGTTGGATAGAGGCAAGTGGAATTCCAT  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264752  ACGTTGTGATGCTATGGAAACTGTTTGACTAGAGTTGGATAGAGGCAAGTGGAATTCCAT  264811

Query  121     GTGTAGCGGTAAAATGCGTAAATATATGGAGGAACACCAGTAGCGAAG  168
               ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264812  GTGTAGCGGTAAAATGCGTAAATATATGGAGGAACACCAGTAGCGAAG  264859
```

Candidatus Phytoplasma mali clone 7 16S ribosomal RNA gene, partial sequence
Sequence ID: MF189568.1Length: 896Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
304 bits(336)	6e-79	168/168(100%)	0/168(0%)	Plus/Plus

```
Query  1    TATTGGGCGTAAAGGGTGTGTAGGCGGTTTAATAAGTCTATGGTATAAGTTCAACGCTTA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  TATTGGGCGTAAAGGGTGTGTAGGCGGTTTAATAAGTCTATGGTATAAGTTCAACGCTTA  314

Query  61   ACGTTGTGATGCTATGGAAACTGTTTGACTAGAGTTGGATAGAGGCAAGTGGAATTCCAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  ACGTTGTGATGCTATGGAAACTGTTTGACTAGAGTTGGATAGAGGCAAGTGGAATTCCAT  374

Query  121  GTGTAGCGGTAAAATGCGTAAATATATGGAGGAACACCAGTAGCGAAG  168
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375  GTGTAGCGGTAAAATGCGTAAATATATGGAGGAACACCAGTAGCGAAG  422
```

Figure 4. 13 Blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) comparison of sequences obtained from sequencing and the reference Phytoplasma mali sequences.

In the case of the sample E21/2/2, the sequence presented 100% of homology with two reference sequences of '*Candidatus* Phytoplasma mali.' The sequenced plasmids (E17/2/4 and E21/2/2) were

aligned using the software Clustal omega with each other and with reference sequences of '*C*. P. mali' and '*C*. P. prunorum to highlight exact matches and mismatches in the sequence (Figure 4.14).

Sequence analysis of three members (AP, PD and ESFY) of the phytoplasma group 16SrX revealed differences of 1.0 and 1.5%. Species distinction between these phytoplasmas was decided based on the molecular markers (16S-23S rRNA spacer region and ribosomal protein), serological comparisons, vector transmission and host range (Seemüller and Schneider, 2004).

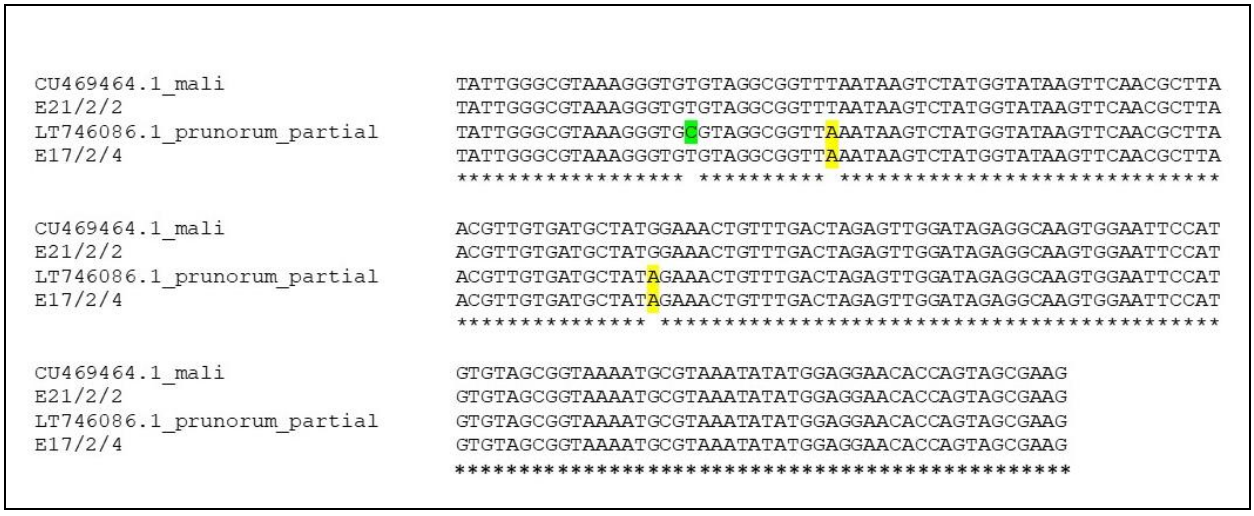


Figure 4. 14 Clustal omega multiple alignments of the 16S rDNA fragments between '*Candidatus* Phytoplasma mali,' E21/2/2 sequence, '*Candidatus* phytoplasma prunorum' and the sequence of the sample E17/2/4 (http://www.ebi.ac.uk/Tools/clustalo). Asterisks indicate the nucleotides in the column that are identical in all the sequences.

The sequence of sample E21/2/2 is identical to the sequence of Phytoplasma mali CU469464.1. Comparing with the sequence of P. prunorum there are two nucleotides in that they differ. On the other hand, the sequence E17/2/2 shows one nucleotide difference compared with P.mali and with the sample E21/2/2.

By comparing the cloned 16S rRNA part of our phytoplasma samples, we could identify differences in the reference and some other isolates of Phytoplasma mali and Phytoplasma prunorum (Figure 4.15).

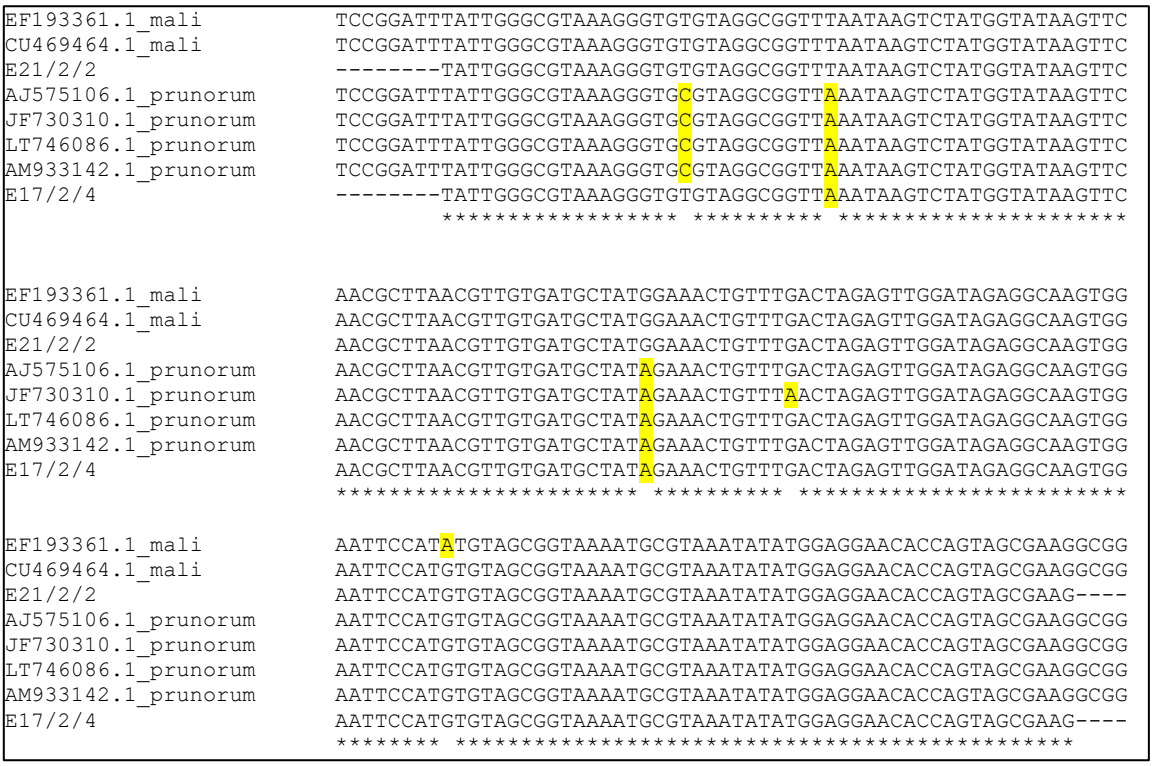


Figure 4. 15 Clustal omega multiple sequence alignment results of the obtained sequences (E17/2/4 and E21/2/2) and some isolates of Phytoplasma mali and Phytoplasma prunorum retrieved from Genbank. Asterisks indicate the residues and nucleotides that are identical in the alignment.

A virtual restriction mapping of the obtained DNA product (amplicon 168 bp) of 16S rDNA sequences of P. mali, P. prunorum, and P. pyri revealed that the sequences of P. prunorum and P. pyri contain a restriction site for the enzyme BfmI, while P. mali didn't, as shown in Figure 4.16. The resulting restriction fragments could help us to discriminate between P. mali and P. pyri and P.mali and P. prunorum and further proved that the cloned 16S rRNA piece originated from a *C.* P. mali phytoplasma.

The multiple alignment made by Clustal omega with the sequences E17/2/4, E21/2/2, P. mali and P. prunorum give us important information. The sequence E17/2/4 presents two sites of different nucleotides comparing with P. mali and these two nucleotides resemble more to the P. prunorum sequence. Since both samples were from apple samples, one from DNA extraction and the other one from crude extract. To determine that is actually P. prunorum more samples will need to be sequenced.

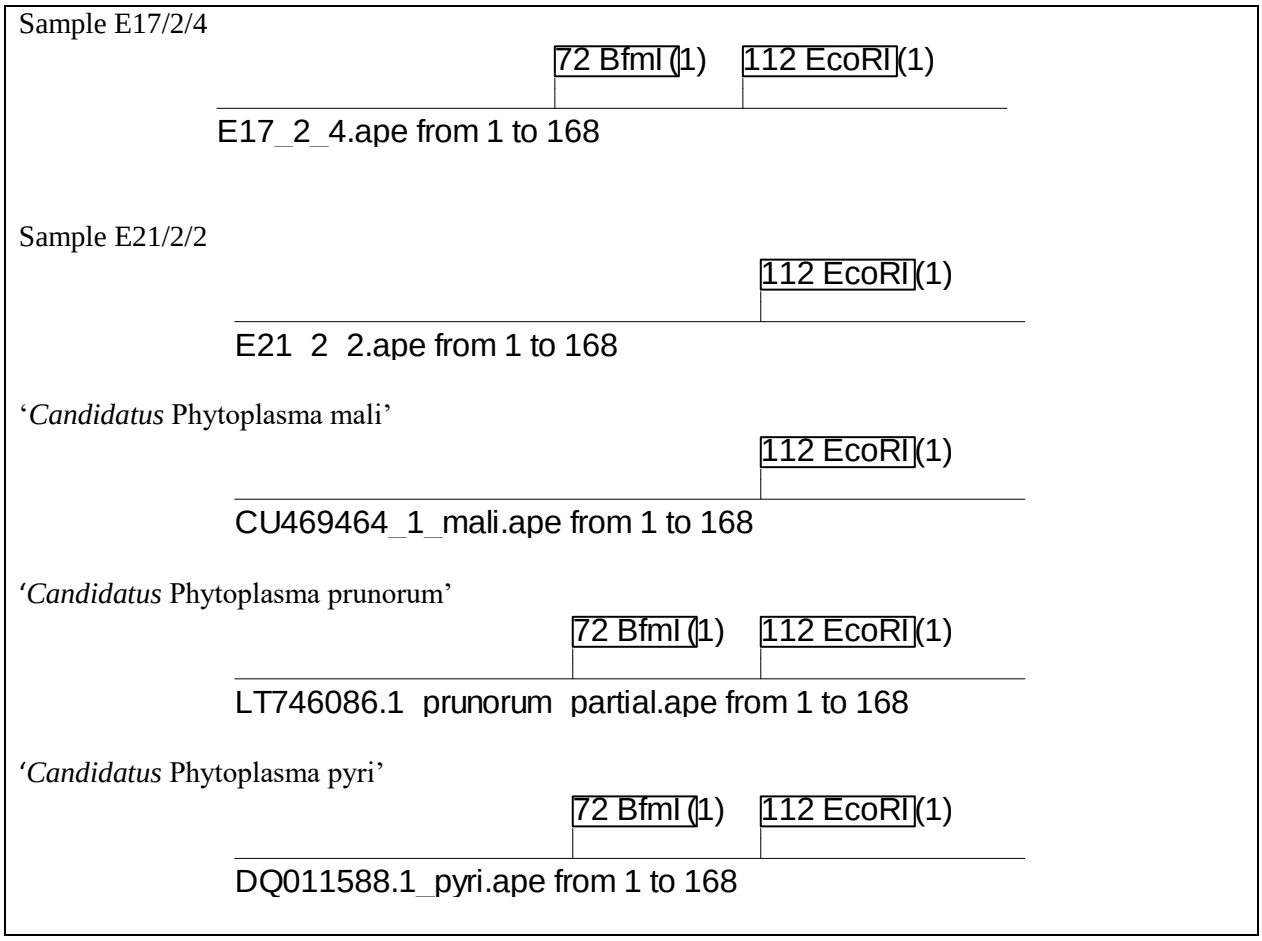


Figure 4. 16 Restriction sites generated by the ApE software present in P. mali, P. pyri, and P. prunorum.

4.7 Real-time fluorescence loop mediated isothermal amplification

LAMP assay combined with simplified DNA extraction techniques, can result in the amplification and diagnosis within one hour (Tomlinson, 2010). In our expriment enzymes and instrument from Optigene UK were used, the analysis was made using real-time detection of fluorescent signal emitted by the fluorescent dye incorporated into the dsDNA produced during amplification. Lamp reaction is easy to assemble, and since the reaction and analysis is made in a closed tube, there is less potential of contamination (Bekele, 2011).

Primers for LAMP have been designed based on the 16S rDNA gene region. During our tests we used either LAMP primers designed by us or published primers reported by (Jonghe, 2017), for detection of ribosomal 16S rRNA in Phytoplasma X group. The Figure 4.17/C shows the amplification curves obtained with these primers. With these set of primers we got the expected

sigmoidal logistic curve. In this reaction amplification was confirmed after 20 minutes, no amplification was observed in the no template control reactions in any case. The use of four primers increase the specificity, and the *Bst* polymerase is less prone to inhibitors. The primers reported by Jonghe (2017) were useful and validated to work on site to detect AP, PD, and ESFY. According to (Notomi, 2000) primer design is a crucial critical point for a LAMP. From our results with the primers of Jonghe et al. 2017, we tried the concentration validated for the primers 0.2µM for the outer primers (F3-B3) and 1µM for inner primers (FIP and BIP).
Positive signals obtained in the figure 4.17 (A, B and C), indicates amplification of the 16S rRNA sequence during the amplification reaction in the investigated samples.

The results obtained from our designed primers were not the expected sigmoidal curve in any of the two concentrations tested. This suggests that further optimization would be needed to determine an optimal primer concentration for the outer inner set of primers by varying the concentrations of both sets between 0,1-0,4 and 0,5- 1µM. We also might consider that an excess of the inner primers is required because of the auto-cycling strand displacement DNA synthesis occurring in the LAMP amplification (Jonghe, 2017). Other parameters which can be optimized are the time of incubation and temperature of the amplification (20 to 45 min and 55 to 75°C, respectively). The dilution of the samples is another factor to take into account for the further optimization of the technique since the detection in undiluted samples does not produce good results. According to the results of (Jonghe, 2017) and stable detection was at 10^{-5} sample dilution. Crude extracts can also be tested to compare and check the ability of this tool to amplify these samples with our primers. Since LAMP allows the use of crude extract samples this technique has potential as on-field technique.

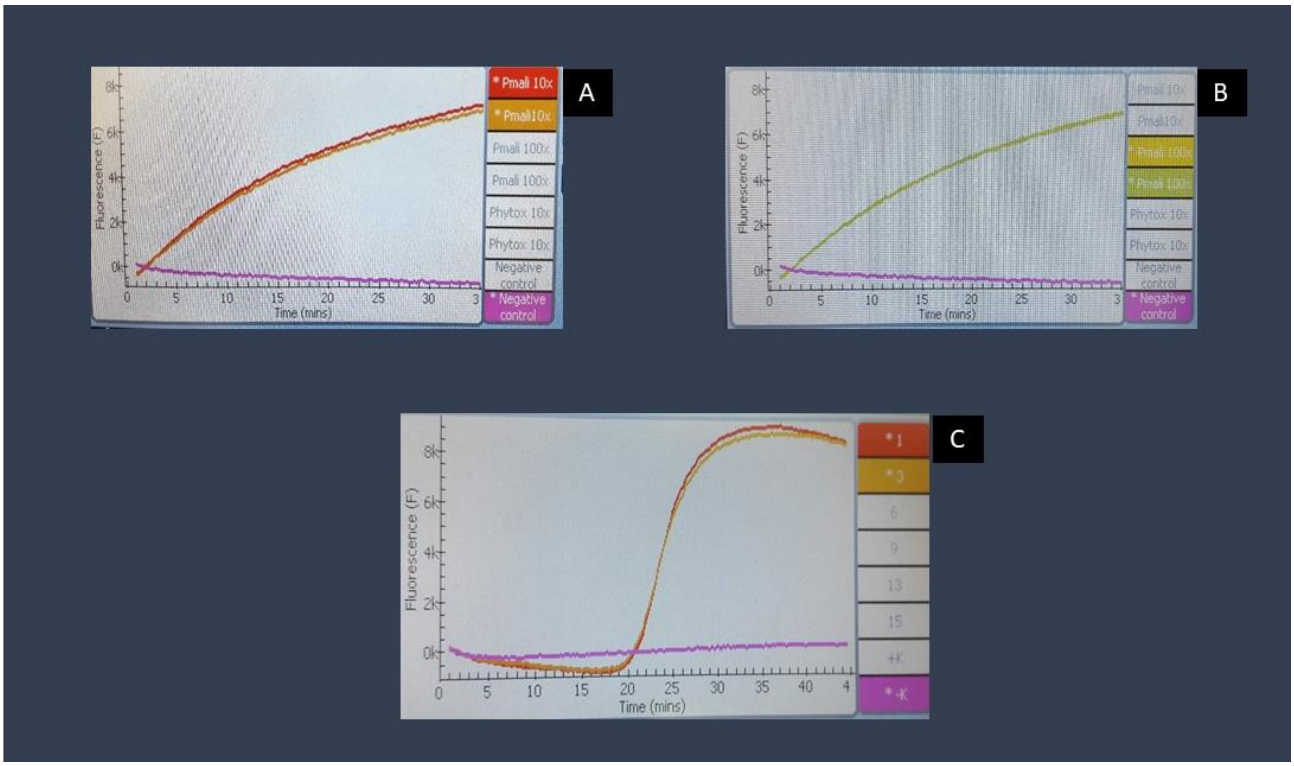


Figure 4. 17 Loop-mediated isothermal amplification results. A. LAMP with designed primers with 10X diluted DNA. B. LAMP with designed primers with 100X diluted DNA. C. LAMP with reported primers with undiluted DNA.

5. CONCLUSIONS AND RECOMMENDATIONS

Conclusions

- Our results confirmed the presence of '*Candidatus* Phytoplasma mali' from the extracted DNA samples and crude extract of the five varieties of apple trees collected from Olcsvaapáti, Hungary.
- A nested PCR that uses a universal phytoplasma primers set P1/P7 followed by a group-specific 16SrX set of primers fO1/rO1 was optimized and can be used for reliable detection of infected '*Candidatus* Phytoplasma mali' apple samples. Our newly designed primers Pmali_16S_542/Pmali_16S_709 for nested PCR in combination with a first round PCR reaction with either set of primers P1/P7 or fO1/rO1 has the advantage to allow the detection of phytoplasmas for the group 16SrX in a reliable way.
- For Lamp, assay a further optimization of the reaction with the newly designed primers is needed. Lamp reaction with the reported primers is a useful and sensitive method that can detect the presence of 16SrX phytoplasma.
- From the isolation of DNA from apple samples from Olcsvaapáti, Hungary we obtained good quality DNA for further assays.
- We could detect the presence of phytoplasma infection by the use of reported and newly designed primers by nested PCR technique.
- By cloning and sequencing of the nested PCR product, we confirmed the usefulness of the newly designed primers and prove that the fragment amplified by these primers belongs to '*Candidatus* Phytoplasma mali.'
- The newly designed Lamp primers needs further optimization to detect group 16SrX phytoplasmas in a reliable way since the results obtained are not adjusted to the sigmoidal curve results as expected for positive Lamp results.

Recommendations

For phytoplasma detection simple and sensitive detection technique is needed to be established and applied for effective prevention. Detection is equally essential as intensive vector control that is the standard control measure used in plantations. Since many apple trees are latently infected and no symptoms are evident, the early detection is needed to avoid an outbreak (Lesnik, 2007).

To test the specificity of the designed primers nested PCR should be tested with different samples of phytoplasmas belonging to the various groups.

A new study can be performed by nested PCR amplifying non-ribosomal protein genes like *secA*, *secY*, *imp* gene (coding for the immunodominant membrane protein), *aceF* and *tuf* gene. These regions appear to have more variation than the commonly used 16S rRNA gene (Marcone, 2000), but their usage must be validated before their use for diagnostics of phytoplasmas.

For LAMP approach, specificity tests to determine if there are false positive results for other phytoplasmas that don't belong to the group 16SrX is needed. Determination of the limit of detection (LOD) is also needed since the concentration of phytoplasma in the infected plants is highly variable. According to the OptiGene optimization manual, the reaction speed can be improved by altering the primer location. Sensitivity can be improved by testing the lowest concentration that the assay can detect and be sure that the LAMP assay will only identify phytoplasma group 16SrX and no other similar organisms. All these parameters can be tested in further analysis and in optimization of loop-mediated isothermal amplification using the designed primers.

For further studies and on-field '*Candidatus* Phytoplasma mali' detection, I recommend validation of colorimetric LAMP assay for routine diagnosis using hydroxy naphthol blue to detect the magnesium pyrophosphate by-product in a successful LAMP amplification.

6. SUMMARY

Diagnostics of '*Candidatus* Phytoplasma mali' in Hungary, with molecular methods

Apple proliferation (AP), is a phytoplasma disease of apple trees (Rosacea: *Malus* spp.), is well known in Europe where it represents one of the most economically important threats to apple plants. The disease affects the overall tree vigor resulting in significantly smaller fruits and can lead to the decline of the tree and the plantation. Hungary is one of the major apple producing countries, covering 27 000 ha equivalent to 6% of the EU apple tree area.

Phytoplasma diagnostics is based on nested polymerase chain reaction (nPCR) to selectively amplify16SrRNA where the first step use P1/P7 primers. As a second reaction, group specific primers (fO1-rO1) are used, selectively amplifying product only if a phytoplasma belonging to the group X is present. Beside these general primers in this study, we design new primers (P_mali16S_542F and P_mali16S_709R) to detect and identify '*Candidatus* Phytoplasma mali.' We used universal primer pairs described in the literature (P1-P7) and (fO1-rO1) as outer primers of the nPCR reaction.

PCR detection of AP agent with (P_mali16S_542F and P_mali16S_709R) was successful. The amplified products were cloned into a vector, purified and sequenced.

To test another tool for diagnostics, we also used a simple and effective phytoplasma diagnostic technique, loop-mediated isothermal amplification of DNA (LAMP). For LAMP, we designed two pairs of primers (Pmali_F3, Pmali_B3, Pmali_FIP, Pmali_BIP) and we tested them on symptomatic and asymptomatic plant samples.

As a result, we successfully detected *C*. P. mali in Hungary and validated the usage of LAMP primers for P. mali diagnostics.

7. ACKNOWLEDGEMENTS

8. DECLARATIONS

DECLARATION

Signed below, Myriam Estefanía Peña Zúñiga, student of the Faculty of Agricultural and Environmental Sciences, Szent István University, at the /MSc Course of Agricultural Biotechnology declare that I have used the cited and quoted literature in accordance with the relevant legal and ethical rules. I understand that the one-page-summary of my thesis will be uploaded on the website of the Faculty/Institute/Course.

Confidential data are presented in the thesis: yes no

Gödöllő, …..day ……month…….year

…….
Student's signature

As primary thesis adviser of the author of this thesis, I hereby declare that review of the thesis was done thoroughly; literature sources cited in the dissertation were used in accordance with the relevant legal and ethical rules. I hereby, approve the thesis for oral defense on Final Examination.

Confidential data are presented in the thesis: yes no

Gödöllő, …..day ……month…….year

…….
Signature of the Primary Thesis Adviser

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