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Polymerase Chain Reaction
for Phytoplasmas Detection

Duška Delić
University of Banjaluka, Faculty of Agriculture
Bosnia and Herzegovina

1. Introduction

This chapter inspired treat caused by phytoplasmas diseases in food production, and increased need for sensitive and accurate detection of these microorganisms. Early and sensitive detection and diagnosis of phytoplasmas is of paramount importance for effective prevention strategies and it is prerequisite for study of the diseases epidemiology and devising of pathogen management.

Phytoplasmas are prokaryotes lacking cell walls that are currently classified in the class *Mollicutes* (2). To the class *Mollicutes* (cell wall-less prokaryotes) belonging both pathogenic groups: mycoplasma-like organisms (MLOs) and mycoplasmas. However, in contrast to mycoplasmas, which cause an array of disorders in animals and humans, the phytopathogenic MLOs resisted all attempts to culture them *in vitro* in cell free media (89). Following the application of molecular technologies the enigmatic status of MLOs amongst the prokaryotes was resolved and led to the new trivial name of "phytoplasma", and eventually to the designation of a new taxon named 'Candidatus phytoplasma' (73).

Diseases associated with phytoplasma presence occur worldwide in many crops, although individual phytoplasmas may be limited in their host range or distribution. There are more than 300 distinct plant diseases attributed to phytoplasmas, affecting hundreds of plant genera (70). Many of the economically important diseases are those of woody plants, including coconut lethal yellowing, peach X-disease, grapevine yellows, and apple proliferation. Following their discovery, phytoplasmas have been difficult to detect due to their low concentration especially in woody hosts and their erratic distribution in the sieve tubes of the infected plants (15). First detection technique which indicated presence of some intercellular disorder was based on graft transmission of the pathogen to healthy indicator plants. The establishment of electron microscopy (EM) based techniques represents an alternative approach to the traditional indexing procedure for phytoplasmas. EM observation (17, 33) and less frequently scanning EM (59) were the only diagnostic techniques until staining with DNA-specific dyes such as DAPI (148) was developed. Lately, protocols for the production of enriched phytoplasma-specific antigens have been developed, thus introducing serological-based detection techniques for the study of these pathogens in plants or insect vectors (65).

Phytoplasma detection is now routinely done by different nucleic acid techniques based on polymerase chain reaction (PCR) (144, 12, 52, 165). The procedures developed in the last 20

years are now used routinely and are adequate for detecting phytoplasma infection in plant propagation material and identifying insect vectors, thus helping in preventing the spread of the diseases and their economical impact.

Therefore, aim of this chapter is to provide an overview of the PCR-based techniques for detection, identification and characterisation of this plant-pathogenic *Mollicutes* (cell wall-less prokaryotes).

1.1 Relevant features of phytoplasmas

Phytoplasmas, previosly known as 'Mycoplasma-like organisms' or MLOs, are wall-less bacteria obligate parasites of plant phloem tissue, and of several insect species (Fig. 1). Phytoplasma-type diseases of plants for long time were believed to be caused by viruses considering their infective spreading, symptomatology, and transmission by insects (84, 85, 86, 119, 90). Etiology of these pathogens was explored accidentally by group of Japanese sciences (45). They demonstrated that the causes agent of the yellows-type diseases are wall-less prokaryotes related to bacteria, pleomorphic incredibly resembling to mycoplasmas.

Phytoplasmas have diverged from gram-positive bacteria, and belong to the '*Candidatus* Phytoplasma' genus within the Class *Mollicutes* (73). Through evolution the genomes of phytoplasmas became greatly reduced in size and they also lack several biosynthetic pathways for the synthesis of compounds necessary for their survival, and they must obtain those substances from plants and insects in which they are parasites (11) thus they can't be cultured *in vitro* in cell-free media.
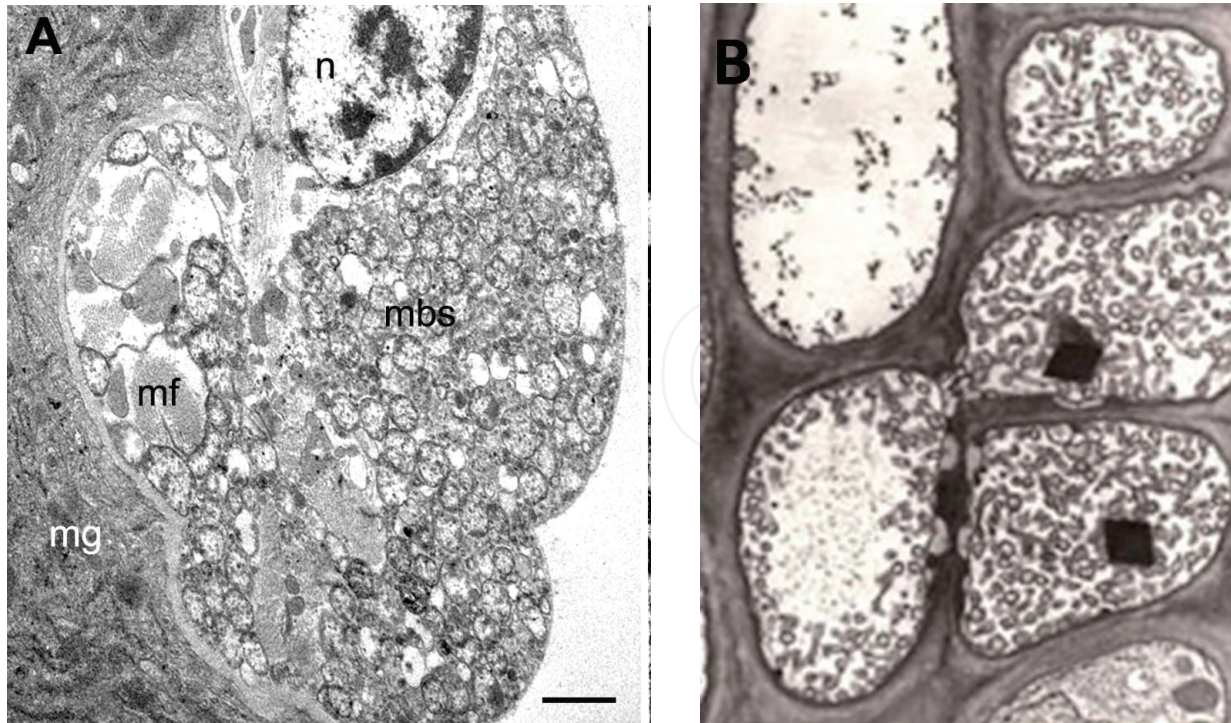

Fig. 1. Electron microscopy: of cross sections: A) of the vector leafhopper muscle cells around the midgut; B) sieve tubes of phytoplasmas infecting plants.
http://www.jic.ac.uk/staff/saskia-hogenhout/insect.htm

Not all plant species infected with phytoplasmas have disease symptoms, but infected plants normally show symptoms such as virescence, phyllody, yellowing, witches' broom, leaf rool and generalized decline (19). The most common symptoms of the infected plants are yellowing caused by the breakdown of chlorophyll and carotenoids, whose biosynthesis is also inhibited (21). Induced expression of sucrose synthase and alcohol dehydrogenase I genes in phytoplasma-infected grapevine plants grown in the field was also recently demonstrated (72).

Phytoplasmas are mainly spread by insects of the families *Cicadellidae* (leafhoppers), *Fulgoridae* (planthoppers), and *Psyllidae*, which feed on the phloem tissues of infected plants acquiring the phytoplasmas and transmitting them to the next plant they feed on (136, 2). They enter the insect's body through the stylet and then move through the intestine and been absorbed into the haemolymph. From here they proceeded to colonize the salivary glands, a process that can take up to some weeks (5, 80). Another pathway of phytoplasma survival and transmission is vegetative propagating plant material. As it mentioned phytoplasma invading phloem tissue and it is mostly find that in woody plants they disappear from aerial parts of trees during the winter and survive in the root system to re-colonize the stem and branches in spring (149, 150, 58).

1.2 Laboratory diagnostic of phytoplasmas

In time when phytoplasmas were discovered as plant pathogens diagnostic was difficult since detection was based on symptoms observation insect or dodder/graft transmission to host plant and electron microscopy of ultra-thin sections of the phloem tissue. Serological diagnostic techniques for the detection of phytoplasma began to emerge in the 1980's with ELISA based methods. However, serological methods weren't always sensitive enough to detect various phytoplasmas (13, 47). Finally, in the early 1990's PCR coupled with RFLP analysis allowed the accurate identification of different strains and species of phytoplasma (127, 91, 145). Nowadays, diagnosis of phytoplasmas is routinely done by PCR and can be divided into three phases: total DNA extraction from symptomatic tissue or insects; PCR amplification of phytoplasma-specific DNA; characterization of the amplified DNA by sequencing, RFLP analysis or nested PCR with group-specific primers (117).

For the DNA extraction of known phytoplasma, several protocols for isolation from infected plant material and insects have been developed. Control samples are drowning from plants commonly infected by phytoplasmas. Reference phytoplasma strain collections are maintained in experimentally infected periwinkle (*Catharanthus roseus*) which is available for research and classification purposes (18, 26).

In the second stage of the testing, DNA extracted from plants or insects is amplifying by using the polymerase chain reaction or PCR. PCR is a standardised technique in gene analysis to provide sufficient genetic material for detection (153). It works through the use of short lengths of DNA called primers that have a known sequence. Double stranded DNA is melting in a heating step exposing two single strands to which the primer can anneal. For the final stage, study of genetic variability is performing in order to differentiate between gene sequences from different phytoplasma.

In adition to sequencing, there are several strategies which allow study of genetic variability in PCR products: Restriction fragment length polymorphism (RFLP) (93, 162); Terminal

restriction fragment length polymorphism (T-RFLP) (66); Heteroduplex Mobility Assays (HMAs) (160); Single Strand Conformation Polymorphisms (SSCP) (126).

Alternative diagnostic methods have been established such as real-time PCR (12, 71, 161) and recently developed method for rapid detection of several phytoplasma species called loop-mediated isothermal amplification (LAMP) (155, 68).

2. Sampling procedure

Quality of DNA is of key importance in molecular diagnostics, since it can affect the final result. On other hand, for preparations of good quality and enriched in phytoplasma DNA, sampling material is of essential importance. Nevertheless, the quality of DNA depends on which plant tissue is examined.

2.1 Sampling of plants

It is generally more accurate sampling in the growing season, and although it can be used in the dormant season, this is not appropriate for the plant health inspections under the certification scheme. Due to the seasonal variation the optimal time for the diagnosis of phytoplasmas is from June to late autumn (30). Phytoplasmas could be detected using the polymerase chain reaction (PCR) from leaf midribs or phloem shaves from shoots, cordons, trunks and roots (117). Phytoplasmas were not always detected in samples from the same sampling area, from one sampling period to the next, firstly due to the uneven distribution, seasonal movement. Having this in mind, when collecting samples the best is to take leaves from different part of plant if it is possible symptomatic one, total amount should be around 20 g. If symptoms are absent phytoplasma detection by PCR can be improved by sampling from shoots, cordons and trunks, especially during October or early spring. In this case the best is to sample roots near to the plant bases though small feeding roots are the best tissue for extraction. Sampling of dry and rotted plant parts is not recommended since phytoplasmas are obligatory parasites. Palmano (2001) (134) demonstrated importance of proper identification of plant parts sampling; in this case the leaves have to show obvious symptoms but without being necrotic or completely yellow. In addition, variance in phytoplasma titters between infected plants of the same species has been observed by Berges et al. (2000) (15) and may be caused by different stages of development and age of plants.

It is recommended to record sampling area and plants by GPS device taking the coordinates and keep samples on cold (4 °C) till laboratory delivery.

2.2 Sampling of insects

Collection of the insect vectors for phytoplasma PCR analyses should be done in period where insects carry phytoplasma, furthermore knowledge about insects host plants and habitats are crucial things for successful collection.

Different traps and sampling techniques can be applied to collect and monitor phytoplasma vectors according to the objective of the study. The most common trapping techniques are sticky chromotropic traps, emergence traps, sweep net and vacuum insect collectors (107, 40). Collected insects should be place in ethanol and/or frozen.

3. Preparation of DNA templates

3.1 Samples preparation for homogenization

Prior to start extraction from collected plant samples, leaf midribs and/or phloem shaves are preparing for homogenization. Homogenization in liquid nitrogen with mortar and pestles is the most used method although some automatic homogenizers such as Fast Prep (MP Biomedicals, USA) (137) and Homex 6 (Bioreba, Switzerland) (52, 131) are available as faster alternative for the standard method.

3.2 DNA extraction

Accuracy of molecular analysis for pathogen detection in plant material requires efficient and reproducible methods to access nucleic acids. The preparation of samples is critical and target DNA should be made as available as possible for applying the different molecular techniques. However the suitability of most of the molecular methods depends closely on the amount of phytoplasma cells or nucleic acid in the extract. Approximately, 1% of phytoplasma DNA is extracted from tissue of total DNA (20). Since the concentration of this phloem-inhabiting pathogens is subjected to significant variations according to season (151), and is very low especially in woody hosts (79, 88), the importance of obtaining phytoplasma DNA at a concentration and purity high enough for precise analysis is aparent.

There are a great many published methods for preparing the plant tissues or other type of samples before molecular detection of phytoplasmas; however, they all pursue access the nucleic acid, avoiding the presence of inhibitory compounds that compromise the detection systems. Target sequences are usually purified or treated to remove DNA polymerase inhibitors, such as polysaccharides, phenolic compounds or humic substances from plants (121, 63, 164, 122).

Depending on the material to be analyzed the extraction methods can be quite simple or more complex. Generally there are three main approaches for obtaining of DNA template: protocols including a phytoplasma enrichment step, CTAB (cetyltrimethylammonium bromide) buffer-extraction protocols and DNA extraction using commercial kits.

Phytoplasma enrichment extraction protocols (1, 138, 108) including preparation of plant extract in the phytoplasma enrichment buffer (PGB), after one or two centrifugations the obtained pellet is dissolving in the CTAB buffer following chloroform and/or phenol extraction and precipitation in isopropanol.

Simple laboratory protocols based on preparation of plant extract in CTAB-buffer have also been published by several authors (35, 46, 6, 106, 165, 120, 152) with few steps and minimal handling, reducing the risk of cross contamination, cost and time, with similar results to those of longer and more expensive protocols.

CTAB based-protocols were also adopted for extraction of phytoplasmas DNA from hemipterian vectors (107, 46, 116, 50, 51).

The use of commercial kits, either general or specifically designed for plant material or for insect individuals, in some cases with magnetic separation has gained acceptance for extraction, given the ease of use and avoidance of toxic reagents during the purification process. Among those: DNeasy Plant kits, Qiagen (52, 42); Genomic DNA Purification kit,

Fermentas (143, 77); High Pure PCR Template Preparation kit, Roche (132); Wizard Genomic DNA Purification kit, Promega (104); NucleoSpin PlantII kit, Macherey-Nagel (135); FastDNA spin kit MP, Biomedicals (10); while InviMag Plant DNA Mini kit, Invitek; and QuickPick Plant DNA kit, Bio Nobile are optimized for extraction with a King Fisher mL Thermo Science workstation (137, 24, 99, 41).

Recently a new method (LFD) (37, 155) has been developed for rapid DNA extraction which processing DNA in loop-mediated isothermal amplification (LAMP) procedure for the detection of phytoplasmas from infected plant material. LFD method allows DNA extraction from leaf and wood material just in two minutes. Plant extract prepared in commercial buffer supplied with the LFD (Forsite Diagnostics Ltd) commercial kit is placing onto LFD membranes of lateral flow devices, and small sections of these membranes are then adding directly into the LAMP reaction mixture and incubating for 45 min at 65 °C. Moreover, Hodgetts et al. (2011) (68) obtained also satisfied results with LAMP using DNA prepared with an alkaline polyethylene glycol (PEG). This DNA extraction method (31) involves gently maceration of a small amount of plant tissue in the PEG buffer and then transfer of the macerate to the LAMP reaction.

Nevertheless, the choice of one or another system for nucleic acid extraction relies in practice on the phytoplasma to be detected and the nature of the sample, the experience of the personnel, the number of analyses to be performed per day, and the type of technique. As there are no universally validated nucleic-acid extraction protocols for all kinds of material and phytoplasma pathogens, those available should be compared before selecting one method for routine.

4. Nucleic acid amplification method

Detection and identification of phytoplasmas is necessary for accurate disease diagnosis. Sensitive methods need to be implemented in order to monitor the presence and spread of phytoplasma infections. Hence, it is necessary to devise a rapid, effective and efficient mechanism for detecting and identifying these microorganisms. Molecular diagnostic techniques for the detection of phytoplasma introduced during the last two decades have proven to be more accurate and reliable than biological criteria long used for phytoplasma identification (95). Polymerase Chain Reaction (PCR) is the most versatile tool for detecting phytoplasmas in their plant and insect hosts (153). One of the most utilized protocols for phytoplasma detection and characterization encompasses nested-PCR and RFLP analyses.

4.1 Nested PCR

Nested-PCR assay, designed to increase both sensitivity and specificity, is the leading method for the amplification of phytoplasmas from samples in which unusually low titer, or inhibitors are present that may interfere the PCR efficacy (56). The use of nested-PCR has been reported for diagnostic purposes particularly in plants when phytoplasmas occur in low titer in the phloem vessels of their host-plants and their concentration may be subjected to seasonal fluctuation (57, 75, 100, 117).

DNA consists of long sequences of paired bases called genes which code for a particular trait. Some of these gene sequences are consistent across bacteria but vary in their detailed

sequence. These differences can be compared and used as a diagnostic test for a particular phytoplasma. Phytoplasma diagnostics has been routinely based on phytoplasma-specific universal (generic) (Table 1) or phytoplasma group specific (Table 2) Polymerase Chain Reaction (PCR) primers designed on the basis of the highly conserved 16S ribosomal RNA (rRNA) gene sequences (1, 38, 44, 61, 77, 144, 153). Nevertheless, to detect phytoplasmas in DNA samples universal phytoplasma primers designed on sequences of the 16S-23S rRNA spacer region (SR) (153) are generally using.

Nested-PCR is performing by preliminary amplification using a universal primers pair followed by second amplification using a second universal primer pair. By using a universal primer pair followed by PCR using a group specific primer pair, nested-PCR is capable of detection of dual or multiple phytoplasmas present in the infected tissues in case of mixed infection (92). Until the reliability of universal primers detecting phytoplasmas is determined, it is advisable to use at least 2 different primer pairs to test a sample (eg P1/P7 (44) and R16F2/R16R2 (91); 6F/7R (146) and fU5/rU3 (102). Unfortunately, some of the primers can induce dimers or unspecific bands. They also have sequence homology in the 16S-spacer region to chloroplasts and plastids increasing the risk of false positives (64). Therefore, more specific universal phytoplasma primers are currently being developed (66, 112) and it may be that these will be more suitable for diagnostics from samples.

Primer set	Location	PCR product length	Reaction	References
P1 P7	16S/23SR	1800 bp	Direct PCR	(44) (153)
R16F2 R16R2	16S/IS	1245 bp	Nested PCR	(91)
R16F2n R6R2	16S/IS	1240 bp	Nested PCR	(55)
F1 B6	16S	1050 bp	semi-nested PCR	(38) (133)
6F 7R	16S/23	1700 bp	Direct PCR	(146)
fU3 fU5	16S	880 bp	Nested PCR	(102)
SecAfor 1 SecArev 3	secA gene	840 bp	Direct PCR	(67)
SecAfor 2 SecArev 3	sec A gene	480 bp	semi-nested PCR	(67)

Table 1. PCR universal primers commonly used for the detection of phytoplasma

Phytoplasma group-specific primers have also been designed on ribosomal protein gene, SecA, SecY genes (coding for the translocase protein) (28, 98), *vmp*1 gene (stolbur phytoplasma membrane protein) (28), *imp* gene (coding immunodominant membrane protein (112, 36), non-ribosomal gene *aceF* (115) and *tuf* gen (encoding the translation elongation factor Tu) (Table 2) (56, 67, 109, 147, 87,).

Primer set	Specificity	Location	Expected size of PCR product	References
fTufAy rTufAy	16SrI	tuf gene	940 bp	(147)
AysecYF1 AysecYR1	16SrI	secY gene	1400 bp	(98)
rp(I)F1A rp(I)R1A	16SrI	Ribosomal protein	1200 bp	(96)
rp(II)F1 rp(II)R1	16SrII	Ribosomal protein	1200 bp	(112)
rp(III)F1 rp(III)R1	16SrIII	Ribosomal protein	1200 bp	(112)
LY 16Sf LY16Sr	16SrIV	16S	1400 bp	(62)
LYC24F LYC24R	16SrIV	nonribosomal	1000 bp	(60)
rp(V)F1A rp(V)R1A	16SrV	Ribosomal protein	1200 bp	(97)
rp(VI)F2 rp(VI)R2	16SrVI	Ribosomal protein	1000 bp	(112)
rp(VIII)F2 rp(VIII)R2	16SrVII, 16SrVIII	Ribosomal protein	1000 bp	(112)
rp(IX)F2 rp(IX)R2	16SrIX	Ribosomal protein	800 bp	(112)
rpStolIF rpStolIR	16SrXII-A	Ribosomal protein	1372 bp	(112)
rpAP15f rp/AP15r	16SrX-A	Ribosomal protein	1000 bp	(114)
AP13/AP10 AP14/AP15	16SrX-A	nonribosomal	776 bp	(27)
f01 r01	16SrX	16S	1100 bp	(102)
AceFf1/AceFr1 AceFf2/AceFr2	16SrX	aceF	500 bp	(115)
FD9R FD9F	16SrV	secY	1300	(35)
FD9F3b FD9R2	16SrV	secY	1300 bp	(29) (6)
STOL11R1 STOL11F2	16SrXII	secY	990 bp	(35)
STOL11R2 STOL11F3	16SrXII	secY	720 bp	(29)
fStol rStol	16SrXII-A	16S/SR	570 bp	(106)
fAY rEY	16S	16SrV	300 bp	(1)

Table 2. Several group specific primers used for phytoplasma detection

The search for phytoplasma-specific primers has led to evaluation of primers based on these regions appears to offer more variation than that of the 16S gene. Nevertheless, design of primers based on various conserved sequences such as 16S rRNA gene, ribosomal protein gene operon, *tuf* and *SecY* genes was the major breakthrough in detection, identification, and classification of phytoplasmas (57, 147, 109, 161, 111, 112).

Primers previously designed for specific amplification of DNA from stolbur phytoplasma were recently found to prime amplification of DNA from other phytoplasmas (39, 77); therefore, it may be advisable to supplement use of phytoplasma-specific primers with RFLP analysis of amplified DNA sequences.

The choice of primer sets for phytoplasma diagnosis by nested PCR mostly depends on the phytoplasma we are looking for. Nested-PCR with a combination of different universal primers (Table 1) can improve the diagnosis of unknown phytoplasmas present with low titter in the symptomatic host. Universal ribosomal primers followed with nested with group-specific primers (Table 2) are extremely useful when the phytoplasma to be diagnosed belongs to a well-defined taxonomic group (117).

PCR products are usually visualised on 1% agarose gel prepared in 1xTAE buffer, stained with ethidium bromide (40).

The efficiency of nested-PCR has shown that it can reamplify the direct PCR product in dilution of 1: 60 000 (81). However, the system has not yet been devised to identify all the taxonomic groups, and this approach requires more than one PCR step, increasing the chances of contamination between samples, and does not provide the rapid and simple diagnostic tool required.

4.1.1 Restriction fragment length polymorphism (RFLP)

For identification of all detected phytoplasmas as well as for molecular characterisation of certain phytoplasmas strains Restriction Fragment Length Polymorphism, or RFLP is commonly used. RFLP is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated.

Phytoplasma amplified PCR products are cutting into fragments at specific sites using enzymes. More specific detection methods involve using phytoplasma-specific primers or differentiation on the basis of phylogenetic RFLP analysis of PCR amplified sequences (91, 145). RFLP analysis of PCR amplified DNA sequences using a number of endonuclease restriction enzymes (93). The pattern of cut DNA is viewing using 5% polyacrilamid gel (95) or 2,5% to 3% agarose gel electrophoresis. Analysis of a known genomic sequence can show what size of fragments to expect depending upon the enzymes chosen for the cuts e.g., providing that 6 or more frequently cutting restriction enzymes are used in the RFLP analysis, specific identification of the phytoplasma may be obtained.

Moreover this analysis is very useful for identification of new phytoplasmas, or phytoplasmas from a poorly studied region or crop. Because the RFLP patterns characteristics of each phytoplasmas are conserved, unknown phytoplasmas can be identified by comparing the patterns of the unknown with the available RFLP patterns for

known phytoplasmas without co-analyses of all reference representative phytoplasmas (94, 162, 163, 25). In this case it is preferable to use bigger number of enzymes to achieve identification (38). Enzymes found valuable for these analyses include AluI, BamHI, BfaI, DraI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, MseI, RsaI, Sau3AI, TaqI and ThaI.

Phytoplasma has not been cultured in cell-free medium, thus cannot be differentiated and classified by the traditional methods which are applied to culturable prokaryotes. The highly conserved 16S rRNA gene sequence has been widely used as the very useful primary molecular tool for preliminary classification of phytoplasmas. A total of 19 distinct groups, termed 16S rRNA groups (16Sr groups), based on actual RFLP analysis of PCR-amplified 16S rDNA sequences or 29 groups based on RFLP with new computer-simulated RFLP *in silico* analysis have been identified (93, 162).

4.1.2 Terminal restriction fragment length polymorphism (T-RFLP)

A protocol based on the Terminal Restriction Fragment Length Polymorphism (T-RLFP) analysis of 23S rDNA sequence using a DNA sequence analysis system has been developed to provide the simultaneous detection and taxonomic grouping of phytoplasmas (66). Terminal-restriction fragment length polymorphism (T-RFLP) analysis is a direct DNA-profiling method that usually targets rRNA (82). This genetic fingerprinting method uses a fluorescently labelled oligonucleotide primer for PCR amplification and the digestion of the PCR products with one or more restriction enzymes. This generates labelled terminal restriction fragments (TRFs) of various lengths depending on the DNA sequence of the bacteria present and the enzyme used to cut the sequence. The results of T-RFLP are obtaining through TRF separation by high-resolution gel electrophoresis on automated DNA sequencers. The laser scanning system of the DNA sequencer detects the labelled primer (141) and from this signal the sequencer can record corresponding fragment sizes and relative abundances. Resulting data is very easy to analyse, being presented as figures for statistical analysis and graphically for rapid visual interpretation.

The method was also designed to allow simple and easy testing of phytoplasmas and at the same time gave indication of their taxonomic group (9, 66). Comparing with the conventional nested-PCR/RFLP, method is less time-consuming and the approach is less expensive than sequencing.

4.1.3 Single Strand Conformation Polymorphisms (SSCP)

Single-strand conformation polymorphism (SSCP) analysis is a broadly used technique for detection of polymorphism in PCR-amplified fragments. SSCP was also assessed for the application in detection of the molecular variability phytoplasmas (125, 126). Amplified phytoplasma regions (16S rDNA, *tuf* gene, and *dnaB* gene), respectively are mixing with denaturing buffer after incubation, results of the SSCP are visualising on a non-denaturing polyacrylamide gel, optimized for each fragment length. SSCP revealed the presence of polymorphism undetected by routine RFLP analyses in all analyzed phytoplasma regions. Advantages of the SSCP in comparison with RFLP are sensitivity, time and cost consumption as well as suitability when large number of samples are screening for molecular variability.

4.1.4 Heteroduplex Mobility Assay (HMA)

Heteroduplex mobility assay (HMA) has been recently developed as fast and inexpensive method for determining relatedness between phytoplasmas DNA sequences. Initially, it was developed by Delwart et al. (1993) (43) to evaluate viral heterogeneity and for genetic typing of human immunodeficiency virus (HIV).

So far, HMA was used in studies for differentiation of phytoplasmas in the aster yellows group and clover proliferation group (159) determination of genetic variability among isolates of Australian grapevine phytoplasmas (32); study of the genetic diversity of 62 phytoplasma isolates from North America, Europe and Asia (160); for phylogenetic relationships among flavescence dorée strains and related phytoplasmas belonging to the elm yellows group (7); and to determine genomic diversity among African isolates of coconut lethal yellowing phytoplasmas causing Cape St. Paul wilt disease (CSPD, Ghana), lethal disease (LD, Tanzania), and lethal yellowing (LYM, Mozambique) (110).

Amplified PCR products from positive phytoplasma strains are combining with the amplified products of reference strain mixing with annealing buffer and submitting to HMA analyses (110, 160) following visualization of HMA products on polyacrylamide gel. Heteroduplexes migrate more slowly than a homoduplex in polyacrylamide gel electrophoresis. The extent of the retardation has been shown to be proportional to the degree of divergence between the two DNA sequences. It was noticed, that presence of an unpaired base influence the mobility of a heteroduplex more than a mismatched nucleotide (158, 157). Performing HMA, Marihno et al (2008) (110) succeeded to identified three groups of phytoplasmas associated with various coconut lethal yellowing diseases. Moreover, this grouping was consistent with the genetic diversity described in the coconut yellowing-associated phytoplasmas detected after cloning, sequencing, and phylogenetic analyses.

Further optimisations of this approach could facilitate phylogenetic study and diagnosis of many other phytoplasmas and development of a comprehensive PCR-based classification system. Considering simplicity and rapidness of the method, HMA could be used for initial screening among a large number of isolates and rapid identification of phytoplasmas as well as other organisms.

4.2 Immuno-capture PCR

Immuno-capture PCR assay, in which the phytoplasma of interest is first selectively captured by specific antibody adsorbed on microtiter plates, and then the phytoplasma DNA is released and amplified using specific or universal primers, can be an alternative method to increase detection sensitivity (139, 64). This method is aimed at avoiding the lengthy extraction procedures to prepare target DNAs. Nonetheless, this method is not suitable for detection of fruit tree and grapevine phytoplasmas.

4.3 Real-time PCR

Since the most universal as well as specific diagnostic protocols rely on nested PCR which, although extremely sensitive, is also time-consuming and posses risk in terms of carry-over

contamination between the two rounds of amplification, real-time PCR has recently replaced the traditional PCR in efforts to increase the speed and sensitivity of detection for mass screening.

The main principle of real-time PCR is based on fluorescent chemistries for labelling of the amplicons. During a real-time PCR run, accumulation of newly generated amplicons is monitored by each cycle by fluorescent detection methods, and so there is no need for post-PCR manipulation such as electrophoresis, which is required at the end of regular PCR. Moreover, the amount of fluorescent, monitored at each cycle is proportional to the log of concentration of the PCR target, and for this reason real-time PCR is also powerful technique for quantification of specific DNA. There are several labelling techniques, most of which specially bind to a target sequence on the amplicon, while others aspecifically stain double-stranded (ds) DNA amplicons. In addition, numbers of protocols have been developed for real-time PCR universal and specific detection phytoplasma.

For preliminary screening, 16S rDNA gene were adapted for the universal diagnosis of phytoplasmas using direct real-time PCR amplification (30, 48, 71) (Table 3) and all of them exploited a TaqMan probe for detection. TaqMan probes are labelled at the 5'end with reporter dye and at the 3' end with a quenching molecule; during each PCR cycle in the presence of the specific target DNA, the TaqMan probe, bound to its target sequence, which is then degraded by the 5'-3' exonuclease activity of the Taq polymerase as it extend the primer. The fluorescence moiety of the probe is therefore freed from its quencher-labelled portion and the fluorescence is detected by the optical system of the apparatus. The sensitivity of the 16S rDNA-based primer/probe system can be used to detect phytoplasmas belonging to several ribosomal subgroups and they showed sensitivity similar to that of conventional nested-PCR.

Group specific phytoplasma primers and probes for real-time PCR system have been designed to overcome problem with the time-consuming methods for phytoplasma strains identification and to further enhance the specificity of detection. Several laboratories have proposed rapid, specific and sensitive diagnostic protocols for detection of quarantine and economically important phytoplasmas of fruit trees and grapevine such as flavescance dorée (FD) and bois noir (BN) phytoplasmas infecting grapevine (22, 48, 8, 53, 71, 14); 'Ca. Phytoplasma mali' (apple proliferation, AP), 'Ca. Phytoplasma pyri' (pear decline, PD), 'Ca. Phytoplasma pruni' (European stone fruit yellows, ESFY) important pathogens of fruit trees (12, 76, 48, 156, 3,4, 113, 23, 128, 41). Most of the primer/probe systems are targeting 16S rDNA gene though some others genes or even randomly cloned DNA fragments to which no specific function is assigned have been used (Table 3). For fluorescent detection SYBR Green I has been applied for the diagnosis of AP, PD, ESFY and FD, all quarantine phytoplasmas of fruit trees and grapevine in Europe. Real-time PCR assays were also developed using TaqMan minor groove binding (MGB) probe to detect AP in plant material (12, 3) as well as for FD, BN and other phytoplasmas less frequently infecting grapevines (71, 128). MGB (minor groove binding) probe has an MGB ligand and non-fluorescent quencher conjugated to the 3' end, plus a fluorescent reporter dye at the 5' end. The MGB ligand allows the use of shorter and more specific probes by increasing the stability of the probe-target bond. This property allows the use of shorter probes, with higher specificity than conventional TaqMan ones and the discrimination of even single nucleotide

mismatched (83, 128). Furthermore, applying the same protocols, phytoplasmas DNA could be also detected in insect samples (113, 76, 48, 71) what is also decisive in the search for other potential vectors.

Specificity	Target gene	References
Universal	16S rDNA	(30)
Universal	16S rDNA	(48)
Universal	16S rDNA	(71)
FD	16S rDNA	(48)
FD	16S rDNA	(8)
FD	Sec Y	(71)
FD	16S rDNA	(22)
BN	Genomic fragment	(48)
BN	16S rDNA	(8)
BN	Genomic fragment	(71)
AP	Nitro reductase	(48)
AP	Genomic fragment	(76)
AP	16S rDNA	(12)
AP	16S rDNA	(4)
AP	16S rDNA	(23)
AP	16S–23S rRNA	(128)
PD	16S–23S rRNA	(128)
ESFY	16S–23S rRNA	(128)
ESFY	Ribosomal protein	(113)
'Ca. P. asteris'(onion yellows)	tuf	(161)
'Ca. P. asteris'(aster yellows)	16S rDNA	(8)
'Ca. P. asteris'(aster yellows)	16S rDNA	(69)
Beet leafhopper transmitted virescence virus	16S rDNA	(34)

Table 3. Oligonucleotide primers and probes used for phytoplasma detection by real-time PCR

A well-optimized reaction is essential for accurate results, which must be further analysed. As it is mentioned before, diagnosis of the pathogens in woody plants is often hampered by the presence of PCR inhibitors such as polyphenolics, polysaccharides and other molecules that may produce false negative results even from heavily infected samples. Additional problem may be also caused by amplification of other bacteria with universal phytoplasma primers/probe which could be present on the surface of some plants (49). Therefore, to avoid false positives specific probe can be included. So far, several sequence-specific detection tools are available: the chloroplast chaperonin 21 gene (8); cytochrome oxidase gene (71); the chloroplast gene for tRNA leucine (12); and the 18S rDNA gene (30, 118, 113,

128) addressed as targets to control the quality of total DNA extracted. SYBR Green I is one of the cheapest chemistry for real-time PCR detection, but the specificity of the reaction is extremely low, and needs to be checked. SYBR Green I dye chemistry will detect all double-stranded DNA, including non-specific reaction products. Therefore, amplification of non specific DNA may occur and analyses of melting curve is usually indispensible (48, 156).

One of the biggest advantages of real-time PCR is suitability of the method for quantification of nucleic acids of many plant pathogens, including phytoplasmas. In past competitive PCR was applied to monitor multiplication of 'Candidatus Phytoplasma asteris' in vector *Macrosteles quadrinlineatus* (101). Quantification was achieved following co-amplification of phytoplasma DNA and several dilutions of an appropriate internal standard. This approach was complex, several steps, such as electrophoresis, image analysis of gel, compensating for differences in intensity due to the different sizes of the product from the pathogen target and the internal standard, were required before the band intensities could be plotted for linear regression analysis. However, nowadays absolute quantification of phytoplasma DNA was achieved per gram of extracted tissue (161, 23) or per insect vector (76). Possibility of the method to quantify amount of phytoplasma DNA in plant tissue and insect vectors gave opportunity to better understand biology and epidemiology of the pathogens, to allow examination of different multiplication rates and to calculate the concentration in their plant and vector host (161, 142, 23) as well as to study interactions of different phytoplasma species or strains present in mixed infection (100, 19). These results will find application in development of resistant plant varieties, a hot topic for economically important woody crops such as palms, fruits and grapevines.

4.4 Loop-mediated isothermal amplification assay (LAMP)

Methods described above require relatively expensive equipment for amplification of the phytoplasma DNA and/or analysis of the results. In addition, standard methods for DNA extraction involve buffers, such as a CTAB buffer combined with phenol / chloroform extraction and isopropanol precipitation (46, 165), which are time-consuming and cannot be performed in the field. Whilst leaf tissue is usually used as the source of DNA for detection of many phytoplasmas, in other cases, such as coconuts, trunk borings or roots are often used, and DNA is then extracted from this woody tissue either by grinding in liquid nitrogen, or when this is unavailable, the sawdust is left in the CTAB extraction buffer for 48 h before the subsequent phenol chloroform extraction and alcohol precipitation (129). For that reason there is increase need for development of the method for a more rapid diagnostic assay for phytoplasmas that can be used to produce a diagnosis within an hour of sampling in the field or on site in case of imported material in quarantine stations.

Several attempts to produce field-based systems, e.g. using phytoplasma-specific antibodies and ELISA-based or lateral flow devices (LFD)-based systems, fall down because of a lack of sensitivity, and whilst a phytoplasma IgG antibody based system is commercially available for few phytoplasmas (103). Recently, Fera (Food and Environment Research Agency) developed isothermal amplification assays, such as the Loop-Mediated Isothermal Amplification (LAMP) procedure for detection of several human and plant pathogens including phytoplasmas (130, 140, 37, 154). In the method the cycling accumulates stem-loop

DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops, and produces up to 10^9 copies of the target in less than 1 h at 65°C. Further, LAMP products can be detected by conventional agarose gel electrophoresis; using spectrophotometric equipment to measure turbidity (124); in real-time using intercalating fluorescent dyes (105); or by visual inspection of turbidity or colour changes (123, 74).

For the routine diagnosis colorimetric assay that uses hydroxyl napthol blue to detect the magnesium pyrophosphate by-product in successful LAMP amplification (54) showed the best suitability. The hydroxyl napthol blue can be incorporated into the LAMP reaction and the colour change visualized immediately after amplification has been completed, and amplification can subsequently be confirmed by agarose gel electrophoresis when necessary.

Two methods for extraction of nucleic acid from plant material were adopted for LAMP application: LFD (37, 155) and an alkaline polyethylene glycol (PEG) DNA extraction method (31, 68).

Primers for the LAMP assays were designed as described in Tomlison et al. (2010) (155) and Bekele et al (2011) (16) based on the 16S-23S intergenic spacer region. In addition *cox* gene primers were used to confirm that all DNA extractions supported LAMP (16). Primers for LAMP assays were designed against range of ribosomal group (16SrI, 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrXI, 16SrXII, 16SrXXII) (68).

Developed protocol for LAMP-based diagnostic for a range of phytoplasmas can be conducted in the field and used to provide diagnosis within 1-hour of DNA extraction (68). According to the same author, PEG extraction method showed several advantages such is rapidness and requires less equipment than the LFD-based method, reducing the likelihood of sample contamination though the disadvantage of this method is that the DNA cannot be stored reliable long-term. Further efforts are doing to develop a hand held device capable of performing extraction, set-up and real-time detection for grapevine phytoplasmas. The device will make a single step homogeneous system from sampling to result, further reducing the risk of sample-to-sample contamination and enabling testing by non-specialists in the field (68).

5. Conclussions

In this review, molecular approaches for phytoplasma detection, identification and characterisation have been discussed. Before molecular techniques were developed, the diagnosis of phytoplasma diseases was difficult because they could not be cultured. Thus classical diagnostic techniques, such as observation of symptoms, were used. Ultrathin sections were also examined for the presence of phytoplasmas in the phloem tissue of suspected infected plants. Treating infected plants with antibiotics such as tetracycline to see if this cured the plant was another diagnostic technique employed. Diagnostic techniques such as ELISA test which allowed the specific detection of the phytoplasma began to emerge in the 1980s. In the early 1990s, PCR-based methods were developed that were far more sensitive than those that used ELISA, and RFLP analysis allowed the accurate identification of different strains and species of phytoplasma. Restriction fragments length polymorphism (RFLP) analysis together with the sequencing of 16Sr phytoplasma genes was the first step on this way enabling the construction of phylogenetic trees. Nowadays, polymerase chain

reaction with primers from sequencing of randomly cloned phytoplasma DNA, from 16S rRNA, from ribosomal protein gene sequences, from SecY and Tuf genes, and from membrane associated protein genes opened new paths for research on phytoplasma identification and classification.

Nested PCR has been applied to overcome problems related to sensitivity of phytoplasma detection, although this approach is more time consuming and subject to template. Unfortunately, nested-PCR also meets some difficulties: unspecific bands, false positives or negatives caused by DNA and contamination of single or nested PCR. Therefore, confirmation of PCR results by using different primer pairs combinations (generic and group-specific) with subsequent RFLP and/or sequencing of PCR amplicons seems to be the way for correct phytoplasma identification in the examined samples.

More recently, real-time PCR has replaced the traditional PCR in efforts to increase the speed and sensitivity of detection and improve techniques for mass screening as well as to bypass post-PCR manipulations. Moreover, the techniques as quantitative real-time PCR (QPCR) have been developed to allow assessment of the level of infection in plants and vectors.

T-RFLP, SSCP and HMA analyses provide simultaneous detection and group characterisation of phytoplasmas.

Isothermal amplification of nucleic acid has recently been described as an alternative to PCR and applied for specific detection of several phytoplasmas. This method has potential for testing in field or in under equipped laboratories.

Despite the developments of all protocols which overcome most of the difficulties of phytoplasma diagnosis, the detection of these pathogens is still quite laborious. Therefore, future work is needed to develop quicker procedures to extract phytoplasma-enriched nucleic acids, giving accent on automation which involving silica or magnetic beads. Furthermore, developments for phytoplasma detection should be stressed on improvements of methods which enable simultaneous detection and taxonomic grouping of phytoplasmas. Use of high-throughput, sensitive, rapid and quantitative techniques will help to understand how phytoplasmas exploit their unique ecological niches.

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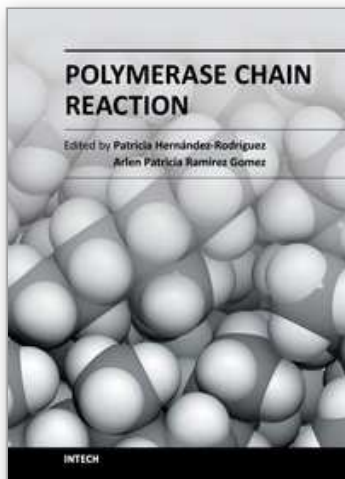
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This book is intended to present current concepts in molecular biology with the emphasis on the application to animal, plant and human pathology, in various aspects such as etiology, diagnosis, prognosis, treatment and prevention of diseases as well as the use of these methodologies in understanding the pathophysiology of various diseases that affect living beings.

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