[Frontiers in Bioscience 12, 673-689, January 1, 2007]

Phytoplasmas: diversity, taxonomy, and epidemiology

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1. ABSTRACT

Phytoplasma associated diseases are spread worldwide, and in several cases are associated with severe epidemic of very often quarantine importance. These plant pathogens are prokaryotes belonging to the *Mollicutes* class since they lack a cell wall; up to now they were not cultivated in axenic culture therefore Koch postulates are only sometimes fulfilled by using alternative tools, such as graft or insect transmission. The possibility to design specific primers for highly conserved genes such as 16S ribosomal gene together with the use of molecular probes randomly cloned from phytoplasma genome, allowed discriminating and molecularly classifying them. Now a certain amount of knowledge is available that allow starting epidemiological studies in order to prevent further spreading of phytoplasma-associated diseases. In this paper molecular, biological and epidemiological characteristics of phytoplasma associated with important diseases worldwide are described.

2. INTRODUCTION

The evidence that several plant diseases, believed to be caused by viruses, were associated with phloem colonization by prokaryotes morphologically resembling mycoplasmas was first shown in 1967 (1). Since then, several hundreds of plant syndromes have been reported to be associated with the so-called mycoplasma-like organisms. Due to the lack of *in vitro* growth, they were poorly characterized until the last ten years, when ribosomal rDNA sequencing provided evidence that these wall-less prokaryotes colonizing plant phloem and insects constitute a large monophyletic group within the class *Mollicutes*. The pathogen identification relied for more than 20 years on microscopic observations (DAPI staining) or electron microscopy detection; however in the last 15 years the applications of DNA-based technology allowed to preliminary distinguish different molecular clusters inside these prokaryotes. The Phytoplasma Working Team of the International Research Project for Comparative

Figure 1. Several plant species show malformations associated with phytoplasma presence: from left to right periwinkle, seed cabbage and hydrangea.

Mycoplasmology (IRPCM) adopted the trivial name 'phytoplasma' to identify the prokaryotes belonging to this group; the "*Candidatus* Phytoplasma" genus has been proposed and adopted in order to start formal classification of these prokaryotes, some of them associated with important or quarantine-subjected plant diseases (2). Up to date satisfaction of Koch postulates has not been achieved, but indirect proof, such as phytoplasma and symptoms eliminating after tetracycline treatments, confirmed that they are associated with many plant diseases worldwide; it was also demonstrated that genetically undistinguishable phytoplasmas can be associated with diseases inducing different symptoms and/or affecting different plant species, and also that different phytoplasmas can be associated with similar symptoms in the same or in different plant host(s).

Plants infected by phytoplasmas exhibit an array of symptoms that suggests profound disturbances in the normal balance of growth regulators (3, 4 and 5). Symptoms include virescence/phyllody (development of green leaf like structures instead of flowers) (Figure 1), sterility of flowers, proliferation of axillary buds resulting in a witches' broom behaviour, abnormal internodes elongation, generalized stunting.

3. PHYTOPLASMA DIVERSITY

Phytoplasmas are wall less prokaryotes with sizes variable from 200 to 800 nm, they are polymorphic (Figure 2), and could survive and multiply only in hysotonic habitats, such as plant phloem or insect emolymph; therefore they are strictly host-dependent, but they could multiply in insect vectors and also infect their eggs. The phytoplasma chromosome is very small (680-1,600 kb) and phylogenetic studies propose that the common ancestor for phytoplasmas is *Acholeplasma laidlawii* in which the triplet coding for tryptophan (trp) is UGG, while in the other prokaryotes, enclosing mycoplasmas and spiroplasmas, trp is coded by UGA.

Phytoplasmas are genetically distinguishable from mycoplasmas infecting human and animal for the presence of a spacer region (about 300 bp) between 16S and 23S ribosomal regions, which codes isoleucine tRNA ($tRNA^{Ile}$) and part of the sequences for alanine tRNA ($tRNA^{Ala}$). Sequencing of complete rRNA genes for two phytoplasma strains shows that tRNA coding for valine and asparagine are located downstream from the 5S rRNA gene, and this is a unique feature of phytoplasmas (6). First phytoplasma identification and classification systems proposed were based on specificity of vector transmission, on range of host plants and, more recently, on symptom expression of a common host (periwinkle). Experimentally determined plant host ranges and ranges of insect vector species are broader than those observed in nature, and show a considerable amount of overlaps. In case of symptom syndromes induced in infected plants similarities and differences in species of insect transmitting phytoplasmas and in species of plant hosts could reflect genetic differences in pathogens as well as the genetic of the plant and insect hosts (7).

Development of polyclonal antisera first, and of monoclonal antisera later, allows to start first differentiations among phytoplasma groups (8, 9, 10, 11, 12, 13, 14, 15). While polyclonal antisera have relatively low specific titres, and are not readily useful for discrimination among phytoplasmas, the monoclonal antisera greatly improved the reliability of immunoidentification techniques, such as ELISA, dot-blot immunoassays and immunofluorescence tests.

Because of the inability to isolate phytoplasmas in pure culture their identification was carried out only in the recent years by serological methods or by the use of specific cloned DNA probes. From these first studies it appeared possible that several phytoplasma groups could be clearly distinguishable for their chromosomal and extra chromosomal DNA sequences. In order to achieve a general and reliable system of phytoplasma detection and identification molecular tools such as PCR/RFLP (polymerase chain reaction/restriction fragment length polymorphism) and nested-PCR on the conserved (16SrDNA) ribosomal phytoplasma region were developed and applied. This detection approach provides rapid and reliable means for preliminary classification towards epidemiological studies on diseases associated with phytoplasma presence (16) (Table 1).

Table 1. Reference strains of phytoplasmas as classified using 16S ribosomal gene

Reference phytoplasma	Ribosomal subgroup	Geographic distribution
Aster yellows	**16SrI**	
Aster yellows	16SrI-A	North America
Aster yellows 'Ca P. asteris'	16SrI-B	Worldwide
Clover phyllody	16SrI-C	America, Europe
Pawlownia witches' broom	16SrI-D	Asia
Blueberry stunt	16SrI-E	North America
Aster yellows apricot strain	16SrI-F	Spain
Aster yellows strain AV976	16SrI-L	Germany
Aster yellows stain AVUT	16SrI-M	Germany
Ipomea oscura witches' broom	16SrI-N	Taiwan
Onion yellows	16SrI-O	USA
Decline of Croatian poplar	16SrI-P	Croazia
Peanut witches' broom	**16SrII**	
Peanut witches' broom	16SrII-A	Asia
Lime witches' broom 'Ca. P. aurantifolia'	16SrII-B	Arabic peninsula
Faba bean phyllody	16SrII-C	Africa, Asia
Papaya yellow crinkle	16SrII-D	Australia, Arabic peninsula
Pichris echoides yellows	16SrII-E	Italy
Cotton phyllody	16SrII-F	Africa
Peach X disease	**16SrIII**	
Peach X disease 'Ca. P. pruni'	16SrIII-A	North America
Clover yellow edge	16SrIII-B	America, Asia, Europe
Pecan bunch	16SrIII-C	USA
Solidago virgaurea yellows	16SrIII-D	USA
Spirea stunt	16SrIII-E	USA
Asclepias yellows	16SrIII-F	North America
Walnut witches' broom	16SrIII-G	USA
Poinsettia branching factor	16SrIII-H	Worldwide
Virginia grapevine yellows	16SrIII-I	USA
Sechium edule yellows	16SrIII-J	Brazil
Coconut letal yellowing	**16SrIV**	
Coconut lethal yellowing 'Ca. P. palmae'	16SrIV-A	Florida, Caribbean
Yucatan coconut lethal yellowing	16SrIV-B	Yucatan
Tanzanian coconut lethal yellowing	16SrIV-C	Africa
Carludovica palmata yellowing	16SrIV-D	Mexico, Texas
Walnut witches' broom 'Ca. P. castaneae'	16SrIV-E	Corea
Elm yellows	**16SrV**	
Elm yellows 'Ca. P. ulmi'	16SrV-A	North America, Europe
Jujube witches' broom 'Ca. P. ziziphi'	16SrV-B	Asia
Alder yellows	16SrV-C	Europe
Flavescence dorée 'Ca. P. vitis'	16SrV-D	Europe
Rubus stunt	16SrV-E	Europe
Clover proliferation	**16SrVI**	
Clover proliferation 'Ca. P. trifolii'	16SrVI-A	North America
Strawberry multiplier	16SrVI-B	Canada, Florida
Sudan periwinkle phyllody	16SrVI-C	Africa
Ash yellows	**16SrVII**	
Ash yellows 'Ca. P. fraxini'	16SrVII-A	America, Europe
Erigeron witches' broom	16SrVII-B	Brazil
Loofah witches' broom	**16SrVIII**	
Loofah witches' broom	16SrVIII-A	Taiwan
Pigeon pea witches' broom	**16SrIX**	
Pigeon pea witches' broom	16SrIX-A	North America
Almond witches' broom 'Ca. P. phoenicium'	16SrIX-B	Libanon
Ruscus decline	16SrIX-C	Italy
Apple proliferation	**16SrX**	
Apple proliferation 'Ca. P. mali'	16SrX-A	Europe
European stone fruit yellows 'Ca. P. prunorum'	16SrX-B	Europe
Pear decline 'Ca. P. pyri'	16SrX-C	Europe, North America
Spartium witches' broom 'Ca. P. spartii'	16SrX-D	Italy, Spain
Rice yellow dwarf	**16SrXI**	
Rice yellow dwarf 'Ca. P. oryzae'	16SrXI-A	Asia
Sugarcane white leaf	16SrXI-B	Asia
Leafhopper transmitted strain BVK	16SrXI-C	Germany
Stolbur	**16SrXII**	
Stolbur 'Ca. P. solani'	16SrXII-A	Europe, South America
Australian grapevine yellows 'Ca. P. australiense'	16SrXII-B	Australia
Mexican periwinkle virescence	**16SrXIII**	
Mexican periwinkle virescence	16SrXIII-A	Mexico
Strawberry green petals	16SrXIII-B	Florida
Bermudagrass white leaf	**16SrXIV**	
Bermudagrass white leaf 'Ca. P. cynodontis'	16SrIV-A	Asia, Italy
Hibiscus witches' broom	**16SrXV**	
Hibiscus witches' broom 'Ca. P. brasilense'	16SrXV-A	Brazil

The production of cloned phytoplasma DNA probes allowed starting some of the features that form a basis for phytoplasma classification. The use of cloned probes shows clear evidence that phytoplasma grouping was possible on the basis of their DNA sequences; total undigested or digested DNAs of strains from different host plants in dot or Southern hybridisation show clear evidence that phytoplasma grouping was possible on the basis of their DNA sequences.

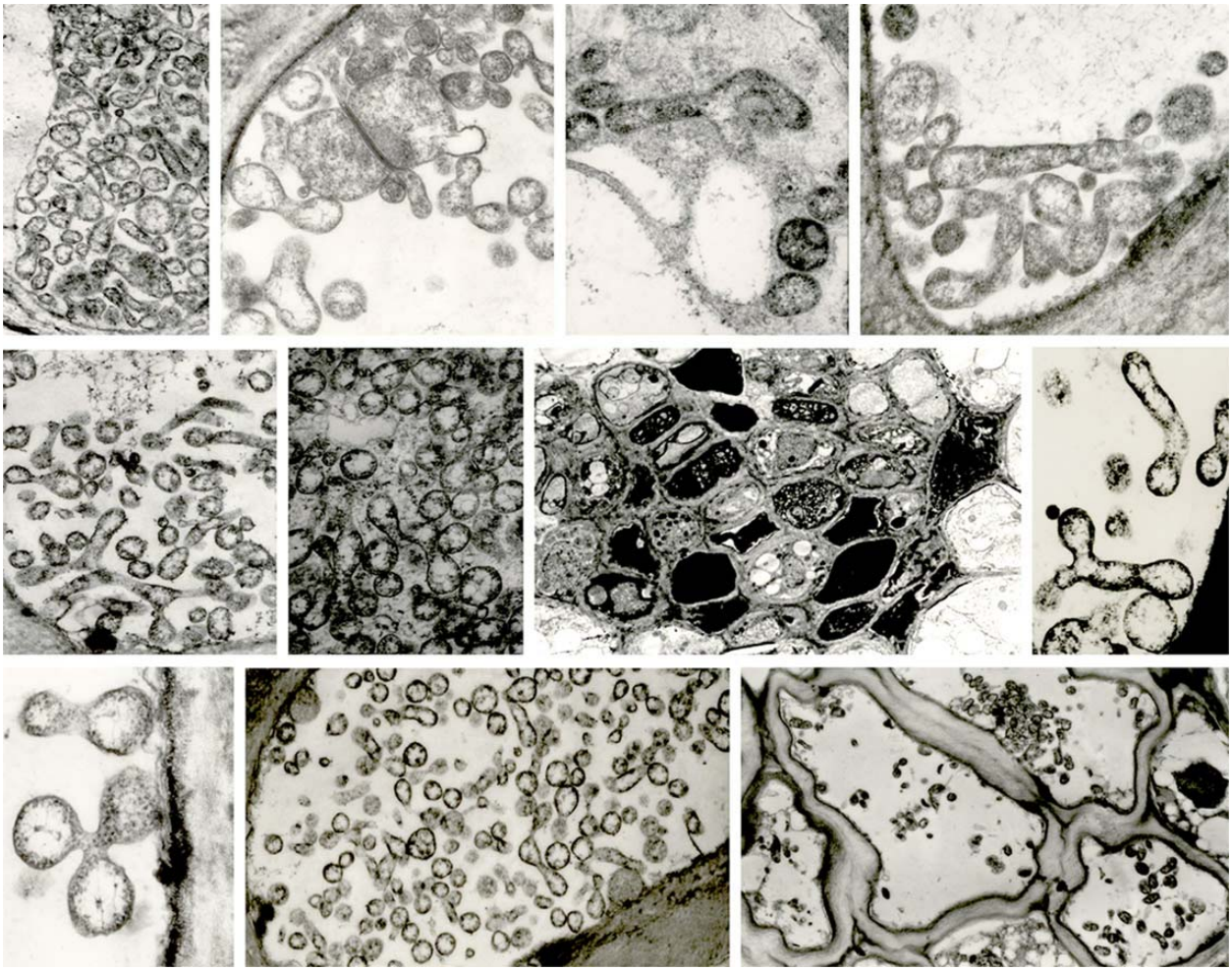

Figure 2. Electron micrographs at different magnifications of sieve tubes cross sections showing the polymorphism in shape and in dimensions of phytoplasmas infecting plants.

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. RFLP analysis together with the sequencing of 16Sr phytoplasma genes was the first step on this way (17, 18, 19, 20, 21, 22, 23, 24) enabling the construction of phylogenetic trees of many microorganisms especially in the *Mollicutes* taxon. Molecular characterisation of the entire phytoplasma genome including its sequencing recently performed (25) will provide, after its full annotation more precise basis for taxonomy, but it will be necessary to do it for several other phytoplasmas in order to achieve comparative genomic that could allow a deeper understanding about physiology of these organisms.

The presence of extra chromosomal DNA, similar to plasmid DNA, has been demonstrated in phytoplasmas by using DNA probes; these DNAs ("double stranded covalently closed circle") could be different in different phytoplasma strains, but their role is still unknown in the majority of the cases (26, 27, 28, 29, 30).

4. MOLECULAR CLASSIFICATION

The use of sensitive techniques such as PCR and nested-PCR appears to be very important to study these prokaryotes, however dot-hybridisation and RFLP analyses of total genomic DNA provided first evidences for phytoplasma differentiation. Sequence analyses of the 16SrDNA allow producing a detailed picture of phytoplasma diversity and of their phylogenetic relationships with other prokaryotes; numerous studies carried out on this gene in several phytoplasmas led to the conclusion that they are a unique monophyletic group of *Mollicutes* that could be indicated by the new name of phytoplasmas. This name emphasises the phylogenetic distance of these prokaryotes from some of the mycoplasmas infecting animals and humans (31). According to the recommendations of the International Committee of Systematic Bacteriology, subcommittee on the Taxonomy of Mollicutes a new *Candidatus* species may be described when a 16S rDNA sequence (longer than 1200 bp) has less than 97.5% identity with any previously described *Candidatus* species. Also two phytoplasmas sharing more than 97.5% of 16S sequence can be designed as separate *Candidatus* species when they meet the

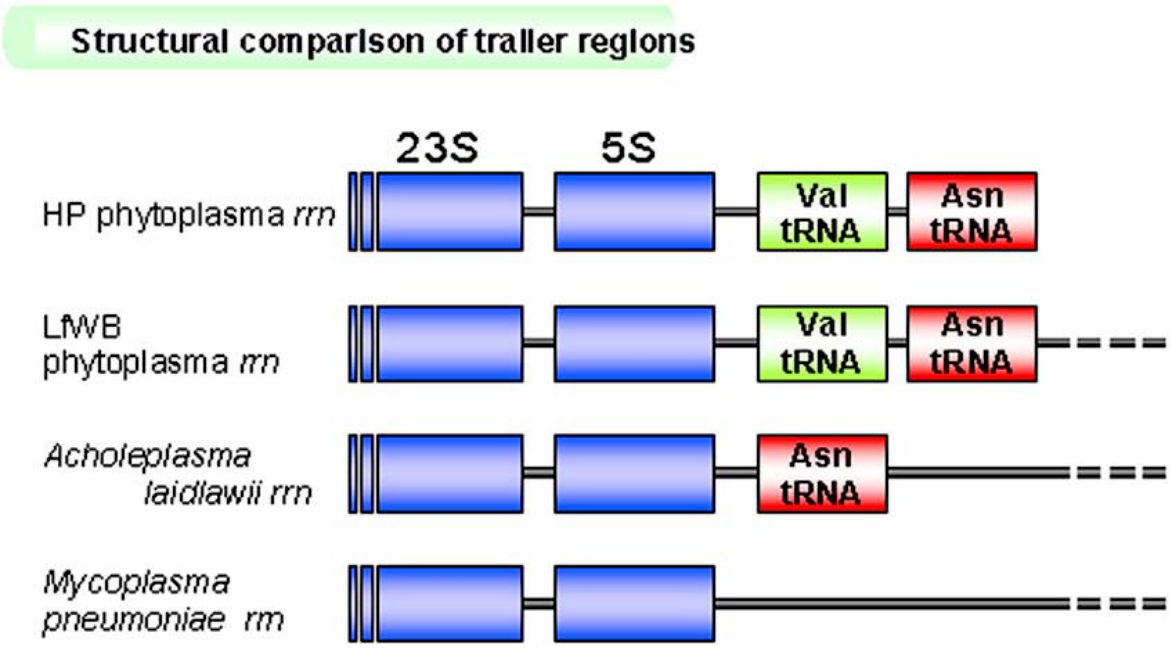

Figure 3. Schematic organization of 23S, 5S and downstream region compared among some prokaryotes (179).

following three criteria: i) they are transmitted by different vectors; ii) they have different natural plant host(s); and iii) there is evidence for molecular diversity between the two phytoplasmas (2). Up to date several phytoplasmas received a '*Candidatus* name' (Table 1) and tentative classification following 16S ribosomal grouping as parameter is now commonly employed for identification in order to study the phytoplasma-associated plant diseases. Beside those described in Table 1 (2, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45) other *Candidatus* were published with not clear reference to the ribosomal subgroups identified till now: '*Ca*. P. japonicum' infecting hydrangea in Japan, '*Ca*. P. ramni', '*Ca*. P. allocasuarinae' infecting those plant species in Germany and in Australia respectively, and '*Ca*. P. pini' infecting a few *Pinus* species in Europe (43, 46, 47).

More than one hundred distinct phytoplasma 16S rDNA genes were sequenced however additional conserved DNA sequences markers can be used as supplemental tools for finer phytoplasma differentiation; preliminary physical map of some phytoplasmas are also available. The rp gene sequences reveal more variation than 16S rDNA (48), the analyses conducted by RFLP or sequencing on *tuf* and/or *SecY* genes show clear indications of phytoplasma strains relationships at least with geographical distribution (6, 49, 50, 51, 52, 53, 54, 55, 56, 57, and 58). The use of the 23S rDNA gene was found to be not useful since it appears more or similarly conserved as the 16S. Studies performed comparing similar regions in two phytoplasmas and in phylogenetically closer relatives showed that different organization at this level clearly distinguish phytoplasmas from other phylogenetically related *Mollicutes* (Figure 3). Hybridization analyses indicated the presence of two sets of 16S rDNA operons, and heterogeneity of these two operons was suggested for some collection maintained phytoplasmas as well as from wild strains field collected (59, 60).

5. MOLECULAR IDENTIFICATION

DNA for phytoplasma detection and identification must be extracted from plant midribs or phloem in which phytoplasma titre is usually higher but always lower than 1% of DNA extracted. Several protocols have been described, but usually a chloroform/phenol extraction followed by isopropanol precipitation protocol provides useful results for the majority of plant species tissues. Shorter procedures can be adopted for routine testing of insects, but the DNA will be difficult to maintain for long-time.

It is advisable to control nucleic acid quality before performing PCR in order to avoid possible inhibitor presence that is quite common, especially when the extraction is performed from woody host plants, in certain periods of the year (Winter/Spring), or after spraying of plants with pesticides. Direct-PCR followed by nested-PCR assays with internal primer pairs designed on 16S ribosomal region of phytoplasmas allows detecting phytoplasma presence in field collected samples from herbaceous as well as from woody host plants, and from insect potential or vector of phytoplasma-associated diseases as well. PCR conditions are slightly different in agreement with primer pairs employed, but for the complete identification of detected phytoplasma, it is necessary to perform RFLP analysis of 16S rDNA amplicons. Using these tools finally researches on phytoplasma have become possible in many laboratories and to start validation of important knowledge about taxonomy and epidemiology (61, 62, 63, 64, 65, 66, 67, and68). Recently, application to phytoplasmas of

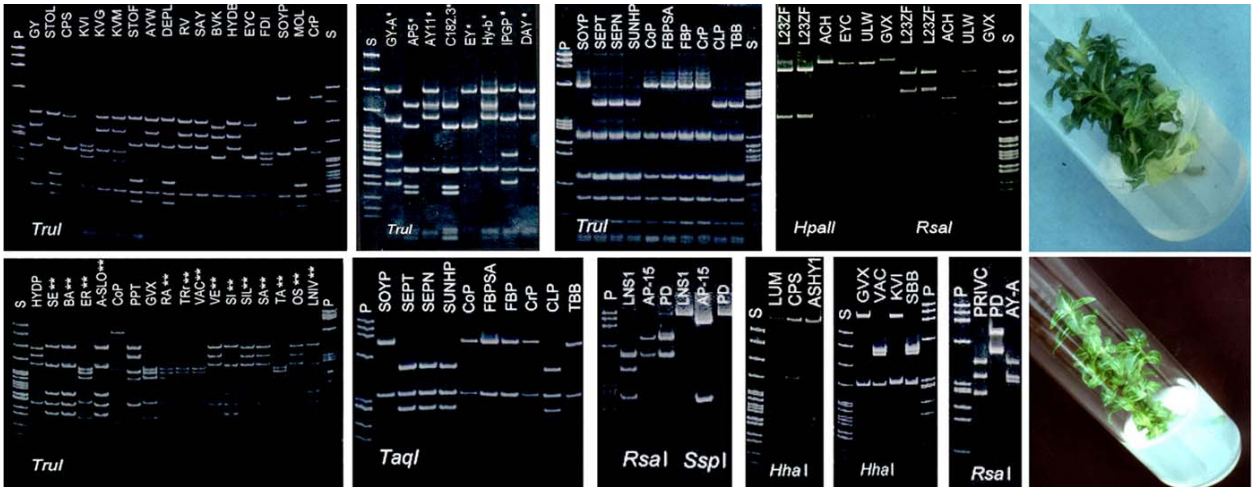

Figure 4. The phytoplasma strains maintained in collection (http://www.dista.agrsci.unibo.it/person/collectionseptember_2003.pdf) are classified by PCR/RFLP of 16Sr DNA gene.

Quantitative assays by real time PCR shows possibility to increase the number of samples that can be tested in order to reduce time and cost of routine analyses (69, 70, and 71). On the other hand the possibility of phytoplasma maintenance in micro propagated shoots (72, 73, 74, and 75) make it possible to organize and maintain a collection of phytoplasma strains in micro propagated periwinkle plants (http://www.dista.agrsci.unibo.it/person/collectionseptember_2003.pdf) (Figure 4) that can be provided upon request for general taxonomic identification purposes worldwide.

6. A CASE STUDY: MOLECULAR CHARACTERIZATION OF FLAVESCENCE DORÉE PHYTOPLASMAS

Flavescence dorée (FD) is a quarantine devastating disease of grapevine widespread in several countries in the European Union (76, 77, 78, 79 80, 81, 82, and 83); preliminary studies at genetic level indicated that at least two types of phytoplasma are involved in the epidemics belonging respectively to ribosomal subgroups 16SrV-C and 16SrV-D with defined geographical distributions. PCR/RFLP analyses on a segment of the conserved ribosomal protein operon, which has been shown to give finer differentiation of phytoplasmas in classification studies, was useful to distinguish among the FD phytoplasmas and to study the phylogenetic relationships within the FD types associated with the diverse epidemics. RFLP analysis of the PCR amplified ribosomal protein fragment, coding the 3' end of *rpl22* and the entire *rps3* genes, differentiated 4 rp-subgroups among the FD strains and 4 subgroups among the reference strains belonging to elm yellows group (16SrV) (Figure 5). A collaborative study conducted on the non-ribosomal DNA fragment FD9 coding for a phytoplasma *SecY* gene, showed that genetic variability among 17 Italian and 3 French FD strains was present after RFLP analyses. Sequencing and phylogenetic analysis of the same ribosomal protein DNA fragment validated the delineation of 4 distinct FD strain types derived by RFLP analyses. All the FD strains together with the reference strains ALY (alder yellows), RuS (rubus stunt), and JWB (jujube witches' broom) formed a cluster very well distinct from the Elm yellows (European and American strains) cluster. Moreover, ALY was shown to be more closely related to three FD strain types either from Italy or from France. All the strains belonging to elm yellows group formed a monophyletic group, paraphyletic to the reference strain OAY (aster yellows from *Oenothera*) that belongs to 16SrI-B subgroup. Within the elm yellows group 8 distinct lineages (subgroups) were identified. The subgroup delineation was generally in agreement with that deduced by nucleotide sequence analysis. Both phylogenetic analyses on the rp DNA sequences and on the deduced amino acid sequences supported the eight RFLP subgroups delineated between the FD and the reference strains within the Elm Yellows group. From the phylogenetic tree based on the nucleotide sequences, a probable evolutionary trend among the FD strains could be drawn. The FD-C type strains, present in a restricted area in northern Italy, are the most distantly related to the other FD strains and probably represent a strain prevailing in previous epidemics, as confirmed by a later report on FD-C epidemic in Serbia (84). Both the Italian and French strains are transmitted by the leafhopper *Scaphoideus titanus* Ball. (85), of which life-cycle is strictly connected with grapevine, resulting in an epidemic spreading of the disease; however, considering the genetic variability of FD-related phytoplasmas, alternative vectors to S. *titanus* as well as occasional vectors must be taken into consideration for disease control.

7. PHYTOPLASMA EPIDEMICS

The infection of plants by phytoplasmas is mainly performed by insect vectors belonging to a few species such as leafhoppers, plant hopper, cixiid, and psyllids. The main characteristics of insect vectoring such prokaryotes from infected to healthy plants is their phloem-feeding ability; sucking such plant liquid the insect can acquire the pathogens, that are then able to infect the

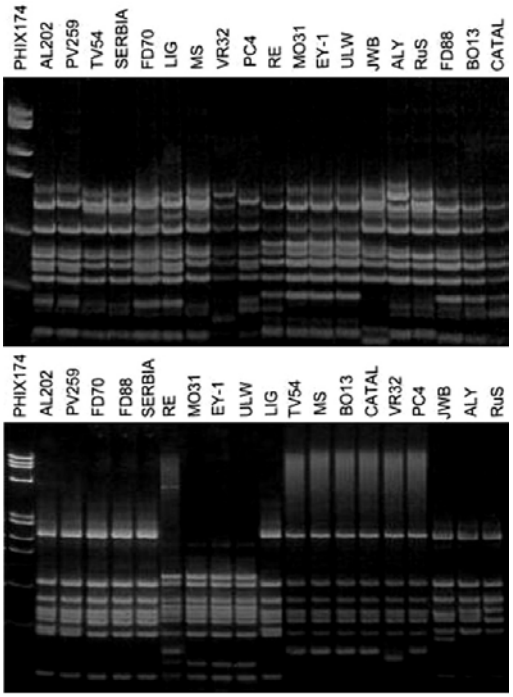

Figure 5. RFLP analyses of rpS3 gene of several strains of Flavescence dorée phytoplasmas compared with elm yellows strains detected in grapevine and with other phytoplasma strains belonging to ribosomal group 16SrV. On the left the enzyme employed is TaiI and on the right Tsp509I (180).

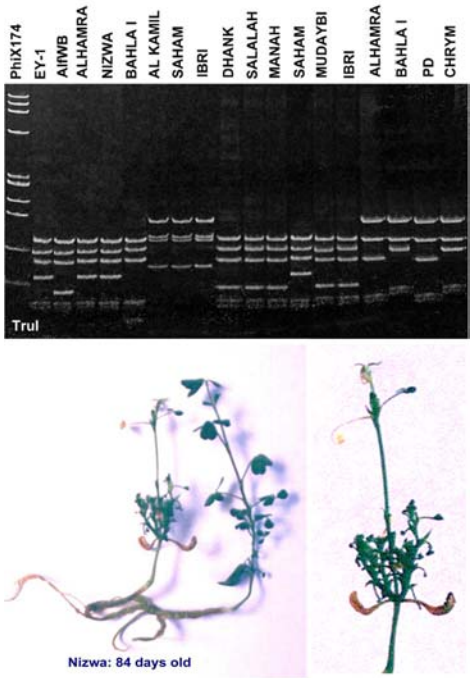

Figure 6. Phytoplasma transmission by seed was not yet confirmed some indications were found in alfalfa from Oman. From left to right: alfalfa plantlets with witches' broom symptoms, RFLP analyses of tested material (some profile not referable to phytoplasmas are also present), close up of witches' broom in a micropropagated growing alfalfa plantlet (181).

alimentary canal, emolymph (with or without multiply in it) as well and the salivary glands enabling insect to transmit pathogens to the healthy plants (86, 87, 88, 89, 90, 91, 92, 93, 94, and 95). There is usually a latent period in which the insect is infected, but it is not able to transmit the pathogen to healthy plants, this period can last from a few hours to a few weeks; for many combinations insect/phytoplasma is not known exactly. Insect vectors are not affected by phytoplasma presence in fact in some cases also transovarially transmission was demonstrated such as for the combinations *Scaphoideus titanus*/aster yellows (96); *Hishimonoides sellatiformis/*mulberry dwarf (97), and *Matsumuratettix hiroglyphicus* (Matsumura)/sugarcane white leaf (98). There are reports about increasing performances of phytoplasma-infected leafhoppers (99), and about performance and concentration of phytoplasma in relationships with environmental temperature (100).

Phytoplasmas are also transmitted by the majority of the dodder species, this transmission is usually important only for research studies since it allows to transfer phytoplasmas to useful experimental plant hosts such as periwinkle (*Catharanthus roseus* G. Don.) that is the host in which the majority of reference strains for *Candidatus* species should be maintained (2). Micro propagation together with other agricultural practices such as grafting, cutting, micropropagation or other ways to propagate plant germoplasm avoiding sexual reproduction are long time known ways of phytoplasma transmission.

Very recently the possibility of phytoplasma transmission by seed was also under investigation. After first suspect related to the epidemiological spreading to coconut lethal yellowing (101) other studies on Oman alfalfa (*Medicago sativa* L.) cultivations severely affected by phytoplasma infection inducing witches' broom and loss of yield were carried out. Fourteen commercial varieties of alfalfa seeds, collected in different regions in which phytoplasma-associated disease was present, were tested after germination in sterile condition in agar: during 50 days after sprouting, nucleic acids were weekly tested by nested-PCR from batches of about 10 plantlets. Tests performed on 2 and 3 weeks old material provided amplification from seedlings growing from several of the varieties tested, and different phytoplasma-related patterns were identified by RFLP analyses. Tests performed on alfalfa plantlets in older growth stages allowed detecting these phytoplasmas in a minor number of seed batches. In just one case an 84 days old plantlet in micro propagation showed witches' broom symptoms (Figure 6) and resulted phytoplasma infected.

Control of epidemic outbreak can be carried out theoretically either by controlling the vector or by eliminating the pathogen from the infected plants by antibiotics, mainly tetracycline for the lack of cell wall of phytoplasmas or by other chemicals (102, 103, 104, 105). Both protection measures resulted quite ineffective under field conditions: the first because it is impossible to eliminate all vectors from environments, and the second because the use of antibiotics is very expensive, not allowed in several countries, and not always effective for

Figure 7. Symptoms of phytoplasma associated diseases in woody plants. First row from left to right: grapevine affected by Flavescence dorée, cherry with quick decline, Japanese plum affected by leptonecrosis showing declining of scion and rootstock proliferation (last two pictures). Second row from left to right: apple fruits produced by plants with apple proliferation, tiny shoots subjected to fungal attack produced by apple with proliferation disease; lime showing witches' broom, and pear with red leaves in August that can indicate the presence of slow decline associate with phytoplasmas.

long-time. Therefore the only real way to control phytoplasma infection is to prevent the outbreaks by producing clean material or by finding phytoplasma resistant varieties or at least, tolerant but these latter can be employed only under restricted and defined environmental conditions (106, 107, 108, 109, 110, and 111). In order to gain informations in this fields research is still not very much developed even if some basic knowledge about epidemiology, and physiopathology of phytoplasma associated to diseases is available: the knowledge about some phytoplasma membrane-protein as well as about plant gene or plant products possibly involved in pathogenetic mechanisms can improve possibilities to better understand the way to eliminate these dangerous and mostly still unknown pathogens (112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, and 129).

8. ECONOMICALLY DANGEROUS PLANT DISEASES

Many of the cultivated plants are affected by phytoplasma infection not only in countries where agriculture is still not very well advanced, but also in the so called more advanced countries where these pathogen are severely damaging both herbaceous and woody plants. The major diseases known from longer time as associated with phytoplasmas are, in tropical areas, coconut lethal yellowing, sandal spike disease, pawlownia witches' broom, corn stunt, rice yellow dwarf diseases; they are among those more economically important. Forest trees are very often severely destroyed by phytoplasma epidemic in countries such as India and Central Africa, but also in US and in Europe. Elm yellows or witches' broom is a disease that almost eliminated historical as well as new elm plantation in Europe and in North America; in particular plant surviving the severe epidemic of Dutch elm disease were killed by successive phytoplasma infections (130, 131, 132, 133, 134, 135, 136, 137, 138, and 139). Among fruit plants grapevine, apple, pear, plum, apricot, cherry, citrus and the majority of small fruit are more or less severely affected by phytoplasma associated diseases described as yellows, decline, proliferation, and witches' broom (Figure 7). In some region of the world such as Western US or some Northern Italian regions from the fifties the pear cultivation were eliminated due to the presence of a pear decline killing plants in fast or slow times. Similar is the situation with the phytoplasma associated yellows diseases in many viticultural regions of Europe: Flavescence dorée (see above) is a quarantine pathogen seriously decreasing quality and yield in France, Catalonia (Spain), North Italy with epidemic spots also in Eastern Europe. In several regions of Middle East citrus

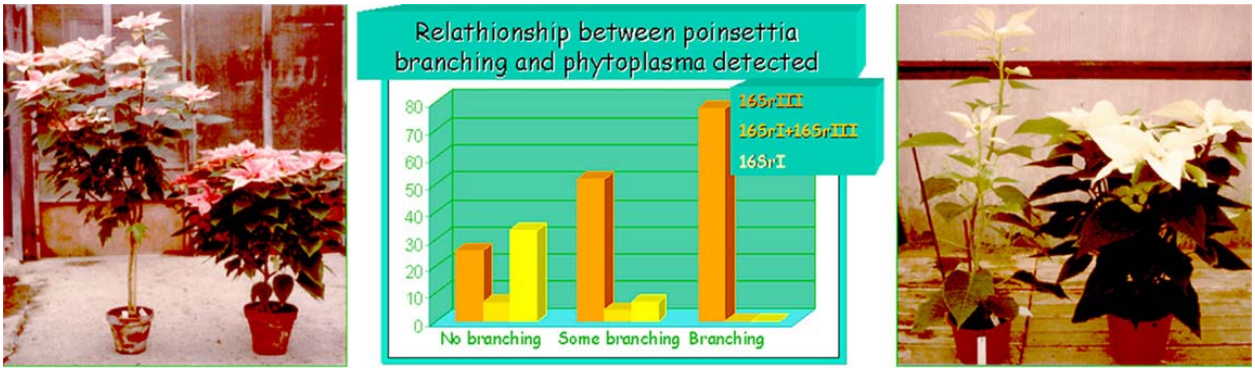

Figure 8. On the left of each figure poinsettia without phytoplasmas: it is clear the size and branching increase in the presence of phytoplasmas, relationships of branching with diverse phytoplasmas is shown in the graph in the middle.

species are affected by phytoplasma diseases such as lime witches' broom, that is almost eliminating traditional lime production in the Sultanate of Oman (140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, and 157). Worldwide there are severe epidemics also in herbaceous plants both cultivated and weeds that are often completely destroyed by epidemics (23, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171 172, 173, 174, and 175).

Phytoplasma associated with these diseases are molecularly distinguishable in most of the cases at the 16Sr DNA level (Table 1) therefore epidemiological studies can be carried out in order to eliminate infected plants and to prevent further epidemic spreading. The main limitation to the real application of these procedures that can be very successful in eliminating or reducing the impact of phytoplasmas diseases is that agricultural-related problems are not under consideration in many countries worldwide for opposite reasons (over production or not qualified production); people working in this field are not always aware of the risk connected with the trading or the maintenance in field of phytoplasma infected plants.

9. ECONOMICALLY USEFUL PHYTOPLASMAS

Poinsettia (*Euphorbia pulcherrima* Willd.; sin. *Poinsettia pulcherrima* Willd.) is a very important potted plant in which the role of phytoplasmas in branch inducing was demonstrated: to obtain marketable plants without phytoplasmas it is necessary to treat the plants with chemicals 6-7 times (176, 177, and 178). The phytoplasmas associated with poinsettia branching belong to 16SrIII-H group, but they have been detected several times in mixed infection with phytoplasmas of the 16SrI group (B and C subgroup) or 16SrXII-A subgroup, without these prokaryotes the plants present restricted branching (Figure 8). Results of nested PCR assays indicate that phytoplasmas show diverse ability to infect poinsettias: one year after grafting only 63% of plants were infected and the infection rate appears to be related with phytoplasma type grafted: plants grafted with 16SrI+16SrIII phytoplasmas show 62% of infection, while in plants grafted with 16SrIII phytoplasmas alone the percentage of transmission was about 24%. It is interesting to underline that the survival of grafting is not necessary to infect plants: 14% of infected plants show lack of survival of graft. In some of the plants that were doubly-infected a phytoplasma population fluctuation was observed: while one year after grafting two phytoplasmas could be detected, later on only the 16SrIII-H phytoplasmas were observed. After three years from grafting phytoplasma-infected plants showed several degrees of branching that correlated with the diverse phytoplasmas identified: 16SrIII-H was associated with normal branching in 78% of plants, while 16SrI phytoplasmas were present only in plants with restricted or no branching. Only one plant with mixed infection was detected and showed restricted branching. Phytoplasmas of 16SrIII-H group were also detected in 3 plants without branching, suggesting population variability for the branch-inducing characteristic among phytoplasmas in this group.

10. PERSPECTIVES

The phytoplasma-related researches are still in their infancy, several tasks could be fulfilled in order to acquire a clear knowledge of the situations for control of disease spreading. The sequencing of complete phytoplasma genome, after its full annotation, will provide more precise basis for taxonomy, but it will be necessary to do it for several other phytoplasmas in order to achieve comparative genomic analysis that could allow a deeper understanding about physiology of these organisms. Currently, it seems to be the only possible research for phytoplasma classification and identification since it is still difficult to fulfil the minimal requirement for a formal taxonomy, not only because they are not cultivable *in vitro*, but also because of their low titre in the infected plants. The dreams and the hopes of some researchers were devoted to phytoplasma cultivations in order to gain more consistent knowledge about these pathogens or in general about these prokaryotes. This should be the next step to be achieved by researchers that will help to better understanding not only plant-pathogen interactions but also safeness of using some phytoplasma for economically useful purposes similarly to what it is done in poinsettia.

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Key Words: Phytoplasmas, Plant Diseases, PCR/RFLP, Molecular identification, Review

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