

Review: Phytoplasmas and phytoplasma diseases: Part I: Characterisation and general properties

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Abstract

Phytoplasmas are phloem- restricted plant- pathogenic bacteria. They cause diseases in a wide range of host plants, resulting in significant economic and ecological losses worldwide. Phytoplasmas are transmitted mainly by insects and vegetative propagation material. Research on phytoplasmas has a long history, with significant progress being made in the past 30 years. Divided into three sections, a series of articles is aimed to provide an overview of the current state of scientific knowledge. The first part, presented here, summarizes discovery and taxonomy of phytoplasmas and includes recent advances in knowledge of phytoplasma genomes, phytoplasma host interaction and transmission, specifically transmission by insect vectors.

Keywords: Plant pathogenic bacteria, taxonomy, phytoplasma-host interaction, insect transmission

Zusammenfassung

Bericht: Phytoplasmen und Phytoplasmenkrankheiten: Teil I: Charakterisierung und allgemeine Merkmale. Phytoplasmen sind pflanzenpathogene Bakterien, die das Phloem ihrer Wirtspflanzen besiedeln. Phytoplasmen verursachen Krankheiten an einer Vielzahl von Wirtspflanzen und führen weltweit zu erheblichen wirtschaftlichen und ökologischen Verlusten. Die Übertragung der Erreger erfolgt hauptsächlich durch Insekten sowie vegetatives Vermehrungsmaterial. Die Forschung zu Phytoplasmen hat eine lange Geschichte und besonders in den vergangenen 30 Jahren wurden bedeutende Fortschritte erzielt. Eine aus drei Teilen bestehende Artikelserie soll einen Überblick über den aktuellen Stand der Phytoplasmenforschung geben. Der hier vorgestellte erste Teil fasst die Entdeckung und Taxonomie der Phytoplasmen zusammen und behandelt zudem aktuelle Erkenntnisse zu Phytoplasma-Genomen, den Wechselwirkungen zwischen Phytoplasmen und ihren Wirten sowie zur Übertragung von Phytoplasmen, insbesondere durch Insektenvektoren.

Schlagwörter: Pflanzenpathogene Bakterien, Taxonomie, Phytoplasma-Wirt-Interaktion, Insektenübertragung

Discovery and classification of phytoplasmas

Phytoplasmas are plant pathogenic bacteria lacking a cell wall and affecting numerous plant species worldwide (Wei and Zhao, 2022). The first records of symptoms clearly attributable to phytoplasmas date back about 1000 years, when tree peonias (*Paeonia suffruticosa*) with floral virescence, a typical phytoplasma symptom, were annually presented to the imperial court in China (Wang and Maramorosch, 1988; Maramorosch, 2011). A first scientific record of a phytoplasma epidemic was generated 200 years ago. At that time, a disease called mulberry dwarf disease threatened the production of mulberry leaves, thereby affecting the silk industry (Ishii 1969, reported in Namba, 2019). In 1967, in electron microscopy studies, Doi et al., (reported in Namba, 2019) observed pleomorphic bodies in the sieve tubes of infected mulberry trees, in yellowing petunias and in potatoes and paulownias with witches-broom symptoms. The organisms were 80–800 nm in diameter and lacked a firm cell wall. Due to their similarity with vertebrate mycoplasmas, the organisms were named mycoplasma-like organisms (MLOs) (Iida 1972, reported by Hogenhout et al., 2008).

The advancement of molecular techniques led to tremendous progress in phytoplasma taxonomy. Comparative analysis of 16S rRNA genes indicated that mycoplasmas, spiroplasmas, and achleoplasmas had evolved from ancestors of Gram-positive bacteria (Woese, 1980). In 1989, Lim and Sears compared the sequence of a fragment spanning the 16S rRNA gene and the spacer between the 16S rRNA and 23S rRNA genes of an MLO (*Oenothera* virescence phytoplasma, aster yellows group) with the sequences of a spiroplasma, several mycoplasmas and an achleoplasma. The authors suggested an assignment of MLOs to the class Mollicutes. The sequence comparisons pointed to a relation of MLOs to mycoplasmas, but differences in the spacer between the 16S rRNA and 23S rRNA genes indicated an early divergent

evolution. The 16S rRNA sequence of the MLO was most similar to the achleoplasma. At the same time, MLOs were already known as causal agents of various diseases worldwide (McCoy, 1979, McCoy et al., 1989). In 1993, the trivial name phytoplasmas for uncultivable phytopathogenic mycoplasma-like organisms from the class Mollicutes was proposed. Phytoplasmas were defined as pleomorphic prokaryotes without cell wall, living in plant sieve tubes, associated with characteristic plant disorders (yellowing, abnormalities in development), and transmitted by phloem feeding leafhoppers, planthoppers and psyllids. In addition, a resistance to penicillin but a sensitivity to tetracycline, a genome size of 530-1350 bp, a low DNA G + C ratio (from 20 to 30 %) and a phylogenetic relation to other members of the class Mollicutes were considered as characteristics of phytoplasmas (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes, 1993; IRPCM (International Research Programme on Comparative Mycoplasmaology), 2004; Wang et al., 2024, for review).

Up to now, the efforts towards axenic cultivation of phytoplasmas were largely unsuccessful (Wei and Zhao, 2022). The only exception is work by Contaldo and co-workers reporting a procedure for the cultivation of phytoplasmas (e.g., aster yellows, flavescence dorée and stolbur phytoplasmas from grapevines; Contaldo et al., 2016). The failure of cultivation on artificial media greatly complicated research on phytoplasma identification and taxonomy, but the continuous progress of nucleic acid technologies has tremendously contributed to the knowledge in this field. Hybridization studies (Kirkpatrick et al., 1987) allowed the detection of the Western X-disease pathogen in infected plants. Moreover, a PCR protocol using primer pairs on basis of 16S rRNA genes of phytoplasmas was developed (Deng and Hiruki, 1991). Restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA genes enabled the

classification of the pathogens into distinct 16S rRNA groups and subgroups according to RFLP similarity coefficients (Lee et al., 1993; Gundersen et al., 1994; Namba et al., 1993; Schneider et al., 1993; Seemüller et al., 1994; Lee et al., 1998). Subsequently, sequencing and phylogenetic analysis of the 16S rRNA gene verified the presumption that phytoplasmas form a distinct, monophyletic clade within the class Mollicutes (Hogenhout et al., 2008, for review). Over time, the 16S rRNA gene RFLP-based classification system was significantly expanded through the use of computer-simulated virtual RFLP analyses. Zhao et al., (2009) developed the online tool iPhyClassifier, which utilizes RFLP pattern similarity coefficients to assign phytoplasmas to established groups and subgroups. Since its introduction, iPhyClassifier has become a widely adopted tool for phytoplasma taxonomy (Wang et al., 2024; USDA 2025).

In 2004, the IRPCM Phytoplasma/Spiroplasma Working Team described a new taxon to allocate phytoplasmas. (IRPCM, 2004). Due to the lack of axenic cultivation, the genus was designated as '*Candidatus* Phytoplasma'. The description of a novel '*Ca. Phytoplasma*' species required less than 97.5 % similarity of 16S rRNA gene sequences longer than 1200 bp to other phytoplasmas. In some exceptional cases, relevant differences in vector species or host plants and a substantial molecular diversity were also used to distinguish between species. Recently, revised criteria for the assignment of '*Ca. Phytoplasma*' species have been proposed. Under these guidelines, the designation of a novel '*Ca. Phytoplasma*' species requires a 16S rRNA gene sequence exceeding 1500 bp and showing less than 98.65% sequence identity to previously described species. For strains sharing higher levels of 16S rRNA sequence similarity, species delineation can be refined using whole-genome average nucleotide identity (ANI), an approach enabled by recent advances in genome sequencing technologies, with a commonly accepted threshold of 95% ANI. Multilocus sequence analysis based on alternative house-keeping genes may also be employed to support species definition (Bertaccini et al., 2022). Other

authors have described new phytoplasma species based on the overall genome relatedness (OGRI) values, including digital DNA-DNA hybridization, G+C content, and ANI (Kirdat et al., 2023, for review). To date, around 50 '*Ca. Phytoplasma*' species have been officially published (Bertaccini et al., 2022).

Currently, phytoplasmas are attributed to the kingdom Bacteria, phylum Mycoplasmatota (originally named Tenericutes), class Mollicutes. As putative taxa defined on the basis of DNA sequences, the '*Candidatus*' status is assigned to the rank of genus and species (Gasparich et al., 2020; Wei and Zhao, 2022).

Properties of phytoplasmas

Genomic aspects

The decoding of the entire genome sequence of onion yellows (OY) phytoplasma ('*Ca. P. asteris*', 16Srl) genotypes in 2004 (Oshima et al., 2004), followed by the sequencing of several other phytoplasma genomes in the years thereafter, demonstrated that phytoplasmas possess one chromosome. The size of '*Ca. Phytoplasma*' chromosomes was described as ranging from 498–959 kb (Wang et al., 2024, for review).

Depending on the chromosome size of the phytoplasma species, between 413 and 876 protein encoding genes have been reported (Kube et al., 2012, Wang et al., 2024 for review). Phytoplasmas possess genes for basic functions, as for DNA replication, transcription, translation, and protein translocation. However, their genomes have lost several genes for metabolic functions, presumably because living in nutrient rich host environments has resulted in reductive evolution. Phytoplasmas lack specific genes involved in the tricarbalic acid cycle, the pentose phosphate pathway, the electron transport/oxidative phosphorylation, the phosphotransferase transport system, the biosynthesis of sterols, fatty acids and most amino acids and in de novo nucleotide synthesis. Furthermore,

no adenosine triphosphate (ATP) synthase gene was identified, indicating that in phytoplasmas ATP synthesis might rely on the glycolytic and malate pathways. However, phytoplasma genomes retained genes encoding transport systems for the uptake of sugars, amino acids, oligopeptides, and inorganic ions from their hosts (Kube et al., 2012, Namba, 2019, for review). Overall, phytoplasmas strongly depend on the metabolism of their host. In addition, selection pressure might have reduced factors inducing host defence such as pathogen associated molecular patterns (PAMPs) (Hogenhout et al., 2008).

Generally, a large part of a phytoplasma genome consists of repeated gene sequences resembling composite transposons, named potential mobile units (PMUs) (Hogenhout et al., 2008; Namba, 2019, for review). Studies indicate that the PMUs encode various effector genes (Sugio and Hogenhout, 2012), proteins, secreted by pathogens, playing a crucial role in host-parasite interaction and virulence (Langin et al., 2020).

In addition to the chromosomal genes, the genomes of most phytoplasmas comprise several plasmids. They, for example, carry genes involved in insect transmission (Namba, 2019, for review).

Phytoplasma host interactions

Phytoplasmas, together with members of the genus *Spiroplasma* have a unique trans-kingdom way of life. They infect and colonize both plants (Kingdom Plantae) and insects (Kingdom Animalia) (Hogenhout et al., 2008, for review). Within the plant, phytoplasmas predominantly live in the phloem. The highest densities of phytoplasmas occur in mature sieve tubes. In addition, phytoplasmas are observed in the cytoplasm of parenchyma cells close to sieve elements (Christensen et al., 2004; Hogenhout et al., 2008, for review). Phytoplasmas easily move within the plant, they smoothly pass the sieve pores and are transported in the phloem sap according to its circadian and seasonal flow (reviewed in Kube et al., 2012 and Namba, 2019). In insect bodies, phytoplasmas multiply in various tissues, both intra- and extracellularly (Hogenhout et al., 2008, for review). Experiments imply that phytoplasmas switching between plant and insect hosts easily respond to the altered environment by differential regulation of gene expression (Makarova et al., 2015).

In plants, phytoplasma infections induce a wide range of specific symptoms, such as excessive shoot proliferation leading to the formation of witches broom, stunting, reduced leaf size, yellowing, reddening of entire plants or upper parts (purple top), phyllody (production of leaf like structures instead of flowers) and virescence (greening of flowers), Fig. 1. Affected plants may decline and show a more or less severe necrosis of the phloem (Namba, 2019, for review). Often, visible disease symptoms alone are not sufficient to identify the involved phytoplasma species. An infection with an identical '*Ca. Phytoplasma*' species may induce highly diverse symptoms in different plant species. On the other hand, different '*Ca. Phytoplasma*' species can induce similar disease symptoms in one host plant species (Bertaccini et al., 2014).



Fig. 1: Typical symptoms of phytoplasma infections in plants: A: Phyllody (production of leaf like structures instead of flowers) and virescence (greening of flowers) in *Catharanthus roseus* (frequently used species for phytoplasma experiments); B: Witches broom, stunting and reduced leaf size of a *Convolvulus arvensis* plant, infected with '*Candidatus* Phytoplasma solani'; C: Yellowing and leaf rolling of a grapevine, infected with '*Candidatus* Phytoplasma solani'; D: Reddening of pear trees infected with '*Candidatus* Phytoplasma pyri'.

To date, the molecular mechanisms of symptom development are not fully understood, however, several studies have proven that phytoplasmas secrete effector proteins for manipulation of host plants (Oshima et al., 2023a, for review). Prokaryotes possess highly specialized systems allowing them to translocate proteins from the cytoplasm to other parts of their cell, the environment or even into other (bacterial or eukaryotic) cells. These machineries, named secretion systems, are large macromolecular complexes embedded in the cell membranes. Several different secretion systems, varying in structure and function have

been identified in prokaryotes. One protein trafficking machinery, highly conserved among classes of bacteria is the Sec secretion pathway. This system enables secretion of proteins that remain in the inner membrane, but also translocation of secreted proteins across the cytoplasmic membrane. The Sec pathway is well-documented in Gram-negative bacteria such as *Escherichia coli*. In *E. coli*, the system comprises a membrane-embedded conducting channel, the so-called SecYEG translocase. Three core proteins, namely SecY, SecE and SecG form the SecYEG translocon. Proteins translocated across the membrane through

the Sec pathway comprise a removable signal sequence recognized by the SecB chaperone. SecB delivers the protein to the ATPase SecA, which provides energy and guides the protein across the SecYEG channel (Tseng et al., 2009; Green and Meccas, 2016). The SecYEG translocon also cooperates with other protein trafficking systems, such as YidC insertase. YidC is a membrane component involved in the insertion of newly synthesized membrane proteins (Oswald et al., 2021). Genes encoding the core proteins of the SecYEG transport channel as well as genes encoding SecA and YidC have been identified in the genomes of several phytoplasma species (Kube et al., 2012, for review), suggesting that phytoplasmas possess a functional SecYEG and YidC system. It is anticipated, that phytoplasmas secrete effector proteins via the Sec pathway (Sugio et al., 2011). A recent study has shown that vines infected with strains of ‘*Ca. P. solani*’ strains containing a secY gene sequence variant (secY52) show mild phytoplasma symptoms only. This suggests that SecY protein structure might influence strain virulence (Pierro et al., 2022).

To date, between 13 and 87 secreted effector proteins have been predicted for different phytoplasma species. The effector proteins characterised to date modulate plant growth and developmental processes and/or interactions between plants and insect vectors. (Wang et al., 2024, for review). Around the year 2000, mutants of OY-phytoplasma leading to milder symptoms than the wild type (OY-W, ‘*Ca. P. asteris*’ 16Srl) were described, namely OY-M (mildly pathogenic mutant of OY-W) and OY-Nim (a non insect transmissible line of OY-M) (Oshima et al., 2001). The determination of the complete genomic sequence of the three OY types for the first time allowed the identification of putative phytoplasma effector genes (Oshima et al., 2004; Bai et al., 2009; Hoshi et al., 2009; Namba, 2019, for review). Due to the lack of axenic phytoplasma cultivation, transgenic plants expressing the effector proteins were generated to relate candidate effectors to distinct plant symptoms. The first factor identified to affect plant morphology was TENGU. Expression of

TENGU in *Nicotiana benthamiana* or *Arabidopsis thaliana* caused stunted bushy plants and induced sterility of flowers. TENGU transformation resulted in downregulation of genes involved in auxin biosynthesis and interfered with the auxin response transcription factors ARF6 and ARF8. The latter factors are involved in the production of jasmonic acid (JA) and the development of mature fertile flowers (Nagpal et al., 2005; Hoshi et al., 2009; Sugio et al., 2011; Oshima et al., 2023ab). Another effector, SAP 11, was identified from aster yellows witches broom phytoplasma (AY-WB, ‘*Ca. P. asteris*’ 16Srl). Expression of SAP 11 led to witches broom symptoms in *A. thaliana*. SAP11 interfered with CINCINNATA (CIN)-related TEOSINTE BRANCHED 1-CYCLOIDEA-PROLIFERATING CELL FACTORS 1 and 2 (TCP) transcription factors (Sugio et al., 2011), which are involved in a range of plant development processes (Lan and Qin, 2020). SAP11 transformation and AY_WB infection led to a downregulation of lipoxygenase (LOX) and jasmonate (JA) synthesis in *A. thaliana*, which enhanced the fecundity of the ‘*Ca. P. asteris*’ insect vector *Macrostes quadrilineatus* (Hemiptera, Cicadellidae, Deltocephalinae) on these plants (Sugio et al., 2011).

Another family of phytoplasma effectors, named phyllogens, has been identified in several ‘*Ca. Phytoplasma species*’. Phyllogens are involved in flower malformations like greening of flowers and phyllody (Oshima et al., 2023a, for review). For example, the phyllogen SAP54 of AY-WB promotes degradation of transcription factors of the MADS-box family, which are involved in regulation of flower development (MacLean et al., 2014). A homologous protein named PHYL1 was identified in OY-phytoplasma and peanut witches’-broom phytoplasma (Maejima et al., 2014, 2015; Liao et al., 2019).

In a work by Huang et al., (2021), another effector of ‘*Ca. P. asteris*’ strain AY-WB, SAP05, was extensively studied. In *A. thaliana*, SAP05 caused leaf rosettes, witches broom and abnormal flowers and delayed senescence. The work showed that SAP05 interacted with RPN10, a subunit of the plant 26S proteasome and target of the poly ubiquitin tag.

In consequence, independent of ubiquitination, SPL and GATA, plant transcription factors were degraded. In contrast, SAP05 did not bind to the RPN10 homolog of *M. quadrilineatus*, although the subunits only differed in two amino acids. In consequence, the authors posed the question whether slight modifications in RPN10 would lead to phytoplasma resistant or tolerant crop plants.

During the last years, homologs of the genes mentioned above have also been detected in other phytoplasma species. For example, a SAP11 homolog is present in '*Ca. P. mali*' (Mittelberger et al., 2022) and '*Ca. P. solani*' (Seruga Music et al., 2019). SAP54 homologs were identified in at least 17 phytoplasma species (Tomkins et al., 2018, for review).

In addition, further (putative) effector genes have been described for a variety of different phytoplasmas (Tomkins et al., 2018, for review). Recently, sequencing and assembly of the '*Ca. P. solani*' strain SA-1 genome identified 38 possible effector genes (Seruga Music et al., 2019). Another study, based on seven genotypes of '*Ca. P. solani*' from insect transmission experiments and vineyards in Austria identified six putative pathogenicity factors/effectors in a first step by aid of bioinformatics. In a next step, corresponding pathogenicity factor/effector constructs were used for an *Agrobacterium*-mediated transient transformation of *Nicotiana benthamiana* leaves. Analysis of the infiltrated *N. benthamiana* leaves using confocal microscopy, pull-down and co-immunoprecipitation, and enzyme assays revealed effects of the putative effectors on the plant carbohydrate metabolism and the ascorbate-glutathione cycle. The virulence factors with the greatest impact on carbohydrate mechanism, named PoStoSP06, PoStoSP013, and PoStoSP28 were localised in the cytosol and the nucleus of *N. benthamiana* cells. PoStoSP28, annotated previously as antigenic membrane protein Stamp directly interacted with grapevine phosphoglucosyltransferases. In *N. benthamiana* cells, expressing PoStoSP28, autophagosome-like structures were

detected, which were rare in control plants (Der-mastia et al., 2023).

Correa Marrero et al., (2024) selected 21 putative effectors, 17 from the phytoplasma strain AY-WB, and four orthologs of SAP11 and SAP54 proteins from other phytoplasmas, and studied their interaction with 1980 *A. thaliana* transcription factors and transcriptional regulators in yeast-two hybrid assays. Widespread interaction between phytoplasma effectors and plant transcription factors related to plant development, particularly to sets of TCP transcription factors was observed. For the effectors SAP48 and SAP06 an interaction with numerous host proteins (in each case more than 200) was identified. The interaction patterns of phytoplasma effectors differed substantially from those secreted by other plant pathogens. Phytoplasma effectors, to an unprecedented extent, targeted plant development related-proteins.

A recent analysis on genomes of 239 phytoplasma species revealed a substantial repertoire of candidate virulence factors, despite the obligate host-associated lifestyle of phytoplasmas, likely restricting horizontal gene transfer. The study demonstrated that the virulence factors, termed by the authors as Phytoplasma Effectors (PhAMEs), frequently act as scaffolds with dual interaction surfaces, enabling them to connect host proteins or integrate signaling pathways. Most phytoplasma effectors seemed unique, however some domains exhibited similarities to effector proteins found in other mollicutes and pathogenic bacteria (Mirkin et al., 2025).

In addition to effectors, also phytoplasma membrane proteins play a relevant role in pathogen transmission as well as in pathogen-host plant interaction. Phytoplasmas lack a firm cell wall, and their membranes are in direct contact with the cytoplasm of the host cell. So far, three groups of immunodominant membrane proteins have been identified and described, specifically immunodominant membrane proteins (Imp), immunodominant membrane proteins A (IdpA) and antigenic membrane proteins (Amp). Due to their direct

interaction with the host cell, these surface exposed membrane proteins are under positive selection pressure. As a result, the proteins are highly variable, even between closely related phytoplasmas (Kakizawa et al., 2006; Boonrod et al., 2012; Konnerth et al., 2016). The Amp protein of ‘*Ca. P. asteris*’, was found to interact with microfilaments in insect cells, indicating that Amp-microfilament complexes are crucial for vector transmission (Suzuki et al., 2006). The protein sequence of Stamp, as already mentioned, the antigenic membrane protein of ‘*Ca. P. solani*’ types, was 26-40% identical with Amp of ‘*Ca. P. asteris*’ (Fabre et al., 2011a). The Imp protein of ‘*Ca. P. mali*’ interacts with plant actin. This finding raises the question if actin binding is relevant for the movement of the pathogen within the plant phloem and in consequence for the colonization of the host plant (Boonrod et al., 2012; Liu et al., 2024). In addition, recent research implied that Imp interacts with peanut witches’-broom phytoplasma effector PHYL1 (Lui et al., 2024).

In addition, several other highly variable surface proteins of phytoplasmas have been identified, such as the variable membrane proteins (Vmp) Vmp1 of ‘*Ca. P. solani*’, and VmpA, similar to Vmp1 in *flavescence dorée* phytoplasma (FDp). VmpA specifically interacts with the leafhopper vector *Euscelidius variegatus*, namely with the perimicrovillar membrane of the insect midgut, suggesting that this protein could be crucial for the colonization of the insect by the phytoplasma (Cimerman et al., 2009; Arricau-Bouvery et al., 2018).

Due to their high variability, genes encoding membrane proteins are suitable targets for multi-locus sequence analysis approaches. They allow a fine tuned characterisation of phytoplasma types. Several studies have exploited genes encoding surface proteins for tracking phytoplasma types in epidemiological studies, e.g., on FDp, ‘*Ca. P. solani*’, ‘*Ca. P. pyri*’, ‘*Ca. P. mali*’, and ‘*Ca. P. prunorum*’ (Fabre et al., 2011b; Danet et al., 2011; Aryan et al., 2014; Murolo and Romanazzi, 2015; Riedle-Bauer et al., 2019, 2022; Malembic-Maher et al., 2020; Mehle et al., 2022).

Transmission of phytoplasmas

Phytoplasmas penetrate their host plants via several ways. Sap sucking insect species transmit the pathogens in a persistent propagative manner. Additionally, infected planting material can be a relevant pathogen source, particularly in the case of vegetatively propagated crop species. Transmission of phytoplasmas via seeds is restricted to some plant species only. In addition, pathogen movement via vascular connections between infected and healthy plants formed by dodder species (*Cuscuta* spp.) is possible. In general, infection via dodder is rare, except in experimental conditions, where this way of phytoplasma transmission to new plants is occasionally exploited (Weintraub and Beanland, 2006; Satta et al., 2019; Trivellone and Dietrich, 2021). Furthermore, transmission via root bridges has been observed, e.g. in *Malus* infected with ‘*Ca. P. mali*’ (Cicotti et al., 2007; Baric et al., 2008).

Insect vectors of phytoplasmas

Taxonomic allocation and life-style of phytoplasma vectors

Phytoplasmas colonize the phloem of their host plants. In consequence, phloem feeding insect species sucking on infected plants can acquire the phytoplasmas. Only a small number of phloem feeding species, however, is capable of phytoplasma transmission. Phytoplasma vectors are solely found in the insect order Hemiptera, suborders Auchenorrhyncha (infraorders Cicadomorpha and Fulgoromorpha) and Sternorrhyncha (superfamily Psylloidea) (Weintraub and Beanland, 2006; Hodkinson, 2009; Weintraub et al., 2019; Trivellone and Dietrich, 2021), Fig. 2.

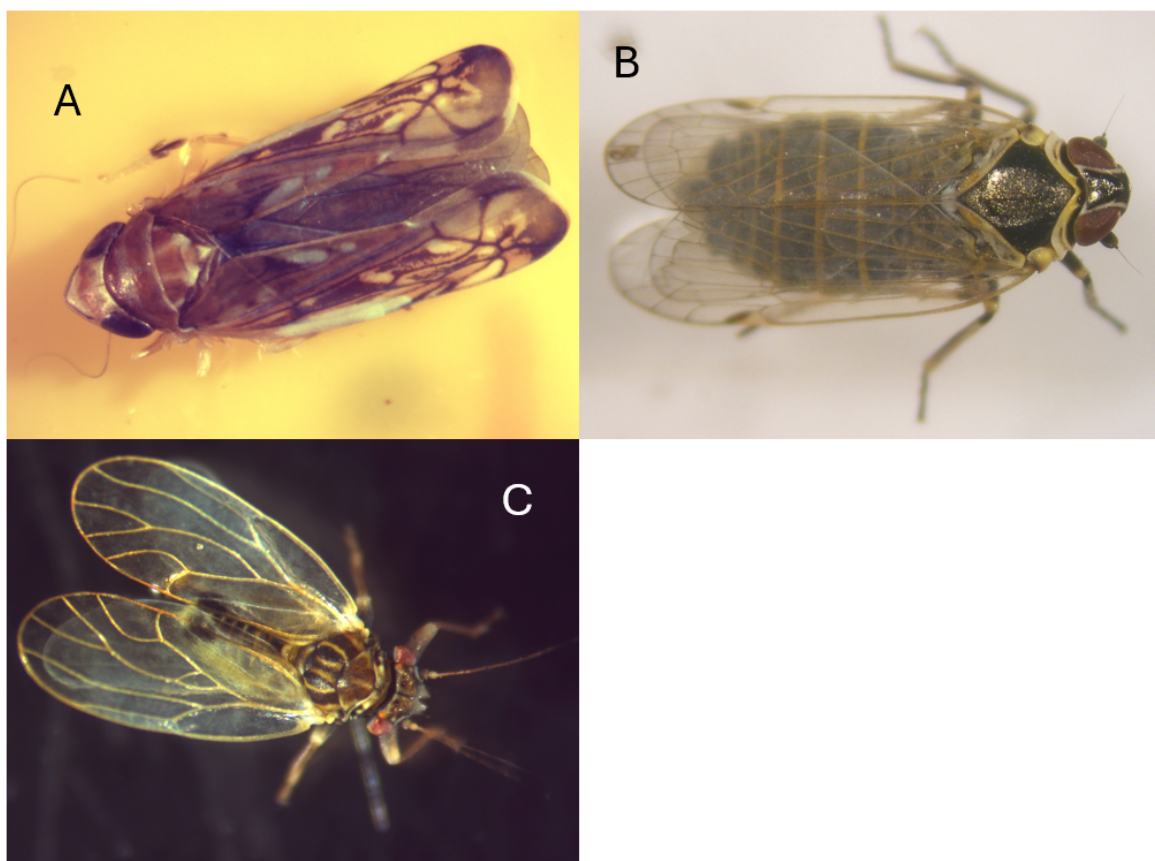


Fig. 2: Insect vectors of phytoplasmas are found in the insect order Hemiptera, suborders Auchenorrhyncha and Sternorrhyncha. A: *Scaphoideus titanus* (Auchenorrhyncha, Cicadellidae, Deltocephalinae), vector of Flavescence dorée phytoplasma; B: *Hyalesthes obsoletus* (Auchenorrhyncha, Cixiidae), vector of , 'Candidatus Phytoplasma solani'; C: *Cacopsylla pyri* (Sternorrhyncha, Psyllidae) vector of 'Ca. P. pyri'.

Among the Cicadomorpha, the largest number of confirmed or putative phytoplasma vectors, around 200 species, belongs to the superfamily Membracoidea, families Cicadellidae and Membracidae. In the Cicadellidae family, confirmed vectors are allocated to the subfamilies Aphrodinae, Cicadellinae, Coelidiinae, Deltocephalinae, Eurymelinae, Iassinae, Macropsinae, Megophthalminae and Typhlocybinae, whereby more than 75% are Deltocephalinae. Fulgoromorpha (superfamily Fulgoroidea) phytoplasma vectors are assigned to the families Cixiidae, Delphacidae, Derbidae and Flatidae (Weintraub and Beanland, 2006; Hodkinson, 2009; Trivellone, 2019; Weintraub et al., 2019; Trivellone and Dietrich, 2021). In addition to their scientific names, several Auchenorrhyncha taxa are designated with trivial names, which refer to their feeding sites, their appearance or their behaviour. For example, the term leafhoppers is used for Cicadomorpha mainly feeding on leaves, planthoppers are

Fulgoromorpha, feeding on leaves (reviewed in Weintraub and Beanland, 2006; Weintraub et al., 2019).

Among the Psylloidea, phytoplasma vectors belong to the genera *Cacopsylla* and *Diaphorina*. Trivial names of psyllids include the terms leaf suckers, leaf flea or psyllids (reviewed in Weintraub and Beanland, 2006; Hodkinson, 2009; Weintraub et al., 2019).

Leafhoppers, planthoppers and psyllids reproduce sexually and develop from eggs into adults over five nymphal instars. As hemimetabolous insects, feeding of nymphs and adults is similar. For specific recognition and location both genders emit vibrational reciprocal courtship signals (Weintraub and Beanland, 2006, for review; Hodkinson, 2009; Alma et al., 2019, for review; Liao et al., 2022).

Leafhoppers hibernate as instars, adults or eggs, whereby most species hibernate as eggs. In general, leafhopper females lay their eggs beneath the epidermis, into crevices on the plant or into the bark of their plant hosts (reviewed in Weintraub et al., 2019). Orientation of leafhoppers seems to rely mostly on visual cues, a wavelength-dependent response was, e.g., reported for the leafhopper *Dalbulus maidis* (Cicadellidae, Deltocephalinae) (Todd et al., 1990). A recent study on host orientation of *Empoasca vitis* (Cicadellidae, Typhlocybinae) indicated that highly developed compound eyes play the major role for host finding. In contrast, relatively few olfactory sensilla on the antennae indicated a lack of ability to detect odours from a long distance (Zhang et al., 2018). Once on a potential host plant, leafhoppers perform probing behaviour with their stylets, which allows them to differentiate between host and non-host plants (Tholt et al., 2015).

In contrast to leafhoppers, Cixiidae females deposit their eggs into the soil. Females of some species even creep into crevices of the soil. Eggs are covered by wax filaments. The nymphs develop on roots of mostly herbaceous host plants in the soil. In most cases, the Cixiid life cycle lasts for one year (Holzinger et al., 2002).

Leaf- and planthopper feeding behaviours differ considerably and range from monophagous to polyphagous species. The host range of a particular vector species strongly determines its efficiency in transmitting phytoplasmas and, consequently, often shapes the host plant spectrum of a given ‘*Ca. Phytoplasma*’ species. Although many insect vectors are so far unknown, it appears that most phytoplasmas are associated with one vector species or genus only. Only few phytoplasmas such as ‘*Ca. P. asteris*’ have competent vectors from different genera or families (reviewed in Trivellone, 2019; Weintraub et al., 2019; Trivellone and Dietrich, 2021).

Psyllids of the genus *Cacopsylla* are the vectors of the genetically closely related fruit tree phytoplasmas ‘*Ca. P. pyri*’; ‘*Ca. P. mali*’ and ‘*Ca. P. prunorum*’

(16SrX or apple proliferation group, Lee et al., 1993, 1998; Schneider and Seemüller, 2004; Jarausch B et al., 2019, for review). Some *Cacopsylla* spp. are monovoltine and migratory. They breed on fruit tree species in late winter and early spring but aestivate and hibernate on conifers, often at higher altitudes. Rising temperatures toward the end of winter of the following season prompt the insects to abandon the conifers, and to migrate back to their developmental hosts, thereby covering long distances. Other species are polyvoltine, produce several overlapping annual generations and overwinter on or close to fruit trees (Jarausch B et al., 2019 for review; Maier et al., 2013; Riedle-Bauer et al., 2022). Studies have shown that the psyllids exploit plant volatiles during their search for suitable host plants and also for finding their aestivation and overwintering hosts (conifers) during migration (Gallinger and Gross, 2020; Gross et al., 2022).

Acquisition and transmission of phytoplasmas by vector insects

Phytoplasma transmission proceeds in a persistent propagative way, generally comprising three phases, namely phytoplasma acquisition, latency and inoculation (Weintraub and Beanland, 2006 for review; Alma et al., 2019, for review). During the acquisition access period (AAP), normally requiring minutes to hours, the vector insect, feeding on an infected plant, takes up the pathogen. Via the stylets, the insect ingests the phytoplasmas in the plant sap into its intestine. In most cases, phytoplasma acquisition, leading to a competent vector, takes place during the insect’s nymphal stage (Alma et al., 2019, for review). Acquisition efficiency strongly depends on the length of the AAP and the phytoplasma titres in the source plants. The phytoplasma titre in the source plants is, e.g., crucial for the ability of the psyllids *C. pyri* and *C. pyricola* to transmit ‘*Ca. P. pyri*’, the causal agent of pear decline (PD). In pome trees, the phloem of the aerial parts declines during winter. Each spring phytoplasma from the roots gradually colonize the newly developing phloem. Therefore, phytoplasma titres in the aerial parts of the trees

are low in spring and rise during the vegetation period (Seemüller et al., 1984 ab). Accordingly, the phytoplasma infection rate and the transmission efficacy of the two psyllid vector species vary strongly during the season, being lowest for springtime generation insects and highest for individuals hatched in autumn (Carraro et al., 2001; Sabaté et al., 2018; Riedle-Bauer et al., 2022). Also in case of *S. titanus*, the vector of FD phytoplasma, a positive correlation of the phytoplasma acquisition by the nymphs and the seasonal phytoplasma load of the vines has been reported (Alma et al., 2019, for review).

In the latency period (LP), lasting from some days to up to 80 days, the phytoplasmas in the vector move from the epithelial cells of the gut into the hemocoel and finally enrich in the secretory salivary gland cells (Weintraub et al., 2019, for review). Recently, the multiplication of OY- phytoplasma in the leafhopper *Macrostelus strifrons* (Cicadellidae, Deltocephalinae) was tracked in 3D imaging and fluorescence staining studies. The pathogen reached the epithelium midgut seven days after the start of the acquisition, spread along the fibres of the visceral muscles, accumulated on the surfaces of the salivary gland cells and entered the relevant cells of the salivary glands after 21–28 days (Koinuma et al., 2020).

The duration of the LP may be influenced by exterior factors such as the temperature or the phytoplasma load taken up by the vector insect (Weintraub and Beanland, 2006, Alma et al., 2019, for review). In some disease cycles, when insect vectors acquire the pathogen within the growing season, the length of the LP determines the start time of pathogen transmission and is thus crucial for successful pathogen control. This is, for example, the case for *S. titanus*. *S. titanus* overwinters in egg stage below the bark of grapevines. From May onwards, the hatching nymphs develop and feed on the vines, from where they take up FD phytoplasmas. In general, the LP for *S. titanus* nymphs lasts for four weeks. In consequence, infectious individuals are present in vineyards

approximately from the second half of June onwards (Chuche and Thiery, 2014).

Only pathogen-vector interactions allowing the steps described above, specifically acquisition, and phytoplasma enrichment in the hemocoel and the salivary glands, lead to a competent vector. Numerous studies have proven that a vector species can be infected with a phytoplasma, but nevertheless, unable to transmit it (Weintraub and Beanland, 2006, Hogenhout et al., 2008, for review). In some vector species, a transovarial phytoplasma transmission (vertical transmission) has been detected. For example, over two generations, laboratory populations of the leafhopper *Matsumuratettix hiroglyphicus* (Cicadellidae, Deltocephalinae) remained infected with sugarcane white leaf disease phytoplasma (16SrXI-B ribosomal group) despite feeding on healthy plants only (Hanboonsong et al., 2002). Vertical transmission has also been reported for the psyllids *Cacopsylla pruni*, transmitting ‘*Ca. P. prunorum*’, and *C. picta*, a vector of ‘*Ca. P. mali*’ (Tedeschi et al., 2006; Mittelberger et al., 2017).

Injection of the phytoplasmas with the saliva into the phloem of a (so far in most cases healthy) host plant takes place when the vector stylets penetrate the sieve element membranes of the host plant (Weintraub and Beanland, 2006). The length of the inoculation access period (IAP), the feeding time necessary for phytoplasma inoculation into a plant, strongly depends on the vector species and the phytoplasma transmitted by it. An IAP of four hours on apricot and plum seedlings sufficed for successful transmission of ‘*Ca. P. prunorum*’ by overwintered *C. pruni* (Riedle-Bauer et al., 2019). In case of transmission of ‘*Ca. P. solani*’ by *Hyalosthes obsoletus* (Cixiidae) to grapevine, an IAP of three to six hours was observed (Bressan et al., 2007). Interestingly, recent laboratory experiments studying the effect of insecticides on transmission of ‘*Ca. P. solani*’ to the experimental phytoplasma host *Catharanthus roseus* suggested that *H. obsoletus* might be able to finish the IAP faster on this plant species. Insecticides reaching 100% *H. obsoletus* mortality after one hour fully

prevented pathogen spread to *C. roseus*, whereas compounds inducing 100% mortality after three hours only, strongly reduced but not fully prevented infections (Riedle-Bauer and Brader, 2023). This observation raises the question of to what extent the required length of the IAP also depends on the susceptibility of the host plant.

The length of the IAP is an important consideration for phytoplasma control by pesticides, notably when infected vector insects migrate into a crop from outside sources. The crucial question is, whether a selected insecticide acts in a shorter period than the IAP and thus prevents phytoplasma transmission by insects moving into the crop (Paleskić et al., 2017, Riedle-Bauer and Brader, 2023).

Predominantly, adult vectors account for significant disease outbreaks. In contrast to instars, adult vector insects are winged and therefore capable of quick movement between more or less distant host plants. Often, instars are monophagous or oligophagous, whereas adults feed on a wider range of plant species (Alma et al., 2019, for review). For example, instars of *H. obsoletus*, the main vector of 'Ca. P. solani', have been detected on few plant species only. In Central Europe, they develop on *Convolvulus arvensis* and *Urtica dioica*. In contrast, the adults feed on many plant species, among them relevant crops such as grapevines, potatoes and many vegetables (Langer and Maixner, 2004; Jović et al., 2019, for review).

In recent years, an increased emphasis has been placed on the multitrophic interactions between insect vectors, their microbial community and plants. These interactions may play a crucial role for the ability of an insect vector species to transmit a phytoplasma. For example, co-occurrence of multiple pathogens in one vector individual may lead to selective transmission (Gonella et al., 2019a, for review). E.g., the leafhopper *Osbornellus horvathi* (Cicadellidae, Deltocephalinae), infected with 'Ca. P. asteris' and 'Ca. P. phoenicium', transmitted 'Ca. P. asteris' only (Rizza et al., 2016). All taxonomic groups of phytoplasma vectors (Auchenorrhyncha and Sternorrhyncha) are colonised

by obligate or facultative endosymbionts. Phloem feeding insect species are particularly dependent on endosymbionts providing them with nitrogen, which is lacking in the phloem sap (Skidmore and Hansen, 2017). Thus, endosymbionts are of crucial importance for the fitness and lastly also for the transmission capability of a vector insect (Skidmore and Hansen, 2017 for review; Gonella et al., 2019a, for review). An investigation of the endosymbiotic microbiota of the leafhopper species *M. striifrons* and *Macrosteles sexnotatus*, both vectors of 'Ca. P. asteris' (16SrI, Aster Yellows group), identified two obligate endosymbionts, 'Candidatus Sulcia muelleri' and 'Candidatus Nasuia deltocephalinicola', five facultative endosymbionts, *Wolbachia*, *Rickettsia*, *Burkholderia*, *Diplorickettsia*, and a novel bacterium belonging to the Rickettsiaceae (Ishii et al., 2013). Analysis of bacterial microbiota of various Auchenorrhyncha species infected and non-infected with 'Ca. P. solani' indicated a prominent effect of the phytoplasma infection on the microbial diversity in vector insects. *Wolbachia* was found in non-infected specimens of all included insect vector species, but in the vast majority of insect species, phytoplasma infection reduced the abundance of *Wolbachia*. 'Ca. Sulcia' was richly present in all insect genera except *Hyalesthes* (Cixiidae), phytoplasma infection greatly reduced its abundance (Moussa et al., 2020). 'Ca. Sulcia muelleri' and an *Ophiocordyceps* related fungal species are universally present in *S. titanus*. In European *S. titanus* specimen, bacteria belonging to the genus *Cardinium* are also part of the insect microbiome (Abbà et al., 2022). Another study identified bacteria of the genus *Asaia* as secondary symbionts of *S. titanus*. *Asaia* putatively affects phytoplasma acquisition by insect vectors and is transmitted to the insect progeny (Gonella et al., 2012).

The vector's immune system may limit phytoplasma multiplication, putatively involving mechanisms like melanization and apoptosis (Gonella et al., 2019a). The establishment and maintenance of in-vitro immunocyte cell cultures of the phytoplasma vectors *C. pyri*, *C. melanoneura* (Psyllidae) and *Euscelis variegatus* (Cicadellidae,

Deltoccephalinae) might contribute to the study of phytoplasmas, symbionts and insect vectors in the future (Monti et al., 2014; Tedeschi et al., 2017). Gonella et al., (2019b) studied the activation of genes related to immunity and the role of the endosymbiont *Asaia* in FDp infected and healthy *E. variegatus*. Groups of insects were allowed to feed on media enriched with *Asaia*. In order to determine the transcript abundance of genes involved in immune pathways (defensin, Raf, a component of the Ras/Raf pathway, phenoloxidase, and kazal type 1 serine protease inhibitor) RNA isolated from whole insects, insect midguts and hemocyte cell cultures was analysed by qRT-PCR. The transcript level of the Raf gene was increased in leafhopper midguts after double infection by *Asaia* and FDp phytoplasma, but not in phytoplasma infected leafhoppers without *Asaia*.

Identification of phytoplasma vectors

The first step in the identification of vector species is a season-long insect monitoring in the infected crop. Molecular means allow the detection of pathogen presence in insects. However, due to the complex vector-phytoplasma relationship illustrated above, lastly, laboratory transmission experiments are required to confirm the vectoring ability of a suspicious insect species. Once a vector species is identified, detailed studies on its life cycle and the developmental stages relevant for pathogen transmission have to follow (Weintraub and Beanland, 2006; Bosco and Tedeschi, 2013).

Phytoplasmas spread via distinct disease cycles

Due to their trans-kingdom life cycle, namely their ability to colonize both, plants and insects, phytoplasmas spread via distinct epidemic cycles, involving plants species that simultaneously act as hosts for the vector insects and for the phytoplasma. The epidemic cycle leading to transmission of a given pathogen strongly depends on the diversity of involved insect vectors, their feeding preferences and the plant species acting as

phytoplasma reservoirs. A closed disease cycle refers to a pathogen transmission that occurs between plant hosts of the same species. If phytoplasma transmission, e.g., involves monophagous vector species only, AAP, LP and IAP take place on one host plant species. In contrast, in open cycles pathogen spread proceeds from some plant species acting as pathogen sources to other plant species, which may act as sinks or dead end hosts. Polyphagous vectors, and in particular, insect species with a migratory behaviour tend to transmit the pathogens in open disease cycles. In nature, however, spread of most phytoplasmas involves both open and closed disease cycles or intermediate forms and in many cases, different insect vectors are involved (Alma et al., 2019, for review).

Conclusions

Up to now, around 50 ‘*Candidatus* Phytoplasma species’ and phytoplasmas allocated to around 300 ribosomal subgroups have been described. This high diversity of phytoplasmas affects a huge number of plant species, including a wide range of annual and perennial crops. The trans-kingdom lifestyle of phytoplasmas, namely their ability to multiply both in plants and in vector insects has led to the evolution of various highly complex epidemic cycles. As phloem parasites, phytoplasmas extract nutrients from hosts and induce major physiological and pathological changes. Current evidence suggests that phytoplasmas secrete a wide range of effector proteins that target key plant proteins, especially transcription factors, and also interact with insect vectors. Phytoplasmas are passively acquired when insects feed on infected plants. In order to establish infection, a vector insect must feed long enough to ingest a sufficient phytoplasma concentration. This period of time is referred to as the acquisition access period, and lasts minutes to hours. After acquisition, a latent period of a few up to 80 days follows, before the insect becomes capable of transmission. During this stage, phytoplasmas colonize the insect gut, disseminate through the hemolymph, replicate in multiple tissues, and ultimately invade the salivary glands. Transmission occurs when an

insect vector, during feeding, injects infected saliva into the phloem of a host plant. The inoculation access period, the feeding duration required for successful transmission, takes hours to days, depending on the insect vector and the phytoplasma species. Insect phytoplasma vectors harbor obligate or facultative endosymbionts.

As phloem sap is deficient in nitrogen, phloem-feeding insects rely heavily on these symbionts for nutrition. Interactions between insect vectors, endosymbionts and pathogens may play a crucial role for the ability of an insect vector species to transmit a phytoplasma.

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