OFFERED REVIEW

CANDIDATUS PHYTOPLASMA PRUNORUM, THE CAUSAL AGENT OF EUROPEAN STONE FRUIT YELLOWS: AN OVERVIEW

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SUMMARY

'*Candidatus* Phytoplasma prunorum' is an important prokaryotic pathogen that infects stone fruits in Europe. It is known to cause several economically relevant disorders of *Prunus* spp. which are collectively referred to as European stone fruit yellows (ESFY). This organism is phylogenetically closely related to other important fruit trees pathogens such as apple proliferation (AP), pear decline (PD) and peach yellow leaf roll (PYLR) agents. Together they form a distinct phylogenetic cluster, the AP or 16SrX group. Like the other fruit tree phytoplasmas of the AP group, '*Ca*. P. prunorum' exhibits a high host specificity. In nature, this pathogen has been reported to infect only plants in the genus *Prunus* and to be transmitted by one insect vector species, the psyllid (Psyllidae) *Cacopsylla pruni*. Also, '*Ca.* P. prunorum' includes strains which greatly differ in virulence. This review summarizes the current knowledge of '*Ca.* P. prunorum' with emphasis on advances that have been made during the last two decades in understanding molecular and epidemiological aspects. Prospects for disease management and future research which could provide insights into the largely unknown mechanisms involved in pathogenicity of '*Ca*. P. prunorum', are also critically discussed.

Key words: phytoplasmas, phytoplasma detection, phytoplasma characterization, phytoplasma transmission, stone fruits, *Cacopsylla pruni*, epidemiology, disease management.

INTRODUCTION

'*Candidatus* Phytoplasma prunorum' is an important prokaryotic pathogen that infects stone fruits in Europe and is known to cause apricot chlorotic leaf roll of apricot (*Prunus armeniaca*), leptonecrosis of Japanese plum (*P. salicina*) and yellows and decline diseases of peach (*P. persica*), European plum (*P. domestica*), almond (*P. dulcis*) and flowering cherry (*P. serrulata*). All these disorders are collectively referred to as European stone fruit yellows (ESFY) (Lorenz *et al*., 1994; Seemüller and Foster, 1995; Seemüller *et al.*, 1998a). The largest economic losses are recorded in apricot and Japanese plum orchards where infection rates of susceptible cultivars can be up to 50% and orchards are rendered unproductive eight to ten years after planting. ESFY has become a major obstacle for successful production of apricot in all central and southern European countries. Due to its economic importance, '*Ca.* P. prunorum' is one of the most intensively studied phytoplasmas. Molecular studies using Southern blot hybridization and restriction fragment length polymorphism (RFLP) and sequence analyses of polymerase chain reaction (PCR)-amplified ribosomal DNA (rDNA) and non-ribosomal DNA showed that '*Ca.* P. prunorum' is a relatively homogeneous organism throughout Europe (Ahrens *et al.*, 1993; Lorenz *et al.*, 1994; Kison *et al*., 1997; Jarausch *et al.*, 1998a, 2000a; Seemüller *et al*., 1998b; Seemüller and Schneider, 2004). This pathogen is phylogenetically closely related to the apple proliferation (AP), pear decline (PD), and peach yellow leaf roll (PYLR) agents, the causes of other major diseases of deciduous fruit trees, which are members of a distinct phylogenetic cluster, the AP or 16SrX group within the phytoplasma clade (Seemüller *et al*., 1998a; IRPCM, 2004; Seemüller and Schneider, 2004). The ESFY agent is distinctly different from the X-disease phytoplasma, a major stone fruit pathogen of North America and from '*Ca*. P. phoenicium' that infects *Prunus* spp. in Asian and North African countries. The ESFY agent also differs from various phytoplasma strains that have been transmitted from diseased apricot, cherry, and Japanese plum trees to the experimental host *Catharanthus roseus* (periwinkle). These strains are members of either the aster yellows or the stolbur or X-disease phytoplasma group (Ahrens *et al.*, 1993; Lorenz *et al*., 1994; Seemüller *et al.*, 1998a; Marcone *et al*., 1999a). '*Ca.* P. prunorum' is known to occur in most southern and central European countries and as far north as in south-east England (Seemüller *et al.*, 1998a, 1998b; Davies and Adams, 2000; Topchiiska *et al*., 2000; Delić *et al*., 2008; Laimer

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and Bertaccini, 2008). It has also been reported in Turkey and Azerbajdzhan (Jarausch *et al*., 2000a; Sertkaya *et al*., 2005; Danet *et al*., 2007). It is likely that the pathogen is present wherever stone fruits are grown in Europe and non-European Mediterranean countries (Seemüller and Foster, 1995). Like the other fruit tree phytoplasmas of the AP group, '*Ca*. P. prunorum' exhibits a very high host specificity. In nature, this pathogen seems to infect only plants in the genus *Prunus* and appears to be transmitted by one insect vector species, the psyllid (Psyllidae) *Cacopsylla pruni* (Scopoli) (Carraro *et al*., 1998a; Weintraub and Beanland, 2006). Also, the pathogen exists in strains which greatly differ in aggressiveness, ranging from avirulent (or nearly avirulent) to highly virulent (Kison and Seemüller, 2001). The aim of this review is to summarize the current knowledge about the ESFY agent '*Ca.* P. prunorum'. Emphasis is placed on advances that have been made during the last two decades in understanding molecular and epidemiological aspects.

MOLECULAR CHARACTERIZATION OF '*CA.* P. PRUNORUM'

Molecular taxonomy. According to the phylogenetic phytoplasma classification, which is largely based on sequence analysis of 16S rDNA, '*Ca*. P. prunorum' is a member of the AP group (IRPCM, 2004). Other members of this group are '*Ca*. P. mali' (the AP agent), '*Ca*. P. pyri' (the PD agent), the PYLR agent, and the newly identified PD (PDTW) agent infecting pear in Taiwan (Lee *et al*., 2000, 2007; Seemüller and Schneider, 2004; Liu *et al*., 2007). Nucleotide sequence comparisons revealed that the 16S rDNA sequences of five '*Ca.* P. prunorum' strains from Germany, Austria and Czech Republic are identical or nearly identical, showing similarity values between 99.8 and 100%. There is no indication of sequence heterogeneity between the two rRNA operons of '*Ca*. P. prunorum' (Seemüller and Schneider, 2004). In interspecific comparisons of the ESFY/AP, ESFY/PD, ESFY/PYLR and ESFY/PDTW agents, differences in 16S rDNA sequences were 1.3-1.5, 1.2-1.3, 1.4-1.6 and 1.2-1.4%, respectively (Seemüller and Schneider, 2004; Liu *et al*., 2007). More distantly related to '*Ca*. P. prunorum' are four other phytoplasmas that cluster in the same subclade as the AP-group members: '*Ca.* P. spartii' (associated with spartium witches'-broom), '*Ca.* P. rhamni' (associated with buckthorn witches'-broom), '*Ca.* P. allocasuarinae' (associated with allocasuarina yellows), and '*Ca.* P. tamaricis' (associated with salt cedar witches'-broom). These phytoplasmas share between 94 and 97.1% 16S rDNA sequence similarity with '*Ca*. P. prunorum' (Marcone *et al*., 2004; Zhao *et al*., 2009). The 16S/23S rDNA spacer region sequences of the abovementioned '*Ca*. P. prunorum' strains are also identical. At 16S/23S rDNA spacer region sequence level, '*Ca*. P. prunorum' differs from the other AP group fruit tree phytoplasmas in 1.2-3.0% of nucleotide positions and from the other phytoplasmas clustering in the AP group in more than 11% of positions (Marcone *et al*., 2004; Seemüller and Schneider, 2004; Liu *et al*., 2007). Also, by RFLP analysis of PCR-amplified rDNA using several restriction enzymes, no genetic polymorphisms were observed among numerous '*Ca*. P. prunorum' isolates collected at various locations in European countries and from various stone fruit species (Marcone *et al*., 1996a; Seemüller *et al*., 1998b; Topchiiska *et al*., 2000; Jarausch *et al*., 2001a; Laimer Da Câmara Machado *et al*., 2001; Delić *et al*., 2008) whereas the ESFY agent can clearly be distinguished from the other AP-group fruit tree phytoplasmas using RFLP analysis of PCR-amplified 16S rDNA sequences employing *Ssp*I, *Bsa*AI and *Rsa*I restriction endonucleases (Marcone *et al*., 1996a; Seemüller *et al*., 1998b).

Genetic characterization. Sequence alignment of less-conserved, non-ribosomal genes has shown a considerable genomic variability among '*Ca*. P. prunorum' isolates in the *imp* gene, which encodes an immunodominant membrane protein, with similarity values ranging from 95.4 to 99.8%. Less genetic variability occurred in the *aceF*, *secY* and *pnp* genes encoding a component of pyruvate dehydrogenase, a translocase protein and a polynucleotide phosphorylase, respectively. For *tuf* and *tlyC* genes, which encode the elongation factor Tu (EF-Tu) and a hemolysin known as a membrane-damaging agent and important virulence factor of many bacteria, respectively, '*Ca*. P. prunorum' isolates proved to be identical or nearly identical with similarity values between 99.7 and 99.9%, and 99.8 and 100%, respectively. No dissimilarities were observed in both *rpsC* and *fol* genes, encoding a ribosomal protein and an enzyme essential for folate biosynthesis, respectively (Danet *et al*., 2007, 2008; Marcone *et al*., 2009). Analysis using *aceF* sequences generated a tree in which French hypovirulent strains of the ESFY agent, namely peach vein clearing, B7 and E22, clustered together and were thus distinguished from the other strains of the same pathogen (Danet *et al*., 2008).

However, phylogenetic trees inferred from *imp*, *secY* and *pnp* genes had different topology and did not allow differentiation of the mentioned hypovirulent strains. Also, phylogenies based on *imp* and *secY* genes clustered some '*Ca*. P. prunorum' strains which are supposed to be non-circulative in a monophyletic group. In phylogenetic trees based on *aceF* and *imp* sequences, an isolate from Azerbajdzhan was delineated as a distinct lineage among the '*Ca*. P. prunorum' strains (Danet *et al*., 2007, 2008). Also, maximal dissimilarities identified between '*Ca*. P. prunorum' and AP and PD agents were 36 and 29% on the basis of *imp* gene, 11 and 12% in

aceF, 7 and 6% in *pnp*, 8 and 7% in *secY*, and 5.4 and 5.9% for ribosomal protein (*rpsV* and *rpsC*) genes, respectively (Morton *et al*., 2003; Danet *et al*., 2007, 2008; Lee *et al*., 2007; Martini *et al*., 2007a). Recent work has shown that '*Ca*. P. prunorum' shares 84.6-87.8% and 89.6% *hflB* gene sequence similarity with '*Ca*. P. mali' strains and '*Ca*. P. pyri', respectively (Schneider and Seemüller, 2009).

RFLP analysis of PCR-amplified *tuf* gene sequences was also employed to differentiate '*Ca*. P. prunorum' strains. Following separate digestion with *Alu*I, *Rsa*I, *Sau*3AI, *Mse*I, *Hha*I, *Hinf*I, *Hpa*II and *Hae*III, more than a hundred isolates infecting peach, apricot and plum trees in southern Italy had similar RFLP profiles. When the same *tuf* gene fragments were digested with *Taq*I restriction endonuclease, two distinct restriction profiles were identified among the '*Ca*. P. prunorum' isolates examined (Marcone *et al*., 2002; C. Marcone, unpublished data). Similar results were obtained by Ferretti *et al*. (2007, 2008, 2009) who employed RFLP analysis of PCR-amplified *tuf* gene sequences using *Nla*III restriction enzyme to examine genetic polymorphism among '*Ca*. P. prunorum' strains which were detected in both stone fruit trees and *C. pruni* individuals. Work by Martini *et al*. (2009) showed that four RFLP subgroups designed Acef-A, -B, -C, and -D, could be identified among '*Ca*. P. prunorum' strains originating from several European countries when *aceF* gene sequences were digested with *Hae*III and *Tsp509*I restriction endonucleases.

One hundred and seventy-five '*Ca*. P. prunorum' isolates, which were collected from 14 *Prunus* species and 4 hybrids in four Mediterranean countries, were analyzed by RFLP using several restriction enzymes of a PCR-amplified non-ribosomal fragment which included a putative nitroreductase gene, an intergenic region and a truncated open reading frame of unknown function. All isolates proved to be identical. However, '*Ca*. P. prunorum' could clearly be differentiated from the AP and PD agents. An overall sequence similarity of 89.6% between '*Ca*. P. prunorum' and '*Ca*. P. mali' occurred on this fragment (Jarausch *et al*., 1994a, 2000a). By Southern blot hybridization of *Hind*III- or *Eco*RI-digested DNA from over 60 '*Ca*. P. prunorum' isolates using a mixture of randomly cloned chromosomal DNA fragements from the '*Ca*. P. mali' as probes, polymorphisms were only observed in two isolates. However, hybridization patterns of '*Ca*. P. prunorum' strains differed significantly from those of '*Ca*. P. mali', '*Ca*. P. pyri' and PYLR strains (Ahrens *et al*., 1993; Lorenz *et al*., 1994; Kison *et al*., 1997; Kison and Seemüller, 2001).

Pulsed-field gel electrophoresis (PFGE) analysis revealed a uniform chromosome size of 630 kb for three strains of '*Ca*. P. prunorum' including the reference strain GSFY1. This size is in the same range as that of '*Ca*. P. mali' and '*Ca*. P. pyri' strains (Marcone *et al*., 1999b; Seemüller and Schneider, 2007). Physical maps have been constructed from the chromosomes of '*Ca*. P. prunorum' and '*Ca*. P. mali' (Lauer and Seemüller, 2000; Marcone and Seemüller, 2001). The physical map of '*Ca*. P. prunorum' strain GSFY1 chromosome is different from that of '*Ca*. P. mali', as evidenced by frequency and location of restriction sites, but the overall genetic arrangement of chromosomes is similar (Marcone and Seemüller, 2001). Work by Kube *et al*. (2008) showed that chomosome of '*Ca*. P. mali', '*Ca*. P. prunorum' and '*Ca*. P. pyri' was linear and not circular as previously known. To prove chromosome linearity, the PFGE-purified, agarose-embedded full-length chromosomes of the three mentioned phytoplasmas were digested with I-*Ceu*I, an intron-encoded endonuclease that cleaves specifically in the 23S rRNA gene of many bacteria. This digestion yielded three macrorestriction fragments, as expected for linear chromosomes having two rRNA operons whereas I-*Ceu*I restriction of the '*Ca*. P. australiense' resulted in only two fragments, as expected for a circular chromosome with two rRNA operons.

Detection. Molecular characterization of '*Ca*. P. prunorum' has also enabled the development of sensitive and specific methods for detection. All attempts to develop direct serological detection methods failed. Currently, PCR technology is the method of choice and has completely replaced the other techniques like dot and Southern blot hybridization. A drawback of molecular detection is the requirement of purified total DNA extracted from infected plants or insects. Several extraction protocols have successfully been applied, e.g., the phytoplasma-enrichment procedure described by Ahrens and Seemüller (1992) or the simplified CTAB-based extraction protocol first published by Maixner *et al*. (1995). Reliable diagnosis is also dependent on the sampled organ and tissue as well as on the sampling time.

The seasonal colonization pattern of '*Ca*. P. prunorum' has been studied in France in apricot, Japanese plum and European plum (Jarausch *et al*., 1999a). Although the pathogen was detectable throughout the year in the aerial parts, the most reliable detection was achieved during summer (July-September) whereas sampling in springtime was not recommended. The pathogen can be detected in roots throughout the year. Detection in phloem preparations from stem and root is usually more reliable than detection in leaf petioles (Jarausch *et al*., 1999a).

PCR technology offers the advantage of the use of primers with different degrees of specificity. Universal phytoplasma primers as well as AP group-specific primers have been designed in ribosomal DNA sequences (Lorenz *et al*., 1995; Seemüller *et al*., 1998b). Due to the close relationship of '*Ca*. P. prunorum' with the other AP-group fruit tree phytoplasmas, primers de-

signed in ribosomal DNA sequences for the specific detection of the ESFY agent cross-amplify DNA from other AP-group fruit tree phytoplasmas, especially PD and PYLR agents (Lorenz *et al*., 1995; Smart *et al*., 1996; Kison *et al*., 1997; Seemüller *et al.*, 1998b). Thus, reliable direct identification of '*Ca*. P. prunorum' could be achieved with non-ribosomal primers selected in the sequence of a putative nitroreductase gene and an intergenic region (Jarausch *et al.*, 1998a, 2000a). Very recently, Yvon *et al.* (2009) published 16S rDNA and 16S/23S rDNA spacer region-based primers which proved to be specific for '*Ca*. P. prunorum' and which did not show PCR signals with several reference strains of '*Ca.* P. mali' and '*Ca.* P. pyri'. As mentioned above, '*Ca*. P. prunorum' can also be distinguished from the other AP-group fruit tree phytoplasmas by RFLP analysis of PCR-amplified rDNA using suitable restriction enzymes (Marcone *et al*., 1996a; Seemüller *et al.*, 1998b). The sensitivity of detection can be increased by nested PCR which is currently one of the most sensitive means suitable for detecting extremely low-titer infections in woody plants. For this, different combinations of ribosomal primers can be used.

A PCR-ELISA procedure has been described by Poggi Pollini *et al.* (1997) using a '*Ca*. P. prunorum'-specific biotinylated probe for detection. This procedure which has been applied for detection of several phytoplasmas other than the ESFY agent, proved to be 10 to 15 times more sensitive than the electrophoretical detection of amplicons (Poggi Pollini *et al*., 1997, 2001).

Unspecific detection of '*Ca*. P. prunorum' infections is also possible by microscopic examination of phloem tissue sections stained with the DNA fluorochrome 4'-6-diamidino-2-phenylindole (DAPI) (Seemüller, 1976). However, this method is not as sensitive as PCR (Jarausch *et al*., 1998a). Visual assessment of characteristic symptoms as described below is possible but not always reliable because symptom expression is largely dependent on the genotype and the season and can be masked by unrelated pathogens or environmental factors.

The correlation between typical symptoms and PCR detection of the pathogen was studied by Jarausch *et al.* (1998a) in France and by Jarausch *et al.* (2008) in Germany. In the first study typical symptoms in a wide range of species and cultivars were highly (95%) correlated to the presence of the pathogen. However, trees showing an unclear symptomatology tested positive for '*Ca*. P. prunorum' in 51% of the cases. These data were confirmed in the study conducted in Germany under different climatic conditions. Early bud break in late winter was the most reliable symptom for ESFY diagnosis in apricot, European plum, almond and flowering cherry.

In certification or quarantine programs, detection still involves indexing using a sensitive indicator host such as peach GF305 seedlings and Japanese plum 'Ozark Premier' and 'Red Heart'. The lengthy procedure and low sensitivity are major drawbacks of the indexing approach. '*Ca*. P. prunorum' detection in quarantine programs might in future rely on the simultaneous detection of a wide range of pathogens by diagnostic DNA microarrays. Prototypes of such a microarray have been developed (Hadidi *et al*., 2004; Pelludat *et al*., 2009).

Quantification of '*Ca.* P. prunorum' by real-time PCR. Determination of absolute quantity of the pathogen in plants and insects has gained increasing importance in recent years to study the plant response in resistance screenings or to characterize insect transmission parameters. The development of real-time PCR technology has greatly facilitated these investigations and several assays for detection and quantification of '*Ca*. P. prunorum' have been published. Torres *et al*. (2005) first reported a real-time assay with ribosomal primers using the fluorescent SYBR Green I dye for the simultaneous detection and quantification of phytoplasmas of the AP group. Absolute quantification was achieved with the standard curve calibration method using a plasmid as template. The measured phytoplasma concentration in the sample was referred to the weight of the plant tissue used for DNA extraction. By this method the authors determined concentrations of $9.7x10^3$ to $3.0x10^5$ phytoplasma cells per gram of tissue of Japanese plum of apricot buds. Martini *et al*. (2007b) were the first to publish a specific real-time assay based on primers selected in the *rplV* (*rpl22*) gene of '*Ca*. P. prunorum'. These authors also used the SYBR Green technology and the standard curve method for absolute quantification. However, they referred the measured concentration of phytoplasma cells to concentration of plant DNA in the analysed extract. The reported phytoplasma concentrations of $1.55x10^2$ to $6.18x10^3$ genome units per nanogram plant DNA of infected apricot trees is therefore not comparable to the data of Torres *et al.* (2005). Martini *et al*. (2007b) determined also the phytoplasma concentration in infested insects of the vector species *C. pruni* and found $1.07x10^7$ to $4.24x10^7$ phytoplasma cells per tested insect. Finally, '*Ca*. P. prunorum' primers ECA1/ECA2 (Jarausch *et al*., 1998) have also been employed in real-time PCR using SYBR Green I dye (Jarausch *et al*., 2009). In this work higher phytoplasma concentrations were determined: up to 10^9 phytoplasma cells per gram freshweight of infected apricot and up to 10^8 phytoplasma cells per individual of *C. pruni*.

TaqMan probes for the specific detection of '*Ca*. P. prunorum' were first described by Pignatta *et al.* (2008) who adapted a multiplex TaqMan assay published by Baric and Dalla-Via (2004) for the simultaneous detection of the phytoplasma and the plant host DNA. Whereas these authors did not quantify the phytoplasma, Thébaud *et al*. (2009) developed a new TaqMan assay with specific primers and probes in the '*Ca*. P.

prunorum' 16S rDNA and specific primers and probes in the *C. pruni* 18S rDNA for the absolute quantification of '*Ca*. P. prunorum' in its vector. These authors could show that the DNA extraction yield from single psyllids varied little as determined with the *C. pruni*-specific TaqMan assay. Therefore, they were able to refer the phytoplasma concentration to the tested insect as it was done in the other reports. Thébaud *et al*. (2009) measured the highest phytoplasma concentration of 4.9x 10^7 cells per insect in overwintered adults of *C. pruni*. Thus, the data of the different authors for the phytoplasma load of psyllids are all in a similar range of 10^7 to 10^8 phytoplasma cells per individual.

The use of real-time PCR for sensitive detection of '*Ca*. P. prunorum' showed that the detection threshold of real-time PCR is largely similar to that of nested PCR while real-time PCR assays greatly reduce the risk to obtain false positives due to contamination of laboratories arising from previous amplifications (Torres *et al*., 2005; Pignatta *et al*., 2008).

INTERACTION OF '*CA.* P. PRUNORUM' WITH THE PLANT

Host range. '*Ca*. P. prunorum' preferentially infects plants in the genus *Prunus*. It occurs in nature mainly on apricot, Japanese plum, and peach. However, the pathogen is also common on almond and flowering cherry (Seemüller *et al*., 1998a). On the basis of primer specificity and RFLP analysis of PCR-amplified DNA, '*Ca*. P. prunorum' infections have also been observed in naturally infected plants of *P. domestica*, *P. avium* (sweet cherry) and *P. cerasus* (sour cherry), *P. mahaleb*, *P. cerasifera*, *P. bokhariensis*, *P. brigantina*, *P. cocomilia*, *P. hollywood*, *P. orthosepal*, *P. simonii*, *P. spinosa*, *P. subcordata*, *P. cerasifera* x *P. munsoniana* (*P*. 'Marianna' GF 8/1), and *P. besseyi* x *P. hortulana* (Jarausch *et al*., 1994a, 1998a, 2000a; Poggi Pollini *et al*., 1995, 2005; Navrátil *et al*., 2001; Paltrinieri *et al*., 2001; Carraro *et al*., 2002). However, its causative role in yellows diseases of these taxa is not as clear, mainly because they are usually less affected and/or tolerant (Carraro *et al*., 1998b; Kison and Seemüller, 2001). By grafting and insect vector, '*Ca*. P. prunorum' was experimentally transmitted to several *Prunus* taxa listed above including *P. insititia*, *P. tomentosa*, *P. padus*, *P. laurocerasus*, *P. cerasus* x *P. canescens*, *P. fructicosa* x *P. avium*, *P. fructicosa* x *P. cerasus* (Kison and Seemüller, 2001; Carraro *et al*., 2004a). It has also been transmitted from diseased stone fruit trees to periwinkle and from periwinkle to tobacco (*Nicotiana tabacum*) via dodder (*Cuscuta campestris* and *C. reflexa*) bridges (Loi *et al*., 1995; Marcone *et al*., 1999a; Marcone and Seemüller, 2001). Moreover, '*Ca*. P. prunorum' was transmitted by grafting from *N. tabacum* to several other *Nicotiana* species and other solanaceous plants, including tomato (*Lycopersicon esculentum*) (Marcone and Seemüller, 2001). By PCR assays using specific non-ribosomal primers and RFLP analysis of PCR-amplified non-ribosomal and/or rDNA sequences, '*Ca*. P. prunorum' was detected in naturally infected plants of ash (*Fraxinus excelsior*), dog rose (*Rosa canina*), hackberry (*Celtis australis*), European hazel (*Corylus avellana*) and grapevine (*Vitis vinifera*) (Marcone *et al*., 1996b; Varga *et al*., 2000; Jarausch *et al*., 2001a; Duduk *et al*., 2004). However, further work based mainly on graft inoculation and vector transmission studies is needed to evaluate the significance of these non-*Prunus* species as alternative hosts of '*Ca*. P. prunorum'.

Symptom expression. '*Ca*. P. prunorum' induces symptoms mainly on leaves and shoots which can vary considerably depending on plant species, rootstock, scion cultivar, strain virulence, phytoplasma concentration, climatic condition, age of trees and insect vector population (Seemüller and Foster, 1995; Kison and Seemüller, 2001; Thébaud *et al*., 2006). The most characteristic symptoms which can be observed in most of the infected *Prunus* species are off-season growth and premature break of leaf buds before flowering. In summer, leaf yellowing or reddening in combination with leaf roll is the most common symptom. Detailed descriptions of symptoms are available elsewhere (Lederer and Seemüller, 1992; Seemüller and Foster, 1995; Laimer and Bertaccini, 2008). Also, trees intensively colonized in the aerial parts usually develop characteristic symptoms (Jarausch *et al*., 1998a, 2008). One major aspect of '*Ca*. P. prunorum' infections is the high mortality rate of apricot, Japanese plum and peach on susceptible rootstocks. Even more tolerant species like European plum may die after strong winter frosts when they are grafted on susceptible rootstocks. Typical ESFY symptoms are common in southern European regions but they have been recently observed in a similar way also in Germany (Jarausch *et al*., 2008).

Colonization behavior. Phytoplasmas differentially colonize plants, depending on the pathogen type, properties of the host and its specific reactions. '*Ca*. P. prunorum' can persist in the aerial parts of diseased trees during the dormant season whereas it is always present at root level throughout the year (Seemüller *et al*., 1998c; Jarausch *et al*., 1999a). Furthermore, Jarausch *et al.* (1999a) proved by tissue culture techniques that the ESFY pathogen colonizing the aerial parts during winter is viable. This behavior is in contrast to AP and PD agents, which owing to the degeneration of sieve tubes in the above-ground parts of apple and pear trees in late autumn and early winter, are, in almost all cases, eliminated in the aerial parts during winter while they persist in the roots where intact sieve tubes are present throughout the year. From the roots both

pathogens may recolonize the aerial parts in spring when new phloem is being formed (Schaper and Seemüller, 1984). However, monitoring of the '*Ca*. P. prunorum' population by DAPI fluorescence method and PCR assays has shown that the pathogen slowly colonized the leaves in spring, yielding thus a colonization pattern like AP and PD agents. Systemic colonization of diseased trees occurred from July throughout late fall.

Responses of *Prunus* genotypes to ESFY infections. It is well known that rootstocks significantly affect the response of grafted trees to '*Ca*. P. prunorum' infections. Thus, 23 established and experimental *Prunus* rootstocks, which are used in Europe were examined by graft-inoculation, PCR assays and DAPI fluorescence method to identify ESFY resistance in stone fruits (Kison and Seemüller, 2001). For inoculation, twenty differently virulent '*Ca*. P. prunorum' strains, which were collected from diseased almond, apricot, flowering cherry, Japanese plum and peach trees, were used. Inoculations were performed by grafting infected tissue onto healthy rootstocks and the inoculated plants were trained in a way that the shoot that developed from the inoculum was grown as scion. Symptom development was monitored over a 5 to 8-year observation period. The rootstocks examined showed considerable differences in their response to '*Ca*. P. prunorum' infections. The symptoms induced included foliar yellowing, foliar reddening, leaf roll, premature defoliation, off-season growth, reduced vigor, phloem necrosis, and death of trees. Also, mortality within the same rootstock and scion often varied considerably, depending on strain virulence. Trees on *P. domestica* stocks Achermann's, Brompton and P 2175 and *P. cerasifera* stock Myrabi (P 2032) were little affected. Most of these trees did not suffer from death of rootstock and scion nor showed foliar symptoms and reduced vigor. Moreover, phloem necrosis was rare and the scion was usually less affected than on other rootstocks, even in presence of severe strains. Slightly more damage occurred in trees on rootstocks GF 677 (*P. dulcis* x *P. persica*), *P.* 'Marianna' GF 8/1, and *P. insititia* stocks St Julien A and St Julien GF 655/2. However, some scion genotypes grown on these stocks were markedly damaged, as evidenced by mortality, foliar symptoms and phloem necrosis. Ishtara [(*P. cerasifera* x *P. persica*) x *P. salicina*], Myrobalan (*P. cerasifera*), and peach rootstocks Higama and GF 305 were found to be moderately to highly susceptible. Mild strains and also some severe strains only affected these rootstocks slightly. However, they were severely damaged by other highly aggressive strains. The most susceptible were peach rootstocks Montclar, Rutgers Red Leaf, Rubira, peach seedlings, apricot seedlings, and *P. insititia* stock St Julien 2. Losses up to 100% were recorded on trees of apricot and all of the most susceptible peach rootstocks. Trees on apricot rootstocks were most severely affected when the scion was apricot and the infecting strain was originating from apricot. In contrast, the susceptibility of trees on peach rootstocks appeared not to be affected by the kind of scion and origin of the inoculum. Off-season growth depended on both host and pathogen. It usually occurred in combinations of the more virulent strains and the more susceptible host. Of the flowering cherry trees grown on various rootstocks, the least susceptible were those on Gisela 3 (*P. fruticosa* x *P. avium*) and F 12/1 (*P. avium*) whereas Gisela 1 (*P. cerasus* x *P. canescens*), Weihroot 158 (*P. cerasus*) and Gisela 5 (*P. fruticosa* x *P. cerasus*) were more affected. The mortality of flowering cherry scions was often higher than that of the rootstocks. Phytoplasmal infections were detected by either DAPI fluorescence test or PCR assays, in all inoculated *Prunus* genotypes, irrespective of symptom expression. The colonization density was lower in little or not affected rootstocks than in susceptible rootstocks. There were also differences between colonization of the roots and the aerial parts of rootstocks. Usually phytoplasma population was higher in the roots than in the stem of the same genotype. In some rootstocks, infections were not found in the stem but only in the roots. Colonization appeared to be persistent over the observation period even in the least affected rootstocks such as *P. domestica* stocks Achermann's, Brompton and P 2175 and *P. cerasifera* stock Myrabi. Since trees on these rootstocks were also slightly damaged, and no recovery was observed, none of the examined genotypes could be considered resistant.

Jarausch *et al*. (2000b) examined the response of sixteen *P. domestica* genotypes which were grown as scion on *P.* 'Marianna' GF 8/1 to a French strain of the ESFY agent. Following graft-inoculation, the plants were observed over a 6-year period. Of the material tested, Prune d'Ente genotypes were shown to be moderately to highly susceptible. Among them, scion cultivars Primacotes, Tardicotes, Lorida and Spurdente were considerably damaged, as expressed by symptoms such as leaf roll, off-season growth, reduced vigor and productivity, and plant death. Little or not affected were several hybrids and X-ray mutants of Reine Claude (greengage) cultivars as they exhibited no mortality while only a few 'Fermareine' and P9184 trees showed off-season growth. By PCR assays, phytoplasmal infections could be detected in all inoculated genotypes, irrespective of symptom expression. However, detection frequency and phytoplasma concentrations were usually lower in the more tolerant hosts than in susceptible genotypes. For the Reine Claude genotypes P1771 and P1119, infections could only be detected in the rootstocks. Experimental inoculations of thirteen sweet cherry cultivars demonstrated a high level of resistance in *P. avium* towards '*Ca*. P. prunorum' (Jarausch *et al.*, 1999b).

Audergon *et al*. (1991) reported that 155 cultivars and selections of apricot grown on apricot seedling

A1236 Manicot, greatly differed in their susceptibility to the strain G 32 Noves of apricot chlorotic leaf roll, following experimental inoculation. Some of them, *e.g.*, 'Tardif de Bordaneuil', 'Caid Azdz 1', and 'Bebeco LA 2-A' proved highly susceptible to be used as indicators whereas other, *e.g.*, 'Jaubert Foulon', 'Max Gold', and 'Chastemi do' resulted tolerant or not infected.

Maintenance of '*Ca.* P. prunorum' in micropropagated plants. So far, biological studies on interactions of '*Ca.* P. prunorum' with its plant host are restricted to field-grown plants which are subjected to the vegetative cycle. As these experiments are long-lasting, attempts have been made to maintain the ESFY agent in micropropagated plants. This can be achieved by introducing ESFY-infected plant material into tissue culture as it has been first reported by Jarausch *et al.* (1994b). These micropropagated ESFY-infected cultures of *P.* 'Marianna' GF 8/1 were maintained up to now proving a stable culture of '*Ca.* P. prunorum' in its host plant for more than 18 years (W. Jarausch, unpublished data). ESFY-infected tissue cultures of *P. domestica*, *P.* 'Marianna', *P. armeniaca* and *P. persica*, have also been established by M. Laimer and co-workers (Laimer, 2003; Laimer and Bertaccini, 2008). These cultures have then been used to develop protocols for the elimination of '*Ca.* P. prunorum' from micropropagated plant material. ESFY-elimination could be achieved applying a combination of *in vitro* thermotherapy and meristem preparation (Laimer, 2003; Laimer and Bertaccini, 2008). In these studies, forty-three out of 48 plantlets regenerated from meristematic tissue of '*Ca.* P. prunorum'-infected peach proved to be healthy while the rate of the pathogen-free plants in regeneration of apricot plantlets was about 80%. Data reported by Jarausch *et al.* (1998b) indicate that simple axillary bud culture of ESFY-infected *in vitro* shoots is not sufficient to eliminate the phytoplasma. Plantlets regenerated from buds of only 2 mm in size remained still infected by '*Ca.* P. prunorum' as demonstrated by PCR detection. These data indicate that micropropagated plants are homogeneously infected with the pathogen and that reliable phytoplasma elimination can only be achieved by combining *in vitro* thermotherapy and meristem preparation. Plant tissue culture has also successfully been used to transmit '*Ca.* P. prunorum' by *in vitro* grafting (Jarausch *et al.*, 1999c). By this approach, ESFY-infected micropropagated *P.* 'Marianna' GF 8/1 scions were grafted onto healthy rootstocks of the same genotype. High transmission rates up to 94% were achieved within three months of growth. Thus, it could be shown that *in vitro* grafting may be useful for a preliminary screening of *Prunus* genotypes for ESFY resistance (Jarausch *et al.*, 1999c). Actually, this approach has regained new interest as the development of quantitative real-time PCR assays for determining the phytoplasma concentration in the inoculated genotype has provided new tools for resistance evaluation (Jarausch *et al.*, 2009).

Recovery. Studies conducted in France and Italy have shown that in apricot orchards severely damaged by '*Ca.* P. prunorum', some trees recovered spontaneously while the vast majority of them declined (Morvan *et al.*, 1986, 1991; Castelain *et al.*, 1997; Loi *et al.*, 2008; Ermacora *et al.*, 2009). In recovered trees a permanent disappearance of symptoms in the above-ground parts but not of the '*Ca.* P. prunorum' infections, occurred. However, the phenomenon of recovery or reduction of strain virulence is still poorly understood especially on molecular and biochemical basis. Musetti *et al.* (2005) investigated the effect of reactive oxygen species (ROS) on the recovery phenomenon in ESFY-affected apricot trees by comparing hydrogen peroxide and related metabolites and enzymes in leaves from recovered, symptomatic, and non-symptomatic infected plants. Substantial amounts of hydrogen peroxide occurred in the sieve tube plasma membranes of leaves from recovered plants but not in those from symptomatic and non-symptomatic plants. Leaves from recovered plants had a guaiacol peroxidase (GPX) activity, which keeps under control ROS generation, lower than that of leaves from symptomatic plants whereas the lowest GPX activity level occurred in non-symptomatic leaves. Also, the reduced glutathione, which can be oxidized to glutathione disulfide in presence of unstable molecules such as ROS, showed a content which was higher in recovered leaves than in symptomatic leaves. In contrast, the highest content of reduced glutathione was recorded in non-symptomatic leaves. From these data the authors concluded that the overproduction of hydrogen peroxide in recovered plants owing to a non-very active oxidant-scavenging system may account for reduction of strain virulence and disease severity, thereby playing a major role in the recovery of ESFY-infected apricot trees. Also, the study by Musetti *et al.* (2005) provided further evidence that '*Ca.* P. prunorum' infections persist in the aerial parts of recovered apricot trees.

Host plant genes differentially expressed upon '*Ca.* P. prunorum' infections. In contrast to progress made in detection, differentiation and phylogenetic classification of phytoplasmas, very little is known about the mechanisms by which phytoplasmas induce disease in plants and the reason for different reactions of the host plants to phytoplasmal infections. Carginale *et al.* (2004) reported on the identification of apricot genes whose pattern of expression changed in response to '*Ca.* P. prunorum' infections by using the differential display technique of mRNAs. Among the genes identified, a gene encoding the heat-shock protein HSP70, a gene encoding a metallothionein and another homologous to the expressed sequence tag 673 cDNA clone of *P. armeniaca*,

whose function was unknown, were up-regulated whereas a gene encoding a protein which showed a significant homology to an amino acid transporter of *Arabidopsis thaliana* was down-regulated. Heat-shock and metallothionein proteins are known to be produced in plants and many other living organisms to a high level in response to a variety of stress conditions. Thus, as concluded by the authors, a possible role of the induced metallothionein proteins in ESFY-infected apricot may be to reduce the concentration of free metal ions in the cell under pathogen attack and preventing an increase in reactive oxygen species. Metal ions may arise from enzymatic degradation of substances contained in the plant cells. Therefore, metallothionein proteins may be involved in sequestering and transport of metal ions from infected tissue to developing areas of the plant. Also, the fact that the expression level of an amino acid transporter gene is greatly reduced by '*Ca*. P. prunorum' infections indicates that the transport of amino acids which are essential for growth and development, to various plant parts including sink organs, is impaired in diseased plants. Amino acid transport also plays a key role in leaf senescence which is a well-controlled process during which the plant recycles substances from cells before death eventually occurs. Thus, the impaired amino acid transport that occurs in infected plants may account for some of the symptoms exhibited by these plants.

TRANSMISSION OF '*CA.* P. PRUNORUM'

Graft transmission. '*Ca*. P. prunorum' can be transmitted from tree to tree by grafting. Usually, it is efficiently transmitted by chip-budding from a suitable donor host to a recipient tree throughout the year including the winter because this pathogen is present in the aerial parts of trees even during the dormant season and is viable and transmissible (Seemüller *et al.*, 1998c; Jarausch *et al*., 1999a). '*Ca*. P. prunorum' is not seed-transmissible. However, a recent work by Necas *et al*. (2008) has shown that '*Ca*. P. prunorum' infections occurred in a few fruits and seeds collected from ESFY-affected apricot trees, as revealed by PCR assays. Also, seeds from '*Ca*. P. prunorum'-infected apricot trees had a viability which was 4.5 times lower than that of healthy seeds while the germination rate was 7 times lower than the healthy control. No phytoplasmal infections occurred in apricot seedlings originating from ESFY-affected trees. The ESFY pathogen has also been transmitted by the *in vitro* grafting technique (Jarausch *et al*., 1999c).

Insect vector transmission. In the recent years, progress has been made in understanding the epidemiology of the ESFY agent, in particular in insect vector transmission. The psyllid *Cacopsylla pruni* has been shown to vector the pathogen in various European countries such as Italy, France and Germany (Carraro *et al*., 1998a, 2001; Jarausch *et al*., 2001b, 2007a, 2007b, 2008). *C. pruni* is a European-Middle Asiatic species which is known to occur in several European countries (Lauterer, 1999). This psyllid is strictly oligophagous on *Prunus* spp., completes one generation per year and overwinters as adult on shelter plants, usually conifers. At the end of winter/early spring, overwintering adults move from the shelter plants back to stone fruit trees (primary hosts) for oviposition (Thébaud *et al.*, 2009). The insects of the new generation feed on the primary hosts until the beginning of July when they leave the stone fruits as adults to move to overwintering hosts. Studies by Carraro *et al*. (2001, 2004b) have shown that both springtime and overwintered adults were able to transmit the ESFY agent to healthy plants, and that the re-immigrant adults transmitted the winter-retained phytoplasma that had been acquired the previous year. In spring, 8.6% of overwintered adults proved to be infested by the ESFY agent and were highly infectious. A similar percentage of springtime adults proved to be phytoplasma-positive but showed a transmission efficiency lower than that of the overwintered adults. The authors concluded that most insects of the new generation, although having acquired the phytoplasma from the primary host, could not complete the latency on them. In experimental transmission trials, a minimum acquisition access period (AAP) between 2 and 4 days, a minimum latent period of 2-3 weeks and a minimum inoculation period of 1-2 days, could be defined. It also was shown that the psyllid transmitted the pathogen in a persistent manner. In Germany, studies performed by Jarausch *et al*. (2007a, 2007b, 2008) revealed that in spring 2-3% of the field-collected overwintered adults were infested by '*Ca*. P. prunorum'. Also, in experimental transmission trials the vector capacity of both overwintered and springtime adults proved to be lower than that described by Carraro *et al*. (2001, 2004a). Similar low infestation and transmission rates were recorded in France by Jarausch *et al*. (2001b) and Thébaud *et al.* (2008). The latter found that in transmission trials, 0.5% of the sampled re-immigrants were infectious whereas springtime adults of the new generation reared on infected plants showed a transmission efficiency of 0.6%. Recent work by Thébaud *et al.* (2009) has shown that the ESFY agent multiplied in its vector after acquisition by the emerging generation also during overwintering, therefore it is transmitted in a persistent-propagative manner. The full acquisition-latency-inoculation sequence could be accomplished only by a few springtime adults before migrating from *Prunus* spp. to conifers. In contrast, most new adults born on infected plants reached their maximum phytoplasma titer only after migrating to conifers in mountainous areas and that, after a latency of eight months, when migrated

back to *Prunus* spp., had a very high transmission efficiency (60%). Thus, secondary spread of the ESFY agent during the growing season appeared to be marginal in comparison to primary infections which originate from outside a given orchard.

Individuals of *C. pruni* which were naturally infested by the ESFY agent as revealed by PCR assays, and for which no transmission data are available, have been found in several European countries like Italy (Carraro *et al*., 1998a, 2001; Pollini *et al.*, 2004; Ferretti *et al*., 2009), France (Jarausch *et al*., 2001b; Yvon *et al.*, 2004, 2009), Spain (Laviña *et al.*, 2004), Czech Republic (Fialová *et al.*, 2004, 2007), Germany (Jarausch *et al*., 2007a, 2007b) and Bosnia and Herzegovina (Delić *et al.*, 2008). A vertical (= transovarial) transmission of the ESFY agent by *C. pruni* was not observed by Carraro *et al.* (1998a). However, Tedeschi *et al.* (2006) found indications for a possible transovarial transmission of '*Ca*. P. prunorum' by *C. pruni*. In this work, different developmental stages of the progeny of infested *C. pruni* females were examined by PCR technology. '*Ca*. P. prunorum' could be detected in eggs, nymphs and newly emerged adults. Also, in transmission experiments using nymphs and newly emerged adults originating from infested females, successful transmission of the ESFY agent to healthy plum plants was achieved in one case. Poggi Pollini *et al.* (2009), were also able to detect the ESFY pathogen in some eggs of *C. pruni*, by using the highly sensitive real-time PCR assays.

Wild *Prunus spp*. play an important role in the epidemiology of '*Ca*. P. prunorum'. Several studies in different European countries have shown that *C. pruni* prefers wild *Prunu*s spp. as main host plants (Carraro *et al.*, 2002; Delić *et al.*, 2008; Fialová *et al.*, 2004, 2007; Jarausch *et al.*, 2001a, 2001b, 2008; Labonne and Lichou, 2004; Laviña *et al.*, 2004; Pollini *et al.*, 2004; Ramel and Gugerli, 2004). During these studies many cultivated and wild *Prunus spp*. have been monitored for the presence of both the ESFY agent and the insect vector. Among the cultivated taxa, *P. armeniaca, P. persica* and *P. dulcis* were the most affected plants by '*Ca*. P. prunorum' but with generally low populations of the vector. In contrast, the highest vector densities were mainly recorded for wild *Prunus* spp. such as *P. spinosa, P. cerasifera, P. domestica* and *P. salicina*. Interestingly, *P. spinosa* and *P. cerasifera* which acted as reservoirs for the pathogen and the vector rarely showed typical symptoms (Carraro *et al.*, 2002; Jarausch *et al.*, 2008). Carraro *et al.* (2004a) investigated the susceptibility of 12 wild and cultivated *Prunus* spp. to '*Ca*. P. prunorum' as well as their capability to host the vector. They found that *P. armeniaca, P. cerasifera, P. domestica, P. persica, P. salicina* and *P. spinosa* can be considered as common and natural hosts of '*Ca*. P. prunorum', *P. avium* and *P. mahaleb* can be considered as non-common natural hosts of the agent, *P. laurocerasus* and *P. padus* can be considered as experimental hosts of the phytoplasma and *P. dulcis* and *P. tormentosa* can be considered as potential common and natural hosts. These findings indicate the epidemiological relevance of some wild *Prunus* genotypes which allow to both the ESFY agent and the insect vector to complete the cycle regardless of the presence of cultivated stone fruit trees.

BIOLOGICAL CHARACTERIZATION OF DIFFERENT STRAINS

Strain differences and strain interference. '*Ca*. P. prunorum' has been shown to exist as several strains, each varying in virulence and the ability to induce specific symptoms. Dosba *et al.* (1991) found differences in virulence when several French strains of '*Ca*. P. prunorum' were characterized pathologically by graft-inoculation to various *Prunus* genotypes. Also, some strains of the ESFY pathogen are known to induce off-season growth and premature bud break in a given rootstock-scion combination while other strains do not (Lorenz *et al*., 1994; Seemüller and Foster, 1995). ESFY-affected peach trees with symptoms which were very similar to those described for PYLR in California, have been observed in southern Italy (Marcone *et al*., 1996a). The most characteristic symptom of the affected trees is a pronounced enlargement of midribs and major lateral veins. Infection of peach by the ESFY agent is known to result sometimes in swollen veins whose occurrence and intensity may vary from year to year and with cultivars. However, it has never been observed in other areas in Europe at the same severity as in southern Italy. Although the cause for the pronounced symptom expression is not clear, it might be due to a specific strain or pathotype (Marcone *et al*., 1996a).

In France, during a 8-year survey of ESFY-infested apricot orchards in the Pyréneés-Orientales, among the diseased apricot trees, some trees were found to be little affected and never declined. From the latter trees, a strain which clearly differed from the more aggressive strains on the basis of symptoms induced in peach GF 305 seedling, following graft-inoculation experiments, was identified. This strain was designed as peach vein clearing (PVC) due to the leaf vein-clearing symptoms induced in peach GF 305 seedlings and subsequently was molecularly identified as '*Ca*. P. prunorum' (Cornaggia *et al*., 1995; Danet *et al*., 2007, 2008). Evidence that variation in symptom expression in a given plant host may be due to the occurrence of different strains also stems from Gentit *et al.* (1998). These authors used differences in symptomatology in peach GF 305 after graft-inoculation to distinguish '*Ca*. P. prunorum' strains from various *Prunus* genotypes from each other and from PVC. A severe strains of the X-disease phyto-

plasma which infects peach in North America could be distinguished from the '*Ca*. P. prunorum' strains as well.

In Germany, work by Kison and Seemüller (2001) has shown that twenty strains of the '*Ca*. P. prunorum' greatly differed in virulence when examined by graft-inoculation of trees on peach (Rubira, Rutgers Red Leaf, Montclar, GF 305), peach hybrid GF 677 and *P.* 'Marianna' GF 8/1 rootstocks. While some strains were nearly avirulent or weakly virulent and induced only mild foliar symptoms and slightly reduced vigor but no mortality, others were highly virulent and caused severe symptoms and a high mortality rate of affected trees. The scions were often more severely affected than the rootstocks. Also, severe strains caused distinctly more phloem necrosis than mild strains. Virulence often depended on the pathogen-scion combination and was in several cases most severe when a strain was maintained in or grafted to its original host which was grown as scion. However, in some instances the origin of the strain and the kind of scion were not crucial for strain virulence, as trees on peach rootstocks were severely affected when grafted with strains from sources other than peach. Furthermore, strains showing pathological differences were indistinguishable with techniques for routine phytoplasma differentiation and characterization such as sequence and RFLP analysis of PCR-amplified rDNA as well as Southern blot hybridization using probes suitable for differentiation of the fruit tree phytoplasmas of the AP group (Kison and Seemüller, 2001). Recent work by Marcone *et al*. (2009) using sequence analyses of less-conserved, non-ribosomal genes has revealed that genetic variability occurred among the above-mentioned strains within some genes. However, the genetic differences observed are neither suitable markers for strain differentiation nor linked to pathological traits.

Differences in virulence were also identified among '*Ca*. P. prunorum' strains infecting apricot trees in an orchard located in northeastern Italy in which infected trees and symptom development under natural infection conditions have been monitored since 1990 (Loi *et al*., 2008; Ermacora *et al*., 2009). Among severely affected trees, non-symptomatic infected trees and trees which showed a complete disappearance of symptoms while being '*Ca*. P. prunorum'-positive (recovered trees), were observed. Healthy apricots were graft-inoculated with scion wood from these three kinds of inocula and monitored for disease development over a 6-year period. The trees inoculated with symptomatic scions developed severe symptoms and these strains were accordingly classified as hypervirulent strains whereas trees inoculated with non-symptomatic scions remained non-symptomatic and these strains were thus regarded as hypovirulent strains. Also, transmission rates in graft-inoculation experiments were higher for hypervirulent strains than for hypovirulent strains. By quantitative real-time PCR assays, the phytoplasma concentration in trees infected by hypovirulent strains proved to be lower than in trees infected by hypervirulent strains (Loi *et al*., 2008; Ermacora *et al*., 2009).

Hypovirulent strains of '*Ca*. P. prunorum' have also been identified in recovered apricot trees in France (Morvan *et al*., 1986, 1991; Castelain *et al*., 1997). Healthy apricot trees inoculated with scions from recovered trees and grown under high infection pressure conditions developed no or only mild symptoms while non-inoculated healthy trees became severely diseased due to natural infection. Thus, recovered trees were harboring avirulent or low-virulent strains (protectant strains) which protect the plant from subsequent infection by virulent (severe) strains. As mentioned above, some of these strains along with PVC are distinguishable from severe strains of '*Ca*. P. prunorum' on the basis of branching patterns using phylogenetic analysis of *aceF* sequences (Danet *et al*., 2007, 2008). It is conceivable that strain interference (or cross protection) play a key role in the recovery phenomenon in which protectant strains may suppress severe strains. Interference between two strains of the '*Ca*. P. prunorum' has also been reported by Cornaggia *et al*. (1995). When peach GF 305 seedlings were graft-inoculated first with the severe ACLR strain and then with the low-avirulent PVC strain, the inoculated plants developed mainly leaf vein-clearing symptoms, indicative of PVC strain infections. However, in plants inoculated first with the PVC strain and then challenged by the ACLR strain, mild symptoms of the first strain developed. Plants inoculated with ACLR strain alone, died within four months. The results indicated a cross protection reaction. Interference between strains of the same taxon is also known for other phytoplasma-plant host combinations (Freitag, 1964; Sinclair and Griffiths, 2000).

MANAGEMENTS APPROACHES

Like other phytoplasmas, direct protection of trees from '*Ca*. P. prunorum' cannot be achieved by chemical control measures. However, the incidence of ESFY agent can be reduced significantly if proper attention is given to several other control measures. These include mainly the use of healthy planting material, effective control of the insect vector, removal of diseased trees and orchard management. Furthermore, data on experimental biological control by cross protection are also available. The most promising approach to control ESFY agent would be through the use of resistant plants.

Healthy planting material. The use of certified pathogen-free trees is recommended for establishing new orchards. Strategies to screen stone fruit crops for graft-transmissible agents including phytoplasmas, have

advanced substantially over the past decade (Rowhani *et al*., 2005). As '*Ca*. P. prunorum' persists in the stem during winter it can also be transmitted by infected dormant bud wood (Seemüller *et al.*, 1998c). This has great implications for epidemiological and quarantine purposes. In the case of no persistence such as for '*Ca*. P. mali' and '*Ca*. P. pyri', dormant scion wood can be grafted and shipped with no risk of pathogen transmission and spread. If the pathogen persists, dormant scion wood should be treated like bud wood during the growing season. Because nearly all stone fruit plants are produced by vegetative propagation, ESFY pathogen spread by latently infected, symptomless planting material is a great risk. Nowadays, nuclear stocks for rootstocks and scion wood have to be tested regularly with a highly sensitive and specific PCR procedure to ensure that they are free from infections. The propagation material in the nursery has then to be protected from natural infections by vector control.

In vitro thermotherapy and meristem tip culture techniques have been used for eradication of '*Ca*. P. prunorum' infections in diseased *Prunus* plants (Laimer, 2003; Laimer and Bertaccini, 2008). Furthermore, cryotherapy of shoot tips which is known as a newly pathogen eradication method, has been successfully applied to eliminate several graft-transmissible pathogens including phytoplasmas from crop plants belonging to six families. Although this method has not yet extended to ESFY-infected *Prunus* genotypes, it has the potential to replace more traditional methods like meristem culture for production of healthy planting material (Wang *et al*., 2009).

Vector control. As overwintering as well as springtime generation of the vector *C. pruni* may transmit the pathogen, vector control has to start in early spring with insecticide treatments directed against the re-immigrant adults which are highly infectious (Carraro *et al.*, 1998a, 2001). A second treatment at the period of egg deposition should prevent the development of a new generation in the orchard. Another spray program may focus on the control of the developing new generation as the new generation is potentially infective when born on diseased trees and is able to transmit the pathogen when leaving the primary hosts. However, insecticide application might not be possible during blossom and has to be selective in late spring and summer in order to minimize damage to beneficial insects. Furthermore, insecticides efficient for the control of psyllids in stone fruits have still to be evaluated and/or are not authorized in every country. Early detection and uprooting of infected trees is of paramount importance to reduce the inoculum sources and the further spread of the pathogen. In addition, wild *Prunus* species such as *P. spinosa*, *P. cerasifera* and *P. domestica* present a further risk for the disease spread. They may harbor the pathogen without obvious symptoms and are known to be preferred hosts of the psyllid *C. pruni* (Carraro *et al*., 2002). Therefore, wild *Prunus* species present within or around ESFY-infested orchards should be carefully monitored and removed if they test phytoplasma-positive.

Orchard management. Another important control measure to slow down the disease spread can be appropriate orchard management. Most of the commonly used rootstock genotypes based on *P. domestica*, *P. cerasifera* or *P. salicina* tend to produce more or less important rootstock suckers. However, these young developing suckers are the preferred host plants of *C. pruni* (Labonne and Lichou, 2004). Much higher psyllid populations have been found on the suckers than on the scion cultivar. A consequent elimination of the rootstock suckers before the migration period of *C. pruni* is therefore strongly recommended (Labonne and Lichou, 2004).

Resistance. Since '*Ca*. P. prunorum' is able to overwinter in the above-ground parts of the trees, resistance of both rootstock and scion cultivars is required to prevent damage by the disease. Also, as rootstocks significantly affect the response of grafted trees to '*Ca*. P. prunorum' infections, the choice of a suitable rootstock may prolong productivity of infected trees. In a previous work, many established and experimental rootstocks and scion cultivars were examined by graft-inoculation, PCR assays and DAPI fluorescence tests to identify resistance in stone fruits. For inoculation, several differently virulent '*Ca*. P. prunorum' strains were used (Kison and Seemüller, 2001). Both rootstocks and scion cultivars showed considerable differences in their response to '*Ca*. P. prunorum' infections. However, satisfactory ESFY resistance could not be detected. Only trees on *P. domestica* stocks Achermann's, Brompton and P 2175 and *P. cerasifera* stock Myrabi were litte affected. Slightly more damage occurred in trees on rootstocks GF 677, GF 8/1, and *P. insititia* stocks St Julien A and St Julien GF 655/2. The remaining tested genotypes including Ishtara, Myrobalan, peach and apricot rootstocks were found moderately to highly susceptible and, thus, cannot be recommended for commercial growing (Kison and Seemüller, 2001). Also, the scions were often more severely affected than the rootstocks. An interesting level of resistance has been observed in several hybrids and X-ray mutants of Reine Claude cultivars (Jarausch *et al.*, 2000b).

Cross protection with avirulent, or weakly virulent strains. Control of ESFY has been attempted by cross protection in France (Morvan *et al*., 1986, 1991; Castelain *et al*., 1997). The cross protection effect among '*Ca*. P. prunorum' strains has been observed over 20 years, indicating therefore a stable establishment of the causal

agent. However, information on a detailed molecular characterization, mode of action, insect vector transmissibility, and safety aspects of protectant strains is still lacking. Also, it is largely unknown whether protectant strains identified in apricot trees in France show cross protection efficiency either on other stone fruit species or against severe strains occurring in other geographic areas of Europe.

CONCLUDING REMARKS

The wealth of molecular and epidemiological data available highlights the unique properties of '*Ca.* P. prunorum', which is relatively homogeneous genetically although it infects a range of different *Prunus* species. Although '*Ca*. P. prunorum' infections have been detected in other plant species than stone fruits, the relevance of the occurrence of ESFY agent in other plants is not clear. Its detection is mainly based on the highly sensitive nested PCR assays with no data from pathological studies. Therefore, '*Ca*. P. prunorum' appears to be a highly *Prunus*-specific pathogen. Also, it is transmitted by only one vector, *C. pruni*, and all attempts to find further vector species failed (J.L. Danet and W. Jarausch, unpublished information). Increasing amount of reports on the occurrence of '*Ca*. P. prunorum' in Europe and neighbouring Mediterranean regions is a clear evidence for a dynamic spread of the pathogen but also for an increased alertness of growers and advisors for this economically damaging agent. The relatively genetic homogeneity and a high plant host and insect vector specificity rise interesting questions about the evolution of the causal agent and the disease. The most susceptible *Prunus* genotypes originate from Asia while tolerant ones are native to Europe. Tolerance or resistance to '*Ca*. P. prunorum' would be expected in regions where the pathogen and its plant hosts have co-evolved, while lack of tolerance or resistance could indicate recent introduction of either the pathogen or the host. Also, the vector *C. pruni* is a European species with limited spread in adjacent Asian countries. Thus, it appears that the distribution of '*Ca* P. prunorum' is tightly linked to the distribution of its vector and that the economic incidence of ESFY has raised dramatically with the introduction of highly susceptible genotypes into Europe. Ongoing phylogeographic studies on the ESFY agent and its vector will shed light into the evolution of the disease in near future. Recent data indicating a monophasic spread of ESFY offers new insights into the efficacity of vector control strategies and the risk assessment and management (Thébaud *et al*., 2009). Despite its apparent genetic homogeneity, several strains of '*Ca.* P. prunorum' with varying degrees of virulence have been described. Although preliminary attempts to exploit avirulent strains in cross protection strategies have been made in restricted areas where these strains were discovered, new research is needed to develop cross protection procedures as an efficient, safe and environment-friendly tool to control ESFY on larger scale. This research can now be addressed since molecular tools to characterize protectant strains and elucidate their mode of action, are available. Natural resistance to '*Ca.* P. prunorum' is still poorly understood and needs further investigations. The role played by the rootstocks for the spread of the disease has not been taken into account sufficiently. Resistant rootstocks may limit the incidence of the disease in the scion cultivar whereas rootstock suckers, especially in susceptible genotypes, may be important entry ports for '*Ca*. P. prunorum' as they are attractive for its vector.

In contrast to progress made in understanding molecular detection and characterization, taxonomy, phylogeny, and epidemiological aspects of '*Ca*. P. prunorum', very little is known about genes mediating pathogenicity or virulence. A number of putative pathogenicity factors are known from the complete genome sequences of the few phytoplasmas which have been determined so far (Hogenhout *et al*., 2008; Kube *et al*., 2008; Tran-Nguyen *et al*., 2008). However, sequence comparisons of the entire genome from a virulent and an avirulent strain of '*Ca*. P. prunorum', would provide insights into the largely unknown mechanisms involved in pathogenicity of this *Prunus*-infecting agent.

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