Assunta Bertaccini · Phyllis G Weintraub
Govind Pratap Rao · Nicola Mori *Editors*

Phytoplasmas: Plant Pathogenic Bacteria - II

Transmission and Management of Phytoplasma - Associated Diseases

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ISBN 978-981-13-2831-2 ISBN 978-981-13-2832-9 (eBook)
https://doi.org/10.1007/978-981-13-2832-9

Library of Congress Control Number: 2019930474

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Phytoplasma-associated diseases are a major limiting factor to the quality and productivity of many ornamental, horticultural, and other economically important agriculture crops worldwide. Annual losses due to phytoplasma diseases vary in many crops, but under pathogen-favorable conditions, they always lead to disastrous consequences to farming communities. There is no effective cure for phytoplasma diseases; the management options emphasize pathogen exclusion, to minimize their spread by insect vectors and propagation materials, and development of host plant resistance. The scientific literature concerning transmission, epidemiology, and management of phytoplasma-associated diseases is growing at a fast pace. Significant advancements have been made on these perspectives in the last decade. Very few compilations are available to show the progress of phytoplasma research on epidemiology and management aspects hence, the major recent research findings are compiled in this book.

The book covers recent and updated information on epidemiology, means of transmission, and management of phytoplasma-associated diseases in 11 chapters contributed by experienced and recognized scientists.

We most sincerely acknowledge all the contributed authors for their earnest efforts in synthesizing the most updated reviews on the subjects. We also like to thank the support and input of the publisher, Springer Nature, for its effort to publish this book. We strongly hope that the book will be useful to everyone interested in phytoplasma research, plant pathology, microbiology, plant biology, and agriculture and serve as an exhaustive and up-to-date reference on the various applied aspects of phytoplasma-associated diseases studied during the past decades.

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About the Editors

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Chapter 1
Insects as Phytoplasma Vectors: Ecological and Epidemiological Aspects

Alberto Alma, Federico Lessio, and Herbert Nickel

Abstract The different aspects involved in the transmission of phytoplasmas by insect vectors (leafhoppers, planthoppers, and psyllids) are presented from an ecological point of view. The epidemiology of phytoplasma-associated diseases is a consequence of the vectors' ability in acquisition, inoculation, dispersal, survival, host range, and habitat colonization. Within the same vector species, acquisition efficiency may depend on the phytoplasma load in source plants and on the vectors' life instar (nymphs *versus* adults). Inoculation may occur earlier or later in the season, depending on the availability of phytoplasma sources and/or possible presence of transovarial transmission. Monophagous and oligophagous species are generally more efficient vectors than polyphagous ones. Among grass feeders, many vector species are considered oligotopic. Ecotones, plant patches, and plant architecture affect the movement and survival of vectors. Vectors' flight activity and spatial distribution, which may differ depending on gender, affect the spread of phytoplasmas and their epidemics may follow an open or a closed cycle. Five examples of diseases, with different phytoplasma cycles (open/closed) and one or more insect vectors involved, are presented: grapevine "flavescence dorée", Palatinate grapevine yellows, grapevine "bois noir" and maize redness in Europe; aster yellows in USA; sugarcane white leaf yellows in South-East Asia; and coconut lethal yellowing in North and Central America.

Keywords Transmission · Open and closed cycles · Host-range · Habitat · Dispersal

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_1

1.1 Introduction

The epidemics of phytoplasma diseases are a consequence of the network formed by pathogens, plants, insect vectors, environmental factors, and farm management. However, although many other aspects of phytoplasma diseases have been widely covered in previous studies, the influence of the vector ecology has often been overlooked. Therefore, insect vectors of phytoplasmas in their ecological and epidemiological aspects are presented in this chapter. The key point will be the transmission process, from an epidemiological point of view, focusing on the sources and sinks of phytoplasmas in insects and/or plants, and on the environmental factors affecting vectors' populations and therefore phytoplasma spread. Five examples of phytoplasmas' epidemics which involve different host plants (e.g. mono and dicothyledons, trees *versus* herbaceous), different plant associations, in different parts of the world, and having different life cycles and behaviour of insect vectors will also be described.

1.2 The Transmission Process: Acquisition, Latency and Inoculation

The typical transmission process of phtyoplasmas consists of three phases: (i) acquisition access period (AAP) when phytoplasmas are sucked from the phloem sieve tubes by the vector's mouth parts (insects are infected); (ii) latency period (LP, or LAP), necessary for phytoplasma multiplication and circulation inside the insect body, including the salivary glands; and (iii) inoculation access period (IAP), when phytoplasmas are injected into the host plant (insects are infective). Therefore, each species has a typical AAP, LP, IAP. Phytoplasmas are transmitted by insect vectors in a persistent-propagative manner, requiring short AAP (a few days), long LP (weeks) and medium-short IAP (Alma et al. 2015). The pathways of phytoplasmas inside the vectors, their influence on vectors' physiology, the possibility of transovarial transmission, the mechanisms regulating the vectors' specificity, the physiology of feeding strategies, have been widely investigated (Weintraub and Beanland 2006; Wilson and Weintraub 2007; Bosco and D'Amelio 2010; Bosco and Tedeschi 2013; Alma et al. 2015) and will be covered in dedicated chapters of the present volume.

Acquisition The acquisition is mainly performed by nymphs, which hatch from the egg on host plants which are already infected (Alma et al. 2015). Nymphs of vectors (leafhoppers, planthoppers, and psyllids) are generally sedentary, moving from plant to plant only by walking or jumping. In some cases (e.g. Cixiidae) they are born and develop underground on the roots, therefore, acquisition may be successful only if adults are laying eggs directly on infected plants. A manipulation of phytoplasma infection on attracting vectors has been observed in *Scaphoideus titanus* Ball, the main vector of "flavescence dorée" (FD) phytoplasmas (16SrV-C and –D): both nymphs and adults are more frequently found on grapevine

FD-infected leaves compared to healthy ones (Chuche et al. 2016). It is not clear if the cues involved are olfactory or visual, although leafhoppers in all stages are more attracted by yellow rather than green. Acquisition may have different efficiency depending on the life instar. Fifth-instar nymphs of *Euscelidius variegatus* (Kirschbaum) are more efficient in acquiring 16SrI-B phytoplasmas from infected daisies than first instar ones, whereas no differences were found in *Macrosteles quadripunctulatus* (Kirschbaum) (Palermo et al. 2001). Nymphs of *S. titanus* are capable of acquiring FD phytoplasmas from grapevine only from the third instar on (Chuche and Thiéry 2014). This important aspect when dealing with pest management strategies may be due also to a lower phytoplasma load in grapevine plants in the early season. In fact, the application of insecticides, particularly insect growth regulators (IGR) require a good timing depending on the life cycle of *S. titanus,* in order to prevent nymphs from acquiring FD phytoplasmas from infected grapevines (Rigamonti et al. 2011; Chuche and Thiéry 2014). However, in certain environmental situations, this could be less important because of infective adults coming from external sources (Lessio et al. 2007b, 2014, 2015) (Example 1). Recent findings suggest that *S. titanus* adults may be capable of acquiring phytoplasmas and transmitting them within just 2 weeks, making pest management of nymphs with IGRs or other active ingredients less important (Alma et al. 2018). In fact, the ability of adult vectors performing AAP has been seldom tested. *M. quadripunctulatus* and *E. variegatus* acquiring the 16SrI-B phytoplasma agents of chrysanthemum yellows (CY) from infected daisy represents another case: for both species, acquisition was successful after 7 days AAP, whereas it was significantly reduced when AAP lasted only 1 day (Palermo et al. 2001). In other cases, it is not clear if acquisition is made by adults or nymphs. For instance *Haplaxius crudus* (van Duzee), the vector of coconut lethal yellowing in Florida and Mexico, is supposed to acquire the phytoplasmas in the adult stage feeding on infected coconut palms, but this aspect has not been clarified yet. The length of AAP varies depending on phytoplasmas, host plant source, and insect vector species. In *S. titanus* the AAP of FD phytoplasmas by nymphs from infected grapevine (*Vitis vinifera* L.) and broadbean (*Vicia faba* L.) is known to last approximately 7 days (Chuche and Thiéry 2014). However, the same species is able of acquiring (at the nymph stage) also 16SrI-B phytoplasmas from infected grapevines with an AAP of 3 days, and from infected daisies (*Chrysanthemum carinatum* L.) with an AAP of 1–3 days (Alma et al. 2001). Sometimes, nymphs stay overtime on the same host plant and therefore the AAP is difficult to measure, but also it is not so important from an epidemiological point of view. For instance, nymphs of *Hyalesthes obsoletus* Signoret feed and overwinter on roots of stinging nettle (*Urtica dioica* L.), where they are able to acquire '*Candidatus* Phytoplasma solani' ("stolbur") (Lessio et al. 2007a), however the length of AAP has never been investigated. Acquisition efficiency also depends on other factors. One of the most important is the phytoplasma load in host plants. In *S. titanus* nymphs, AAP's efficiency increases along with phytoplasma load in grapevine, depending on the season (Galetto et al. 2014; Roggia et al. 2014), the cultivar (Roggia et al. 2014; Bressan et al. 2005; Galetto et al. 2016), and the status of the disease (e.g. recovered plants are a poor phytoplasma source) (Roggia et al. 2014). Acquisition of more than one

phytoplasma strain is possible, at least from a physiological point of view (Alma et al. 2015). For instance, *E. variegatus* is able of acquiring and inoculating both FD and chrysanthemum yellows (CY, 16SrI-B) phytoplasmas feeding on different sources, with little competition on salivary glands colonization and no competition on transmission efficiency (Rashidi et al. 2014). However, in nature it seems more difficult for a single insect vector (especially in the nymph stage) to acquire different phytoplasmas from different plant sources. Therefore, mixed infections in the same insect under field conditions may not result in inoculation ability of both phytoplasmas, but may be due to random acquisition possibly by adults moving from one plant to another.

Latency The length of latency period depends on the multiplication kinetics of phytoplasmas in the vector's body. For instance, it has been demonstrated that 16SrI-B (CY) phytoplasma multiplies faster in *M. quadripunctulatus* (LP = 18 days) than in *E. variegatus* (LP = 30 days) (Bosco et al. 2007). Factors influencing the LP include temperature and carbon dioxide (Galetto et al. 2011) and this may result in shorter/longer LP depending on the season, with consequences on the diseases' outbreak. Recently, it has been demonstrated that adults of *S. titanus* are capable of acquiring phytoplasmas from infected broad beans, and of transmitting them to healthy broad beans after short LP (Alma et al. 2018). In this case under lab conditions, adults acquired FD phytoplasmas (subgroup 16SrV-C, FD-C) after an AAP = 7 days on experimentally infected broad bean plants, and inoculated it after only 7 days of IAP on healthy broad bean plants, with overlapping of AAP, LP and IAP. Actually, LP lasted at least 14 days including AAP and IAP (AAP + LP + IAP $\leq$ 14 days), whereas previously it was thought that *S. titanus* required a LP of 35–42 days, or a minimum of 21 days under laboratory conditions (Chuche and Thiéry 2014). This shorter LP may be due to many different factors; for instance, an AAP performed by adults may have permitted a higher phytoplasma intake, or temperature may have accelerated phytoplasma multiplication.

Inoculation is generally made by adults. Nymphs cannot fly and are unable to move from an infected to a healthy host plant; moreover, usually they become adults during LP. Inoculation may occur in different moments of the season, depending on the biology of vectors and their infective status. Early-season inoculation happens when adult vectors arriving into crop fields are already infective. This is possible, for instance, in migratory species like the aster yellows vector leafhopper, *Macrosteles quadrilineatus* (Forbes), which arrives in Ohio from Southern States as migrating infective females responsible of triggering the epidemics in lettuce crops (Hoy et al. 1999). Another chance of early inoculation occurs when phytoplasmas are transmitted to the progeny. Transovarial transmission allows the vector to maintain a source of inoculum throughout generations, without relying on host plants as sources. This is particularly important for phytoplasmas affecting annual crops. One of the most important examples is the sugarcane white leaf disease: phytoplasmas (16SrXI-B group) are maintained transovarically by *Matsumuratettix hiroglyphicus* (Mastumura). On the other hand, when phytoplasmas are acquired by insect vectors

within the growing season, the starting of inoculation depends mainly on the length of latency period. For instance, when *S. titanus* acquires FD phytoplasma in the nymph stage, it takes generally 4 weeks to complete LP and to turn into adults capable of flying and spreading the disease (Chuche and Thiéry 2014). Inoculation takes place from the end of July and is more frequent in August and September because of the higher phytoplasma load in insects. However, since new findings have demonstrated that under laboratory conditions *S. titanus* adults are capable of completing AAP + LP + IAP on broad beans within 2 weeks (Alma et al. 2018), inoculation may occur following AAP by adults on grapevine regardless of the growing season. The major concerns are about late summer and beginning of autumn, before harvest, when no or little insecticides are sprayed.

1.3 Host Plants of Insect Vectors

Host range and host plant species The degree of specialization or plasticity of vectors with respect to their host plants drives their ability of spreading phytoplasma diseases. According to Nickel and Remane (2002) and Nickel (2003), Hemiptera are classified as follows depending on their host range: first degree monophagous (m1): 1 plant species; second degree monophagous (m2): 1 plant genus; first degree oligophagous (o1): 1 plant family; second degree oligophagous (o2): 2–5 plant families, or up to 5 plant species; polyphagous (p): other. Monophagous species (m1 and m2) are often more efficient vectors than polyphagous ones, leading to a closed epidemiological cycle (Constable 2010; Alma et al. 2015). One of the most important cases is *S. titanus*, a grapevine specialist that may be considered as m2 since its host plants include *V. vinifera*, and other *Vitis* species as well as rootstock hybrids (Chuche and Thiéry 2014). Generally, specialist insects are more likely to be reduced by increased management pressure e.g. pesticides and/or mowing, whereas polyphagous species are more likely to adapt. This goes for instance about *Psammotettix alienus* (Dahlbom), another vector of CY (16SrI-B) (Alma et al. 2015), which becomes dominating in low biodiversity habitats, whereas *H. obsoletus* and *S. titanus* are negatively influenced by it (Trivellone et al. 2012).

After Nickel (2003) who analysed the Auchenorrhyncha fauna on the Central European flora, species-rich plant families such as Poaceae, Cyperaceae, Rosaceae and Asteraceae also, tend to have more Auchenorrhyncha species than species-poor plant families, which may be explained by a facilitated insect radiation on closely-related host plants. Even more important is the host plant size, with trees generally holding a higher load of insects than most herbaceous plants. The proportion of monophagous species (first and and second degree together) is highest on Pinaceae, Salicaceae, Cyperaceae, and Poaceae. This pattern of host specialisation is difficult to interpret and may be caused by different factors for different plant families, e.g. secondary compounds in the former two and neural constraints (Bernays 2001) in the latter.

According to the "Resource Concentration" hypothesis, an insect should tend to remain in dense stands of its host plant (Root 1973). Therefore, specialists rely on plants that are capable of dominating, whereas generalists may be more likely of exploiting assemblages of diverse host plants. Concerning vectors, the majority of oligo- and polyphagous species are associated to herbaceous hosts. Some species live on weeds, although adults may occasionally move to trees and shrubs: this happens with *H. obsoletus* moving from nettle and bindweed (*Convolvolus arvensis* L.) to grapevine (Alma et al. 1988; Weber and Maixner 1998; Lessio et al. 2007a). The same behavior may be observed in *Dictyophara europaea* (L.), an occasional vector of 16SrV phytoplasmas to grapevine (Filippin et al. 2009), which lives on many weeds including pigweed, *Amaranthus retroflexus* L., and occasionally moves onto grapevines (Lessio and Alma 2008; Krstic et al. 2016). *Euscelis incisus* (Kirschbaum) is polyphagous on grasses and weeds, but it transmits 16SrIII-B phytoplasmas following an open epidemiological cycle from *Lathyrus* spp. (source) to *Cirsium arvense* L. (sink) (Jakovljevic et al. 2015). Another species, *Neoaliturus fenestratus* (Herrich-Schaeffer) transmits 16SrII phytoplasmas to *Picris hieracioides* L. plant-to-plant (closed epidemiological cycle), but only the second generation seems to have vector ability (Mitrovic et al. 2012). *N. fenestratus* may be considered a first-degree oligophagous (o1), feeding and breeding mainly on species in the family Asteraceae (Nickel and Remane 2002; Minuz et al. 2013; Lessio et al. 2017b). Oligophagous species seem to be quite sensitive to plant, and particularly to sward, architecture (Blake et al. 2011), and their fluctuations in time may be due to changes in vegetation height and cover. An exception is *Orientus ishidae* (Matsumura), a polyphagous species related to many trees and shrubs (Lessio et al. 2016). To date, there is no evidence that populations of *O. ishidae* are different from one host to another. In fact, it may be found in great numbers on single trees or shrubs, provided that food resources remain available. *Fieberiella florii* (Stål), an occasional vector of the apple proliferation phytoplasma (16SrX-A) (Tedeschi and Alma 2006) is also considered polyphagous, feeding and breeding on trees and bushes of many broadleaf species, including especially Rosaceae (Nickel and Remane 2002). Both *O. ishidae* and *F. florii*, however, are considered as occasional vector species.

Pioneers, aurytopic, oligotopic and stenotopic species Regarding their life strategies, grassland leafhoppers and allies can be divided into four subgroups, respectively: (i) pioneer species (highly mobile generalists, polyphagous or broadly oligophagous), (ii) eurytopic species (widespread generalists, usually oligophagous), (iii) oligotopic species (specialists of certain habitats, usually oligophagous), and (iv) stenotopic species (specialists of habitats, monophagous) (Nickel and Achtziger 2005). Among vectors, oligotopic species include *H. obsoletus*, whereas *E. incisus* is eurytopic and *P. alienus*, *Macrosteles cristatus* (Ribaut) and *M. laevis* (Ribaut) are pioneers (Trivellone et al. 2012; Nickel and Achtziger 2005). On the other hand, *Macrosteles septemnotatus* (Fallén) is reported as stenotopic (Nickel and Achtziger 2005), however its vector ability has not been proven. It is possible that few or no stenotopic species are phytoplasma vectors because of their strict host and environmental needs. From an evolutionary point of view, this may result in

little vector efficiency as stenotopic species are less likely to build up great populations, which makes it difficult to spread phytoplasmas.

1.4 Movement and Dispersal

Ecotones and movement of insect vectors among habitats Phytoplasma diseases are not restricted to a single host plant or crop. Because of the movement of vectors, the same phytoplasma may be carried from one plant species to another, and from one patch to another within the same ecosystem or even – through aerial drift – across long distances up to hundreds and thousands of kilometres. The proximity of habitats may influence the movements of vectors between crops or from spontaneous vegetation to crops. This happens sometimes for tree feeders moving between different host plants depending on their degree of specialization/plasticity. It is the case of *S. titanus* which moves from wild to cultivated grapevine (Lessio et al. 2014), of *O. ishidae* from hazelnut or willow to grapevine (Lessio et al. 2016), and of *Oncopsis alni* (Schrank) moving from alder to grapevine (Maixner and Reinert 1999). A more specialized alternation of tree hosts is observed in many psyllids. For instance, *Cacopsylla melanoneura* (Foerster) and *C. picta* (Foerster), vectors of apple proliferation, overwinter on pine trees (family Pinaceae) and move to apple (*Malus* spp.: *C. melanoneura* and *C. picta*) and/or hawthorn (*Crataegus* spp.: only *C. melanoneura*) trees for feeding and breeding (Lauterer 1999; Tedeschi et al. 2009; Alma et al. 2015). On the other hand, *Cacopsylla pyricola* (Foerster) and *Cacopsylla pyri* (L.) are considered monophagous (m2), being related only to pear trees (*Pyrus* spp.) (Lauterer 1999; Tedeschi et al. 2009; Alma et al. 2015). In many other cases, feeders of grasses and herbs are compelled to move on trees or shrubs when the herb layer dries up during the hot season. In Israel, *H. obsoletus* completes two generations per year, and the adults of the second one move onto grapevine because the weeds disappear due to drought stress (Orenstein et al. 2003). As well, in Europe it moves from nettle to grapevine (Mori et al. 2015) or from clary sage (*Salvia sclarea* L.) to lavender (Hossard et al. 2018) although in the latter case there is no grass/tree host alternation. *H. crudus* in the nymph stage lives on the roots of grasses within coconut plantations, whereas the adults move on palm leaves to feed and mate. In this case, it seems however that the movement of adults on palm trees is compulsory for this species to feed and mate. Many other species are known to feed occasionally on grapevine in the adult stage, apart from the already-cited *D. europaea* (Lessio and Alma 2008), and *N. fenestratus* (Bosco et al. 1997; Minuz et al. 2013). *P. alienus* relies mainly on monocotyledons (Gramineae) (Lindblad and Areno 2002; Nickel and Remane 2002; Manurung et al. 2005; Landi et al. 2013). Since soil coverage of vineyards influences and enhances the presence of some leafhopper species (Mazzoni 2006), *P. alienus* is more abundant in vineyards with a grassy (monocotyledon) ground cover. *E. variegatus* is also highly polyphagous (Nickel and Remane 2002) and develops on a wide range of host plants either in field margins or in the vineyard interrow (Lessio et al. 2017b). However, both *E. variegatus* and *P. alienus* are not caught in great numbers on the grapevine canopy

(Bosco et al. 1997). Some species, on the other hand, lay eggs on woody plants but nymphs move onto grasses. This is the case of *Anoplotettix fuscovenosus* (Ferrari) which lays eggs under the bark of grapevine but nymphs move on grasses in the interrow lanes (Alma 1995; Alma et al. 2015). Recently, it has been demonstrated that *S. titanus* nymphs may leave grapevine and move on weeds, especially *Trifolium repens* L. and *Ranunculus acris* L., in the inter-row (Trivellone et al. 2013) that may however, not be considered as host plants since no oviposition occurs. Habitat diversification has an influence on the dispersal and abundance of vectors: for instance, the aster leafhopper *M. quadrilineatus* is more abundant in carrot fields when surrounded by spelt (*Triticum spelta* L.) or other cereals with respect to broadleaf weeds (Szendrei 2012), and *Psammotettix* spp. becomes a dominant species due to a loss of habitat conservation (Hollier et al. 2005; Trivellone et al. 2012).

The influence of plant patches and plant architecture Other important factors affecting vectors' populations are the size and shape of plant patches and the diversification of vegetation layers. In other terms, they are influenced by both horizontal and vertical distribution of their host plants. Species that lay eggs into the ground are usually favored by patches of bare soil, alternated to plant swards. This is the case of *D. europaea*, polyphagous and feeding on many weeds (Lessio and Alma 2008; Krstic et al. 2016). This species is common in xerothermic habitats with isolated grass patches and portions of bare soil, used for laying eggs (Nickel and Remane 2002), and may move on trees and shrubs such as wild and cultivated grapevine (Lessio and Alma 2008) and *Clematis vitalba* L. (Krstic et al. 2016) during the dry season. Other important factors influencing leafhoppers and planthoppers communities are the diversification of the vegetation layer, and the structure of plant swards, the latter having an influence especially on oligophagous species (Blake et al. 2011). For instance, in pastures, *M. laevis* and *E. incisus* respond positively to sheep grazing as they rely mainly on short sward architecture (Brown et al. 1992). On the other hand, structural complexity of plants promotes diversity of associated insect guilds. For instance most tussock grasses have more leafhoppers than non-tussock grasses and tall tree species have more than smaller ones (Nickel 2003).

The flight boundary layer A definition of the "flight boundary" layer in insects, especially leafhoppers, was given by Taylor (1974), as "a layer of air where the flight speed of insects is faster than wind speed", and therefore insects are capable of active movement. Species relying on grasses and weeds, usually are not caught in great numbers higher than ground level. This has been observed on grapevine for *N. fenestratus, H. obsoletus, Euscelis* sp. and *P. alienus* (Minuz et al. 2013), and also for *D. europaea* (Lessio and Alma 2008).

Movement of the insect vectors is one of the most important factors influencing epidemiology of phytoplasma diseases. Leafhoppers and planthoppers tend to disperse mainly in the adult stage. Two main patterns may be distinguished: "free-air"

movement, and "road map" movement. The former happens when a vector species disperses in any direction, regardless of the environmental constrictions. It is typical for grass-feeding species, and for small species that tend to rely on wind. On the other hand, tree-dwellers are not likely to be often carried by the wind, and rely mainly on active movements. In particular, they need the presence of ecological corridors to move along. It is the case of *S. titanus*, that needs a network of wild grapevine for moving along grapevine-growing areas (Lessio et al. 2014). As well, the intercropping lettuce/endive or lettuce/escarole limits the movement of *M. quadrilineatus* with respect to lettuce monoculture (Zhou et al. 2003).

Spatial patterns of plants and insect vectors Spatial distribution of vectors often reflects the displacement of their own host plants. *S. titanus* adults are generally aggregated at the edges of vineyards, because of specimens moving from neighbouring stands of wild grapevines (Lessio et al. 2014; Pavan et al. 2012). Moreover, generally they are more likely to infest the same rows within the vineyard rather than moving from row to row (Lessio et al. 2009a). As well, *O. ishidae* adults are more concentrated at the edges of vineyards because they are moving from host plants outside e.g. willow and hazelnut. However, in this case fewer individuals may be found within the vineyard because grapevine is not a favourite host plant (Lessio et al. 2016). Other species which have a spatial distribution matching that of their own host plants are those living on grasses and weeds: *N. fenestratus,* and *P. alienus* (Minuz et al. 2013), and also *H. obsoletus* (Mori et al. 2012; Minuz et al. 2013).

1.5 The Influence of Vector Sex Ratio on Phytoplasma Epidemics

A different feeding and/or dispersal behaviour between genders may have an influence on the epidemics of phytoplasma diseases. Generally males of leafhoppers and planthoppers hatch earlier in the season than females. In *S. titanus,* the *sex ratio* is more male-biased at the beginning of the season, whereas in late summer and autumn many more females are found (Lessio et al. 2009b). Since the phytoplasma load in grapevines increases with the growing season, late born nymphs and/or late emerged adults (mainly females) are more likely acquiring them. Moreover, females have a longer lifespan compared to males (70 *versus* 50 days) (A. Alma, unpublished), consequently they are capable of transmitting for a longer period of time. Females of *M. quadrilineatus* are more likely transmitting agents of aster yellows diseases (16SrI) with respect to males; however, they spread less than males in lettuce fields, and this results in a more clustered infection pattern in crops (Beanland et al. 1999). In particular, mated females are much more sedentary, whereas unmated ones are able of performing vertical flights, and males are more engaged in local movements (Hoy et al. 1999).

1.6 Modelling the Epidemics

The challenge of modelling epidemics of phytoplasma diseases and their vectors has been faced many times, with respect both to space (spatial models, diffusion models, etc.) and time (phenology models, outbreak models, etc.). Post-embryonic development of *S. titanus* has been modelled as a function of temperature, in order to make pest management more efficient (Rigamonti et al. 2011; Falzoi et al. 2014). A similar model was applied to *C. pyri* (Schaub et al. 2005). An attempt was also made to predict infestations of *S. titanus* during different seasons (Maggi et al. 2013). Other models have been elaborated to forecast FD epidemiology. A deterministic model taking into account the influence of different factors such as recovery, plant replacement, insecticidal sprays, and the presence of hotbeds has been developed. In this model, the insect vector is not present explicitly, but has been introduced as a coupling factor between healthy and infected grapevines (Lessio et al. 2015). A stochastic epidemiological model was implemented taking into account the variations of *S. titanus* within the vineyard due to survival from one year to another, but without considering immigration sources (Maggi et al. 2014). The infection of carrots by aster yellows has been modelled too, as a function of the abundance and infectivity of *M. quadrilineatus*, the main vector of this phytoplasma in the study area (Frost et al. 2013). Finally, another important issue is the forecast of the potential spread of a vector when introduced into a new geographical area. Models of this kind have been proposed about the spread of *S. titanus* in China (Ge and Wen 2006) and Chile (Quiroga et al. 2017).

1.7 Open *versus* Closed Cycle

The epidemiology of phytoplasmas may follow either an open or a closed cycle. Open cycles occur when the phytoplasmas are transmitted by vectors from one plant species (source) to another (sink, or dead-end), and not the other way round. Conversely, closed cycles occur when the phytoplasmas are transmitted always between host plants of the same species (Constable 2010; Alma et al. 2015). However, a phytoplasma rarely follows just one type of cycle: in many cases, both cycles are present, although they may rely on different insect vectors. Feeding specialization has an influence on vector ability. Generally, monophagous vectors lead to a closed epidemiological system of the pathogen, with AAP, LP and IAP spent on the same plant species, whereas polyphagous species are more likely to become occasional vectors, within the frame of an open epidemiological system (Alma et al. 2015). Five examples are presented below about phytoplasma epidemics. A summary of phytoplasma groups and vectors, type of cycle, overwintering strategy of phytoplasmas, and plant source is given in Table 1.1.

Example 1 "Flavescence dorée" phytoplasmas and other phytoplasmas of group 16SrV in grapevine: one vector for closed cycle and three vectors for open cycle.

Table 1.1 Epidemiological cycles of selected plant diseases associated with phytoplasma presence

Phytoplasma ribosomal group/ subgroup	Insect vector	Epidemiological cycle	Overwintering sources	Plant sources
16SrI-B	*Macrosteles quadrilineatus*	Closed	Migrating females	Lettuce and carrots
16SrIV	*Haplaxius crudus*	Closed	Unknown	Coconut palm trees
16SrV-C/−D	*Scaphoideus titanus*	Closed	*Vitis* spp.	
	Dictyophara europaea	Open	*Clematis vitalba*	
	Orientus ishidae	Open	Broadleaf trees?	
16SrXI-B	*Matsumuratettix hiroglyphicus Yamatotettix flavovittatus*	Closed	Transovarial transmission	Sugarcane
16SrXII-A	*Hyalesthes obsoletus*	Open	Nymphs on weeds' roots	Nettle, bindweed and other weeds
	Reptalus spp.	Open		Unknown

FD is associated with the presence of phytoplasmas enclosed in the 16SrV ribosomal group (subgroups 16SrV-C and 16SrV-D) (Martini et al. 1999). The main vector is *S. titanus* (Fig. 1.1), which has been introduced into Europe from North America in 1958 (Chuche and Thiéry 2014). It is a second-degree monophagous (m2) species, feeding and breeding on *Vitis* spp. including *V. vinifera* (European grapevine), American grapevines such as *Vitis labrusca* (L.), *Vitis riparia* Michx, and *Vitis berlandieri* Planchon, as well as rootstock hybrids (Chuche and Thiéry 2014), which are also a source of FD phytoplasmas although they might not show any symptoms (Lessio et al. 2007b). *S. titanus* is monovoltine (Fig. 1.2), and overwinters in the egg stage laid under the bark (Chuche and Thiéry 2014). During summer, adults may move from wild grapevines into vineyards (Pavan et al. 2012; Lessio et al. 2014). This behaviour has been demonstrated with mark-capture techniques, by applying a protein marker (chicken egg whites, or milk) directly on wild grapevine stands and placing traps at different distances from them (Lessio et al. 2014). The majority of adults were captured within 20 m from the source, however some individuals have covered distances up to 350 m (Lessio et al. 2014). Successive experiments showed that in a few cases *S. titanus* may travel at distances up to 2.5 km. Immigrant adults may be already infected, having acquired phytoplasmas on wild grapevines (Lessio et al. 2007b). Moreover, a recent research carried out under laboratory conditions suggested that they may acquire phytoplasmas directly from infected cultivated grapevines (Alma et al. 2018). Either way, *S. titanus* transmits FD phytoplasmas only grapevine-to-grapevine (closed cycle) (Chuche and Thiéry 2014). However, as *S. titanus* has been introduced into Europe from North America, where FD is not known to occur, it has been suggested that other vectors

Fig. 1.1 *Scaphoideus titanus*: second instar nymph (left), fifth instar nymph (middle) and adult (right) (DISAFA, Entomology unit, University of Torino, Italy)

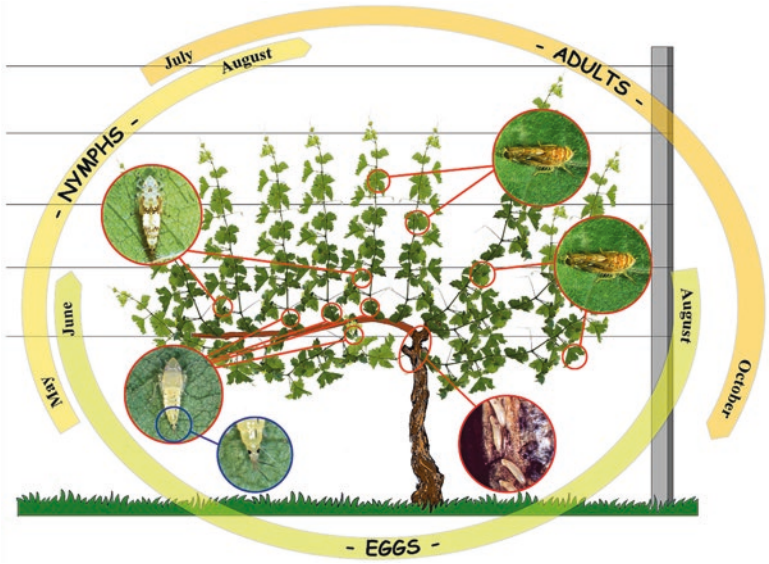

Fig. 1.2 *Scaphoideus titanus*: life cycle (Artwork L. Picciau)

had a role in introducing the phytoplasma from wild plants to grapevine (Belli et al. 2010). In fact, FD phytoplasma may also rely on insect vector species that are bound to other host plants but are able of moving and feeding on grapevines too. These are the cases of *O. alni* (Maixner et al. 2000), *D. europaea* (Filippin et al. 2009), and *O. ishidae* (Lessio et al. 2016). Just like *S. titanus,* all these species are monovoltine and overwinter in the egg stage.

O. alni (Fig. 1.3) lives on alder maintaining alder yellows phytoplasma (AldY) in a closed cycle, but is able to move and feed on grapevines where in Germany the phytoplasma is associated with Palatinate grapevine yellows disease (PGY) (Maixner and Reinert 1999). However, this movement is not so frequent since *O. alni* is a second-degree monophagous species (m2) being strictly associated with alder (Nickel and Remane 2002), therefore it is likely that other vectors are involved in transmitting PGY from alder to grapevine. The genetic variability of AldY has been recently reviewed by Hotz et al. (2016), and a recent survey (Jarausch et al. 2017) has demonstrated that phytoplasmas in the 16SrV group were found at high rates (65%) in *Allygus* spp. and *O. ishidae*.

D. europaea (Fig. 1.4) is able to transmit 16SrV-C phytoplasmas from *C. vitalba* to grapevine under experimental conditions (Filippin et al. 2009). In Piedmont (North West Italy), it was found infected mainly by 16SrV-C phytoplasmas (Gonella et al. 2017). However, adults do not move very often onto grapevine (Lessio and Alma 2008; Lessio et al. 2017b). Eggs are laid into the soil, especially in presence of bare ground patches, and host plants include many grasses and weeds e.g. *A. retroflexus, Solidago canadensis* L., and *Urtica dioica* L. (Lessio and Alma 2008; Krstic et al. 2016). *D. europaea* is considered therefore as a polyphagous species (Nickel and Remane 2002). When placing yellow sticky traps on the grapevines' canopy, few adults are captured (Lessio and Alma 2008). A recent research on

Fig. 1.3 *Oncopsis alni* (Schrank): nymph (left) and adult (right) (Courtesy of G. Kunz)

Fig. 1.4 *Dictyophara europaea* (L.): nymph (left) and adult (right) (Courtesy of G. Kunz)

leafhopper fauna of foraging strip crops (a mix of alfalfa and other legumes) close to vineyards demonstrated that *D. europaea* is abundant only if the strip is poorly covered by the seed mix, and infested by weeds; on the other hand, it is absent or irrelevant when the seed mix provides a good cover of the strip (Lessio et al. 2017b). For these reasons, *D. europaea* may not be considered as an important vector of FD phytoplasmas to grapevine.

Finally, *O. ishidae* (Fig. 1.5) is an Asian species, introduced into the USA in the early nineteenth century, probably along with ornamental plants of the genus *Aralia* sp., and reported for the first time in Europe (Switzerland) in 2002 (Guglielmino 2005). Its importance has been overlooked until some specimens were found to be positive to 16SrV phytoplasmas in Slovenia (Mehle et al. 2010), and in northern Italy (Gaffuri et al. 2011; Zambon et al. 2018). Recently its ability of transmitting 16SrV-C phytoplasmas from broad bean to grapevine under laboratory conditions has been demonstrated (Lessio et al. 2016). At the moment, sources of phytoplasma inoculum for *O. ishidae* in nature are still uncertain. Nymphs are able of acquiring FD phytoplasmas from infected grapevine, too (Lessio et al. 2016), and they have also hatched from eggs laid under the bark of wild and cultivated grapevine under laboratory conditions (Lessio et al. 2016, 2017a). However, nymphs were seldom found on grapevine leaves (Lessio et al. 2017a), which makes an acquisition of FD phytoplasmas less likely from this source. This leafhopper is polyphagous, relying on many trees and shrubs including willow, hazelnut, birch, hornbeam, apple, plum, bramble, and so on (Lessio et al. 2016) (Fig. 1.6). Recently, the same phytoplasmas have been detected both in specimens of *O. ishidae* and in willow and hazelnut (Casati et al. 2017). High populations of *O. ishidae* were also found on *Ailanthus altissima* L., which is also a host for 16SrV phytoplasmas (Filippin et al. 2011; Forte et al. 2013). It is therefore likely that this leafhopper is responsible for an open epidemiological cycle of FD phytoplasmas, by an AAP from other plant sources and IAP to grapevine, although further studies are needed to confirm this hypothesis.

All these species (apart from *O. ishidae*, which is not native from Europe) may have triggered the infection on grapevines moving from their own host plants. Then, when *S. titanus* appeared in European vineyards, the newly-born vector-pathogen association led to heavy disease outbreaks.

Fig. 1.5 *Orientus ishidae*: nymph (left) and adult (right) (Courtesy of G. Kunz)

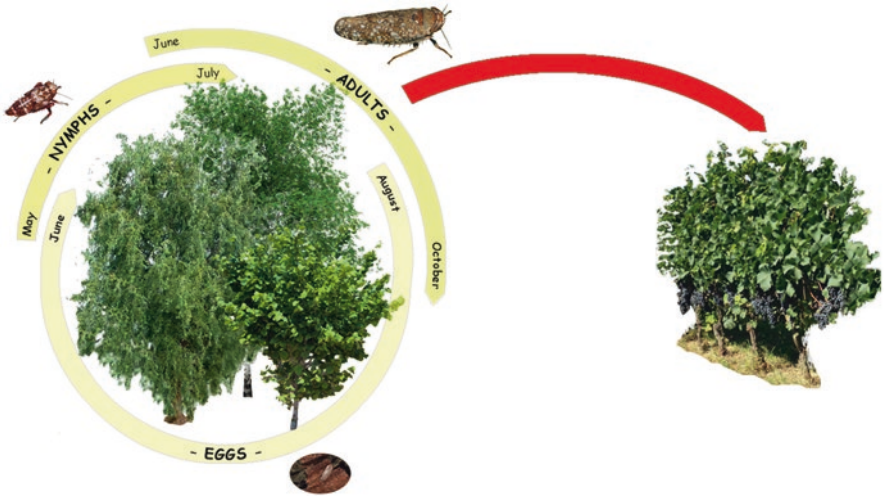

Fig. 1.6 *Orientus ishidae*: life cycle (Artwork L. Picciau)

Example 2 "Bois noir" (BN) in grapevine: same vector(s), open and closed cycle on different host plants. In other cases, the same vector is responsible for maintaining the same phytoplasma in both open and closed cycles. This happens with *H. obsoletus* (Fig. 1.7) and "stolbur" phytoplasmas (16SrXII-A), transmitted plant-to-plant to bindweed and to stinging nettle (closed cycles), and occasionally from the former host plants to grapevine (open cycle) (Sforza et al. 1998; Weber and Maixner 1998; Lessio et al. 2007a). Hence, weeds may be a reservoir for BN phytoplasma, favouring its diffusion in vineyards, and grapevine is a dead-end host for this pathogen. In Israel, where no other sources of BN phytoplasmas have been detected, grapevines are supposed to act as reservoirs (Sharon et al. 2015). However, no current evidence of grapevines being a source of inoculum for *H. obsoletus* are available. Indeed, while nettle, bindweed, and also lavender (Hossard et al. 2018) are the main host plants for *H. obsoletus* in Europe, in Israel this planthopper builds up great populations on *Vitex agnus-castus* L. (Sharon et al. 2005; Zahavi et al. 2007). However, this plant is not a source of phytoplasmas and may also be used in push-and-pull strategies for controlling *H. obsoletus* (Zahavi et al. 2007). BN may also be transmitted by *Reptalus panzeri* (Löw) and *Reptalus quinquecostatus* (Dufour) (Fig. 1.8) (Alma et al. 2015). However, to date the host plants for nymphs of these species are still little known. Adults are found on many trees and shrubs including willow, hornbeam, elm (Picciau et al. 2008), but the sources of inoculum remain unclear. An intermediate kind of cycle concerns maize redness (MR), associated with the presence of the same phytoplasma. In Serbia, MR is transmitted by *R. panzeri* which relies on the rotation between corn and wheat. Infective adult planthoppers feed and lay eggs on corn during summer, and nymphs perform AAP on roots of infected plants. However, when corn is harvested, it is replaced by wheat, which provides food for nymphs performing LP. Adults emerge at the end of the spring and the cycle starts again (Jovic et al. 2009).

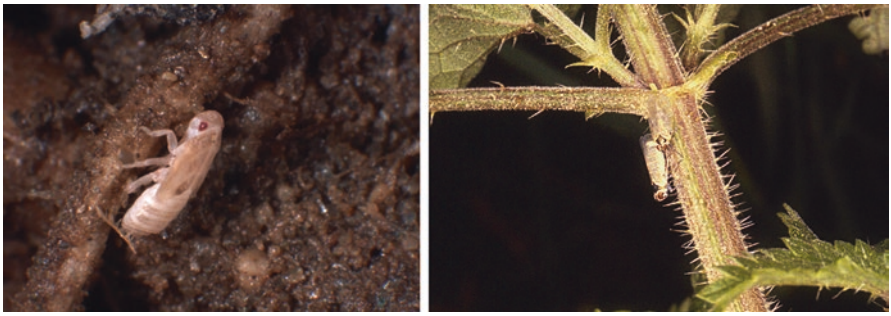

Fig. 1.7 *Hyalesthes obsoletus*: nymph (left) and adults (right) (DISAFA, Entomology unit, University of Torino, Italy)

Fig. 1.8 *Reptalus panzeri*: nymph (left) and adult (middle), and *Reptalus quinquecostatus* adult (right) (Courtesy of G. Kunz)

Example 3 Aster yellows phytoplasmas: a migrating vector. Aster yellows phytoplasmas (AY), belonging to 16SrI ribosomal group, include a huge number of subgroups and are responsible for infecting a wide number of host plants (Duduk and Bertaccini 2009; Alma et al. 2015). They are transmitted worldwide by many different insect vectors. In the European and the Mediterranean area, ten or more species are listed including members of the genera *Macrosteles, Euscelidius, Euscelis* (Alma et al. 2001, 2015)*, N. fenestratus, Psammotettix* spp. (Alma et al. 2015), as well as *Empoasca decipiens* Paoli and *S. titanus* under laboratory conditions, the former with a very low transmission efficiency and possibly without any significance in the field (Alma et al. 2001, 2015). However in the USA the main vector appears to be *M. quadrilineatus*, which is absent in Europe. *M. quadrilineatus* (Fig. 1.9) is a migratory species: overwintering females, bearing AY phytoplasmas, move from Texas and other south States to Ohio, and are responsible for a primary transmission on vegetable crops such as carrot and lettuce (Hoy et al. 1992, 1999;

Fig. 1.9 *Macrosteles quadrilineatus* adult (Courtesy of T. Murray)

Beanland et al. 2005). Immigrant females are clustered at the edges of crop fields, according to patterns of AY infected plants in early spring (Beanland et al. 2005). Moreover, females appear to be more efficient vectors than males at least under laboratory conditions (Beanland et al. 1999). Meanwhile, resident populations overwinter in Ohio in the egg stage, taking advantage of winter cover crops (small grains) (Hoy et al. 1992). These populations, however, do not bear phytoplasmas as no transovarial transmission occurs. Afterwards, insects from the incoming generations start transmitting AY phytoplasmas plant-to-plant. In this case, LP is shorter and permits a closed epidemiological transmission cycle. A similar result has been noted in *M.quadripunctulatus*, which has a LP of 18 days for AY phytoplasma whereas *E. variegatus*, another vector, has a LP of 30 days (Bosco et al. 2007).

Example 4 Sugarcane white leaf phytoplasmas, an insect vector with transovarial transmission and a synergy between two vectors. Sugarcane white leaf disease (SCWL or SWL) is one of the most threatening diseases of sugarcane (*Saccharum officinarum* L.) in South-East Asia. It is associated with the presence of phytoplasmas in the 16SrXI-B ribosomal group (rice yellow dwarf) (Soufi et al. 2013; Zhang et al. 2016). Demonstrated insect vectors are the two leafhoppers *Matsumuratettix hiroglyphicus* (Matsumura) (Hanboonsong et al. 2002) and *Yamatotettix flavovittatus* Matsumura (Hanboonsong et al. 2006) (Fig. 1.10). Transmission seems to occur plant-to-plant in sugarcanes, without alternative hosts since phytoplasmas identified in alternative host plants (e.g. grasses) are genetically different. Therefore, the epidemiology of SCWL must be considered as a closed cycle. However, since sugarcane has an annual cycle, the survival of inoculum must take place elsewhere. In fact, phytoplasma agents of SCWL are transmitted to the progeny of *M. hiroglyphicus* (Hanboonsong et al. 2002). The two insect vector species act somehow in synergy. In Thailand, the flight peak of *M. hiroglyphicus* in sugarcane fields occurs approximately a month earlier with respect to *Y. flavovittatus* (April *versus* May).

Although both species share similar efficiencies of SCWL transmission (55% *versus* 45%), the former is more capable of inoculation to young sugarcane plants than the latter. Therefore, SCWL epidemics in sugarcane plantations is triggered by *M. hiroglyphicus,* but is continued by *Y. flavovittatus* (Hanboonsong et al. 2006).

Fig. 1.10 *Matsumuratettix hiroglyphicus* (left), and *Yamatotettix flavovittatus* (right) adults (Courtesy of A. Cymru)

Dispersal is also different between the two vector species: *Y. flavovittatus* is able to travel downwind at twofold distances than *M. hiroglyphicus* (380 *versus* 160 m) (Thein et al. 2012). Many other leafhopper species are known to bear SCWL phytoplasmas, but their vector ability has not been proven, besides the fact that their biology e.g. abundance in sugarcane crops do not make them likely to be putative vectors. *Exitianus indicus* (Distant) was the only tested species (along with the two proven vectors), and did not transmit SCWL phytoplasmas in Thailand (Hanboonsong et al. 2006).

Example 5 Coconut lethal yellowing: closed cycle for phytoplasmas, different host plants for the insect vector. Phytoplasma-associated diseases in palms have been detected in the Southern States of the USA (Texas, Florida, and Louisiana), Mexico and Caribbean, Western and Eastern Africa, Australasia and Oceania (Gurr et al. 2016; Bander et al. 2017; Harrison et al. 2008; Harrison and Elliott 2016; Myrie et al. 2007). Phytoplasma diseases of coconut palm, *Cocos nucifera* L., belong mainly to subgroups in 16SrIV ribosomal group (Brown et al. 2007; Gurr et al. 2016). Among different phytoplasma diseases of palm trees, the most widely studied is coconut lethal yellowing (CLY) for which the only reported insect vector is *H. crudus* (Fig. 1.11). This planthopper is heterovoltine: nymphs live on roots of grasses such as Bermudagrass (*Cynodon dactylon* L.) and especially St. Augustine grass [*Stenotaphrum secondatum* (Walt.)] within coconut plantations, whereas adults move onto the leaves of palms for feeding and mating, and females go back to grasses for egg laying (Tsai and Kirsch 1978). In this case, grasses do not seem to be a reservoir for phytoplasmas. It is therefore possible that AAP is performed by

Fig. 1.11 *Haplaxius crudus* adult (Courtesy of J-L. Dzido)

adults feeding on infected palms, and subsequent inoculation on healthy palms. However, the movement of turf grass may contribute to spread infected vectors around (Harrison and Elliott 2016). The first attempt of transmission was done by Howard et al. (1983) by caging coconut palms along with (or without) adult planthoppers. Only exposed plants developed symptoms (34 months after exposure), and young palms seemed more likely to be infected than older ones. Both the removal of grasses and the spray of insecticides on coconut palms resulted in a decrease of the symptom severity. Later on, the phytoplasma presence in *H. crudus* was confirmed by PCR (Harrison and Oropeza 1997). All the available information provides evidence that CLY has a closed epidemiological cycle, from palm to palm, relying on *H. crudus*. Recently, the same phytoplasmas have been identified in *Cedusa* sp. (Brown et al. 2007). As well, nymphs of *H. crudus* have been found also on roots of palm trees (Reinert 1977). However, *H. crudus* seems to rely mainly on grasses for oviposition, as documented by Howard (1990). It is not clear if AAP is performed by adults feeding on infected palm leaves, or nymphs on infected palm roots, although the first hypothesis seems to be more likely. *H. crudus* is also able of vectoring 16SrIV-D to date palms in Texas (Dzido et al. 2012). According to Halbert et al. (2014), *H. crudus* is able of survival beyond the northern limit of CLY distribution; therefore, the geographical limitations may be due to the pathogen itself and not to the absence of an insect vector.

1.8 Conclusions

The epidemiology of phytoplasma diseases is strictly related both to the ecological needs and to the biology of insect vectors. The first key point appears to be the phytoplasma sources that trigger the epidemics. Acquisition on infected plants at the beginning of the season, migration of overwintering infective vectors, and transovarial transmission are some of the pathways that phytoplasmas follow in order to reach host plants. The choice of a specific strategy depends on the biology of plants and insect vectors: migration and transovarial transmission are successful for inoculating annual crops, whereas phytoplasma diseases of tree crops are easily

maintained in plants along years and they often follow a closed epidemiological cycle. Likewise, the degree of specialization influences the efficiency of insect vectors, monophagous species being more active than polyphagous ones. That is why phytoplasmas transmitted by generalist species may share more than one vector. The second key point is related to the mobility of insect vectors, as long-range flyers are capable of spreading phytoplasma diseases very quickly. Clustering or random distribution of symptomatic plants are also the result of vector movement, and flight activity of a vector species may differ depending on gender. Finally, vector population growth depends on the host plant assemblage and architecture. The "pathogen-vector-plant-habitat" complex must therefore be considered as a single issue when dealing with the epidemics of phytoplasma diseases.

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Chapter 2
The Biology and Ecology of Leafhopper Transmission of Phytoplasmas

Phyllis G. Weintraub, Valeria Trivellone, and Kerstin Krüger

Abstract Leafhoppers (Hemiptera: Cicadellidae) have long been known to transmit a number of plant pathogens, although the elucidation of the vector-host plant-pathogen relationships are far from well-defined and irrefutable. Due to their small size, the phloem-limited bacterial pathogens in the taxon '*Candidatus* Phytoplasma' were only visualized some 50 years ago. They are difficult to culture, hence their relationships with both their insect vectors and host plants present an ongoing scientific struggle. Precise phylogenetic knowledge of the vector and bacteria may eventually allow the prediction of potential vector-phytoplasma associations. As leafhoppers are poikilothermic, abiotic factors figure strongly in the development of both the insect host and the bacteria within, which in turn affects pathogen transmission. As a group, their life cycle is varied from univoltine to multivoltine and monophagous to polyphagous, and their phytoplasma-associations are equally varied. Furthermore, adult leafhoppers are strong flying insects and some have been documented to move thousands of kilometers. When aided by human conveyance, both the vectors and the pathogens have been transported among continents. In this chapter all these interactions are explored.

Keywords Cicadellidae · Phylogenetic · Host-pathogen · Behavior · Movement

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_2

2.1 Introduction

There are hundreds of plant species, both monocots and dicots, that show yellows disease symptoms, but without apparent source of pathogen entry; such as, graft junctions, mechanical damage, chewed leaves, parasitic plant presence. While there are a number of wind or soil/water borne pathogens, notorious for stealthy entry into the plant vascular system are the hemipterans. With their slender mouthparts, no apparent marks are left on the plant after they have imbibed sap; and if in the process of feeding they transmitted a pathogen, it may be weeks or months before symptoms of disease are apparent in the plant, long after the insect vector has left. In a historical context, before Doi et al. (1967) first visualized what they thought were mycoplasmas in phloem elements, the pathogen was thought to be a virus because of its small size. The first experimental transmission of a pathogen by a leafhopper, *Recilia* (=*Inazuma*) *dorsalis* (Motschulsky), (reported as a virus) was in Japan in 1883 where it was confirmed again in 1893 (Fukushi 1969). However the early history of leafhopper transmission of pathogens may be confusing (Ou 1985), in addition to phytoplasma *R. dorsalis* transmits *Rice dwarf virus*, *Rice tungro virus* (Arocha Rosete and Jones 2010), *Rice gall dwarf virus* (Chen et al. 2016) and *Rice stripe mosaic virus* (Yang et al. 2017). This one vector species serves to illustrate the complex relationship between vector, host plant and pathogen transmission and the need for detailed studies to ferret out all the interactions.

Of the approximately 20,000 identified leafhoppers (Dietrich 2008) there are relatively a few species (ca. 100) that are confirmed vectors, so care must be taken when trying to understand specific pathogen-vector relationships. In this chapter the systematics and taxonomy of the Cicadellidae especially in relation to (potential) vector capacity, the biology of leafhoppers in general, leafhopper-host plant and leafhopper-phytoplasma relationships, and the means of movement and dispersal on local and inter-continental levels are explored.

2.2 Taxonomy of Phytoplasma Insect Vectors

Auchenorrhyncha (Fulgoromorpha and Cicadomorpha) are a suborder of Hemiptera comprising four superfamilies and 32 families. Not considering cicadas, the main groups of taxa are more commonly referred to with five trivial names which reflect, to some extent, host and feeding site preferences, biological habits and/or appearance: sharpshooter, leafhopper, treehopper, planthopper, froghopper or spittlebug. As such, sharpshooters comprises two tribes in the subfamily Cicadellinae (Cicadellini and Proconiini) and the name refers to type of damage observed for the first time on *Homalodisca vitripennis* (Gemar); leafhoppers comprising all species belonging to Cicadellidae indicating those species which mainly feed on leaves. Treehoppers comprises all species belonging to the families Aetalionidae,

Melizoderidae, and Membracidae, and planthoppers comprising all families in the superfamily Fulgoroidea, these last two trivial names suggest their remarkable resemblance to arboreal plants or to leaves in their feeding habitat. However, the above mentioned common names are not necessarily appropriate for all taxa in each particular group.

The phytoplasma vectors all belong to the order of Hemiptera, Auchenorrhyncha and Sternorrhyncha suborders. In the Auchenorrhyncha, the superfamily containing the largest number of competent and potential vectors are the Membracoidea with about 200 species allocated in Cicadellidae and Membracidae families. However, it is worth mentioning that although the monophyly of the superfamily Membracoidea has been proved by recent phylogenetic studies (Hamilton 1983a; Dietrich et al. 2001; Cryan 2005), actual and potential vectors were nearly all detected in the family of Cicadellidae. Species of leafhoppers can be easily distinguished from their relatives in the superfamily Membracoidea by the presence of a suture which divide the mesothorax in two pieces, anepisternum and katepisternum (except for some Typhlocybinae), and the mesepisternum without hook-shaped process dorsally (Dietrich and Deitz 1993).

At the time of the last Nielson's update (1979) on leafhopper vectors of phytoplasma the diagnostic tools to classify and distinguish the phytoplasmas from other pathogens were not available; overall, he reported 128 leafhopper vectors of plant pathogenic agents for which the vector capability has been confirmed by means of experimental trials, often lasting several years. Recently, Weintraub and colleagues provided a list of actual vectors of phytoplasmas (Weintraub and Beanland 2006; Wilson and Weintraub 2007). The ongoing systematic review proposed by Trivellone et al. (2017a) reports the list of confirmed and potential vectors of phytoplasmas which is now partially linked to the taxonomic relational databases named 3I, Internet-accessible Interactive Identification (Dmitriev 2003). In this study the analysis of metadata provides a snapshot of the knowledge on actual and potential vectors worldwide, and highlights the fact that the information on vectors is still lacking for about half of the described ribosomal phytoplasma groups, this concerns mainly some biogeographic realms (e.g. Australasia and Neotropic). Currently, about 200 leafhopper species were recorded as confirmed or potential vectors of phytoplasmas, and the vector competence has been properly demonstrated for about half of them.

As summarized in Fig. 2.1, competent vectors were recorded in 8 out of 19 subfamiles (Aphrodinae, Cicadellinae, Coelidiinae, Deltocephalinae, Eurymelinae, Iassinae, Megophthalminae, Typhlocybinae) of Cicadellidae, and about 80% of them are allocated in the subfamily Deltocephalinae. Thirteen tribes out of 36 described for Deltocephalinae shall comprise phytoplasma vectors, and those which including the largest number are Opsiini, Macrosteliini and Athysaniini. Although significant progress has been recorded, basic knowledge of leafhopper taxonomy, phylogeny and ecology still needs to proceed forward to identify new vectors, and importantly, to clarify the epidemiology of the infection and vector-pathogens

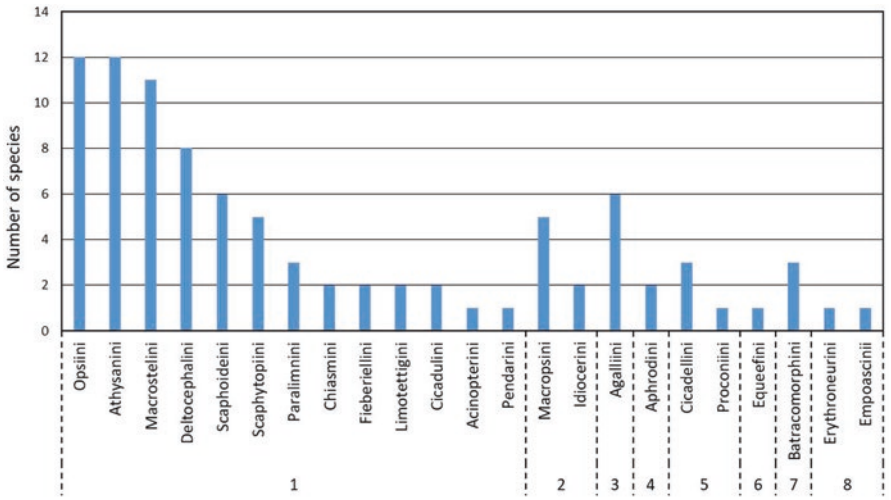

Fig. 2.1 Number of vectors of phytoplasmas recorded in the family of Cicadellidae (Hemipera: Auchenorrhyncha), and their allocation to the subfamilies and tribes. 1: Deltocephalinae; 2: Eurymelinae; 3: Megophthalminae; 4: Aphrodinae; 5: Cicadellinae; 6: Coelidiinae; 7: Iassinae; 8: Typhlocybinae

specificity (Dietrich 2013). One of the most important prerequisites for reliable detection of phytoplasma vectors is their proper taxonomic identification. Albeit the revision of taxonomic changes and the misidentifications of the confirmed vectors are not the present objective of this work, it is worthwhile to briefly highlight some important milestones and progress that have occurred in recent years, as well as challenges to be tackled. For the period from 1895 to 1963, Nielson (1968) summarized the most important changes in the generic and species status of leafhoppers known as vectors of phytopathogenic viruses (also included phytoplasmas at that time recognized as viruses). Revisionary studies to verify the species (taxa) identity are constantly ongoing as essential task for taxonomists. Although taxonomic stability was increased significantly in the last 50 years, many changes in the higher classification are still ongoing and improving such information is a crucial step for the reliable use of the phylogenetic relationship as tool to infer which of the groups of leafhoppers could be considered as potential vectors (Dietrich 2013). In this view, the growing use of information processing tools (modern cybertaxonomy) hugely simplifies managing and dissemination of the information (e.g. TaxonWorks 2015). With respect to the issue of the mis-determined vector species, Nielson (1968) also hinted that was difficult to obtain species tested in experimental transmissions in order to verify the correct species identity. Since the 1980s, the advent of the new technologies of molecular analyses have exacerbated the lacking of reference material owing to usage of the grinding the entire insect bodies subjected to molecular analyses. Although the preservation of the body of reference specimens using the proteinase K digestion is a widely used method in biological studies or those related to museum collections (Asghar et al. 2015), in agronomic and

phytophatological studies this method struggles to take root, even if few exceptions have been already recorded.

Many authors advocated very strongly the use of phylogenetic analyses integrating cladistic approach to make predictions concerning pest species (Dietrich 2013; Trivellone et al. 2017a), because phylogenetic conservatism in certain behavioral traits, and consequent predictability of their expression, gives promise to be able to understand the evolution of vectoring ability. The vector range of phytoplasmas could be phylogenetically constrained; in other words, is more likely that closely related Auchenorrhyncha species will be vectors of the same phylogenetically related phytoplasma groups. Such a phylogenetic signal would make it possible to use the phylogenetic distance as surrogate to assume the vector competence, and predict the risk of spreading phytoplasmas, in particular in those cases where there are incomplete host records (as in the case of leafhoppers).

Recent papers are devoted to clarify relationships between major lineages (Dietrich et al. 2001, 2005; Zahniser and Dietrich 2008), rather than among lower taxa (e.g. subfamily, genus) and explicit phylogenetic analyses by using a cladistic approach are quite scarce and dated (Whitcomb and Hicks 1988; Oman et al. 1990; Hamilton 1994). The Deltocephalinae subfamily (*sensu lato*, including some taxa recently treated as separate subfamilies) phylogeny is based on conserved 28S ribosomal subunit DNA sequences and nuclear histone H3 (Dietrich et al. 2001; Zahniser and Dietrich 2013). A reinterpretation of Deltocephalinae morphological characteristics (Nielson 1979) strongly supports monophyly; although, one of its largest tribes, Athysanini (277 genera and 1965 species), is polyphyletic (Zahniser and Dietrich 2010). Despite their economic importance, there are surprisingly many gaps in the knowledge on the phylogeny, taxonomy, life history and biology of this subfamily.

2.3 Leafhopper Life Cycle

As hemimetabolic (i.e. gradual metamorphosis, lacking a pupal stage) insects, leafhoppers develop from eggs via nymphs to adults. Reproduction is usually bisexual. Adult males and females locate each other through acoustic communication with species- and sex-specific specialized reciprocal courtship calls (duets) via substrate (host plant) – borne vibrational signals (Claridge 1985; Čokl and Virant-Doberlet 2003). Females tend to lay their eggs singly or in batches beneath the epidermis of leaves or into incisions in soft plant tissue or bark of the host plant (DeLong 1971). The eggs can remain dormant for periods ranging from a few weeks to a year or hatch within a few days or weeks. Nymphs undergo five instars (DeLong 1971). Development from egg to adult may take weeks or months depending on temperature and other environmental conditions. Leafhoppers can hibernate in all three life stages, *viz.* as eggs, nymphs, or adults. Most cicadellids hibernate in the egg stage (Waloff 1973). For example, the deltocephaline *Scaphoideus titanus* Ball overwinters as an egg beneath the bark of grapevine (Chuche and Thiéry 2014), whereas *Dalbulus maidis* (DeLong and Wolcott) from the same subfamily overwinters as

nymphs and adults (Summers et al. 2004). Leafhoppers may be univoltine, bivoltine, or multivoltine (one, two or several generations per year) (Maramorosch and Harris 1979). Waloff (1973) noted that univoltine and bivoltine species tend to overwinter as eggs and nymphs. Voltinism is influenced by environmental conditions and region. Insect development is largely dependent on temperature, with higher temperatures, within limits, leading to faster development. Rising temperatures due to global warming may lead to an increase in the number of generations of multivoltine species (Reineke and Thiéry 2016).

Host plant specificity, voltinism and hibernation strategies, together with dispersal patterns, impact on insect vector, and indirectly on phytoplasma, dispersal. The univoltine "flavescence dorée" vector *S. titanus* has a limited dispersal ability (Lessio and Alma 2004) and completes its life cycle exclusively on *Vitis* (Chuche and Thiéry 2014). The polyphagous aster leafhopper *Macrosteles quadrilineatus* (Kirschbaum), on the other hand, is known to transmit several phytoplasma species and has several generations per year as well as a high dispersal capability (Meade and Peterson 1964; Hoy et al. 1992). Thus, the spread of a phytoplasma would be expected to be more limited for a univoltine mono- or oligophagous feeder with limited dispersal capability than for a multivoltine polyphagous species with a higher rate of dispersal.

2.4 Feeding Strategies/Host Plant Selection

Leafhoppers have a close relationship with their host plants. These not only provide food but also a habitat in which to live, mate, and oviposit. Understanding the biology of leafhoppers, how leafhoppers find and locate their host plants together with the tritrophic interactions between plant, phytoplasma and insect vector is essential for devising disease and vector management methods.

Feeding strategies Leafhoppers display a range of feeding strategies. They have highly adapted piercing-sucking mouthparts to feed on the phloem sap (e.g. most Deltocephalinae), xylem sap (Cicadellinae) or the mesophyll tissue (Typhlocybinae) (Wilson and Weintraub 2007). However, these categories are not rigid, as phloem feeders might feed on xylem sap and vice versa, and the feeding preference can also be sex-specific (Chuche et al. 2017).

Many leafhopper species are phloem sap feeders and thus form a pool of potential vectors for phloem-limited phytoplasmas that depend on their insect host for dispersal and transmission to plants. Weintraub and Beanland (2006) suggest that phloem-feeding Hemiptera are well adapted to be efficient vectors because: (i) they are hemimetabolous, i.e. both nymphs and adults have the same feeding habits and can transmit phytoplasmas; (ii) they feed non-destructively and selectively on the phloem sap; (iii) the phytoplasma-insect vector relationship is propagative (reproduction in insect host) and persistent (individuals retain the pathogen for life once infected); and (iv) the same mechanisms that are responsible for transovarial

transmission of obligate symbiotic prokaryotes enable transovarial transmission of some phytoplasmas.

All members of the subfamily Cicadellinae are xylem sap-feeders, with some being vectors of *Xylella fastidiosa* Wells et al. the bacterium causative agent of diseases in grapevine (Pierce's disease), citrus (citrus variegated chlorosis) and several other plant species (Redak et al. 2003). This is the only known bacterial pathogen that is xylem-restricted and transmitted by leafhoppers. None of the xylem-feeding leafhoppers have been reported to transmit phytoplasmas, with exception of a possible single experimental transmission of western X-disease by the cicadelline *Graphocephala confluens* (Uhler) (Maramorosch and Harris 1979).

Members of the Typhlocybinae are mesophyll feeders, but some may also feed on phloem sap (Wilson and Weintraub 2007). Leafhoppers belonging to this subfamily are not only destructive feeders causing "hopperburn", but may also transmit phytoplasmas. Examples are *Empoasca papayae* Oman, a vector of papaya bunchy top-like phytoplasma, and *Empoasca decipiens* Paoli, a vector of chrysanthemum yellows ('*Candidatus* Phytoplasma asteris', 16SrI-B) (Pérez et al. 2010; Galetto et al. 2011). In comparison to phloem-feeding leafhoppers they are less efficient vectors, not least because their feeding behavior may be plant dependent (Galetto et al. 2011), and it is literally a hit or miss affair as to whether or not they acquire the pathogen during feeding.

Host plant selection behavior Like other herbivorous insects, leafhoppers rely on a series of consecutive steps and complex cues to find and select their host plants. This process has been best studied in aphids (Hemiptera: Aphididae). It involves searching for a host plant from a distance during the pre-alighting phase, and once contact with a plant has been made, assessment of surface cues and probing of plant surface cells (Powell et al. 2006), location and insertion of stylets at appropriate feeding sites and salivation followed by committed sap ingestion (Fereres and Moreno 2009). The process can be abandoned at any of these steps (Powell et al. 2006). During these consecutive steps herbivorous insects have to make active choices and may rely on visual, chemical, mechanical and gustatory sensory plant cues as well as background cues such as light and humidity (Saxena et al. 1974). The search for the host plant may be triggered by the need to acquire food, finding mates, selecting oviposition sites, etc., and may differ depending on whether a species is a specialist or generalist herbivore.

The first step for flying insects generally involves the detection of visual (color, shape, contrast) or chemical *stimuli* that can be perceived from a distance (Powell et al. 2006; Fereres and Moreno 2009). These enable insects to orient themselves towards the source of the *stimuli*. Insect herbivores using olfactory cues primarily locate and select host plants based on specific ratios of compounds using highly sensitive olfactory receptor neurons in sensilla located mostly on the antennae (Bruce and Pickett 2011). Members of the Auchenorrhyncha are thought to be less responsive to olfactory cues when searching for a host plant than their relatives in the suborder Sternorrhyncha (e.g. psyllids) (Todd et al. 1990). Accordingly,

olfactory stimuli seem to play a lesser role than visual cues for some leafhopper species in host plant finding and may be largely supplementary (Todd et al. 1990; Bullas-Appleton et al. 2004; Lessio and Alma 2004; Patt and Sétamou 2007; Mazzoni et al. 2009; La Grange et al. 2017). For example, Mazzoni et al. (2009) recorded weak electroantennogram (EAG) responses of *S. titanus* to odors from grapevine rootstock *Vitis riparia* x *rupestris,* which could be explained by a reduction of olfactory antennal sensilla (Stacconi and Romani 2012). Likewise *Mgenia fuscovaria* (Stål), a vector of aster yellows in grapevine, responded weakly to odors from this plant in Y-tube olfactometer assays and plant extracts in EAG tests (La Grange 2016; La Grange et al. 2017). Both species appear to use chemical cues as an enhancement of visual stimuli for host plant finding (Mazzoni et al. 2011; Krüger et al. 2015).

Once on a potential host plant, leafhoppers rely on mechanical and chemical plant cues that may act as stimulants or deterrents for host plant selection. The stereotypical behavioral sequence after making contact with a plant and before stylet penetration involves the evaluation of plant surface characteristics (trichomes, epicuticular waxes, topology) and short-distance chemical cues, stylet probing, plant sap ingestion, and probe termination (Backus 1985). Because of their short antennae, leafhoppers do not explore the plant surface with their antennae but rather through maxillary stylets probing, stylet penetration, short feeding probes and phloem injection (Backus 1988). The mechanisms used in evaluating sensory cues by leafhoppers and other Hemiptera once contact with a plant has been made have been reviewed by Backus (1988). The feeding behavior of some leafhopper species can be host plant-dependent and thus influence transmission of phytoplasmas (Bosco et al. 1997; Galetto et al. 2011).

Host plant specificity Leafhoppers display a range of host plant specializations. Specialist feeders may be monophagous (feeding on one or a few closely related species) or oligophagous (feeding on a number of species within one family). Generalists or polyphagous taxa are able to utilize host plants across multiple families. The feeding strategies of the Deltocephalinae, the subfamily with the largest number of identified vectors, range from mono- to polyphagy (Wilson and Weintraub 2007). The specialist feeder *S. titanus* completes its life cycle exclusively on *Vitis* species but may feed on other plant taxa temporarily (Chuche and Thiéry 2014). Other phytoplasma vectors are generalists, for example several members of the deltocephaline genera *Macrosteles* and *Orosius* and the beet leafhopper, *Circulifer tenellus* (Baker), feed on several plant species in different families (Weintraub and Beanland 2006).

Members of the Deltocephalinae transmit phytoplasmas associated with diseases in various monocot and dicot perennial crops such as sugarcane, grapevine and stone fruits, and annual crops including cereals (e.g. maize, rice, and wheat), vegetables and ornamental plants (Weintraub and Beanland 2006). The feeding strategy of the Macropsini, a tribe within the subfamily Eurymelinae, ranges from mono- to oligophagous; they preferentially feed on woody plants (Wilson and Weintraub 2007). The number of the vectors identified in a family or subfamily may reflect the

degree of economic importance of their host plants and could be higher in some groups than the number of vectors discovered so far (Weintraub and Beanland 2006; Wilson and Weintraub 2007).

2.5 Phytoplasma Specificity

The host range of insect vectors and the phytoplasmas they transmit are interlinked. Just as insects display a range of host plant specializations, phytoplasmas vary in their insect vector and plant host specificity (Bosco and D'Amelio 2010; Bertaccini et al. 2016). Whereas some leafhopper vector specialists have been reported to be natural vectors of a single phytoplasma taxon (e.g. *S. titanus* transmitting "flavescence dorée"), several oligo- and polyphagous leafhoppers are vector generalists and may transmit several phytoplasma taxa, such as polyphagous members of the genera *Macrosteles* and *Orosius. Macrosteles striifrons* Anufriev (= *orientalis* Vilbaste) has been reported to transmit eight, and *Orosius albicinctus* Matsumura and *Orosius argentatus* (Evans) at least five different phytoplasma taxa each (Weintraub and Beanland 2006; Esmailzadeh-Hosseini et al. 2007). Some phytoplasmas have a high vector specificity and have been reported to be transmitted by a single leafhopper species, e.g. transmission of beet leafhopper-transmitted virescence disease (BLTV) by its only known vector *C. tenellus* (Bosco and D'Amelio 2010). On the other hand, phytoplasmas with a low vector- and plant host specificity are transmitted by several leafhopper species to several plant species. The most diverse strains of aster yellows, '*Ca.* P. asteris' subgroup16SrI-B, can be transmitted by more than 24 insect vector species, albeit with varying efficiencies, and has been reported to cause more than 70 diseases in numerous plant species (Lee et al. 1998, 2004).

The relationship between a phytoplasma, its leafhopper vector and host plant can be very specific. A vector may be able to acquire a specific phytoplasma from one plant species but not from another, or may acquire a phytoplasma from one plant species, but may then not be able to transmit it to a different species (Bosco et al. 1997; Galetto et al. 2011). The typhlocybine *Empoasca decipiens* Paoli could acquire and transmit chrysanthemum yellows ('*Ca.* P. asteris', 16SrI-B) from and to daisies [*Chrysanthemum carinatum* Schousboe (Asteraceae)], but was unable to acquire or transmit the same phytoplasma from or to broad bean [*Vicia faba* (L.) (Fabaceae)] (Galetto et al. 2011). Furthermore, a phytoplasma may be transmitted to a plant species but the vector may then be unable to acquire it again from the same plant host species, referred to as a dead-end host, provided there is no other vector (Bosco and D'Amelio 2010). The ability of a vector to transmit a phytoplasma could be related to its feeding biology. Bosco et al. (1997) suggest that the ability to acquire a phytoplasma may be of greater importance than that of inoculation, based on the observation that the feeding behavior of a vector may differ depending on plant species. At the molecular level the antigenic membrane protein

(AMP) of phytoplasmas may be involved in determining transmissibility and vector specificity (Suzuki et al. 2006; Pacifico et al. 2015).

In addition, phytoplasma specificity is also determined by the host and geographical range of the insect and plant hosts involved (Bosco and D'Amelio 2010; Foissac and Wilson 2010). The actual host range of a phytoplasma may be much broader than the host plant range of their insect vectors (Weintraub 2007; Hogenhout et al. 2008). Experimentally, some phytoplasmas have been transmitted to more plant species than occur in the natural host range of their insect vectors (Lee et al. 1998; Weintraub and Beanland 2006).

Leafhoppers may also become co-infected with and transmit more than one phytoplasma taxon by feeding on multi-infected plants or when feeding sequentially on plants infected with different taxa (Bosco and D'Amelio 2010). Infections with multiple pathogens can result in interactive or non-interactive (independent) transmission (Bosco and D'Amelio 2010). For example, the leafhopper *Euscelidius variegatus* (Kirschbaum), a natural vector of chrysanthemum yellows (CY) and an experimental vector of "flavescence dorée" (FD), interactively transmitted both phytoplasmas to *V. faba* when doubly infected after feeding on singly-infected plant hosts sequentially. However, the leafhopper transmitted the FD phytoplasma with far less efficiency when co-infected with the CY phytoplasma than when infected singly with FD phytoplasma, whereas co-infection with the latter had no influence on the CY phytoplasma transmission efficiency (Rashidi et al. 2014).

2.6 Effects of Phytoplasmas on Leafhopper Vectors

Phytoplasmas are obligate endosymbionts with a reduced genome and lacking genes required for major metabolic processes (Oshima et al. 2004). They rely on their insect vector(s) for dispersal and may influence the vector's biology and behavior directly once infected and indirectly through the plant host (Hogenhout et al. 2008; Eigenbrode et al. 2018). In order to meet their requirements and to improve their fitness, phytoplasmas, like other insect-transmitted plant pathogens, manipulate their insect and plant hosts in their favor by suppressing host immune responses, altering host cell processes and interfering with plant development (MacLean et al. 2014; Bendix and Lewis 2018). Makarova et al. (2015) screened the gene expression of AY-WB ('*Ca.* P. asteris') using the model plant *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) and the vector *Macrosteles quadrilineatus* L. and recorded upregulation of more than 74 genes in the phytoplasma-infected vector and 34 genes in the infected host plant. The genes have been linked with metabolic processes and interaction within the host environment. However, the specific function of these genes in the plant and insect hosts still requires confirmation.

Phytoplasmas are acquired passively when feeding on the phloem sap in the sieve tube elements where they reside (Weintraub and Beanland 2006; Orlovskis et al. 2015). They have to pass through and multiply in the insect vector before they can be transmitted during feeding to the phloem of plants (Fig. 2.2). Before vectors

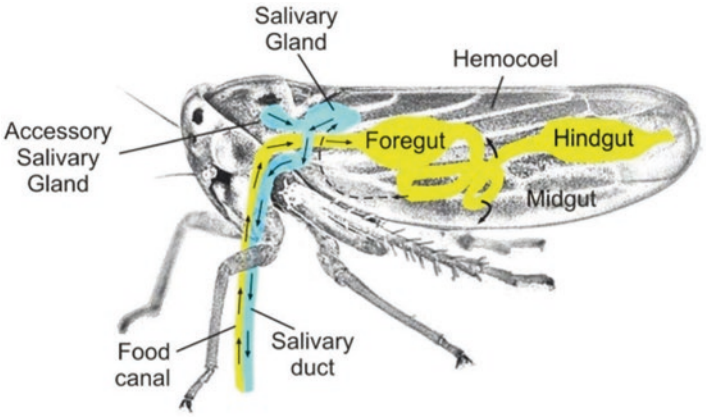

Fig. 2.2 Pathways of phytoplasmas through the insect body. Insect vectors acquire phytoplasmas from infected plants when feeding on the phloem sap. They are transported through the food canal to the intestinal tract where they migrate through the midgut epithelium, circulate in the haemocoel and invade and multiply in various internal organs (e.g. Malpighian tubules, gut and fat bodies, muscle cells). The phytoplasmas then migrate to the salivary glands from where they are transmitted to the phloem of plants during feeding

become infective, phytoplasmas undergo a latent period (the time between acquiring the pathogen and the vector becoming infectious) of 7–80 days (Hogenhout et al. 2008; Bosco and D'Amelio 2010). Recent studies have commenced to unravel the mechanisms which determine host-pathogen specificity; Bosco and D'Amelio (2010) propose that successful colonization of, and multiplication in, the insect body is prompted by the recognition and adhesion of phytoplasmas to insect membrane proteins and that the phytoplasmas have to pass two critical barriers, the midgut and the salivary glands. It has been subsequently demonstrated *in vivo* that during these crucial phases of vector infection the antigenic membrane protein (Amp) of *chrysanthemum* yellows phytoplasmas is involved in the movement through the midgut epithelium and colonization of salivary glands (Rashidi et al. 2015). Furthermore, it has been suggested that the plasmid-encoded transmembrane protein ORF3 may be required for the survival of phytoplasmas in the insect host (Ishii et al. 2009).

Transovarial transmission of phytoplasmas has been suggested for some leafhopper species, including *S. titanus* transmitting aster yellows, *Hishimonoides sellatiformis* Ishihara transmitting mulberry dwarf, and *Matsumuratettix hiroglyphicus* (Matsumura) transmitting sugarcane white leaf (Danielli et al. 1996; Alma et al. 1997; Kawakita et al. 2000; Hanboonsong et al. 2002). In general, this mode of phytoplasma transmission has been shown only for a few vector species and may be related to a long-term association between pathogen and vector (Bertaccini et al. 2016).

Direct effects Phytoplasma infection may affect leafhoppers directly by influencing their biology. It has been postulated that selection pressure reduces pathogenicity. The longer a pathogen and its host have been associated with each other, and

that the relationship may evolve from pathogenic to mutualistic over time (Purcell 1982). To promote their spread phytoplasmas can influence the abundance of their insect vector. A long evolutionary history between aster yellows phytoplasma and *M. quadrilineatus* improved the fitness of the leafhopper by increasing survival rates, lifespan, and fecundity; however, this may be strain-dependent (Beanland et al. 2000). On the other hand, "flavescence dorée" infection may lead to reduced survival and fertility of *S. titanus* (Bressan et al. 2005a), which in turn may be attributable to a recent association between the possibly Palearctic phytoplasma and the Nearctic vector (Bressan et al. 2005a; Arnaud et al. 2007). Accordingly, the direct influence of phytoplasmas on the biology of their leafhopper vectors is variable as phytoplasma infection may reduce survival and fecundity or fertility (Bressan et al. 2005a, b; D'Amelio et al. 2008), increase survival (Murral et al. 1996; Beanland et al. 2000; Ebbert and Nault 2001; Kingdom and Hogenhout 2007) and fecundity or fertility (Beanland et al. 2000), or have no influence on the vector's survival (D'Amelio et al. 2008). Recent studies suggest an indirect influence of phytoplasmas on the reproduction of insect vectors through manipulating the plant defense system (Sugio and Hogenhout 2012).

Infection with phytoplasmas can also influence the behavior of insect vectors directly. *S. titanus,* when infected with "flavescence dorée" phytoplasmas, tend to disperse less than uninfected individuals (Papura et al. 2009), whereas male *M. quadrilineatus,* when infected with aster yellows from symptomatic aster and lettuce plants, displayed greater mobility than uninfected males in a laboratory study (Hoy et al. 1999).

The interaction between phytoplasmas and their hosts has been shown to be temperature dependent. The latent periods in the insect vector are longer and the rate of phytoplasma transmission is lower at 15°C than at 25°C for *D. maidis* transmitting maize bushy stunt (Moya-Raygoza and Nault 1998), *M. quadrilineatus* transmitting aster yellows (Murral et al. 1996) and *M. quadripunctulatus* transmitting chrysanthemum yellows (Maggi et al. 2014), for example. High temperature (30°C) compared to 25°C, on the other hand, may lower (Murral et al. 1996; Moya-Raygoza and Nault 1998), or increase transmission efficiency (Maggi et al. 2014). Greater transmission efficiency at higher temperatures could be attributable to higher rates of phytoplasma replications in the vector, or higher feeding rates of the vector (Maggi et al. 2014). As poikilotherms, insect feeding rates in general are influenced by ambient temperature (Chown and Nicolson 2004).

Dual infection of insect vectors with phytoplasmas may lead to competition and the suppression of the transmission of one of the pathogens (cross-protection) in the insect host (Bosco and D'Amelio 2010; Rashidi et al. 2014). Co-infection of *E. variegatus* with *Chrysanthemum* yellows and "flavescence dorée" resulted in lower transmission efficiency of the latter compared to single "flavescence dorée" infections. However, the presence of FD phytoplasma did not influence the transmission efficiency of CY phytoplasma. *E. variegatus* is a natural host of CY and an experimental host of FD phytoplasmas. The reduced transmission efficiency was attributed to the consistent presence of CY phytoplasma but not FD phytoplasma in the

salivary glands of the insect vector. FD phytoplasma, which does not have a longer-term association with its experimental host *E. variegatus,* was less successful in multiplying in the insect vector than CY phytoplasma (Rashidi et al. 2014).

Not only may leafhoppers acquire multiple phytoplasmas, but the host range of phytoplasmas may overlap with that of other pathogen taxa (e.g. spiroplasmas, viruses) that may share the same insect vector and plant host (Bosco and D'Amelio 2010; Ishii et al. 2013). For example, the plant host range of maize bushy stunt phytoplasma coincides with that of *Spiroplasma kunkelii,* the causative agent of corn stunt disease and *Maize Rayado Fino Virus* (MRFV); all three are transmitted by the leafhopper *D. maidis* (Hruska and Peralta 1997). In addition, multiple pathogen infections in the insect vector can influence phytoplasma transmission; co-infection with a phytoplasma and a Gram-negative bacterium in *E. variegatus* reduced transmission efficiency of X-disease phytoplasma to celery (Purcell and Suslow 1987).

Indirect effects Phytoplasmas may indirectly manipulate the vectors' behavior through infected host plants. By changing the volatile composition of its plant host a phytoplasma may increase its attractiveness to the insect vector (Mayer et al. 2008a), and together with a potential increase in settling time and manipulation of feeding behavior (Eigenbrode et al. 2018) may potentially increase the likelihood of the dispersal of the pathogen.

Little is known about the influence of plant volatiles emitted from phytoplasma-infected plants on the behavior of insect vectors, particularly vectors within the Auchenorrhyncha. Alteration of the host plant volatile profile after phytoplasma infection has been shown for some phytoplasmas (Mayer et al. 2008a; La Grange 2016). The first studies to demonstrate the differential attraction of a phytoplasma vector to infected plants were by Mayer et al. (2008a, b), who reported the attraction of the psyllid vector *Cacopsylla picta* (Förster) (Hemiptera, Sternorrhyncha) to the odor of apple plants [*Malus domestica* (Rosaceae)] infected with '*Ca.* P. mali'. However the leafhopper *M. fuscovaria* has been shown to be more attracted to aster yellows-infected grapevine, alteration of the volatile profile of infected grapevine appeared to have little influence on attraction (La Grange 2016; La Grange et al. 2017). Instead, leaf reflectance may play a role in the preferential attraction of the leafhopper to aster yellows-infected grapevine (Krüger et al. 2015).

Phytoplasmas and other pathogenic bacteria release effector proteins into their host cells to manipulate cell processes which in turn influence the insect vector (Sugio et al. 2011a). Tan et al. (2016) showed that the release of the phytoplasma effector protein SAP11 may alter plant volatile emission by destabilizing a subset of TCP transcription factors in the model plant *Nicotiana benthamiana* infected with '*Ca.* P. mali'. MacLean et al. (2014) proposed that the formation of leaf-like flowers (phyllody) due to infection of aster yellows – witches' broom (AY-WB; 16SrI-A) is caused by the release of AY-WB protein 54 (SAP54), leading to the degradation of plant MADS-box transcription factors (MTFs) in plants. These leaf-like flowers were initially thought to attract *M. quadrilineatus* adults to infected plants (MacLean et al. 2014). However, Orlovskis and Hogenhout (2016) subsequently discovered

that the preference of leafhoppers to phytoplasma-infected *A. thaliana* occurred independently of AY-WB inducing phyllody. These authors postulated that the main role of the phytoplasma effector SAP54 was to attract leafhopper vectors to enhance the spread of phytoplasmas and that phyllody may be a side effect.

Further changes mediated by phytoplasma-infected plants once the insect vector has settled include alterations in plant nutritional quality and suppression of herbivore-induced defense responses to promote insect feeding and oviposition. Adult *M. quadrilineatus* laid more eggs, and a higher number of nymphs was recorded on AY-WB-infected *A. thaliana* plants compared to uninfected control plants (Sugio et al. 2011b). The AY-WB effector protein 11 (SAP11) may not only mediate changes in plant volatile profiles (Tan et al. 2016), but may also influence leafhopper reproduction through the down-regulation of the jasmonate-dependent plant defense system (Sugio et al. 2011a).

Feeding on phloem sap or xylem sap, with carbohydrates or minerals as the main components, respectively, poses nutritional challenges to insect herbivores in terms of acquiring lipids and proteins (Trivedi et al. 2016). Thus sap-feeding insects, including members of the Auchenorrhyncha, rely on symbiotic relationships with microorganisms to provide the required essential amino acids and other nutrients (Hansen and Moran 2014; Fatima and Senthil-Kumar 2015). Ishii et al. (2013) detected multiple infections with obligate and facultative endosymbionts in *Macrosteles* spp., including the obligate endosymbiont '*Candidatus* Sulcia muelleri', which may be involved in encoding genes for biosynthesis of essential amino acids (Wu et al. 2006). Multiple infections are likely to influence pathogen transmission, due to potentially complex interactions between phytoplasmas and obligate and facultative endosymbionts in the insect vector (Trivedi et al. 2016). Microbial symbionts as a source for symbiotic management of insect vectors of phytoplasmas have been reviewed by Alma et al. (2010).

2.7 Movement and Dispersal

Host plant specificity, voltinism and hibernation strategies, together with dispersal patterns, impact on insect vector and movement. Leafhopper movement includes three aspects: within the plant, among plants in a field or a field and adjacent area, and over large geographical regions. All three of which are important in terms of phytoplasma transmission. Typical crop landscapes are changing from what was seen 50 or 100 years ago; a relatively new area of research of habitat or landscape management has emerged in recent years. This research is primarily concerned with methods to increase biodiversity, to improve and maintain crop pollinators, and variety and numbers of natural enemies while maintaining good yields of crop plants (Phalan et al. 2011). While it is recognized that sometimes achieving the goals of improved landscape management may, in fact, also bring pests closer to crop areas, generally the overall benefits outweigh any adverse effects.

Intra-plant movement *Graminella nigrifrons* Forbes, the vector of bushy maize stunt in corn and suspected vector of phytoplasmas to *Prunus* and *Pyrus* spp. (Arocha Rosete et al. 2011), has a very complicated ecology. Mated females tend to exhibit little interplant movement, while males are highly mobile during daylight hours calling for females (Hunt and Nault 1991). These researchers showed that virgin females tend to remain on the upper plant canopy so calling males will land on the lower canopy and search upward for the female. Since mated females would be older and more likely infected with phytoplasmas, it is possible that inoculation in corn starts on the lower leaves. Hoy et al. (1999) found a similar difference in behavior between sex in *Macrosteles. quadrilineatus* and also that males and virgin females fly above the canopy at night. Furthermore, they found that phytoplasma-infected males move more frequently than healthy males.

Inter-field movement *S. titanus* feeds and develops exclusively on the cultivated *V. vinifera*, or the wild grape such as *V. labrusca* and *V. riparia*, but is occasionally found on *Vitaceae* and other plants (Chuche and Thiéry 2014). Therefore, with this species, movement into a vineyard is primarily relevant when there are wild grape growing in the vineyards' vicinity and within a vineyard it is commonly aggregated with adults and nymphs appearing together (Bosco et al. 1997; Decante and van Helden 2006). Since *S. titanus* is primarily distributed in the canopy (Lessio and Alma 2004), there is little to no vertical movement on the plant and grapevine plant density greatly affects spread within a vineyard.

The polyphagous aster leafhopper *M. quadrilineatus*, on the other hand, is known to transmit several phytoplasma species and has several generations per year as well as a high dispersal capability (Meade and Peterson 1964; Hoy et al. 1992). Thus, the spread of a phytoplasma would be expected to be more limited for a univoltine, mono- or oligophagous feeder with limited dispersal capability than for a multivoltine polyphagous species with a higher rate of dispersal. Plant density was also found to be a factor in the distribution of *Nephotettix virescens* (Distant) in rice fields (Ishii-Eiteman and Power 1997). When rice that was planted by seed broadcasting, (hence fields were dense) leafhoppers quickly invaded and colonized the entire field as opposed to fields where panicles were hand planted and evenly spaced.

The effect of manipulations of strip crops within and near vineyards was examined by Altieri et al. (2005) and Lessio et al. (2017). The former research group examined the effects of vegetational corridors (consisting of a variety of plant proving pollen, nectar and beneficial habitat) extending into organic vineyards from the surrounding habitat. In effect, these corridors broke the vineyard monoculture and substantially enhanced biological control of leafhoppers generalist predators and especially by a parasitoid, *Anagrus epos* Girault. The latter group planted strips of various flowering plants in and near vineyards to determine the effects on leafhopper vectors in the vineyards. They found that in most cases there were no significant differences in the number of leafhoppers captured in the vineyards and concluded that the refuge areas for predators and parasitoids do not adversely affect vineyards in terms of vectors. In fact, they found that *Neoaliturus fenestratus* Herrich-

Schaeffer, a known phytoplasma vector in several crops (Salehi et al. 2007; Mitrovic et al. 2012) and frequently found in vineyards (Bosco et al. 1997; Orenstein et al. 2003; Landi et al. 2013) was more abundant on planted strips, than in the vineyards. Further strengthening the effect of vegetation corridors into vineyards was a study by Gaigher et al. (2015) in which they compared the high parasitoid diversity found in remnant natural vegetation which occurred within or near vineyards. Their study emphasized that in the absence of some form of permeability into the vineyard, parasitoids remained segregated in the islands of remnant native vegetation.

Long distance movement There are probably many examples of long distance leafhopper movement in which phytoplasma is spread over hundreds or thousands of kilometers, although these can be difficult to document. In the Great Lakes region of the United States of America and Canada, fields lay fallow under snow the entire winter. Crops can only be planted when the ground is arable, but yellows disease was frequently seen in carrots, celery and lettuce during the summer, meaning that the pathogen was transported from outlying areas. The source region of the phytoplasmas was determined to be the Gulf Coast states (Chiykowski and Chapman 1965; Hoy et al. 1992) and northern Mexico, some 3000 or more kilometers. In the Gulf states, phytoplasma is present year round, and as *M. quadrilineatus* moves northward during the spring, the phytoplasma has time to develop and circulate through the insect such that upon arrival in the Great Lakes region they are infective. Once in the region, other leafhoppers may additional transmit the phytoplasmas.

2.8 Human Activities and Vector Spreading

The account from the consortium DAISIE (Delivering Alien Invasive Species Inventory for Europe) reported a total of about 1600 exotic terrestrial invertebrates, most of them are arthropods belonging to the Hemiptera order (Roques et al. 2009). In the suborder Auchenorrhyncha, the majority of the species introduced from other biogeographical region follow the pathways of trade and international exchange of people and goods. Human activities mediate the dispersal of leafhoppers worldwide, and often the new introductions to new geographic ranges occur unintentionally. Accidental introductions over great distances are facilitated by their overwintering strategy, which occurs through the production of dormant eggs often laid under bark, in wood or buds. Over short distances, the human-mediated dispersal could be promoted by the tendency of certain species to hide and survive as adult in sheltered places.

It is common knowledge that the main exponential growth of introductions of nonindigenous leafhoppers are due to the increasing of the international travel and trade and to their increased efficiency. Clues for role of human activities in spreading leafhopper are suggested by the observation of a disjunct distribution across realms (Hamilton 1983b). The spreading of Hemiptera in general is associated with traffic in plant materials (National Research Council 2002). Many other authors also attributed new introductions along with ornamentals plants trade (Hamilton 1983b;

Rabitsch 2010; Trivellone et al. 2015). The outdated but still relevant Hamilton's account (1983b) on introduced leafhoppers pointed out that several introduced species have in common the characteristic to be monophagous on ornamental plants in the early stages of invasion, and eventually they could explore and spread across different habitat.

New introductions of alien species could result in negative effects particularly in agricultural and forest ecosystems, commonly combined with economic losses (Binimelis et al. 2007; Seljak 2013). However, most of the time the species is not harmful in its native area whereas it might cause economic impact in the region of introduction. The most significant example for role of human activities in spreading potential vectors is *S. titanus* suspected to have been moved from north America to Europe in the stage of egg hidden in the planting material, and then it spread throughout Europe in the same way (Bertin et al. 2007). Because the ability of *S. titanus* to successfully transmit "flavescence dorée" phytoplasma from grapevine to grapevine is very high, it caused enormous damage and still poses a threat for grapevine industry in Europe.

The grass-feeding Palearctic leafhoppers *Elymana sulphurella* Zetterstedt and *Athysanus argentarius* Metcalf, have been introduced in in the American continent and both were discovered to be competent vectors of the aster yellows agent (Chiykowski 1981; Chiykowski and Sinha 1969). *Athysanus argentarius* was introduced to eastern North America from Europe in the 1920s, later it has spread to eastern Canada and westward to Manitoba and Montana, and southward to Iowa and Kentucky (Beirne 1956; Hamilton 1983b). it feeds on a variety of wild grasses such as *Poa* spp. sand in north America it was considered a secondary vector of aster yellows phytoplasmas to crops and its role seems limited to maintain the source of inoculum in wild grasses (Chiykowski 1979).

Another example is the immigrant polyphagous leafhopper species, *C. tenellus*, which feeds on many dicotyledous species. This leafhopper originated in the Mediterranean region of Europe and North Africa, but probably immigrated to the Americas with early Spanish explorers, presumably bringing some their plant materials with microbial community with them. These introductions may have occurred multiple times based upon curly top virus genome analysis of old and new world strains (Briddon et al. 1998). Although the regions of *C. tenellus* colonization in the Americas are ecologically similar, host utilization patterns and population dynamics of European and American *C. tenellus* are different. Populations in New Mexico seem to be more similar to the European populations (small populations and similar host plants), but populations in California have exploited the plant communities and are considered a pest and they transmit plant pathogenic viruses, phytoplasma and spiroplasmas.

Some others species moving across continents could represent a potential risk for phytoplasma spreading as they are known to cause economic damages in the native region. The Nearctic species recently introduced in Palearctic region, *Osbornellus auronitens* Provancher was recorded as a possible vector involved in transmission of grapevine yellows diseases in Virginia (USA) (Beanland et al. 2006), posing a

potential risk of spreading phytoplasmas in the new range of colonization (Trivellone et al. 2017b).

Despite quarantine regulations to prevent entry and spread of pathogens, occasionally phytoplasmas are unwittingly introduced in area where their presence was not previously known. In the new environment the phytoplasmas might find indigenous leafhopper species able to spread them from plant to plant. In Israel, a new phytoplasma in *Limonium latifolium* was detected in 2000 and Weintraub et al. (2004) demonstrated that four of the most common and abundant leafhoppers [*Orosius orientalis*, *Circulifer haematoceps*, *C. tenellus*, and *Exitanus capicola* (Stål)] collected in Arava Valley, Israel vectored phytoplasma to *Limonium* in experimental trials.

2.9 Conclusions

The complex insect vector-pathogen-host plant relationships are being illuminated in pace with the advancement of molecular and biochemical techniques in science for the most part. Complete phylogenies of both vectors and phytoplasmas may allow prediction of which leafhopper species will be associated with only one phytoplasma and which with several, including viruses. However, at this point, transmission efficacy can only be determined through laborious transmission trials; there are many barriers from acquisition in the midgut to the leafhoppers' salivary glands, and not all species that acquire phytoplasmas can transmit them. The environmental cues that attract leafhoppers to infected plants are still being discovered; changes in plant volatiles, leaf color, physical characteristics of the plant, and potential changes in acquisition from phloem as visualized by wave forms on electrical penetration graphs. Finally, the effect of the phytoplasma on its insect host is also uncertain; do evolutionarily long host-pathogen relationships lead to beneficial effects and shorter relationships to detrimental ones to the vector? These questions would be far easier to address in the absence of the movement of leafhoppers and infected plants out of endemic areas. While much progress has been made since the first association of leafhoppers transmitting pathogens, there is still much to be done.

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Chapter 3
Psyllid Vectors

Barbara Jarausch, Rosemarie Tedeschi, Nicolas Sauvion, Jürgen Gross, and Wolfgang Jarausch

Abstract '*Candidatus* Phytoplasma' species are mostly transmitted from plant to plant by phloem feeding hemipterans, primarily leafhoppers (Cicadellidae) and planthoppers (Fulgoroidea) (Hemiptera, Auchenorrhyncha). However, there is one group of phytoplasmas, the 16SrX or apple proliferation group, whose members are transmitted by psyllid vectors of the superfamily Psylloidea (Hemiptera, Sternorrhyncha). These psyllid-transmitted phytoplasmas are genetically closely related and are associated with economically important diseases of fruit trees such as pear decline, apple proliferation and European stone fruit yellows. The psyllid vector species of these phytoplasmas are also closely related and all belong to the genus *Cacopsylla*. Both, phytoplasmas and psyllid vectors, are geographically limited to the Palaearctic region, mainly Europe. Only pear decline and peach yellow leaf roll phytoplasmas have probably been introduced to America along with their vectors. As phytoplasma-infected trees cannot be cured and resistant plant material is not available to the growers, preventive control measures such as vector control are of paramount importance to limit the spread of these diseases. Thus, detailed

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_3

knowledge about the biology and ecology of the vector species, their host plants as well as knowledge about the transmission parameters is crucial.

Keywords Phytoplasmas · Psyllid vectors · Epidemiology · Pear decline · Apple proliferation · European stone fruit yellows

3.1 Introduction

The jumping plant-lice or psyllids form the well-defined superfamily Psylloidea which belongs to the suborder Sternorrhyncha of the order Hemiptera. About 4.000 species of this superfamily are described worldwide including about 400 species in Europe (Burckhardt 1994; Burckhardt and Ouvrard 2012). All recognised phytoplasma vectors are found in the genus *Cacopsylla* which is part of the subfamily Psyllinae within the family Psyllidae (Weintraub and Beanland 2006; Burckhardt and Ouvrard 2012). An electronic determination key for the most important *Cacopsylla* species found on Rosaceae in Europe is available at the internet by www.psyllidkey.eu (Burckhardt et al. 2008). Table 3.1 gives an introductive overview of the most important phytoplasma diseases and their agents which are

Table 3.1 Phytoplasma diseases of fruit crops, their associated agents, psyllid vectors and vector's host plant

Psyllid species	Phytoplasma	Disease	Host plant
Cacopsylla picta	'*Candidatus* Phytoplasma mali'	Apple proliferation	*Malus* x *domestica*
Cacopsylla melanoneura	'*Candidatus* Phytoplasma mali'	Apple proliferation	*Crataegus* spp., *Malus* x *domestica*
Cacopsylla pruni A and B[a]	'*Candidatus* Phytoplasma prunorum'	European stone fruit yellows	*Prunus* spp.
Cacopsylla pyri	'*Candidatus* Phytoplasma pyri'	Pear decline	*Pyrus* spp.
Cacopsylla pyricola	'*Candidatus* Phytoplasma pyri'	Pear decline	*Pyrus* spp.
Cacopsylla pyricola	'*Candidatus* Phytoplasma pyri'	Peach yellow leaf roll (USA)	*Prunus persica*
Cacopsylla pyrisuga[b]	'*Candidatus* Phytoplasma pyri'	Pear decline	*Pyrus* spp.
Cacopsylla chinensis	'*Candidatus* Phytoplasma pyri' strain PD-TW	Pear decline-Taiwan	*Pyrus* spp.
Cacopsylla qianli[b]	'*Candidatus* Phytoplasma pyri' strain PD-TW	Pear decline-Taiwan	*Pyrus* spp.

[a]Complex of two cryptic species A and B
[b]Presumed vectors

transmitted by psyllid vectors. So far, all important fruit crop diseases associated with the presence of phytoplasmas of the group 16SrX are vectored by psyllids.

The basic geographical distribution of the most important psyllid vectors in Europe and neighbouring regions has been assessed during the COST action FA0807 and is available online (Costphytoplasma 2013).

Psyllids are phloem feeders and both nymphs and adults feed on plant sap. Depending on the species, the eggs are laid on the new buds, in crevices of the bark or on leaves where they can produce pit-like deformations on the leaf blade (Hodkinson 2009). The nymph development passes through five instars which are more or less strongly flattened dorso-ventrally (Burckhardt 1994). Phytoplasma vector species on apple and stone fruits are – like other North temperate psyllids – univoltine whereas pear psyllids with the exception of *Cacopsylla pyrisuga* (Foerster 1848) are mostly polyvoltine with overlapping generations. Polyvoltine vector species usually overwinter as adults on or near to their host plants. Univoltine vector species have an obligate emergence as imagines (= emigrants) to their overwintering plants, such as conifers, and return to their respective reproduction host plants in spring (= remigrants) (Mayer et al. 2011; Burckhardt 1994). This applies for *Cacopsylla pruni*, *C. melanoneura* and *C. picta* (Thébaud et al. 2009; Mayer and Gross 2007; Cermák and Lauterer 2008; Tedeschi et al. 2002; Jarausch et al. 2013; Jarausch and Jarausch 2014, 2016). In these cases overwintering was observed only on conifers at higher altitudes (Mayer and Gross 2007; Thébaud et al. 2009; Pizzinat et al. 2011; Ulubaş Serçe et al. 2011). Migration to the respective reproduction or overwintering plant seems to be direct and may take place even over long distances. Plant volatiles are exploited by the psyllids to find their reproduction host plants or overwintering plants during migration (Gross 2016; Mayer and Gross 2007; Mayer et al. 2011).

As phytoplasmas are phloem-limited, only phloem-feeding insects can potentially acquire and transmit the pathogen (Weintraub and Beanland 2006). The insects acquire the phytoplasma during feeding in the phloem of infected plants. The following process of phytoplasma passage and multiplication in the insect body comprises the latent or incubation phase and the infectivity period where the insect can transmit the pathogen. Detailed descriptions of the cellular processes of transport and multiplication in the insect body have been reported for leafhoppers (Weintraub and Beanland 2006; Hogenhout et al. 2008). So far, these mechanisms of phytoplasma transmission remain to be demonstrated for psyllid vectors as well. Several publications show that psyllids transmit the pathogen in a persistent propagative manner (Carraro et al. 2001a, b; Thébaud et al. 2009). The length of time needed for an individual to become infectious seems to differ among the psyllid vectors. However, it is of paramount importance for the disease spread by univoltine vectors: vectors with a long latent phase predominantly transmit the phytoplasma after overwintering when remigrating into the orchards (monocyclic disease spread on regional scale). In contrast, vectors with a short latent phase transmit the phytoplasma already before migration as well as after overwintering (polycyclic disease spread on local and regional scale) (Jarausch et al. 2013).

3.2 *Cacopsylla picta*

Cacopsylla picta (Foerster 1848) (Figs. 3.1 and 3.2) is distributed only in Europe and is monophagous on *Malus* spp. (Jarausch and Jarausch 2010; Ouvrard 2017). The insect completes one generation per year and overwinters as adult on conifers. At the end of winter (March/April), *C. picta* remigrants move from the overwintering sites to apple trees for oviposition (Jarausch and Jarausch 2014). The insects of the new generation (emigrants) feed on their reproduction host until the beginning of July when they leave the apple trees as adults (Mattedi et al. 2008; Jarausch et al. 2011). Findings of phytoplasma-infected individuals of *C. picta* have been reported from many different countries: Italy, Germany, Bosnia-Herzegovina, France, Switzerland, Finland, Czech Republic, Bulgaria, Spain and Croatia (Frisinghelli et al. 2000; Jarausch et al. 2003, 2011; Carraro et al. 2008; Delić et al. 2008; Lemmetty et al. 2011; Ludvikova et al. 2011; Etropolska et al. 2015; Miñarro et al. 2016; Križanac et al. 2017). In north-east Italy, the natural infection rate of *C. picta* was found between 9% and 13%, respectively for overwintering and offspring adults (Carraro et al. 2008). In the main apple growing regions of Italy, Trentino and Alto Adige (South Tyrol) (Italy), mean infection rates of 6% (Mattedi et al. 2008), 11% (Baric et al. 2010) and 20% (Fischnaller et al. 2017) for remigrants were reported. In Germany, about 10% of overwintered *C. picta* were found naturally

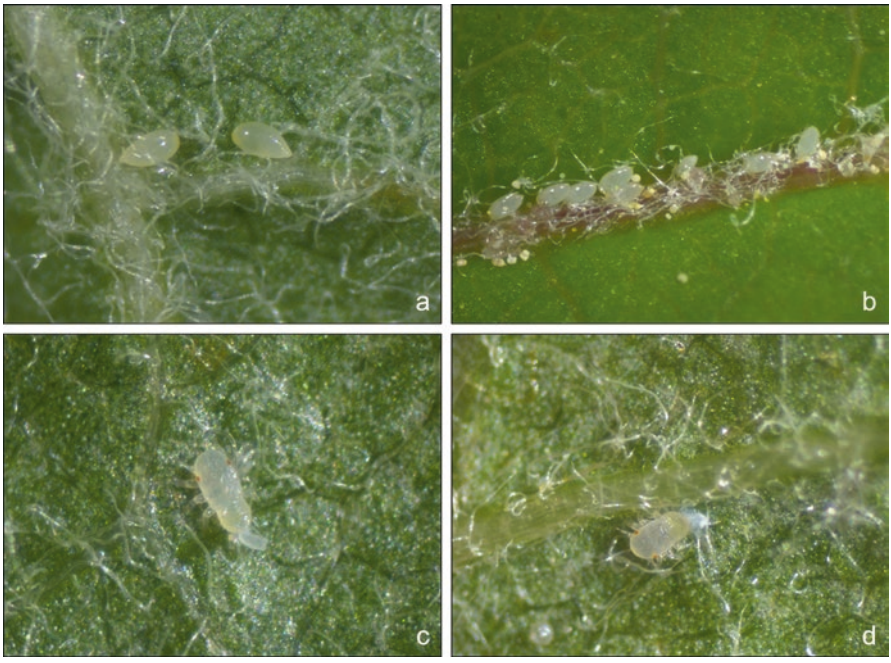

Fig. 3.1 *Cacopsylla picta* pre-imaginal stages: (**a** and **b**) eggs, (**c** and **d**) nymphs (Courtesy by L. Görg)

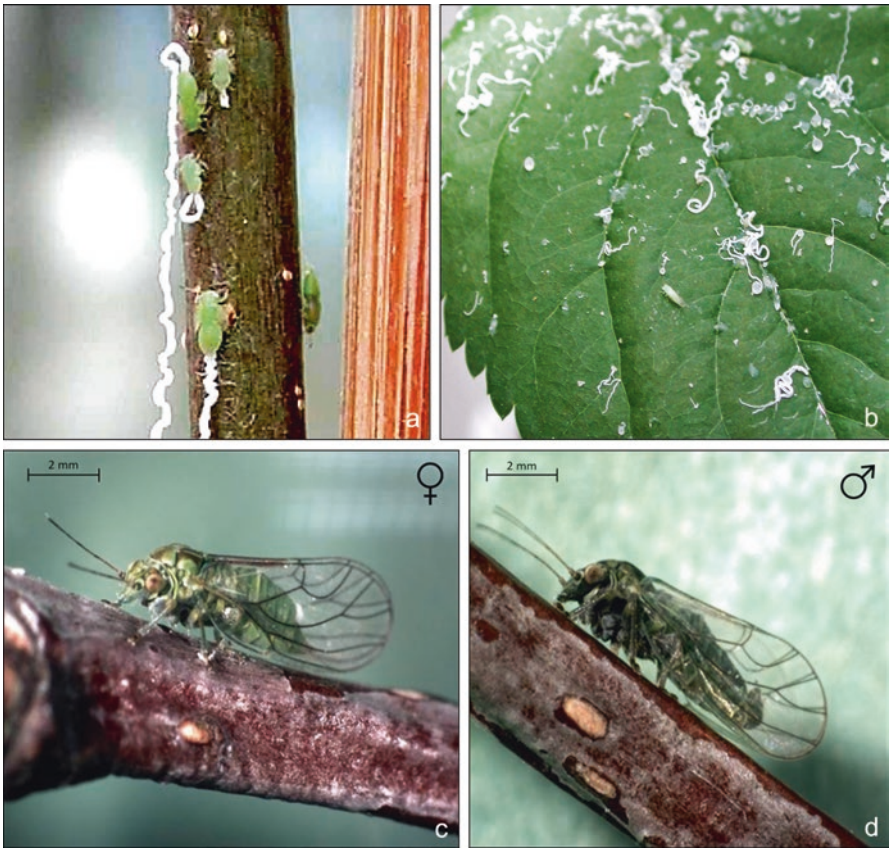

Fig. 3.2 *Cacopsylla picta*: (**a**) 5th stage nymph with waxy secretions, (**b**) newly emerged adult, (**c**) overwintered female and (**d**) overwintered male

infected with the pathogen every year (Jarausch et al. 2004, 2007a, 2011). Data for Northern Switzerland (10%) (Jarausch et al. 2011), Western France (Alsace: 14%) (Jarausch et al. 2011), Finland (11%) (Lemmetty et al. 2011) and Spain (13%) (Miñarro et al. 2016) were in a similar range. A lower infection rate of roughly 3% was reported from Bulgaria (Etropolska et al. 2015). Jarausch et al. (2011) found no significant relationship between the infection status of the orchard and the infection rate of *C. picta* remigrants captured within these orchards, although a tendency of higher infection rates in abandoned orchards was observed.

These data can be explained by a regional dispersal of remigrant individuals and by a certain percentage of transovarial transmission of the phytoplasma as detected by Mittelberger et al. (2017). During experimental transmission trials in different laboratories in Germany and Italy it was confirmed that both emigrants and remigrants of *C. picta* can transmit the agent efficiently (Jarausch et al. 2004, 2007a, 2011; Carraro et al. 2008; Mattedi et al. 2008). However, the transmission efficiency

was usually higher with remigrants (range of 8–45% with groups of 5 individuals) than with emigrants (range of 2–20% with groups of up to 20 individuals) (Jarausch et al. 2011). Both, males and females, can transmit the phytoplasma and are naturally infected to a similar percentage. As males and females are present in the orchards at a ratio of 1:1.4 (Jarausch et al. 2011), both genders can contribute to the spread of apple proliferation phytoplasma (‘*Ca*. P. mali’).

The phytoplasma concentration in the infected individuals of *C. picta* was extremely high and ranged between 10^6 and 10^8 as measured by quantitative PCR (Jarausch et al. 2011; Mayer et al. 2009). Phytoplasma-infected and infective psyllids appeared among the first remigrants of *C. picta* in apple orchards in early spring, indicating winter-retention of the pathogen (Jarausch et al. 2011; Mattedi et al. 2008). Furthermore, phytoplasma concentrations in the remigrants were constantly high and did not differ significantly during the first 7 weeks after arrival in the orchard (Jarausch et al. 2011). Thus, remigrants are considered to be infective during their whole life span on apple after overwintering. Although remigrants readily transmitted the phytoplasma under experimental conditions, this could not be verified by bait plant trails in the orchard: natural transmission in the orchards in Trentino was found to be situated during the migration period of the new generation of *C. picta* (Mattedi et al. 2008). Transmission by emigrants before leaving the orchard is not also supported by experimental transmission trials, but also by quantitative PCR data which indicate that ‘*Ca.* P. mali’ multiplies very quickly to high concentrations after experimental acquisition (Pedrazzoli et al. 2007). Individuals born on infected plants acquired the phytoplasma to nearly 100% while new adults emerged on healthy plants and fed thereafter on infected plants had only an acquisition rate of about 10% (Jarausch et al. 2010).

In conclusion, *C. picta* is able to transmit ‘*Ca*. P. mali’ during the entire period when they are present on apple trees. As remigrants as well as emigrants are able to transmit, a polycyclic disease spread is supposed in which remigrants disperse the pathogen on a regional as well as local scale and emigrants on a local scale (Jarausch et al. 2011). Although the population density of *C. picta* in the orchards is usually low (Jarausch et al. 2009, 2011), this species is considered to be the main vector of apple proliferation wherever it is present.

In Germany, *C. picta* transmits all three ‘*Ca.* P. mali’ subtypes AT-1, AT-2 and AP as defined by Jarausch et al. (2000) (W. Jarausch, unpublished data) while in Alto Adige (South Tyrol) (Italy) the spread of apple proliferation seems to be predominantly linked to the transmission of subtype AT-2 by *C. picta* (Baric et al. 2011). All three subtypes were also identified in *C. picta* of North East Italy (Martini et al. 2008). *C. picta* individuals captured in Finland were infected with subtypes AT-2 and AP (Lemmetty et al. 2011). Jarausch et al. (2010) also reported differences in acquisition, multiplication and transmission efficiencies among different strains of ‘*Ca.* P. mali’. Interestingly, strains which multiplied best in the insect exhibited the lowest titers in apple plantlets.

3.3 *Cacopsylla melanoneura*

Cacopsylla melanoneura (Fig. 3.3) has a holo-Palaearctic distribution and is oligophagous on Rosaceae plants such as *Crataegus* spp., *Malus domestica*, *Mespilus germanica* and *Pyrus communis* (Ouvrard 2017). This species was originally described as hawthorn psyllid (Lal 1934; Ossiannilsson 1992; Lauterer 1999), while successively it has become an economically injurious pest of apple trees in particular in relation to the spreading of the apple proliferation disease (Alma et al. 2000;

Fig. 3.3 *Cacopsylla melanoneura* life stages: (**a**) eggs, (**b**) 1st and 2nd stage nymphs, (**c**) 5th stage nymph, (**d**) newly emerged female, (**e**) overwintered female, (**f**) overwintered male (DISAFA, Entomology unit, University of Torino, Italy)

Tomasi et al. 2000; Tedeschi et al. 2002). In most of the areas where the presence of the disease has been recorded, *C. melanoneura* and *C. picta* occur sympatrically (Jarausch et al. 2003; Delić et al. 2005; Mattedi et al. 2008; Miñarro et al. 2016), in some others (Northwestern Italy and Norway) only *C. melanoneura* has been reported (Tedeschi et al. 2002; Brede 2017).

Several studies on natural infection rate and vector ability of this species revealed a diversified scenario. Generally in apple growing regions where *C. melanoneura* and *C. picta* coexist, the latter has a major vector role (Frisinghelli et al. 2000; Mattedi et al. 2008; Jarausch et al. 2003, 2004, 2007a), while *C. melanoneura* has been considered ineffective in transmitting '*Ca.* P. mali' (Mayer et al. 2009). On the contrary in the areas where *C. picta* does not occur, in particular in Northwestern Italy, *C. melanoneura* has an important role in the spreading of the disease (Tedeschi et al. 2002, 2003; Tedeschi and Alma 2004). Indeed, natural infection rates in apple orchards is very low, less than 1% in Germany, Northern Switzerland, Eastern France (Mayer et al. 2009); but ranging from 4.2% (0,6% in average) in South Tyrol (Northeastern Italy) (Baric et al. 2010; Fischnaller et al. 2017) to 5–6.2% in Trentino region (Northeastern Italy) where the role of this insect as vector of '*Ca.* P. mali' has been recently reviewed (Malagnini et al. 2010; Tedeschi et al. 2012), and reaching around 4% and up to 45% in a 100% infected orchard in Northwestern Italy (Tedeschi et al. 2003). Moreover, against a non-transmission reported in Germany, 0.36% of infected plants were obtained after transmission trials with *C. melanoneura* from Trentino (Northeastern Italy) (Mattedi et al. 2008), while in Northwestern Italy 29.4% of plants inoculated with naturally infected overwintered adults (88.9% in the case of insects collected in a 85% infected orchard) tested positive to '*Ca.* P. mali' (Tedeschi and Alma 2004). Due to the fact that these transmission trials were carried out using batches of around 20 specimens/test plant, a range of 1.4–8.4% of probability of transmission by a single *C. melanoneura* was estimated in Northwestern Italy (Tedeschi and Alma 2006). In particular it was highlighted the crucial role of overwintered individuals in comparison to newly emerged adults due to a higher percentage of '*Ca.* P. mali'-positive individuals (3.6% vs. 0.8%) and a longer period spent in apple orchards (14.6 vs. 6 weeks) (Tedeschi et al. 2002, 2003). These differences in relation to vector attitude of *C. melanoneura* are confirmed also by the phytoplasma titre in the insect. In Germany, the maximum phytoplasma concentration never exceeded 40,000 copies/individual in field collected insects, far below the minimum titre threshold found for an effective transmission by *C. picta* (10^6–10^8 phytoplasma DNA copies) (Jarausch et al. 2007a, 2011; Mayer et al. 2009). However, in Northwestern Italy '*Ca.* P. mali' reached a concentration of 10^4–10^6 copies/individual *C. melanoneura* (Monti et al. 2013). The life cycle is similar to that of *C. picta*, but the overwintering adults appear earlier in the year on *Crataegus* spp. or apple trees (Mayer et al. 2011). This migration is dependent on a temperature threshold, recorded in the orchards, which in Northeastern Italy has been found to be around 9.5°C (Tedeschi et al. 2012). Likewise the new generation abandons the host plant earlier (end May–June) than *C. picta* to migrate to the aestivation and overwintering plants (Mattedi et al. 2008; Tedeschi et al. 2002). The overwintering plants are reached, thanks to warm ascending currents, at different

altitudes depending on the geographical area, 462–535 m a.s.l. in South Moravia, mainly 1350–1650 m a.s.l. in Northwestern Italy (Cermák and Lauterer 2008; Pizzinat et al. 2011). Different conifer species were recorded as overwintering plants and by caging on them newly emerged adults from hawthorn or apple plants it was possible to follow the whole life cycle of the psyllid throughout the year (Pizzinat et al. 2011). No correlation between immigration dynamics and apple phenology could be demonstrated; however, oviposition occurs at bud burst while egg peak and hatchings are always before the first flowering. This confirmed a good degree of synchrony between *C. melanoneura* and host-plant growth, being linked with temperatures as stated for psyllids in general by Hodkinson (2009).

In order to apply well-timed control strategies, Tedeschi et al. (2012) defined an immigration index based on the maximum temperatures of the 7 days registered in the apple orchard, to predict the arrival of the overwintered adults. In practice, in the Valsugana valley (Northeastern Italy) where this study was carried out, psyllids start to reach the apple orchards when this threshold is 9.5°C. In other areas this threshold should be adjusted either according to historical collection data or by programming periodical field collections. Moreover, other geographical factors associated with the winter sites location (e.g., the regional orography, the main air streams and distance from apple orchards) may differently affect the psyllid migration process and influence its presence or absence, both in terms of time and quantity, in a given apple orchard (Tedeschi et al. 2012).

Concerning host plants, so the plants on which *C. melanoneura* feed, copulate, lay eggs and completes its immature to adult life cycle, several studies attributed diverse roles to hawthorn or apple, depending on different geographical areas. In Northwestern Italy, *C. melanoneura* is equally common in both apple orchards and on *Crataegus monogyna* plants; moreover hawthorn plants have been found infected with '*Ca.* P. mali' entailing a possible role of this plant as a phytoplasma source of inoculum (Tedeschi et al. 2009). On the contrary in Germany this psyllid preferred hawthorn as host plant which, however, was not found infected with the phytoplasma (Mayer et al. 2009).

All the different roles assigned to *C. melanoneura* (i.e. efficiency in acquiring and transmitting '*Ca.* P. mali') by the studies conducted to date suggest the existence of different populations at both geographical and host plant scale (Malagnini et al. 2010, 2013; J. Gross and R. Tedeschi, unpublished results), but also of different combinations of psyllid populations and phytoplasma strains (Baric et al. 2011). In particular there is a strict co-occurrence of AT-1-associated subtypes of '*Ca.* P. mali' and *C. melanoneura* in several regions of Northwestern Italy, where *C. melanoneura* is considered to be the most important vector of apple proliferation (Casati et al. 2010), while in other regions where *C. picta* is the principal vector there is the dominance of '*Ca.* P. mali' subtypes AT-2 (Cainelli et al. 2004; Jarausch et al. 2000; Baric et al. 2011) or AP (Jarausch et al. 2000, 2004; Martini et al. 2008).

3.4 *Cacopsylla pruni*

Cacopsylla pruni (Scopoli 1763) (Fig. 3.4) is widespread in its native Western Palearctic area (Ossiannilsson 1992; Steffek et al. 2012; Ouvrard 2017). This psyllid species is strictly oligophagous on *Prunus* spp., completes one generation per year and overwinters as an adult on conifers. At the end of winter – early spring, according to the climatic conditions and the geographic zones, adult remigrants move from the overwintering plants back to *Prunus* spp. for oviposition. They prefer wild, uncultivated *Prunus* spp. as host plants such as *P. spinosa* (blackthorn) or *P. cerasifera* (myrobolan) (Labonne and Lichou 2004; Jarausch et al. 2008) but they

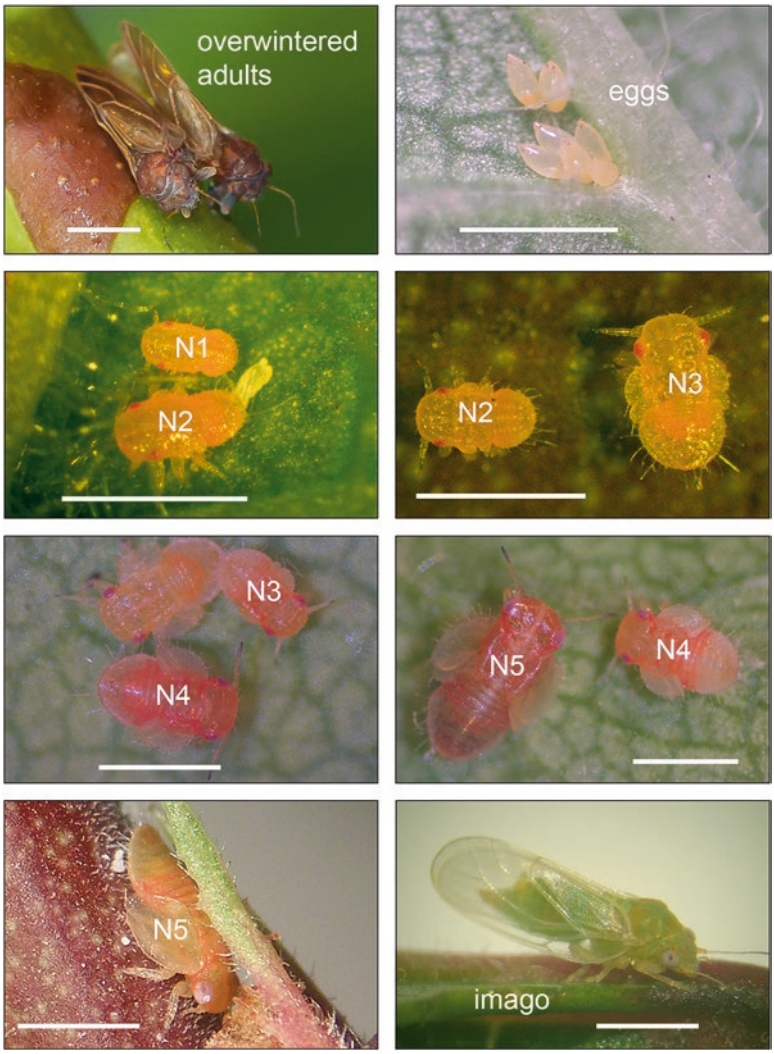

Fig. 3.4 *Cacopsylla pruni* life stages Bar = 1 mm

can also reproduce on cultivated *Prunus* e.g. apricot, peach or European and Japanese plum. Interestingly, remigrants were highly attracted by rootstock suckers of apricot trees where high oviposition rates could be observed and population densities of newly emerged adults were high (Labonne and Lichou 2004). The adults of the new generation feed on their reproduction hosts until the beginning of July, when they leave the *Prunus* plants to move to their overwintering plants (Carraro et al. 2001b, 2004; Thébaud et al. 2009). So far, *C. pruni* is the only psyllid species described as vector of '*Ca*. P. prunorum' (Carraro et al. 1998; Jarausch et al. 2001, 2007b, 2008; Thébaud et al. 2009; Marcone et al. 2010). A comprehensive overview of the distribution of '*Ca.* P. prunorum' and its vector *C. pruni* in European fruit-growing areas was reviewed by Steffek et al. (2012). Recently, Peccoud et al. (2013) proposed that *C. pruni* is a complex of two cryptic species, provisionally named *C. pruni* "A" and *C. pruni* "B". Population genetics analysis with microsatellite markers (Sauvion et al. 2007) showed that the two species overlap over a large geographical area around the Mediterranean (N. Sauvion, unpublished data) and there are indications that both cryptic species are potential vectors of '*Ca*. P. prunorum'.

Naturally infected individuals of *C. pruni* were found in several European countries like Italy (Carraro et al. 1998), France (Yvon et al. 2004), Spain (Laviña et al. 2004), Czech Republic (Fialová et al. 2004), Switzerland (Ramel and Gugerli 2004), Germany (Jarausch et al. 2007b), Bosnia-Herzogovina (Delić et al. 2008), Turkey (Ulubaş Serçe et al. 2011), Austria (Lethmayer et al. 2011) and Bulgaria (Etropolska et al. 2016). Interestingly, the natural infection rate of *C. pruni* highly varies among different geographical zones and in most of the described cases very few individuals were carrying the phytoplasma in the field. In Germany, Jarausch et al. (2007b, 2008) found 2–3% of the field collected overwintered adults naturally infected by '*Ca*. P. prunorum'. Similar low infection rates of only 0.6% were confirmed in France by Jarausch et al. (2001) and Thébaud et al. (2008). In contrast, Ermacora et al. (2011) reported infection rates in the first remigrants in apricot orchards in Northeastern Italy of 56.4% reaching a plateau slightly exceeding 80% in the last two captures. Ulubaş Serçe et al. (2011) found a mean percentage of 23% infected individuals of *C. pruni* collected on *P. spinosa* or wild plum. In a recent study, Maier et al. (2013) ascertained the phytoplasma in 0–11.5% of the remigrants and in 0–3.44% of the springtime generation insects in lower Austria.

The transmission of '*Ca*. P. prunorum' by different developmental stages of *C. pruni* was studied in detail under controlled conditions (Carraro et al. 1998, 2001b, 2004). The overwintered adults (remigrants) as well as the adults of the new generation (emigrants) of *C. pruni* were able to transmit the agent to healthy test plants. The remigrants were often already infected and infectious when they reached their *Prunus* hosts in early spring. The authors concluded that *C. pruni* transmits the winter-retained phytoplasma that had been acquired the previous year. The overwintered psyllids continued to transmit the pathogen in a persistent manner until their death. During transmission trials under controlled conditions conducted in Germany by Jarausch et al. (2007b, 2008), the vector capacity of overwintered and new generation adults of *C. pruni* was consistently lower than that described by Carraro et al. (2001b, 2004). Similar low infectivity and transmission rates of only 0.6%

were confirmed in France by Jarausch et al. (2001) and Thébaud et al. (2008). Thébaud et al. (2009) demonstrated that the population of *C. pruni* has an extremely long "effective latency" period which lasts the overwintering period. During this time the phytoplasma concentration within the insects continuously rises reaching a maximum of 10^7 phytoplasmas per insect at remigration. They concluded that only overwintered adults can efficiently transmit the agent and, thus, the disease spread is monocyclic. The vertical (transovarial) transmission of '*Ca.* P. prunorum' was not observed by Carraro et al. (1998) and Thébaud et al. (2009) whilst Tedeschi et al. (2006) proved the existence of this passage in *C. pruni*. Many studies showed that wild *Prunus* spp. play an essential role in the epidemiology of the disease, not only as a reservoir of the psyllid vectors but also of the phytoplasma, in particular blackthorn (*P. spinosa*) and myrobalan plum (*P. cerasifera*) (Jarausch et al. 2001, 2008; Carraro et al. 2002; Fialová et al. 2004, 2007; Labonne and Lichou 2004; Laviña et al. 2004; Poggi Pollini et al. 2004; Yvon et al. 2004; Ramel and Gugerli 2004; Delić et al. 2008; Maier et al. 2013). Recently, Sabaté et al. (2016) pointed out the role of *P. mahaleb* as potential reservoir of '*Ca*. P. prunorum' and its vector *C. pruni* in Spain. Whereas low populations of *C. pruni* were found on cultivated *Prunus spp.* such as *P. armeniaca, P. persica, P. amygdalus* and *P. domestica,* much higher vector densities were reported from different wild *Prunus* spp. such as *P. spinosa, P. cerasifera, P. domestica* and *P. salicina*. Interestingly, the wild *P. spinosa* and *P. cerasifera,* which represented reservoirs for the pathogen and the vector, rarely showed typical symptoms (Carraro et al. 2002; Jarausch et al. 2008). In conclusion, many wild *Prunus* spp. play an important role in the epidemiology of ESFY disease as the cycle of '*Ca*. P. prunorum' as well as of its vector *C. pruni* can be completed independently from the presence of infected cultivated stone fruit trees. In order to determine the distance that psyllids could spread the pathogen by natural means, Maier et al. (2013) tracked the dispersal of *C. pruni* in a model apricot orchard in lower Austria during a mark, release and recapture experiment. The study proved a fast and frequent tree-to-tree movement of *C. pruni* adults. Insects easily covered distances from row to row or even farther (ca. 13 m) within 24 hours after release and were present in a large part of the model orchard after 8 days (up to 24 m from the release point).

3.5 Pear Psyllids

In Europe, three recognized or presumed vectors of pear decline disease (PD) live on pear: *Cacopsylla pyri* (Linné 1758) (Fig. 3.5), *C. pyricola* (Foerster 1848), and *C. pyrisuga* (Foerster 1848). *C. pyri* is reported from Europe, the Caucasus, Central Asia, the Russian Far East and China; *C. pyricola* naturally occurs in the Western Palaearctic and has been introduced into the USA and Canada in the early nineteenth century (Ossiannilsson 1992; Ouvrard 2017). The two species are oligophagous on *Pyrus* species such as *P. communis*, *P. eleagrifolia*, *P. pyraster*, *P. amygdaliformis* and *P. salicifolia* (Burckhardt 1994). The biology of *C. pyri* and *C.*

Fig. 3.5 *Cacopsylla pyri* life stages: (**a**) eggs, (**b**) nymphs, (**c**) adults, (**d**) female (DISAFA, Entomology unit, University of Torino, Italy)

pyricola is similar since both are polyvoltine (Burckhardt and Hodkinson 1986). Thus, *C. pyri* can complete 4–5 generations in Central Europe and up to 8 generations in Southern France. Two morphologically distinct forms can be distinguished: a darkish winter form (*C. pyri* f. *pyri*) and a light summer form (*C. pyri* f. *pyrarboris*). *C. pyricola* has 4–5 generations in France and 3–4 in the USA with the darker winter form (*C. pyricola* f. *simulans*) appearing as one and the lighter summer form (*C. pyricola* f. *pyricola*) as 3–4 generations per year, respectively. The first oviposition of the winter form coincides with raising temperatures in early spring on leave buds and midribs of the leaves (Burckhardt 1994). In contrast, *C. pyrisuga* is univoltine; the adults overwinter on conifers and remigrate to *Pyrus* spp. by middle March to April. Egg deposition takes place in two different steps at the beginning of April and second in the middle of May followed by a 6 week lasting nymph development and the emergence of new adults in June. All three pear psyllids can cause direct damage on pear trees: the nymphs affect plant growth by sucking phloem-sap, while the secreted honeydew burns plant tissue and favours the growth of sooty mold.

First reports of pear psyllids as vectors of phytoplasmas came from the Pacific coast of North America. Jensen et al. (1964) identified *C. pyricola* as the vector of '*Ca.* P. pyri' at a time when the disease was thought to be virus-borne. Since then no

further vector has been described for the USA. However, the distribution of the putative vectors of '*Ca.* P. pyri' in Europe and the whole Palearctic region is diverse: while for Great Britain only *C. pyricola* has been described as vector (Davies et al. 1992), *C. pyri* was identified as main vector in France (Lemoine 1984), Italy (Carraro et al. 1998) and Spain (Garcia-Chapa et al. 2005). In Czech Republic (Kucerova et al. 2007; Ludvikova et al. 2011) and in lower Austria (Lethmayer et al. 2011) all three pear psyllid species were found naturally infected with '*Ca*. P. pyri', while in Turkey only *C. pyri* carried the phytoplasma albeit all the three species were present in the investigated regions (Kaya et al. 2016). In contrast to *C. pyri* and *C. pyricola*, the vector capability of *C. pyrisuga* is so far not yet confirmed (Jarausch and Jarausch 2010). Recently, a fourth pear psyllid, the polyvoltine species *Cacopsylla bidens* (Šulc 1907) has been found infected with '*Ca.* P. pyri' in Bulgaria (Etropolska et al. 2015). Also for this species its vector capability still needs to be proven.

After the identification of *C. pyricola* as vector for '*Ca*. P. pyri' in California (Jensen et al. 1964), many investigations in the USA and Europe followed in order to determine the infection rate of the psyllids and to analyse the transmission parameters. In United Kindgdom transmission trials carried out with field-collected *C. pyricola* yielded transmission rates between 3–61% depending on the collection site of the psyllids (Davies et al. 1992). Acquisition of '*Ca*. P. pyri' by *C. pyricola* from experimentally infected pear seedlings was best in August and lowest in winter. In California, Blomquist and Kirkpatrick (2002a) detected the pathogen in both winter and summer forms of *C. pyricola*, but without a clear seasonal trend. The number of phytoplasmas per psyllid was estimated to range from 1×10^6 to 8.2×10^7 with higher titre in the winter form. They concluded that psyllid-mediated spring infections could happen well before '*Ca*. P. pyri' would normally recolonize the upper part of the tree from the roots. In Italy, Carraro et al. (1998, 2001a) detected '*Ca*. P. pyri' in 55% of groups of *C. pyri* collected from March to October in the orchards and 30% of the inoculated test plants became infected. They could furthermore show that *C. pyri* retained the phytoplasma during winter, but could not transmit PD to dormant plants. Raddadi et al. (2011) tested *C. pyri* collected in Italian pear orchards detecting '*Ca.* P. pyri' in 26% of the specimens (in 27% of males, and in 25% of females) with nested-PCR and in 51% of the individuals through quantitative PCR with a number of '*Ca.* P. pyri' cells ranging from 1.43×10^1 to 8.50×10^5 per *C. pyri* individual. Moreover, fluorescent *in situ* hybridization (FISH) allowed to detect '*Ca.* P. pyri' in Malpighian tubules and salivary glands of *C. pyri*. Garcia-Chapa et al. (2005) found that the percentage of infected individuals in Spain is similar from June to August but reaching a rate of almost 100% in September coinciding with the maximum phytoplasma titre in the aerial plant parts. The highest transmission rate to an artificial sucrose medium was obtained in August and also in October. Although the percentage of infected psyllids was similar for both genders, '*Ca*. P. pyri' transmission by females was significantly higher than by males. During transmission trials under controlled conditions Caglayan et al. (2010) showed the capability of *C. pyri* to transmit PD from infected pears to healthy periwinkles and confirmed it as vector of '*Ca*. P. pyri' in Turkey.

PD has also been found in Taiwan (PD-TW) where the European species *C. pyri* and *C. pyricola* are not present. Liu et al. (2007) found two other *Cacopsylla* species, *C. qianli* and *C. chinensis*, infected with the PD-TW phytoplasma. Their role in transmission of PD-TW phytoplasma in Taiwan remains to be clarified. But recently Liu et al. (2011) proved the transmission capacity of *C. chinensis* during transmission trials in Taiwan. Based on PCR detection and symptom development they showed that pear trees were either infected by PD-TW or PD-TWII phytoplasma strains, or co-infected by both, when exposed to *C. chinensis* specimens.

Insect vectors for peach yellow leaf roll (PYLR) phytoplasma have been searched intensively in California in the 1980s and 1990s. As two similar diseases, western X and PYLR, associated with two genetically distinct phytoplasmas exist in the same region, only the application of molecular methods enabled to proof that a psyllid is the main vector of PYLR phytoplasma. Experimental transmission of PYLR phytoplasma to peach seedlings was achieved with field collected *C. pyricola* from naturally infected peach trees (Guerra 1997). In field surveys for leafhoppers and psyllids in diseased peach orchards only *C. pyricola* proved to be infected with PYLR phytoplasma as confirmed by molecular means (Blomquist and Kirkpatrick 2002b). Ten to 25% of groups of 10 individuals were positive indicating a high infection rate. Infected psyllids were captured from peach as well as from pear grown in the neighbourhood. The population dynamics of *C. pyricola* was similar in peach and pear with low densities in summer and an increase in autumn. Thus, the spread of PYLR is dependent on adjacent pear orchards where presumably the vector reproduces (Purcell et al. 1981).

3.6 Genetics

Morphological differentiation is problematic due to extensive resemblance of some psyllid species especially among females and is error-prone for nymphs (Oettl and Schlink 2015). DNA-based techniques for the identification of insect species have recently become of particular interest either to support or even to replace traditional morphological discrimination (Jenkins et al. 2012). Molecular methods offer in particular advantages for the identification of immature stages where distinct morphological characters are lacking or are not sufficient for discrimination at the species level. Furthermore, molecular methods can help to identify insects which are damaged by removal from sticky traps. One approach is PCR-RFLP based on the cytochrome c oxidase subunit I (COI) region. This gene is widely used as barcoding marker for species determination and provides therefore a valuable tool also for the identification of invasive insect species and quarantine pests. This method has been successfully applied for the molecular discrimination of *C. melanoneura* and *C. picta* from other *Cacopsylla* species which are difficult to distinguish by morphological means (Oettl and Schlink 2015). This rapid and cost-effective approach allowed also a reliable identification of nymphal stages.

Tedeschi and Nardi (2010) developed a molecular tool based on the mitochondrial control region (CR) to distinguish psyllid species living together on hawthorn:

C. melanoneura and *C. affinis*. This PCR assay allowed the species identification by using specific primers and different sizes of the PCR products. By testing individuals of *C. melanoneura* from different regions in Italy, two genetic variants were detected by this method. The variants differed by the presence (WI, with indel) or the absence (WOI, without indel) of a 56 bp indel. Whereas the WOI type was dominant in all tested regions the WI type was consistently found at low percentages ranging from 5% to 31%. Recently, WI and WOI variants of *C. melanoneura* were also detected by this approach in Bulgaria (Etropolska et al. 2016). A distinction of different populations of *C. melanoneura* was enabled by the development of microsatellite markers for this species (Malagnini et al. 2007). With these SSR markers populations from apple could be differentiated form those of hawthorn indicating that two different host races of *C. melanoneura* may exist (Malagnini et al. 2013). Whether these genetic differences are linked to the observed differences in phytoplasma vectoring ability is not known.

Microsatellite genotyping was also applied to analyse the population structure of *C. pruni* (Sauvion et al. 2007, 2009). Based on the analysis of nine microsatellite *loci* two distinct populations of *C. pruni* – named A and B – were identified. Peccoud et al. (2013) demonstrated that both groups can be phylogenetically separated on their ITS2 sequences. These authors developed specific PCR primers for each group enabling an easy molecular typing of *C. pruni* A and B. It is important to note that both groups cannot be distinguished morphologically. As far as known, both populations occur sympatrically in Southern France while type B is predominant in most of the other European regions. Etropolska et al. (2016) found only *C. pruni* type B in different regions of Bulgaria.

3.7 Rearing

Although all Psylloidea pass through 5 instars, the period for complete nymph development under natural conditions can be highly influenced by variations of ambient temperature, humidity and voltinism status (Hodkinson 2009). This inconvenience can be eliminated by the establishment of rearings under controlled and standardised conditions. Breeding of polyvoltine species without host alternation such as *C. pyri* can be started from field collections on *Pyrus* cv. all around the year. However, also multivoltine species such as *C. pyricola* or *C. pyri* undergo a reproductive diapause in the autumn generation (Hodkinson 2009). Compared with polyvoltine psyllid species, the austere life cycle and obligate host change of univoltine species makes collection and rearing difficult. However, efforts undertaken during the last years identified the key factors for the establishment of permanent rearings of the univoltine species *C. picta* and *C. pruni*.

The most common way to establish psyllid colonies usually starts from pairs or groups of field collected adults which are caged on specific host plants for copula-

tion and oviposition. In order to obtain healthy psyllid populations for diverse laboratory use, standardised plant material from tissue culture or from seedlings was used, e.g. from *Malus* cultivars Golden Delicious or Royal Gala for *C. picta* and *C. melanoneura* colonies (Jarausch et al. 2004; Mayer et al. 2009; Tedeschi et al. 2003) or from *Prunus* cultivars *P. marianna*, *P. salicina or P. cerasifera* for *C. pruni* (Carraro et al. 2001b, 2004; Jarausch et al. 2008; Thébaud et al. 2009), respectively. Since transovarial transmission of the phytoplasma has been demonstrated for *C. picta* (Mittelberger et al. 2017) and *C. pruni* (Tedeschi et al. 2006), PCR testing of individuals has to be done to ensure a phytoplasma-free psyllid population. For subsequent acquisition trials, rearings were directly installed on phytoplasma-infected *Malus* or *Prunus* cultivars under similar rearing conditions.

Breedings of the most important univoltine *Cacopsylla* vector species *C. picta, C. melanoneura* and *C. pruni* were installed in cages, bugdorms or glass vessels infested with mature females and males collected from their natural host plants from February until April (Carraro et al. 2002; Jarausch et al. 2004, 2008; Mayer et al. 2008a; Tedeschi et al. 2003). The preimaginal development under recommended rearing conditions for these northern temperate species (20–25°C day and around 15°C night, relative humidity between 50–80% and natural day light conditions of light:dark, 16:8 hours) (Jarausch and Weintraub 2014) takes about 5 weeks until emergence of new adults. Thus, psyllid colonies could be maintained on the reproduction host plant under experimental conditions for several months, at least until autumn of the same year. However, as univoltine vector species have an obligate alternation of plants for reproduction and overwintering, a permanent rearing was not possible. For a long time it was believed that conifers are used by migrating *Cacopsylla* species just for shelter during winter time (Burckhardt 1994; Burckhardt et al. 2014). It remained unclear whether overwintering psyllids actually fed on conifers (Hodkinson 2009). Recent studies confirmed that these species have a feeding activity on overwintering plants (Gallinger and Gross 2018), and die when isolated on a deciduous conifers plant such as *Larix decidua* (Pizzinat et al. 2011). Thus, Jarausch and Jarausch (2014) succeeded for the first time to maintain a hibernating continuous colony of *C. picta* until the next year and hence they could establish a permanent rearing of this vector species. Based on empiric data from field observations in combination with results obtained during laboratory experiments they elaborated the following parameters and key factors for a successful overwintering of *C. picta* and the establishment of a continuous rearing: (i) smooth host plant switch, (ii) moderate summer temperatures and sufficient humidity, (iii) natural winter climate conditions with cold and frost and (iv) suitable conifer species, in this case spruce or pine. The model suggests a direct migration of emigrants from the reproduction host apple to conifers as aestivation and overwintering plants in summer and vice versa to apple orchards for remigrants in early spring. However, the host plant switch does not occur abrupt, but is characterized by a smooth adaptation phase from its host for reproduction to its overwintering plants as experimentally demonstrated. Despite some species-specific particularities, the same approach was adopted for rearing the vector of '*Ca*. P. prunorum', *C. pruni* (Jarausch and

Jarausch 2016). These studies confirmed that both psyllid species are univoltine and cannot reproduce on conifers.

A further rearing approach for *C. pruni* was applied by Thébaud et al. (2008) using sleeve cages on healthy or infected *Prunus* plants. Thébaud et al. (2009) found *C. pruni* on *Picea abies* at an altitude of 1260 m a.s.l. and were able to overwinter them in sleeve cages on these conifers but survival rates ranged only between 1.2 and 8.9%. Using a similar approach, Pizzinat et al. (2011) could hibernate *C. melanoneura* in branch cages on different coniferous species at an altitude between 1442 and 1636 m a.s.l. in Northwestern Italy.

3.8 Multitrophic Interaction

The secret language of the earth's ecosystem involves a multitude of players that populate the playing field and occupy different rungs on the food chain (Wartenberg 2016; Gross 2016; Sauvion et al. 2017). The actors include plants, plant feeding insects, insect feeding insects, plant parasites, insect parasites, pathogenic and beneficial microorganisms, vectoring organisms, pollinators, and more (Gross 2016; Corcket et al. 2017; Kaiser et al. 2017; Giron et al. 2017). Thus, fundamental research on the biology and ecology including chemically mediated multitrophic interactions of vectoring psyllids is needed for understanding vector epidemiology, plant pathogen transmission and for developing appropriate and sustainable control measures of vectors and pathogens (Gross 2016).

The natural enemies of phloem-feeding insects (predators, parasitoids, or entomopathogens) may have large effects on speciation, community composition and ecosystem processes (Elzinga et al. 2007). Hence, these plant-pathogen–vector systems are of particular interest, in which a transmitted pathogen infects both, its host plant and vector insect, because pathogens and their differing hosts (plant and insect) must develop co-adapted strategies to avoid deleterious effects of each other (Gross 2016). In general, the pattern of volatile organic compounds (VOCs) released from plant modules (leaves and flowers) can change both quantitatively and qualitatively with abiotic (e.g. warming, drought) and biotic stressors (e.g. herbivore feeding, pathogen infection).

The role of allelochemicals for psyllid behavior and the influence of phytoplasma infections on volatile production of psyllid host plants were studied during the last 12 years (Gross 2016). For identifying their particular host plants for feeding and reproduction, volatile signals are used in many species during migration (Gross and Mekonen 2005; Mayer et al. 2008a, 2008b, 2009; Soroker et al. 2004; Weintraub and Gross 2013). Also non-volatile phloem/xylem components influence host choice and maybe oviposition behaviour (Mayer et al. 2011). By analysing the VOCs emitted by the leaves of apple trees, it was shown that '*Ca*. P. mali' changed the odour of infected trees compared to healthy ones (Mayer et al. 2008a). In pear trees infected with '*Ca*. P. pyri', the expression of ethylbenzoate differed significantly between infected and healthy trees. The virulence of different strains of '*Ca*. P.

mali' was proven to influence the pattern of produced volatiles in the model plant tobacco as well as in apple trees both quantitatively and qualitatively (Rid et al. 2016). The influence of volatiles on the interactions between the pathogen '*Ca*. P. mali', the host plants for reproduction (apple trees) and overwintering (conifers) of its vector *C. picta* and the proposed epidemiology of '*Ca*. P. mali' is the most studied so far: *C. picta* reproduces on apple and new adults emerge at phenology stages 69–71 (late blossom, early fruit development) of the apple plant. These emigrants are attracted by β-caryophyllene (Mayer et al. 2008b), which is mainly produced by infected apples during this time (Mayer et al. 2008a), and both males and females are lured to infected plants (J. Gross, unpublished data), increasing the number of psyllids, which are able to acquire the phytoplasma. Witches' broom produced exclusively by infected plants increase their leaf surface and may support the emission of volatile β-caryophyllene (Mayer et al. 2011). Shortly after feeding on apple, the adults emigrate to conifers where they stay until spring (Mayer and Gross 2007). After overwintering, the psyllids return to apple trees (remigrants), but now prefer to lay their eggs on uninfected plants, which increases the opportunity to transmit the phytoplasma (Mayer et al. 2011). Which signals may regulate this egg-laying behaviour still remains unknown and are the focus of ongoing research. By developing on apple plants infected by '*Ca*. P. mali', the nymphs of *C. picta* suffered higher mortality and remained smaller compared to the ontogenetic development on uninfected plants (Mayer et al. 2011). In contrast, infection by '*Ca*. P. mali' was tolerated by adults and seems to have no detrimental effect. Thus, females of *C. picta* evolved mechanisms to minimize harmful effects for their offspring emanated by the phytoplasma by avoiding oviposition on infected plants. In this context it seems possible that non-volatile signals from phloem sap could be involved in the psyllids' final decision (J. Gross, unpublished data). This behavior ensures the development of a new, vital vector generation, which is important for the spread of the phytoplasma. In conclusion, the complex multitrophic interactions between phytoplasma, plant and vector may result in both higher numbers of transmitting vector insects and a very effective transmission of the phytoplasma within the insect population.

The outcome of the research on multitrophic interactions is the key for developing innovative and sustainable applications in phytomedicine. Besides intraspecific active pheromones, also interspecific active allelochemicals such as β-caryophyllene or ethylbenzoate can be used as lures in traps. Different classes of infochemicals (attractants, arrestants, and repellents) can be combined to attract-and-kill strategies, push-and-pull or push-pull-kill strategies (Gross and Gündermann 2016). By using allelochemicals instead of pheromones, the emission rates of the attractive compounds, often plant produced kairomones, has to be multiple times higher than by using pheromones, requiring new types of dispensers or microencapsulated infochemicals (Gross 2017). Signals triggering interactions between psyllids, phytoplasma and plants and within psyllid species are not restricted to volatile and non-volatile infochemicals, as the existence of acoustic communication in courtship behaviour of *C. pyri* has been shown recently (Eben et al. 2014). This may enable innovative attempts for the control of *C. pyri* by extending push-and-pull strategies to acoustic signals and including the use of both attractive chemical and acoustic

signals in combination with repellent signals (also acoustic or chemical) (Gross 2017). Based on the results on multitrophic interactions the development of chemically lured traps for monitoring and mass trapping has recently started (Eben and Gross 2013; Gross and Gündermann 2016).

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Chapter 4
Vector Role of Cixiids and Other Planthopper Species

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Abstract Cixiid planthoppers are primary vectors of several phytoplasma associated diseases on economically important crops as well as on herbaceous plants that act as pathogen reservoirs. The main phytoplasma transmitted by the cixiid species is '*Candidatus* Phytoplasma solani', also known under the trivial name of "stolbur" phytoplasma. The principal vector of '*Ca.* P. solani' is *Hyalesthes obsoletus*, while three additional cixiid vectors are assumed to have minor or middle role in transmission, or have local importance: *Reptalus panzeri*, *R. quinquecostatus* and *Pentastiridius leporinus*. "Bois noir" disease of grapevine is the most studied and widespread '*Ca.* P. solani'-associated disease transmitted by *H. obsoletus.* Studies on spread and epidemiology of this disease resulted in identification of several epidemiological cycles in the different parts of Europe. Each cycle is associated with specific plant-hosts of the vector population: *Urtica dioica*, *Convolvulus arvensis*, *Vitex agnus-castus* and *Crepis foetida*. However, in France *H. obsoletus* is associated with cultivated plant-hosts, *Lavandula* spp. and *Salvia sclarea*, acting as pathogen reservoirs. *R. panzeri* is a vector of '*Ca*. P. solani' involved in its transmission to grapevine, maize and potato. However, its epidemiological role is up-to-date determined only in south-eastern Europe, i.e., the Balkan region; presumably due to limited geographical distribution of this cixiid species and its feeding preferences being different in central Europe. *R. quinquecostatus* is a vector of '*Ca*. P. solani'; but, without clear defined epidemiological role in any of the '*Ca*. P. solani'-

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_4

associated diseases. *P. leporinus* is a major vector of '*Ca*. P. solani' to sugar beet in France, involved in a specific epidemiological cycle dependent upon annual crop rotation including winter cereals. Role of *Dictyophara europaea* and planthopper putative vectors of diverse genera harboring phylogenetically diverse phytoplasma groups are also described.

Keywords '*Candidatus* Phytoplasma solani' · *Hyalesthes obsoletus* · *Reptalus* spp. · *Pentastiridius leporinus* · *Dictyophara europaea*

4.1 *Hyalesthes obsoletus*

Taxonomy, Biology and Distribution The cixiid planthopper *Hyalesthes obsoletus* Signoret 1865 (Hemiptera: Cixiidae) (Fig. 4.1) is the principal insect vector of '*Candidatus* Phytoplasma solani' with a major role in the disease epidemiology (Maixner 1994; Langer and Maixner 2004; Johannesen et al. 2008; Kosovac et al. 2016a). *H. obsoletus* is a polyphagous species feeding in diverse plant hosts, many of which are herbaceous plants involved in '*Ca.* P. solani' epidemiology as natural reservoirs and inoculum sources (Hoch and Remane 1985; Langer and Maixner 2004; Sharon et al. 2005; Kosovac et al. 2016a). The insect is native to southern-central Europe, Mediterranean, south-Russia, Kazakhstan and Asia Minor; it is polyphagous, thermophilic and occurs on stony or crumby soils, in abandoned land, on sunny embankments and dry grassland (Holzinger et al. 2003). The species reaches its northerly distribution limit in Germany and northern France where it is known from xerothermic habitats such as river valleys up to 350 ma.s.l. (Nickel 2003; Hoch and Remane 1985). The adults are polyphagous on herbaceous and occasionally also on woody plants, but reproduction occurs on few perennial herbaceous host species only (Lessio et al. 2007). In the insect's northern range field bindweed (*Convolvulus arvensis*) used to be the main host for reproduction.

Fig. 4.1 *Hyalesthes obsoletus* on *Urtica dioica* (stinging nettle) in the field (left) and on the plant (right)

Occasionally the species also reproduces on *Ranunculus* spp., *Cardaria draba* and *Calystegia sepium* (Holzinger et al. 2003; Langer et al. 2003); in Italy *H. obsoletus* was reported to reproduce on *Urtica dioica* (Alma et al. 2002). In the eastern Mediterranean, *H. obsoletus* is associated with diverse plants specific for this climatic area, among which *Vitex agnus-castus* (chaste tree or monks pepper) is the most frequently found to host insect adults (Hoch and Remane 1985; Sharon et al. 2005). During the last 15–20 years, however, in the northern distribution range a change of host plant occurred, as more and more *H. obsoletus* populations are linked to *U. dioica* as main developmental host. Occasionally adult *H. obsoletus* feed on grapevines and other crop species, but these species are not utilized for reproduction (Alma et al. 1988; Sforza et al. 1998; Maixner et al. 2008; Maixner 2011). In central Europe *H. obsoletus* is univoltine, while in eastern Mediterranean coast of Israel has two generations per year (Sharon et al. 2005). Eggs are deposited in groups on the soil close to their host plants. The hatching instars move into the soil where the instars feed on the roots of the host plants. The emergence of adults in the next year takes place in the soil and the adults leave the soil by crawling between rocks and earth (Alma et al. 1988). Adult flight periods in central Europe last for 6–8 weeks during the months of June to August (Sforza et al. 1998; Bressan et al. 2007; Forte et al. 2010).

H. obsoletus is, due to its relevance as phytoplasma vector, the most known representative of the south Palaearctic cixiid planthopper genus *Hyalesthes*. In addition, *H. obsoletus* is the type species, i.e., the name bearing type of the genus. However, there are number of taxonomic and nomenclature issues related to this species which are often neglected and are burdening the applied research on its role in phytoplasma diseases transmission, epidemiology and management strategy. According to the original description (Signoret 1865) the *H. obsoletus* type specimen was collected in south France (Chambéry), but a description of the host-plant is lacking. Attempts to locate the type material have failed (Hoch and Remane 1985), leading to the general conclusion that it is probably lost. In addition, type material of a single *H. obsoletus* synonym, *Liorhinus albolimbatus* Kirchbaum 1868, collected in Dalmatia is also probably lost (Hoch and Remane 1985) and there is no information of its host plant. Given the known diversity of host plant associations of *H. obsoletus* and its potential for genetic segregation and host-race formation (Johannesen et al. 2008; Imo et al. 2013; Kosovac et al. 2016b, 2018a) attributing a type specimen to any plant association would be speculative. Additionally, *H. obsoletus* represents only one of the seven species within the *Hyalesthes obsoletus* species group (Hoch and Remane 1985; Dlabola 1994) which further contributes to the difficulty of determining its exact host-plant associations and vector role across a wide geographic distribution. All species within the group are of identical outer morphology (distinguishable only based on male genitalia) and of sympatric occurrence with circum-Mediterranean *H. obsoletus sensu stricto* which make research and exact delimitation of *H. obsoletus* adults and immatures and their developmental host plants difficult (Kosovac et al. 2018a).

Epidemiology of '*Ca.* P. solani' in Central Europe Planthopper species belonging to the Cixiidae family are considered as the main vectors of '*Ca.* P. solani' known also as "stolbur" phytoplasma (subgroup 16SrXII-A). This pathogen is largely distributed in Europe and the Mediterranean area (COST FA0807 2014) and it affects a wide range of wild and cultivated plants such as solanaceous crops, many vegetables, maize, lavender and also grapevines where it induces a disease referred to as "bois noir" (BN) or "Vergilbungskrankheit" (VK). The spread of '*Ca.* P. solani' occurs via a disease cycle including herbaceous host plants as phytoplasma reservoirs and the transmitting insect vectors (Maixner et al. 1995; Maixner 2011).

Planthoppers transmit phytoplasmas in a circulative-propagative mode, a latent period between phytoplasma acquisition and the ability to transmit the pathogen is therefore required (Weintraub and Beanland 2006). In case of *H. obsoletus* it is generally assumed that phytoplasma acquisition occurs during the nymph stages which acquire the pathogen when feeding on the roots of infected host plants. Adults emerging from the soil are already infective (Maixner 2011). In transmission assays a minimum inoculation access period (IAP) between 3 and 6 h was reported (Bressan et al. 2007). *C. arvensis* and *U. dioica* (Fig. 4.1) are suitable hosts for *H. obsoletus* development and at the same time for phytoplasma. Nymphs feeding on infected *C. arvensis* and *U. dioica* roots may take up the phytoplasma nad become pathogen vectors. Nettle and bindweed therefore play a crucial role for phytoplasma spread in central Europe. Grapevine, in contrast, is a dead end host in disease cycles including *H. obsoletus* as main or sole vector because the instars never feed on grapevine and thus do not acquire the pathogen from grapevine plants (Maixner 2011).

The relevance of '*Ca.* P. solani' as relevant pathogen is attributable to several factors, namely the ability of the phytoplasma to infect a broad range of plant hosts, the ability of *H. obsoletus* to exploit some of these as developmental hosts and the polyphagy of adult *H. obsoletus* resulting in phytoplasma transmission to various crops.

In western and central Europe BN has been known by grapevine growers for over 50 years. In eastern France it was described at the beginning of the 60s, in Germany in the Rhine and Mosel valleys in 1965. In the 1990s, however, BN gradually became one of the most important diseases of grapevine with infection rates reaching 50–80% in some areas. An extended geographical range and increased populations of *H. obsoletus* were assumed to be the causes for the frequent and serious disease outbreaks (Boudon-Padieu 1999). Epidemic outbreaks alternated with endemic periods. In Germany, e.g. an epidemic outbreak during the 1990s with a peak incidence in 1997 was followed by a period of decreasing disease incidence in the years thereafter (Maixner et al. 2008). In Germany and in France BN epidemics in the 1990s were associated with bindweeds as reservoir host plant (Maixner et al. 1995; Sforza et al. 1998; Boudon-Padieu 1999). In contrast, a close association of *H. obsoletus* populations to nettle was reported from northern Italy (Alma et al. 2002). In 2004 Langer and Maixner observed that the phytoplasma types present in *U. dioica* were molecularly distinguishable from those detected in *C. arvensis*. Based on RFLP analysis of the *tuf* gene a pattern named tuf-type a was ascribed to

nettle-associated "stolbur" types, and of a pattern tuf-type b to bindweed-associated phytoplasmas. A tuf-type c pattern was detected during this study in *Calystegia sepium* and seemed to be restricted to the Mosel area and of local importance. Quite predominantly the *H. obsoletus* specimens caught on each of the plant species harbored the weed host specific '*Ca.* P. solani' type. The authors concluded that specific phytoplasma types are associated with nettle and bindweed respectively, and in the field nettle- and bindweed-type phytoplasmas are transmitted in different epidemiologic cycles. The same study, however, also reported that in Germany at this time (2004) bindweed phytoplasma types were predominant and a wide dissemination of nettle types was observed. In contrast investigations in Piedmont and Valle d'Aosta between 2002 and 2005 revealed an exclusive presence of nettle-associated "stolbur" types in the insect vectors, and confirmed the strong links between *H. obsoletus*, BN and *U. dioica* in north-western Italy (Lessio et al. 2007). In the following years in western and central Europe (France, Germany, Switzerland, northern and north-eastern Italy) the proportion of vector populations associated with *U. dioica* and infected with nettle-associated phytoplasma types increased substantially (Angelini et al. 2008; Carraro et al. 2008; Foissac et al. 2008; Kehrli et al. 2008; Maixner et al. 2008). Outbreaks of BN during the last 10–15 years such as those in Württemberg and (Maixner et al. 2008) in Austria (Aryan et al. 2014) or in Emilia Romagna (Contaldo et al. 2016) were predominantly associated with nettle phytoplasma types.

In 2008 Johannesen and co-workers carried out genetic analyses of *H. obsoletus* populations including specimens from several European countries and Israel. Mitochondrial DNA data strongly suggested a geographical expansion and a circum-Alpine invasion of *H. obsoletus* populations into German, northern Swiss and northern French grapevine growing areas in coincidence with BN outbreaks. No affiliation of host plant and insect mitochondrial DNA haplotype was found implying that *H. obsoletus* is able to exploit both host plant species. Nevertheless the study indicated a light but significant differentiation of host plant populations by random amplified polymorphic DNA (RAPD) markers. Further studies by microsatellite markers (Imo et al. 2013) provided strong evidence that the novel use of *U. dioica* as host plant in the north-western border of the species range had in fact resulted in a genetically divergent host population (host race) of *H. obsoletus* in mid-west Germany. A further study indicated that the tuf-type a pathogen had been introduced into northern viticultural areas by secondary, plant unspecialized insect vectors. The rapid dissemination of the tuf-type a phytoplasmas, however, was possible through the nettle specialized vector populations resulting from prior migration events. Thus in the northern areas the evolution of vector host races together with the introduction of a new phytoplasma type resulted in severe disease outbreaks in grapevine (Johannesen et al. 2012). The sudden infestation of nettle by *H. obsoletus* in northern regions could be related to an increase in temperatures. This theory is supported by the finding that development of *H. obsoletus* nymphs on *U. dioica* is retarded compared to *C. arvensis* (Cargnus et al. 2012). The global warming during the last 50 years led to longer vegetation periods and probably also

to longer feeding periods for the *H. obsoletus* nymphs and this might explain the shift to nettle of the insect populations (Imo et al. 2013).

A disease scenario similar to the one illustrated above for Germany and northern France, namely the sudden use of *U. dioica* by *H. obsoletus*, high planthopper densities and BN outbreaks involving a single *U. dioica*-associated "stolbur" strain was observed some years later in central to east European countries. In 2010 a dramatic increase of *H. obsoletus* populations on nettle was detected in southern Moravia, Czech Republic, whereas only a sporadic occurrence of a few individuals of *H. obsoletus* had been noted in 2008–2009 (Šafářová et al. 2011). In Austria, in accordance with Czech observations, investigations between 2003 and 2008 revealed absence or very low densities of *H. obsoletus* in most parts of the country (except in the southern, Slovenian border region) and solely bindweed-associated pathogen types (Riedle-Bauer et al. 2006, 2008). From 2012 onwards, however, a mass occurrence of *H. obsoletus* almost exclusively on stinging nettle all over eastern Austria was recorded (Aryan et al. 2014). The high population densities of *H. obsoletus* on nettle were accompanied by the frequent occurrence of '*Ca*. P. solani' in nettles, planthoppers and grapevines. Analysis of the phytoplasma types by aid of four molecular markers (*secY*, *stamp*, *tuf* and *vmp1* genes) revealed the presence of a single genotype named CPsM4_At1 in stinging nettles, and more than 64% and 90% of its abundance in grapevine and *H. obsoletus,* respectively. This genotype showed a tuf-type b restriction pattern previously attributed to bindweed-associated '*Ca*. P. solani' strains, but a different sequence compared to reference tuf-type b strains (designated as tuf-type b2) due to a substitution in the *Hpa*II restriction site of this type compared to tuf-type a. All other marker genes indicated that this was a distinct nettle phytoplasma genotype (Aryan et al. 2014). In the following years CPsM4_At1 continued to spread in Austria (Riedle-Bauer et al. 2016) and several studies revealed the wide dissemination of nettle-associated '*Ca*. P. solani' types showing a tuf-type b2 RFLP pattern in central, as well as in eastern Europe (Atanasova et al. 2015; Plavec et al. 2015; Kosovac et al. 2016a).

Genetic analyses of the insect vectors involved in the Austrian disease scenario assigned three Austrian nettle-associated populations to two regional European (Pannonian and Adriatic) reference populations and proved that the vector's exploitation of *U. dioica* was not due to the expansion of an ancestral *U. dioica*-associated host race of the vector. The spread of the vector and the pathogen was equivalent to the reported situation in central-western Europe. In both cases disease incidence was low and coupled to *C. arvensis* until the rise of a *U. dioica*-associated phytoplasma type and vector population. However, the vector populations and their associated '*Ca*. P. solani' types in Austria and in central-western Europe differed and thus the dissemination histories were independent. It remains to be tested whether the new use of *U. dioica* in Austria leads to separate insect host races. If verified, this would be an independent case of host-plant specialisation (Johannesen and Riedle-Bauer 2014).

In parallel to the evolution of the nettle-associated vector populations, bindweed-associated *H. obsoletus* (Fig. 4.2) populations accounted and still account for significant disease outbreaks in central Europe. Epidemics are not restricted to

Fig. 4.2 *Convolvulus arvensis* (field bindweed)

grapevine; frequently solanaceous crops and vegetables area also affected. In north eastern Italy (Trieste area) in 2002 and in the following years the abundant presence of *C. arvensis* within and around vegetable fields entailed significant *H. obsoletus* populations and severe "stolbur" outbreaks in celery and tomato (Carraro et al. 2008). A major disease outbreak on solanaceous crops and celery associated with bindweed reservoirs was recorded in south Moravia from 2006 onwards (Navrátil et al. 2009). In northern lower Austria at present (2017) bindweed-associated vector populations account for severe "stolbur" outbreaks in potatoes and vegetables. Interestingly these bindweed-associated vector populations occur in a region where absence or very low densities of *H. obsoletus* have been recorded in 2003–2008 (Riedle-Bauer et al. 2006, 2008). All studies examining nettle-associated populations confirm high insect densities on this plant species as, e.g., in the Verona province (Italy) (Bressan et al. 2007), Switzerland (Johannesen et al. 2013), Mosel (Germany) (Maixner and Johannesen 2013), Serbia (Mori et al. 2013), and Austria (Aryan et al. 2014; M. Riedle-Bauer, unpublished). It seems that the enormous *H. obsoletus* densities on nettle contribute to the BN outbreaks associated with nettle-BN types during the last 15 years.

The host species utilized for reproduction influences the phenology of *H. obsoletus*; in general, delayed phenology of populations on *U. dioica* as compared to *C. arvensis* has been reported. A study in northern Italy revealed that nymphs on *C. arvensis* overwintered as third instars only, whereas nymphs on *U. dioica* mostly overwintered as second instars. Subsequently on field bindweed the fifth instars were recorded by early June, on stinging nettle by mid-late June (Cargnus et al. 2012). In accordance to these observations the vector flight activity on stinging nettle starts approximately 2–4 weeks later than on bindweed (Maixner et al. 2009; Forte et al. 2010).

Phytoplasma infestation rates of *H. obsoletus* populations fluctuated greatly between studies, locations and sampling years but, in general, a significant percentage of the planthoppers carried the phytoplasma. Rates of PCR positive individuals

from 8% to 80% have been observed for nettle-associated populations (Bressan et al. 2007; Maixner and Johannesen 2013; Johannesen et al. 2013; Šafářová et al. 2013; Riedle-Bauer et al. 2016). Infection levels of bindweed affiliated *H. obsoletus* populations were comparable, during epidemic phases levels between 13% and 44% were recorded (Maixner et al. 1995; Sforza et al. 1998; Riolo et al. 2007). The high infection levels together with the reported short IAP allow a rapid phytoplasma spread despite the fact that grapevines are poor hosts for the planthopper (Bressan et al. 2007). Several investigations suggest that vector densities, and therefore disease pressure, are predominantly influenced by the species surrounding the crop. Usually *U. dioica* is rarely present in vineyards, while frequently nettle patches of varying size grow in roadside ditches, in fallow areas, along embankments or close to walls or wood piles. High nettle densities can be associated with high BN incidences. In most cases *H. obsoletus* do not fly distances of more than a few meters (Bressan et al. 2007) in consequence BN incidence is highest at the edges of the vineyards and/or when the distance between grapevines and the nettle sources is short (Mori et al. 2008, 2012). Bindweed in contrast, is a creeping, stolon-producing plant, spreading both inside and outside fields and vineyards. Bindweed in green covers of vineyards or inside fields may therefore result in development of *H. obsoletus* directly in the crop or the vineyard (Maixner 2006). Several studies, however, indicate a significant role of the vineyard surroundings also in case of bindweed-associated disease cycles. Sforza and co-workers (1998) observed a frequent presence of bindweed and regular captures of *H. obsoletus* on fallows in France. Investigations inside and outside vineyards in Austria showed higher *H. obsoletus* captures in fallows as compared to green covers inside vineyards (Riedle-Bauer et al. 2011). These findings are supported by the observation that also in case of bindweed cycles disease incidence is usually highest in the border rows of the crop.

Adult *H. obsoletus* prefer open soil with sparse vegetation. This behavior can result in high infection pressure for vineyards and field crops. Due to the shortage of other alternative food plants the planthoppers feed on the crop species (Maixner et al. 2008). This scenario was confirmed by vineyard experiments including different soil covers. Cixiids were regularly captured in the canopy of plots with open soil, but not in those with green cover (Riedle-Bauer et al. 2011).

Epidemiology of '*Ca.* P. solani' in South-Eastern Europe The cixiid planthopper *H. obsoletus* is a principal vector of '*Ca.* P. solani' associated with several diseases threatening diverse agricultural crops in south-eastern Europe and western Asia, with a major role in pathogen spread and epidemiology (Aleksić et al. 1967; Cvrković et al. 2014; Atanasova et al. 2015; Kosovac et al. 2016a). As in central Europe, *U. dioica* and *C. arvensis* are considered to be the main host-plants of *H. obsoletus* and '*Ca.* P. solani' reservoir plants and inoculum sources (Langer and Maixner 2004; Johannesen et al. 2008; Imo et al. 2013; Kosovac et al. 2016a). Each plant harbors a specific pathogen strain according to the elongation factor Tu gene, tuf-type a (type I; including its variants tuf-type b2 or tuf-type ab) and tuf-type b (type II), respectively (Langer and Maixner 2004; Aryan et al. 2014; Atanasova

et al. 2015; Kosovac et al. 2016a). Hence, the source plants determine the epidemiological pathways of the transmitted phytoplasma diseases as well as the pathogen genotype. Both tuf-type a and tuf-type b '*Ca.* P. solani' strains were detected in infected plants (mostly grapevine) in Macedonia, Montenegro and Georgia (Atanasova et al. 2015; Kosovac et al. 2016a; Quaglino et al. 2016), while solely tuf-type b strains were identified in Hungary, Serbia, Bosnia and Herzegovina, Romania, Russia, Azerbaijan and Israel (Duduk et al. 2004; Cvrković et al. 2014; Delić et al. 2016; Balakishiyeva et al. 2018; Ember et al. 2011, 2018). However, *H. obsoletus*-transmitted '*Ca.* P. solani' strains in south-eastern Europe, including the Mediterranean coastal regions, show the occurrence of additional regional-specific vectors' host-plants harboring the tuf-type b strain further redirecting disease spread and epidemiology (Kosovac et al. 2013, 2016a; Sharon et al. 2005, 2015).

In the Mediterranean coastal zone the *Vitex agnus-castus* (monk's pepper) plants (Fig. 4.3) are a preferred host of *H. obsoletus* (Hoch and Remane 1985; Sharon et al. 2005). First record of association with this host-plant is dated to year 1890 (Horváth 1891), but it was since forgotten until recent epidemiological studies in Israel (Sharon et al. 2005, 2015) which confirmed the tight association of *H. obsoletus* adults and nymphs with the *V. agnus-castus*. According to the literature and recent faunistic data *H. obsoletus* populations of this host-plant association are found from north Dalmatia (Horváth 1891) through the east Adriatic and Aegean coast of Bosnia and Herzegovina, Montenegro and Greece (Hoch and Remane 1985; Kosovac et al. 2016a, 2018a; Đurić et al. 2017) to the eastern Mediterranean coast of Israel (Sharon et al. 2005, 2015). *V. agnus-castus*-associated *H. obsoletus* in Israel is characterized by two flight periods per year (April–July and September–November) (Sharon et al. 2015), while on the Adriatic coast of Montenegro it has one prolonged flight period starting at the end of June, with peak activity in mid-July and declining until the end of September or the beginning of October (Kosovac et al. 2016a). The epidemiological significance of *V. agnus-castus* as pathogen source plant is unclear for Israel (Sharon et al. 2015) and undetermined in Bosnia and Herzegovina, Greece and

Fig. 4.3 *Vitex agnus-castus* (monk's pepper, chaste tree or Abraham's balm) as host-plant of *Hyalesthes obsoletus* in the Mediterranean

Croatia. However, its role as a reservoir plant and the vector role of associated *H. obsoletus* populations in Montenegrin vineyards were recently demonstrated and the related tuf-type b epidemiological cycle was defined (Kosovac et al. 2016a). In the Mediterranean vineyards of Montenegro, as well as on natural habitats, *V. agnus-castus* is found to host several (*tuf*/*stamp*/*vmp1* genes) genotypes of '*Ca.* P. solani' and to act as a source plant for pathogen transmission routes which are intermixed or interconnected with routes of transmission vectored by *C. arvensis*-affiliated *H. obsoletus* or *R. panzeri* (Kosovac et al. 2016a). The percentage of naturally phytoplasma-infected *V. agnus-castus* was 16–25%, while the percentage of associated *H. obsoletus* populations harboring phytoplasmas was 4–10%. No symptoms were observed in any of the naturally '*Ca.* P. solani'-infected *V. agnus-castus*. In laboratory controlled transmission assays *V. agnus-castus* -affiliated populations of *H. obsoletus* successfully transmitted '*Ca.* P. solani' genotypes to grapevines confirming their epidemiological role. Four of the six transmitted genotypes were also identified in the naturally BN-infected grapevines, therefore confirming the identity of naturally occurring and experimentally transmitted genotypes of the pathogen. These findings indicate that *V. agnus-castus* can act as a source plant of '*Ca.* P. solani' for the vector acquisition in the Mediterranean area and as natural reservoir plant of the pathogen. Laboratory controlled transmission assays combined experimental and molecular data and demonstrated that the *V. agnus-castus*-associated populations of *H. obsoletus* are transmitting '*Ca*. P. solani' to grapevine; whereas, the identification of naturally occurring '*Ca*. P. solani' in asymptomatic *V. agnus-castus* confirms its role as source of the pathogen (Kosovac et al. 2016a). This study presents molecular and experimental evidence of one-way-path of the epidemiological cycle sourced by *V. agnus-castus*, implying its importance as phytoplasma reservoir, but raising the question whether *H. obsoletus* populations cannot only acquire '*Ca*. P. solani', but also transmit it to healthy *V. agnus-castus* plants which requires further investigation. Experimental confirmation of the role of the *Vitex*-associated *H. obsoletus* in '*Ca*. P. solani' and the BN transmission in Montenegrin vineyards indicates its possible vector role in the wider area of the Mediterranean where some of the major wine-producing regions are located. Thus further research to determine the occurrence of *Vitex*-associated *H. obsoletus* populations, their infection degree and epidemiological significance, as well as a determination of the vector population life cycles in different regions of Mediterranean is still to be studied. This is especially important for the eastern Mediterranean coast of Israel where the preferred host-plant for *H. obsoletus* is *V. agnus-castus* (Sharon et al. 2005). In this situation the vectors' populations collected on *V. agnus-castus* are found harboring phytoplasmas in a substantial rate (14–50%; Sharon et al. 2005, 2015), but the role of *V. agnus-castus* as source plant remained unresolved (Sharon et al. 2015).

Three epidemiological cycles of '*Ca.* P. solani' vectored by plant-associated populations of *H. obsoletus* in south-eastern Europe are reported, each associated with specific host-plants of the vector and pathogen sources: *U. dioica*, *C. arvensis* and *V. agnus-castus* (Maixner 1994; Langer and Maixner 2004; Kosovac et al.

Fig. 4.4 *Crepis foetida* (stinking hawk's-beard) as host-plant of *Hyalesthes obsoletus* in the Balkans

2016a). Survey of the distribution, host association and '*Ca.* P. solani' infection of *H. obsoletus* populations in south-eastern Europe, led to the discovery of a new herbaceous plant hosting high-density populations of the planthopper in the Balkans – the biannual herb *Crepis foetida* (stinking hawk's-beard) (Kosovac et al. 2013) (Fig. 4.4). Occurrence of this host-association was confirmed in a number of locations in south-eastern Serbia including several syntopic with *C. arvensis*- or *U. dioica*-associated populations. Preliminary data on the genetic peculiarities of *C. foetida*-associated *H. obsoletus* populations, based on mitochondrial and nuclear marker genes, indicated the presence of a genetic differentiation to the populations associated with the other two host plants. Subsequent preliminary insights into genetic divergence relative to Mediterranean *V. agnus-castus*–associated populations raised questions regarding the cryptic differentiation potential of *H. obsoletus* (Kosovac et al. 2016b, 2018a). In addition, recent study revealed the '*Ca.* P. solani' infection of *C. foetida*-associated populations and their role as vector in independent epidemiological cycle (Kosovac et al. 2018b). *H. obsoletus* populations associated with *C. foetida* were found harboring a tuf-type b '*Ca.* P. solani' in the average rate of 33% within the insect populations (Kosovac et al. 2013). These findings have implications for possible ecological speciation and cryptic species arise related with host-plant associations within the *H. obsoletus* species group. The incidence of this '*Ca.* P. solani' strain in populations associated with *C. foetida* raises questions related to the ability of this host-associated *H. obsoletus* population to transmit the phytoplasma and its role in the epidemiology of '*Ca.* P. solani'-associated diseases in south-eastern Europe. The most recent data provided evidence of genetic segregation between host-plant specialized *H. obsoletus* populations associated with *C. foetida* and *V. agnus-castus* as well as compared to *C. arvensis* and *U. dioica*, leading to cryptic speciation diversification (Kosovac et al. 2018a).

Epidemiology of '*Ca.* P. solani' in South-Western Europe In France, lavender (*Lavandula angustifolia*) and lavandin (*Lavandula x intermedia*) are strongly affected by the "stolbur" phytoplasma (Fig. 4.5). Contrary to most crops that are dead-end hosts for this phytoplasma, *L. angustifolia* and *L. intermedia* are both hosts for '*Ca.* P. solani' and its insect vector, *H. obsoletus* (Boudon-Padieu and Cousin 1999). As a consequence in large diseased areas are present high vector population levels in both lavender and lavandin fields (Yvin 2011). The first lavender fields were planted in the 1930s in south-eastern France (Meunier 1999).

Fig. 4.5 Lavender field affected by '*Candidatus* Phytoplasma solani'

Lavender decline disease, associated with "stolbur" phytoplasma presence, is known from the 1970s and became an agronomic problem in the middle of the 1990s due to the important increase of the cultivated areas and the use of susceptible cultivars (Meunier 1999). This disease is the main threat for lavender production with strong economic consequences and is responsible of a 50% drop of the lavender essential oil production between 2005 and 2012 (CIHEF 2012). Lavender plants were usually cultivated from 10 to 12 years, but presence of lavender decline constrains the grower to uproot the plants within 4–5 years of planting (Moreau et al. 1970). Symptoms of lavender decline are yellowing and either standing up or rolling down of the leaves, and reduction and abortion of inflorescences (Boudon-Padieu and Cousin 1999) (Fig. 4.5). Like in other phytoplasma diseases, symptoms may be located only on some branches or affect the whole plant. After yellowing, the affected branches dry, resulting in plants with mixed dead and still green branches. After several growth cycles, the plants become completely brown and dry (Boudon-Padieu and Cousin 1999).

The main ways to control a phytoplasma epidemic are to suppress the pathogen transmission from one plant to another by killing the vector (Chuche and Thiéry 2014a, 2014b) or destroying the vector host plant if the crop is a dead-end host (Kehrli and Delabays 2012). Using insecticides to control adults is impossible in the *H. obsoletus*/"stolbur"/lavender system because lavender is a very attractive crop to pollinators (honeybees, bumblebees) and bee protection rules out the use of insecti-

cides. Moreover nymphs develop on the roots and can be found at several tens of centimeters deep (Boudon-Padieu and Cousin 1999), and are thus not affected by insecticide sprays. The main actions undertaken are the use of healthy planting material and the selection of less susceptible lavender and lavandin cultivars, up to now, no cultivar was found resistant to the phytoplasma infection (Gaudin et al. 2011). Other prophylactic methods are currently under investigation such as spraying kaolinite clay or inter-cropping plants. Most of the resistance to lavender decline seems to be linked to the unsuitability and/or poor attractiveness of the plant for *H. obsoletus*. In this way less susceptible cultivars to disease Diva (lavender) and Grosso (lavandin) host very low numbers of adults and nymphs, while important populations can be found on the very susceptible C15/50 (lavender) and Abrial (lavandin) plant cultivars (Yvin 2013).

For years, resistant lavandin cultivars such as Grosso were not really affected by lavender decline due to the very few *H. obsoletus* completing their life cycle on them. Although some adults could be collected on these cultivars, very few nymphs were found on the roots. So, the key point in the resistance of this plant to the vector was the low number of the nymphs. Because lavandin and lavender are rarely cultivated on the same area, the absence of sustainable *H. obsoletus* populations on resistant lavandin meant that phytoplasma propagation in the crop was avoided. Most of the decline symptoms observed were probably due to inoculation by infected vectors from wild plants that are quite rare in lavandin plots.

Clary sage, *Salvia sclarea*, is a semi-perennial crop generally with a 3-year lifespan. Stems are harvested during years 2 and 3, then a new crop, usually wheat or lavandin, is grown in place of clary sage. Since clary sage is only present for 3 years, this means that *H. obsoletus* can quickly colonize and adapt to it. The increase in *S. sclarea* cultivation in which high populations of *H. obsoletus* were found in areas where resistant lavandin cultivars are grown, most probably allowed the breakdown of the lavender decline resistance (Chuche et al. 2018). Clary sage plots can constitute a reservoir for the pathogen and its vector close to the lavandin plots (Chuche et al. 2018). An experimental transmission trial conducted with *H. obsoletus* sampled on lavender demonstrated that clary sage can also be a host plant for the phytoplasma (Chuche et al. 2018). Laboratory infected plants present typical symptoms of phytoplasmas as stunted and very small leaves. However, naturally-infected *S. sclarea* collected in the field did not show obvious symptoms, hence it is difficult to estimate the proportion of plants harboring '*Ca.* P. solani'. The secY genotype of '*Ca.* P. solani' transmitted (S1) was identical to those of a phytoplasma sampled from lavender. Thus, *H. obsoletus* from lavender are able to transmit '*Ca.* P. solani' to *S. sclarea* in which it could multiply. The role of *S. sclarea* as a reservoir of '*Ca.* P. solani' for lavender crop is also supported by the numerous "stolbur" diseased lavandin found in the neighboring plot of the clary sage (Chuche et al. 2018). Thus, infective adults from clary sage could transmit the pathogen to Grosso cultivar that would be a dead-end-host for the phytoplasma. The clary sage harvesting method, consisting of harvesting all the plants in summer during the *H. obsoletus* flight period, probably forces the cixiids to temporarily desert the plots in search of food.

Currently high level of decline symptoms has been observed on the lavandin cultivar Grosso only in the Valensole area (Chuche et al. 2018). Agricultural practices seem to be a major factor driving the vector exchanges between lavender/lavandin and clary sage plots by increasing the dispersal of the adult carrying phytoplasma and the inoculation probability to crops.

The origin of the population of *H. obsoletus* on lavender and clary sage is unknown. The genetic polymorphism comparison between different host plant populations in France revealed that *H. obsoletus* from *S. sclarea* are closer to individuals from field bindweed and nettle that were collected from distant areas, rather than insects from lavender of the same region (Chuche et al. 2018). Indeed, mithocondrial DNA haplotypes of insects from *S. sclarea* are all bb. according to *H. obsoletus* haplotype nomenclature established by Johannesen et al. (2008, 2012), like half of the *H. obsoletus* from *C. arvensis*, while those from *L. angustifolia* are all ab. Either *H. obsoletus* from field bindweed colonized clary sage, or the haplotype diversity in the lavender population is wider than that shown by the sample studied. According to Johannesen et al. (2008), south-eastern France is in the area of the ancestral haplotypes ab where the haplotype diversity is the highest. The homogeneity of *H. obsoletus* mitochondrial DNA haplotype from clary sage suggests that a small number of insects initiated the clary sage population and/or that the bb haplotype is the only one that can develop on this plant. The morphological study showed that individuals from lavender and clary sage are of similar size and are very small compared to insects from nettle and field bindweed (Chuche et al. 2018). This could be influenced by the similarity of metabolites produced by these plants, which belong to the same family, the Lamiaceae. Host plant quality can greatly affect phytophagous insects (Bernays 2001; Awmack and Leather 2002) and such an effect was reported on the wing length of *H. obsoletus* living sympatrically on *U. dioica* and *C. arvensis* (Johannesen et al. 2008).

Bindweed seems to be the only other host plant of *H. obsoletus* and "stolbur" phytoplasma in lavender agroecosystem (Cousin et al. 1969; Hossard et al. 2018) and is suspected to be a source of infection in newly planted lavender fields (Maixner 2010). Plant genotyping showed that bindweed may not be responsible for yellow decline of lavender, as the main phytoplasma strains found in lavandin differed from that of bindweed and other wild plants (Danet et al. 2010). Moreover it is unclear if the *H. obsoletus* populations and phytoplasma strains found in *S. sclarea* plots have the same plant origin (Chuche et al. 2018). So, the origin of both '*Ca.* P. solani' strains and *H. obsoletus* populations is still unknown and supplementary genotyping of plants and insects would then be required.

4.2 *Reptalus panzeri*

Taxonomy, Biology and Distribution The distribution of *Reptalus panzeri* (Löw 1883), according to literature data, is covering a wide area in the southern parts of central Europe, England, south-eastern Europe, Mediterranean region, Asia Minor

and Caucasus (Holzinger et al. 2003). However, recent faunistic data confirmed its wide distribution only for the central and south-eastern Europe (Holzinger et al. 2003; Palermo et al. 2004; Jović et al. 2009; Kovačević et al. 2014; Lang et al. 2016; Mitrović et al. 2016). Records of its distribution and occurrence in the Mediterranean region should be presumed as erroneous misidentification of a closely related congener *R. quinquecostatus*, as it was confirmed after re-examination of museum collections for specimens recorded in England (Webb et al. 2013). Recent efforts to collect *R. panzeri* in the Mediterranean have failed and only confirmed occurrence of *R. quinquecostatus* (J. Jović and I. Toševski, unpublished data). Identification of *R. panzeri* and differentiation from closely related congeners primarily relies on the shape of the male genitalia (Holzinger et al. 2003), while fast and reliable molecular assays are available for female and/or nymph identification (Bertin et al. 2010). *R. panzeri* has a complex taxonomic and nomenclature history which was recently summarized by Webb and coauthors (Webb et al. 2013). This species was first illustrated by Panzer in Panzer 1799, however as a visual description of a misidentified species described by Linnaeus in 1761 as *Cicada leporina* from Sweden. After several revisions of European Auchenorrhyncha, Löw has questioned the identity of Panzer's concept of *C. leporina* based on revisions made by several authors (see in Webb et al. 2013) and concluded that a species with a short crown (as illustrated by Panzer) was not found anywhere in Scandinavia. In addition he concluded that *C. leporina* is the only species with five scutellar keels (characteristic for today genera *Pentastiridius* and *Reptalus*) that is present in the whole of Scandinavia. Hence, based on illustration of *C. leporina* by Panzer and the type locality given by Linnaeus, Löw has concluded that the species described by Panzer was a new one. For this purpose, Löw chose the name panzeri (*Oliarus panzeri*), after the man who has first figured it (Webb et al. 2013). Later, the species was transferred from genus *Oliarius* to *Reptalus* by Emeljanov (1971). The type locality of Panzer's specimen used for description of *R. panzeri* was reported as "Habitat in Austria. Dn. de Megerle" (Webb et al. 2013), i.e., that material was collected by Megerle, a collector from Vienna and that type material was probably originating from nowadays eastern Austria. This is important because the type material of Panzer's collection has not been found and is probably lost (Webb et al. 2013).

R. panzeri habitat preferences, feeding behavior and host-plant preferences indicate that it is a polyphagous species generally found on diverse shrubs and herbaceous plants in hot and dry areas, on xerothermic waysides, sunny hillsides or on plateaus (Holzinger et al. 2003). In central Europe, adults are often found on diverse woody plants and shrubs of *Rosa*, *Prunus spinosa*, *Clematis*, *Salix*, *Crataegus*, *Pinus* and others (Nickel 2003). In south-eastern Europe feeding preferences further diversifies. Although individuals of this cixiid can be found in association with woody plants of the genera *Prunus*, *Rubus* and *Ulmus* (I. Toševski, J. Jović and T. Cvrković, unpublished data), large populations are found frequently on maize (Jović et al. 2009; Cvrković et al. 2014). *R. panzeri* has associated its life cycle with maize fields under the agronomic practice of maize-winter wheat rotation (Jović et al. 2009). In years of favorable environmental conditions its populations can be found in very high densities on maize fields with up to 50 adults observed per single

maize plant (J. Jović and I. Toševski, unpublished data). Similar to other cixiids, *R. panzeri* nymphs are subterranean feeders found in maize fields with Johnsongrass (*Sorghum halepense*) and winter wheat (*Triticum aestivum*) acting as reservoirs hosting both the vector and the pathogen population (Jović et al. 2009). In contrast to that, in Germany *R. panzeri* immature stages are found only on the roots of *Ranunculus* spp. while adults are present on *U. dioica, Prunus, Salix, Hedera helix* and *Clematis vitalba* as a preferred host-plant (Lang et al. 2016). This discrepancy in feeding preferences of adults and nymphs in the two regions influences the acquisition of the pathogen ('*Ca.* P. solani') in terms of efficiency (rate of individuals harboring the pathogen) and phytoplasma genotype acquired (Cvrković et al. 2014; Lang et al. 2016).

The biology of *R. panzeri* is little known because prior to the discovery of its vector role in the maize reddening pathogen transmission, this cixiid was not considered of agronomic importance. The life cycle of *R. panzeri* has been studied only in maize fields of south Banat region of Serbia (Jović et al. 2009). It is an univoltine species, with the eggs being placed in soil next to the roots of host-plant (maize) in July/August, the nymph period develops between September of a year and June of the following year (on winter wheat and Johnsongrass) and adults are observed in June/July. Johnsongrass is as weedy reservoir plant of the pathogen and suitable inoculum source for vector acquisition in the maize fields (Jović et al. 2009). However, in the "bois noir"-infected vineyards of the north-eastern Serbia where *R. panzeri* was found as a major vector of '*Ca.* P. solani', the source plant for the pathogen acquisition is still unknown. It is presumed to be in the woody surroundings of vineyards of this viticultural region comprised of *Prunus*, *Rubus* and *Ulmus* plant. In the potato fields of north Serbia affected by potato "stolbur" disease diverse possible source plants were confirmed as '*Ca.* P. solani' reservoirs, including Johnsongrass and field bindweed (*C. arvensis*) (Mitrović et al. 2016). However, their role in the vectors' life cycle and disease epidemiological cycle needs to be verified.

Epidemiology of '*Ca.* P. solani' in three Agro-ecosystems *R. panzeri* is a competent and highly efficient vector of '*Ca.* P. solani' in south-eastern Europe (Jović et al. 2007; Cvrković et al. 2014; Mitrović et al. 2016), while its role in the pathogen transmission is inconclusive for central Europe (Lang et al. 2016). As a vector of '*Ca.* P. solani', *R. panzeri* is known to transmit at least three diseases affecting economically important agricultural crops: maize redness (MR) disease of maize, "bois noir" (BN) disease of grapevine and "stolbur" disease of potato (Jović et al. 2007, 2009; Cvrković et al. 2014; Mitrović et al. 2016). Its major role in transmission and epidemiology of these diseases was confirmed by both experimental and molecular data, as well as in field investigations. The MR pathogen of maize is transmitted primarily by *R. panzeri*, whilst the latter two diseases are as transmitted by *H. obsoletus* and tentatively by *R. quinquecostatus* (Maixner 1994; Mitrović et al. 2016; Pinzauti et al. 2008; Chuche et al. 2016). The epidemiology of these diseases is tightly connected with the phenological cycle of the vector, its host-plant prefer-

ences, the diversity of plants acting as pathogen reservoirs within and surrounding affected fields and the agricultural practices.

The similar rate of *R. panzeri* adults harboring '*Ca.* P. solani' in the three agro-ecosystems of south-eastern Europe (maize and potato fields, and vineyards; 17–28%) the same tuf-type b and a single multilocus genotype STOLg (Cvrković et al. 2014; Mitrović et al. 2016) transmitted by the vector, indicates shared source and epidemiological connection among transmission routes in this region. In contrast, the *R. panzeri* populations in Germany are associated with different host-plants (*U. dioica* and *C. vitalba*) and are infected with tuf-type a as well as tuf-type b of '*Ca.* P. solani'. Its rate of infection with '*Ca.* P. solani' is low (1.1%), while only specimens originating from *U. dioica* transmitted the pathogen to periwinkle and this was a tuf-type a. Differences in rate of infection and pathogen strain harbored by *R. panzeri* populations in Serbia and in Germany suggest several possible causes underlying different behavior of these populations: geographical and habitat peculiarities, changes in plant preferences (host-shift) and/or genetic differentiation among populations. These population differences needs to be addressed in future studies, along with *R. panzeri* biology in diverse natural and agro-ecosystems and feeding preferences towards diverse host-plants acting as '*Ca.* P. solani' reservoirs.

4.3 *Reptalus quinquecostatus*

Taxonomy, Biology and Distribution *Reptalus quinquecostatus* (Dufor 1833) was described as *Cixius quinquecostatus* by Dufour in 1833 (reviewed in Webb et al. 2013), while Fieber in 1876 made a complete revision of the European Auchenorrhyncha fauna and described again several cixiids by placing them in genus *Oliarus*, including *O. quinquecostatus* (Webb et al. 2013). Later, the species was transferred from *Oliarius* to *Reptalus* genus by Emeljanov (1971). Description and illustration of *R. quinquecostatus* male genitalia, by which it can be reliably distinguished from closely related congeners, was recently given by Holzinger et al. (2003) and Bertin et al. (2010). In addition, same as for *R. panzeri*, fast and reliable molecular assays are available for female and/or nymph identification (Bertin et al. 2010). However, during an identification work on some early described European cixiids and resolving misidentification of species occurring in the United Kingdom a new information on the identity of *R. quinquecostatus* was provided (Webb et al. 2013). The authors examined Dufour's type series of *R. quinquecostatus* and found that the types didn't match with the above identifications but in fact matched the figures given by authors of *R. melanochaetus* (Fieber 1876). This situation requires, as explained by Webb et al. (2013), that either a new type (neotype) should be designated and that the type of *C. quinquecostatus* by Dufor should be disregarded, or that a new name be given for the species previously identified as *R. quinquecostatus* in continental Europe (e.g., as described by Holzinger et al. 2003). The first action

Fig. 4.6 *Reptalus quinquecostatus*

seems more appropriate and it is the one argued and recommended by Webb et al. (2013), i.e., the retention of the current identity of this species in continental Europe.

The cixiid planthopper *R. quinquecostatus* (Fig. 4.6) was until just recently (Chuche et al. 2016) a potential vector that was abundant and constitute one of the main cixiid species in some crops affected by "stolbur" such as carrot, potato, maize and grapevine (Mazzoni 2005; Trivellone et al. 2005; Jović et al. 2007; Alma et al. 2008; Picciau et al. 2008; Cvrković et al. 2014; Mitrović et al. 2016). This is a polyphagous species and adults can be collected on a huge diversity of grassy and woody plants (Picciau et al. 2008; Pinzauti et al. 2008; Taszkowski et al. 2015). *R. quinquecostatus* is a mesophilic species that occurs in most of the European countries and can be found from Portugal to Ukraine, and from the United Kingdom to Greece. It was also described in Caucasus, Middle East and China (Taszkowski et al. 2015). The biology and behaviour of this species remain poorly known. As with other cixiids, females lay their eggs in the soil near the base of host plants. After hatching, the five nymph instars live underground and feed on roots. Acquisition of '*Ca*. P. solani' can be achieved by nymphs feeding on infected root phloem while transmission to cultivated and wild plants is done by flying adults during summer. *R. quinquecostatus* is a univoltine species in temperate latitudes (Taszkowski et al. 2015).

Epidemiology of '*Ca.* P. solani' The first proof of the ability of *R. quinquecostatus* to transmit '*Ca.* P. solani 'was provided by Pinzauti et al. (2008) who achieved an inoculation efficiency of 40% into an artificial feeding medium. This method does not consider any crucial interactions occurring between the vector, the phytoplasma and the plant during the establishment of a vector-borne disease (Alma et al. 2001; Bressan et al. 2005; Hogenhout et al. 2008), however later transmission trials succeeded to inoculate '*Ca.* P. solani' phytoplasma to *Catharanthus roseus* (Chuche et al. 2016; Ember 2016) while transmission trials to grapevine, tobacco and lavender were unsuccessful (Pinzauti et al. 2008; Cvrković et al. 2014; Chuche et al. 2016). Many reasons can explain the difference in inoculation success between *C. roseus* and the different tested crops. Transmission success depends greatly on the

relationship between the insect vector, the host plant and pathogen strains (Alma et al. 2001; Bressan et al. 2005; Lopes et al. 2009). Sap-feeding Auchenorrhyncha test less into phloem vessels and more into the xylem sap when feeding on resistant plants and thus may not be capable of injecting enough phytoplasmas into phloem to initiate an infection (Lopes et al. 2009). Moreover, in the Chuche et al. (2016) study, *R. quinquecostatus* has its best survival rate on *C. roseus* compared to the other tested plants. Because inoculation efficiency depends also on the period that an infective vector has access to a host plant (Purcell 1982), the transmission capability of *R. quinquecostatus* on crop plants could have been underevaluated. The lack of success in transmission studies leads to questions about a direct role of *R. quinquecostatus* in '*Ca*. P. solani' phytoplasma transmission in grapevine and potato fields (Pinzauti et al. 2008; Cvrković et al. 2014; Mitrović et al. 2016). The rate of infected *R. quinquecostatus* observed varied between 8% and 76% (Pinzauti et al. 2008; Cvrković et al. 2011; Chuche et al. 2016). Rates of infection for males and females are similar and '*Ca.* P. solani'-highly infected *R. quinquecostatus* adults are frequently found in vineyards and are often observed feeding on young green grapevine shoots and on leaf midribs (Pinzauti et al. 2008; Chuche et al. 2016). The ability to transmit '*Ca.* P. solani' to plants and observations of adults feeding on crop plants consolidate the hypothesis that *R. quinquecostatus* could be a specific vector. The characterization of '*Ca.* P. solani' strains carried by *R. quinquecostatus* revealed that this cixiid can harbor a high diversity of strains. Some of these seem to be specific to *R. quinquecostaus* and were never found in '*Ca.* P. solani' insect vectors, but can be detected in potato field and vineyards (Cvrković et al. 2014; Chuche et al. 2016; Mitrović et al. 2016). So, the finding of phytoplasma genotypes present in *R. quinquecostatus* and not in *H. obsoletus* or *R. panzeri*, suggests that *R. quinquecostatus* could have a role in '*Ca.* P. solani' epidemiology.

Thus the discovery of the ST58 '*Ca.* P. solani' strain in *R. quinquecostatus* and in grapevines but not in *H. obsoletus*, supports the hypothesis of a specific role of this cixiid in "stolbur" epidemics (Chuche et al. 2016). Assuming that the difference observed between *R. quinquecostatus* and *H. obsoletus* is not due to sampling, the relationship between former and new phytoplasma strains could be the result of (i) a differential feeding behavior of *R. quinquecostatus* on wild host plants harboring this strain and not visited by *H. obsoletus*; (ii) a better acquisition of this strain by *R. quinquecostatus*, or (iii) the incapacity to *H. obsoletus* to acquire this strain. In the first case, it is possible to hypothesize that there is an unidentified plant species that could constitute a reservoir for '*Ca.* P. solani'. *R. quinquecostatus* would perform its life cycle on this plant and would acquire phytoplasmas as nymph instars by feeding on root phloem. Once adults and infective, they would transmit phytoplasmas to other plants, such as grapevines. *R. quinquecostatus* could also transfer the new strain to plants where *H. obsoletus* can acquire it, creating new epidemic cycles. It cannot be excluded that the ST58 strain could be transmitted to grapevine by another vector species, while *R. quinquecostatus* propagated this strain only to wild plants. In the same study by Chuche et al. (2016) the similar composition of '*Ca.* P. solani' genotypes in *R. quinquecostatus* and grapevines, particularly the presence of ST6 and ST58 genotypes, associated with the absence of this genotype in *H. obsoletus*, seems to be an indirect proof of the ability of *R. quinquecostatus* to

transmit '*Ca.* P. solani' to grapevine. *R. quinquecostatus* could also have an indirect role in contributing to create and/or maintain alternative '*Ca.* P. solani' cycles in weeds. Different polyphagous vectors involved in the diverse transmission cycles of "stolbur" phytoplasma can share some of the same host plants and the same "stolbur" phytoplasma genotype. This assumption would explain the presence of the same '*Ca*. P. solani' genotype in *R. quinquecostatus* and in the "stolbur" phytoplasma vectors *H. obsoletus* and *R. panzeri*, the wild host plant *C. arvensis*, grapevine and potato fields (Cvrković et al. 2014; Chuche et al. 2016; Mitrović et al. 2016). The presence of *R. quinquecostatus* in the agroecosystems could lead to an increased pathogen presence allowing subsequent acquisition by other vectors able to transmit '*Ca.* P. solani' strains (Cvrković et al. 2014; Chuche et al. 2016). *R. quinquecostatus*, probably as numerous other polyphagous vectors, can contribute to genetic differentiation and local adaptation of phytoplasma strains and consequently to different transmission scenarios. Characterization of '*Ca*. P. solani' genotypes associated with different crops and insects could provide evidence on pathways underlying the epidemics of "stolbur" in the field (Mitrović et al. 2016). Indeed, some '*Ca*. P. solani' strains detected in one crop matched the strains from other crops, wild plants, as well as different cixiids species. Results from molecular studies would help to understand the different routes of transmission and exchanges of '*Ca*. P. solani' strains among the plants via polyphagous vectors (Mitrović et al. 2016). Since *R. quinquecostatus* can transmit '*Ca.* P. solani' to plants and was found carrying the same "stolbur" genotypes found in diverse crops, its role in "stolbur" epidemiology in diverse agroecosystems deserves to be further investigated. Even if no successful transmission has been made to crops, this cixiid should not be discarded as a potential link in the propagation of "stolbur" phytoplasma within vineyards and potato fields.

4.4 *Pentastiridius leporinus*

Taxonomy, Biology and Distribution *Pentastiridius leporinus* (Linnaeus, 1761) is described from Sweden as a species with Palearctic distribution. It is widespread throughout Europe, Middle East, Middle and East Asia, and Northern Africa (Bressan et al. 2009). Little is known about its host-plant range, but data regarding its feeding behavior indicate its common association with *Phragmites australis* (Nickel 2003; Holzinger et al. 2003). *P. leporinus* is one of the first cixiid planthoppers described in Europe. It was named *Cicada leporina* from Sweden after its fluffy wax tail, a characteristic of nymphs and females in this group (Linnaeus 1761): there has been no disagreement on the identity of *P. leporinus* in recent times, however, only a fragment of the type remained in the museum collection of Linnaeus (London), so there is no possibility of confirming its identity (Webb et al. 2013).

Epidemiology of '*Ca.* P. solani' *P. leporinus* is a competent and efficient cixiid planthopper vector of '*Ca.* P. solani' (Gatineau et al. 2001; Quaglino et al. 2013). As

phytoplasma vector, this species was first tentatively identified and treated as *P. beieri*, later referred to as *Pentastiridius* sp. and finally reliably confirmed as *P. leporinus* (Gatineau et al. 2001, 2002; Sémétey et al. 2007; Bressan et al. 2009). The disease induced by *P. leporinus*-transmitted '*Ca.* P. solani' and another phloem-restricted bacterium is called "syndrome des basses richesses" (SBR) and is affecting sugar beet crops in eastern France (Gatineau et al. 2001, 2002; Bressan et al. 2008, 2009). The disease has a complex etiology which after several studies was determined to be associated with a γ-3 proteobacterium (SBR proteobacterium) and the "stolbur" phytoplasma (Sémétey et al. 2007; Bressan et al. 2008). The SBR causes significant economic losses on sugar beet crops due to the poor sugar content of affected roots. Symptoms include yellowing and curling of old leaves and proliferation of small leaves with narrow and twisted chlorotic laminae (Gatineau et al. 2001; Sémétey et al. 2007). In the first study on the vectors of SBR disease up to 13% of *P. leporinus* specimens were found harboring '*Ca.* P. solani' (Gatineau et al. 2001). However, in all the following studies the dominant pathogen infecting the vector was the SBR proteobacterium (Sémetéy et al. 2007; Bressan et al. 2008). Nonetheless, *P. leporinus* successfully transmits both SBR-inducing pathogens. Symptoms of SBR disease associated with '*Ca.* P. solani' and with SBR proteobacterium are very similar, based on field collected material and experimentally infected plants (Gatineau et al. 2001; Sémétey et al. 2007), while those induced by '*Ca.* P. solani' alone can even be more severe and induce greater reductions in taproot biomass and sugar content (Bressan et al. 2008). Little is known about host-plant range and biology of *P. leporinus*, however in the SBR-affected sugar beet fields of east France, *P. leporinus* seems to have adapted its life cycle to sugarbeet-winter wheat annual crop rotation (Bressan 2009; Bressan et al. 2009). This is intriguingly similar to the afore-described adaptation of *R. panzeri* to maize-winter wheat rotation in MR-affected maize fields of north-eastern Serbia (Jović et al. 2009). *P. leporinus* instars successfully develop on the winter wheat leading to large populations of adult vectors migrating towards the nearby sugar beet fields; same as *R. panzeri* to maize fields. Both diseases, SBR of sugar beet and MR of maize, are characterized by fluctuations in occurrence and severity, in space and time, with cycles of epidemics and epiphytotic appearance (Bressan 2009; Jović et al. 2007, 2009). Hence, environmental conditions seem to play an important role in occurrence, incidence and severity of these diseases. Changing of agronomic practice by introducing another crop (that is not a winter one) in annual rotation, e.g. spring barley, has proven to be very effective in SBR-affected sugar beet fields by reducing number of emerging planthoppers for 80% (Bressan 2009). Similar change in agronomic practice is proposed for *R. panzeri* in maize fields (Jović et al. 2009). Attempts to identify the alternative host-plant of *P. leporinus* in east France have failed, but the host-shift to the sugar beet from an ancestral host-plant is hypothesized (Bressan et al. 2009), same as for *R. panzeri* to maize (Jović et al. 2010). The number of similarities between the two pathosystems indicates a general potential of the cixiids to exploit temporary habitats and adapt to annual cropping rotation system. This should be causing a concern given their role as effective phytoplasma vectors.

4.5 Biology and Vector Role of *Dictyophara europaea* in Phytoplasma Transmission

Taxonomy, Biology and Distribution *Dictyophara europaea* (Linnaeus 1767), the European lantern fly (Fig. 4.7), is one of the rare planthopper vectors of phytoplasmas belonging to a family other than Cixiidae, namely, Dictyopharidae. According to length, thickness and shape of cephalic process, Emeljanov (2003) subdivided the genus *Dictyophara* into five subgenera and placed the European lantern fly to the subgenus *Dictyophara s. str.* Germar 1833. *D. europaea* is an autochthonous, alternative vector of "flavescence dorée" (FD) disease of grapevine in the European vineyards (Filippin et al. 2009a) which enables infection by transmitting the pathogen from a wild reservoir, *Clematis vitalba*, to grapevine. *D. europaea* is a polyphagous phloem-feeding planthopper commonly found throughout the western Palaearctic, with a single report from north-western China (Song and Liang 2008). Interest in the biology, behavior, life history and host-plant associations of *D. europaea* has increased in recent years due to the evidence of its involvement in the epidemiology of FD phytoplasma disease in the viticultural regions of north-eastern Italy and the Balkans (Filippin et al. 2009a; Krstić et al. 2016, 2018).

D. europaea is a univoltine species which overwinters in the egg stage (Nickel 2003) and has five instars (Krstić et al. 2016). The 1st- and 5th- instar nymphs show stable color patterns, bicolored and entirely green, respectively; while nymphs of the 2nd, 3rd and 4th instar exhibit highly variable color patterns (Fig. 4.7). Krstić et al. (2016) gave detailed insights into the oviposition behavior of *D. europaea* as a complex process directed towards the optimal protection of eggs, hidden in soil particles and placed onto the soil surface as "soil nests". This oviposition strategy provides a good protection for eggs which are camouflaged within the soil particles, and become an integral part of the soil superficial layer which makes the detection by predators difficult. This may facilitate egg dispersal by wind which could contribute to the successful spread of the *D. europaea* throughout environments (Krstić et al. 2016). In addition, the females have been observed depositing 2–4 eggs per single oviposition event, which represents a way of minimizing the risk and increasing the survival, acting according to the general predictions of the bet-hedging strategy.

Fig. 4.7 *Dictyophara europaea*. Fourth-instar nymph (left), fifth-instar nymph (center), adult (right)

Seasonal occurrence, spatio-temporal distribution and host-plant associations of *D. europaea* indicate its wide dispersal in herbaceous and shrub layer and its polyphagous feeding pattern (Nickel 2003; Krstić et al. 2016). In the vineyard agroecosystems of northern Italy *D. europaea* was found exploiting diverse herbaceous plants and grasses as feeding sources, but it also shows a level of close association with *Amaranthus retroflexus* and *U. dioica* as preferred host plants for nymphs and adults (Lessio and Alma 2008). In the same region, *D. europaea* was also found to be associated with *C. vitalba* plants, a reservoir plant of the FD-C phytoplasma strain (Filippin et al. 2009a). In the subsequent study performed in Balkans, Krstić et al. (2016) confirmed the polyphagous feeding nature of *D. europaea*, for all instars and adults, as well as the adult horizontal movement during the growing season to the temporarily preferred feeding plants where they aggregate during the dry season. In south-eastern Europe *D. europaea* adults aggregate in late summer on *C. vitalba*, however, this could be the consequence of the migration caused by desiccation of herbaceous vegetation rather than a higher preference to *C. vitalba* as a feeding plant (Krstić et al. 2016). Given the polyphagous feeding behavior of *D. europaea*, its potential to acquire and tentatively spread phylogenetically distinct phytoplasma groups has made this planthopper a tentatively important link for elucidating some of the epidemiological cycles of phytoplasma-associated plant diseases.

Epidemiology of "flavescence dorée" Strain FD-C and Other *Dictyophara*-Carried Phytoplasmas The role of *D. europaea* in the epidemiological cycle of FD-C phytoplasma strain (16SrV-C subgroup) was confirmed combining evidences of experimentally obtained data on transmission ability from naturally-infected *C. vitalba* to healthy grapevine seedlings and of molecular tracing the phytoplasma genotypes from source plant to affected plant by multilocus typing (Filippin et al. 2009a). Later, Filippin et al. (2009b) and Cvrković et al. (2010) reported *D. europaea* harboring '*Ca.* P. solani' in the vineyard ecosystem of Serbia and Italy which raised the questions of its role also in the "bois nor" disease and in other '*Ca.* P. solani'-associated diseases. Moreover, Mitrović et al. (2012) described *D. europaea* populations harboring '*Ca.* P. solani' and identified the presence of a strain associated with bushy stunt in *Picris hieracioides* (*Picris hieracioides* bushy stunt−PHBS, 16SrII-E subgroup). The recent finding of substantially higher rates of FD-C phytoplasmas in populations of *D. europaea* in vineyards of Montenegro (>60%), as compared to previously reported infection rates in Serbia and Italy (3%), initiated a study on the population genetics peculiarities of this planthopper and interaction with the pathogen and the endosymbionts associated with it (Krstić et al. 2018).

Recent epidemiological studies in south-eastern Europe have revealed *D. europaea* populations that are heavily FD-C infected (Montenegro), as well as populations with low FD-C infection rates that are colonized by the endosymbiont *Wolbachia* (Italy and Serbia), and highly FD-infected populations in the absence of *Wolbachia* (Krstić et al. 2018). Several possible causes underlying the differences in vector infection rates were examined: (i) population genetic characteristics of *D. europaea*, (ii) *Wolbachia* effects on fitness components of *D. europaea* laboratory

colony, and (iii) rate of FD-C infection in *C. vitalba* reservoir plant and differences in FD-C genotypes harbored by low and high *Wolbachia*-infected vector populations. The genetic diversity level of *Wolbachia*-infected populations of *D. europaea* was found to be lower than the one in uninfected populations and to exhibit a different evolution of fixed haplotypes. The FD phytoplasma was found to be genetically diversified in both populations, but this had no relation to the FD-C infection rates of the vector. In addition, no evidence of fitness upgrades (based on fecundity, longevity and body weight) with regard to *Wolbachia* presence was found in infected populations which could explain the transmission pattern of this facultative endosymbiont. Vector's population genetic parameters, fitness response, field data and the observed negative correlation between FD-C infection and *Wolbachia*-presence rates indicate that *Wolbachia* either competes with FD-C within *D. europaea*, or that it confers host protection against FD-C phytoplasma (Krstić et al. 2018). These finding gave a promising start for investigation on the relations between *Wolbachia* and phytoplasma insect vectors.

4.6 Other Possible Planthopper Vectors

Reptalus cuspidatus *R. cuspidatus* (Fieber 1876) is abundantly present in Croatian vineyards (Mikec et al. 2006) and it is reported as the most common and abundant *Reptalus* species in vineyards in Switzerland (Trivellone et al. 2016). The species has also been frequently found in vineyards of north-western Italy (Picciau et al. 2008). In Croatia *R. cuspidatus* consistently harbors 16SrXII-A phytoplasmas and could play a role in the spread of "bois noir" (Mikec et al. 2006). In Switzerland recent investigations revealed in *R. cuspidatus* specimens the presence of phytoplasmas of the tuf-type b2 previously associated with nettle-affiliated disease cycles (Aryan et al. 2014; Atanasova et al. 2015; Plavec et al. 2015; Kosovac et al. 2016a). Its *secY* gene sequences, however, were different from those of the nettle type phytoplasmas, in addition *R. cuspidatus* was never observed on nettle. These findings raise the question whether this peculiar phytoplasma type is hosted by an up to now unknown plant species from which it can be acquired by *R. cuspidatus* (Trivellone et al. 2016). Given this and the case that two of its congeners, *R. panzeri* and *R. quinquecostatus*, are phytoplasma vectors, the role of *R. cuspidatus* as potential BN vector should be further investigated.

Cixiids Vectors of '*Candidatus* Phytoplasma phoenicium' '*Ca.* P. phoenicium' (16SrIX-B) is associated with almond witches' broom, a disease rapidly spreading in the Mediterranean area and in Iran (Verdin et al. 2003). It was also identified in peach (*Prunus persica*) and nectarine (*P. persica* var. *nucipersica*) in southern Lebanon (Abou-Jawdah et al. 2009) and in GF-677 (*P. amygdalus* x *P. persica*) in Iran (Salehi et al. 2011). Since 2017 '*Ca.* P. phoenicium' was added to the EPPO A1 list of pests recommended for regulation as quarantine pests in Europe (OEPP/EPPO 2017). Surveys of cixiids in affected peach and almond orchards in Lebanon

revealed that individuals from the genera *Tachycixius, Cixius, Eumecurus* and *Hyalesthes* were infected with the phytoplasma. Transmission experiments identified *Tachycixius* spp. as a vector: field collected individuals from this genus were able to transmit the pathogen to healthy potted peach seedlings. *Cixius, Tachycixius* and *H. obsoletus* have two flight-peaks in Lebanon, one in spring and one in the autumn which might be related to their feature of accomplishing two generations per year. In case of *Eumecurus* only one flight peak was observed (Tedeschi et al. 2015).

Fulgoroid Vectors of Palm Lethal Yellowing Diseases Phytoplasma associated with lethal yellowing diseases of palms have caused the loss of millions of coconut and other palms around the world. Based on 16S rRNA gene sequences the phytoplasmas in this disease complex are placed into diverse taxonomic groups 16SrIV, 16SrXI, 16SrXIV, 16SrXXII and 16SrXXXII (Bertaccini et al. 2014; Gurr et al. 2016). Due to the diversity of agents involved in this disease and the diversity of the geographical locations many different pathosystems exist and a range of confirmed or putative insect vectors has been reported. Transmission experiments under controlled conditions have been carried out since the 1960s. Only a minority of them, however, have been successful, therefore knowledge of vectors is still very incomplete (Gurr et al. 2016). To date successful transmission experiments allowed the identification of two vectors species, both of the superfamily Fulgoroidea. In southern Florida caged coconut palms, *Veitchia merrillii* and *Pritchardia thurstonii* repeatedly exposed to field collected cixiid planthoppers, *Haplaxius* (= *Myndus*) *crudus* (van Duzee), for a period of 34 months developed lethal yellowing disease (Howard et al. 1983). Molecular analyses of the associated agent revealed a homogeneous population of subgroup 16SrIV-A phytoplasmas (Harrison et al. 2002, 2008). In surveys of insects on palms in northern Florida *H. crudus* was shown to be heterovoltine, with the number of generations affected by temperature. The largest numbers of adults were collected in winter although according to literature; the species was thought to overwinter as subterranean nymphs (Halbert et al. 2014). In India the phytoplasmas contribute to palm decline and significant yield losses. Experiments under controlled conditions including electron microscopic analyses proved that the derbid planthopper *Proutista moesta* (Westwood) transmitted phytoplasmas to coconut seedlings. The majority of these seedling developed specific disease symptoms within 8–24 months (Rajan 2013).

Molecular analyses proved the presence of phytoplasmas in several other planthopper species, which are therefore regarded as putative vectors. In Jamaica lethal yellowing group phytoplasmas were detected in members of the *Cedusa* genus (Derbidae) (Brown et al. 2006). Phytoplasmas associated with a lethal yellowing-type disease of coconuts in Tanzania were indentified in *Diastrombus mkurangai* Wilson (Derbidae) and in *Meenoplus* spp. (Meenoplidae) (Mpunami et al. 2000). In the coastal region of Mozambique *D. mkurangai* carried phytoplasmas with 99% identity on the 16S rRNA gene with the coconut phytoplasmas detected in the same

area that are '*Ca.* P. palmicola' (16SrXXII-A) (Harrison et al. 2014; Bila et al. 2017).

The "Bogia" coconut syndrome is a serious plant disease of palm species in Papua New Guinea. It is associated with a phytoplasma related to, but distinct from, the coconut lethal yellowing group (16SrIV) (Lu et al. 2016) and was recently described as '*Ca.* P. noviguineense' (Miyazaki et al. 2018). A wide range of Hemiptera taxa was tested from sites affected by the "Bogia" coconut syndrome and Pilotti and co-workers (Pilotti et al. 2014) found the Fulgoroid planthopper species *Zophiuma pupillata* (Stål) (Lophopidae) infected with the phytoplasma. Another study combined molecular testing of phytoplasmas in insect bodies with feeding assays on sucrose solutions. Six Hemiptera taxa and their feeding solutions were positive for the phytoplasma: *Z. pupillata* and *Lophops saccharicida* (Kirkaldy) (Lophopidae), one unidentified species of the tribe Zoraidini (Derbidae), one unidentified species of the Ricaniidae family and two Flatidae species, *Taparella amata* (Walker) and *Colgar* sp. The case that six species were capable of introducing the phytoplasma DNA into their feeding medium suggests that the phytoplasma associated with "Bogia" coconut syndrome is potentially transmitted by a wide range of insect vectors (Lu et al. 2016).

Cixiid Vectors of '*Candidatus* Phytoplasma australiense' '*Ca.* P. australiense' (16SrXII–B) is associated with several phytoplasma diseases in New Zealand and Australia such as Australian grapevine yellows, sudden decline of cabbage tree, strawberry lethal yellows, papaya die-back and yellow leaf disease of New Zealand flax (*Phormium* spp.) (Davis et al. 1997; Liefting et al. 1998; Padovan et al. 2000). In New Zealand two vector species have been identified by transmission experiments, both of them belong to the genus *Zeoliarus* (previously *Oliarus*) (Cixiidae) (Larivière and Fletcher 2008). *Zeoliarus atkinsoni* (Myers), a planthopper considered to be monophagous on New Zealand flax (*Phormium* spp.) transmitted the pathogen from infected to healthy *Phormium* (Liefting et al. 1997). In recent transmission studies the polyphagous species *Z. oppositus* (Walker), transmitted the '*Ca.* P. australiense' to both *Coprosma robusta* (karamu) and *Cordyline australis* (New Zealand cabbage tree) (Winks et al. 2014).

Fulgoroid Vectors of Sugarcane Phytoplasmas Sugarcane is affected by yellows and decline diseases associated with phytoplasma presence. These diseases cause more or less similar symptoms but differ in the identity of the associated phytoplasmas, vector relationship and geographic distribution (Marcone 2002; Rao et al. 2012). Literature predominantly links leafhopper species to the transmission of sugarcane phytoplasmas, in some areas, however, Fulgoroids have been reported as confirmed or putative vectors (Rao et al. 2012). In Cuba *Saccharosydne saccharivora* (Westwood) (Delphacidae) was the prevalent Auchenorrhyncha species in the sugarcane fields. In transmission experiments, this species transmitted a phytoplasma to healthy sugarcane seedlings. Phylogenetic analysis of 16S rRNA gene sequences identified the phytoplasmas present in sugarcane, in *S. saccharivora* and in *Cedusa* spp. (Derbidae) as '*Ca.* P. graminis' (16SrXVI-A) (Arocha et al. 2005a, b). In Mauritius the sugarcane leaf yellows phytoplasma is a commonly encoun-

tered disease. The sugarcane Delphacid planthopper, *Perkinsiella saccharicida* (Kirkaldy), could be involved in pathogen transmission as molecular analyses led to the detection of phytoplasmas from two ribosomal groups 16SrIII and 16SrI group in the insects (Joomun et al. 2007; Rao et al. 2012).

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Chapter 5
Transovarial Transmission in Insect Vectors

Rosemarie Tedeschi and Assunta Bertaccini

Abstract Phytoplasma ability to infect a new generation of insects by transovarial transmission was demonstrated in some insect vector/plant host combinations mainly by molecular evidence coupled with biological assays. *Scaphoideus titanus* was the first one in which phytoplasma detection in eggs, newly hatched nymphs and adults (reared on phytoplasma-free *Vicia faba* seedlings) was demonstrated. This kind of transmission was proved also for mulberry dwarf phytoplasmas and for the agent of white leaf disease of sugarcane, transmitted respectively by *Hishimonoides sellatiformis* and *Matsumuratettix hiroglyphicus*. Recently *Cacopsylla pruni*, vector of '*Candidatus* Phytoplasma prunorum' and *Cacopsylla picta* one of the main insect vectors of '*Candidatus* Phytoplasma mali' were also shown to have phytoplasma transovarial transmission, therefore this kind of transmission should be taken into consideration when epidemiological studies are performed on phytoplasma-associated diseases. The fact that the insect is not only the vector, but also a reservoir of the phytoplasma has implications for disease management, increasing the difficulty of disease control. Up to now only a few phytoplasma ribosomal groups such as 16SrI, 16SrX and 16SrXI have been demonstrated to be transferred transovarially in their insect vectors, very likely those capable of better adaptation to both plant and insect environments. Therefore, it can be speculated that only strains of phytoplasmas with specific genetic characteristics have become transovarially transmissible and probably only after a long host–parasite relationship.

Keywords Ovary · Eggs · Progeny · Phytoplasmas · Epidemiology

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_5

5.1 Introduction

Transmission process by insect vectors is a capital step for every plant pathogen because it determines the pathogen dispersal in the space and by the time, influencing its ecology and the disease epidemiology. Even more interesting and of greater epidemiological significance is the transovarial transmission, that is the transfer of pathogens to succeeding generations through invasion of the ovary and infection of the eggs. Transovarial transmission is also called vertical transmission as opposed to direct and horizontal (i.e. contact or venereal) and to indirect and horizontal (through plant transmission). Phytoplasmas are found in most of the major organs of an infected insect host once they are established. They enter the insect's body through the stylet and then move through the intestine wall and into the haemolymph. From here they proceed to colonize the salivary glands, a process that can take up to some weeks. The clover phyllody phytoplasma was the first reported as transovarially transmitted (Posnette and Ellenberger 1963) by nymphs of *Euscelis incisus* (Kirschbaum) reared from eggs laid by infective females on wheat plants without a prior acquisition. Wheat plants however, were successively found to be susceptible to this phytoplasma (Chiykowski 1967) therefore, this study requires to be reconfirmed using other plant species.

Some years later, Sinha and Chiykowski (1967) indicated the presence of aster yellows (AY) phytoplasmas in ovaries of the leafhopper *Macrosteles fascifrons* (Stål) injecting tissue extracts into healthy leafhoppers, then testing the latter singly for inoculativity on aster seedlings. A similar experiment carried out on oat plants failed to allow transmission and the authors explained the results with transovarial transmission, since wheat was considered immune to the pathogen. These data have only historical relevance since it was not possible to identify phytoplasmas in the sixties, when these diseases were believed to be caused by viruses. Surveys for phytoplasma presence in different tissues of infective leafhoppers using serological techniques showed the presence of aster yellows phytoplasmas in the ovaries of *M. fascifrons* (Sinha and Chiykowski 1967), but failed to show whether the phytoplasmas were able to reach the oocytes. More recently, using both ELISA and gold immunolabeling techniques, the "flavescence dorée" phytoplasmas were observed in several tissues of *Euscelidius variegatus* (Kirschbaum), including the mycetome, but not in their genital organs (Lefol et al. 1994).

5.2 Factors Affecting the Transovarial Transmission

Transmissibility to eggs is a complex mechanism and may depend on many factors: permeability of membranes in the vector; genetic variability; long association between the pathogen and the vector with special adaptations in the vector; the symbionts. Transovarial transmission generally concerns microbes that are transmitted in a persistent-propagative way. In these cases, after acquisition, the

microorganism able to replicate/multiplicate in the insect, moves from the gut lumen into the hemolymph and other tissues such as the Malpighian tubules, fat bodies and brain, or reproductive organs, and finally reaches the salivary glands. When the microorganism is one of the insect transmitted plant pathogen such as a virus or a bacterium (and thus also a phytoplasma) all these passages require specific interactions between pathogen and vector components to overcome the midgut infection barrier, to carry out dissemination (including midgut escape and salivary gland infection); to colonize salivary gland (escape barrier) and in some selected cases to pass the transovarial transmission barriers (Hogenhout et al. 2008; Bertaccini et al. 2016). The ability of the pathogens to bind to their insect host tissues and in particular to the reproductive structures may allow the transovarial transmission.

Important roles to insect transmissibility of phytoplasmas were linked to molecular interactions such as those with the membrane proteins such as Imp, IdpA, Amp that interact with the insect proteins (Kakizawa et al. 2006; Suzuki et al. 2006; Galetto et al. 2011; Rashidi et al. 2015; Konnerth et al. 2016). The analysis of promoter sequences on phytoplasma plasmids revealed that the absence of these sequences in a plasmid of the strain OY-NIM allows to lose its insect-transmissibility (Ishii et al. 2009a, 2009b). Furthermore, during the maintenance of OY-NIM by plant tissue culture for 10 years, the plasmid was gradually lost suggesting that the plasmid is not essential for the phytoplasma viability in a plant host, but it may be a key element for phytoplasma to adapt to insect hosts.

The possibility to cross the barriers in order to transmit phytoplasmas to progeny should be associated to their genome size and plasticity (Kawakita et al. 2000). Anyhow, considering that phytoplasmas with similar genome size did not show similar competence in terms of transovarial transmission, perhaps more important than the size of the genome, could be its variability. The phytoplasma genome plasticity is also due to integration of foreign DNA such as the potential mobile units (PMUs) also named sequence variable mosaics (SVMs) that are believed to be involved in the emergence and adaptive evolution of phytoplasmas. This integration can be a process enabled by ancient and recurrent phage attacks, site-specific chromosomal integration of prophage genomes, and subsequent genetic recombination and duplication events. The absence of phage-derived SVMs in *Acholeplasma* genomes (phytoplasma ancestors) supports the concept that a phage-mediated gene exchange enabled phytoplasma transkingdom parasitism, helping them in insect colonization (Wei et al. 2008) that may also affect the frequency and prevalence of transovarial transmission (Arismendi et al. 2015).

It has been ascertained that some insect vectors may be negatively affected by the presence of phytoplasmas, while some others show increased fitness when phytoplasma-infected (Sugio et al. 2011). This can be considered as a consequence of the period of co-evolution between the two organisms, with long term co-evolution leading to an increased fitness of vectors as results of a selection/adaptation process (Beanland et al. 2000; Bressan et al. 2005). To ensure a successful transovarial transmission, the pathogen should not be harmful to its insect host because the reproduction and fecundity are essential also for the pathogen spreading.

From this point of view, transovarial transmission can be an indication of a long lasting co-evolution of insect vectors with phytoplasmas and it could be expected in increasing numbers of insect species and phytoplasmas, but always with very specific characteristics for the diverse binomia.

Finally it is well known that insect symbionts can influence transovarial transmission, enhancing immunity and resistance to pathogens. Moreover Jia et al. (2017) showed that an insect symbiotic bacterium directly harbours a viral pathogen and mediates its transovarial transmission to offspring. They proved that the *Rice dwarf virus* (RDV) is able to bind to the envelopes of the bacterium *Sulcia muelleri*, a common obligate symbiont of leafhoppers, allowing the virus to exploit the oocyte entry path of *Sulcia* in rice leafhopper insect vectors. It is plausible that such a model of virus–bacterium interaction is a common phenomenon in nature and may influence the phytoplasma transovarial transmission.

5.3 Ovary Structure in Insect Vectors and Mechanisms of Transovarial Transmission

The ovaries of insects are paired structures composed of a cluster of ovarian or egg tubes called ovarioles, in which full growth of the eggs takes place (Fig. 5.1a). Each ovariole contains a series of developing oocytes each surrounded by a layer of follicle cells forming an epitelium (the oocyte and its epitelium are termed follicle). Two basic types of ovaries are found among insects: panoistic and meroistic. In panoistic ovaries, considered the ancestral ones and typical of the most primitive insect orders, all oogonia are transformed to oocytes and there are not specialized nutritive cells. In meroistic ovaries, on the other hand, both oocytes and nurse cells (called trophocytes) are generated, so in addition to the epitelium, each oocyte is connected with one or several nurse cells until the end of its growth period. The meroistic type of ovary can be subdivided into polytrophic and telotrophic types. In the polytrophic meroistic ovary, trophocytes and oocytes alternate along the length of the ovariole, while in the telotrophic meroistic ovary, the trophocytes are restricted at the upper end of the ovaries and are connected to oocytes in the early stages of their development by cytoplasmic processes called nutritive or cytoplasmic cords (Gullan and Cranston 2010; Kot et al. 2016).

Hemipteran insects, which include all reported phytoplasma vectors, have a telotrophic meroistic ovary with ovarioles characterized by: (i) a terminal filament, (ii) a tropharium in which mitosis gives rise to primary oocytes and where trophocytes reside; (iii) a vitellarium in which linearly arranged oocytes sequentially grow through previtellogenesis, vitellogenesis and choriogenesis and (iv) a pedicel or stalk (Fig. 5.1b-d). Nutrients and maternal gene products pass through the cytoplasmic cords to the developing oocytes. The terminal filaments from all ovarioles fuse into a suspensory ligament that anchors the ovary to the fat body; whereas, proximally, the pedicel joins the ovariole with the lateral oviduct.

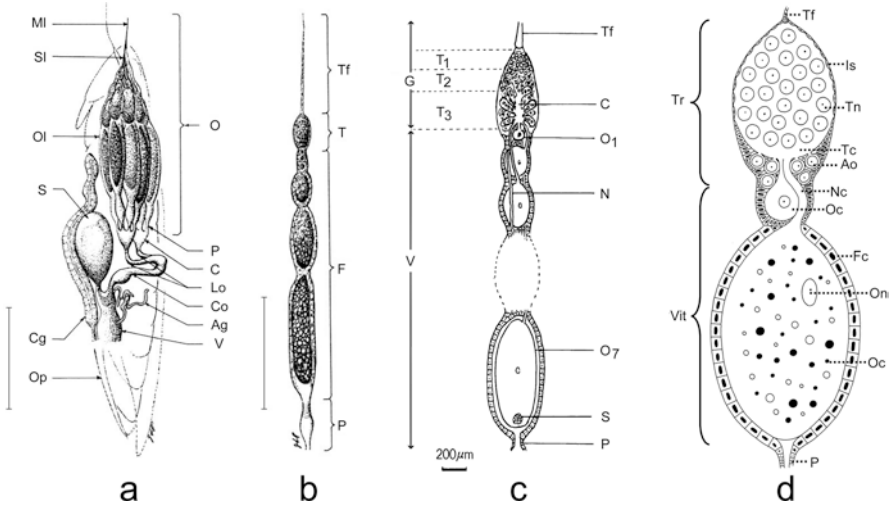

Fig. 5.1 (**a**) Lateral view of female reproductive system of the leafhopper *Dalbulus maidis*. O, ovary; Ol, ovariole; Sl, suspensory ligament; Ml, median ligament; P, pedicle; C, calyx; Lo, lateral oviduct; Co, common oviduct; S, spermatheca; Cg, colleterial gland; Ag accessory gland; V, vagina; Op, ovipositor. Vertical bar = 1.0 mm. (**b**) Detail of a *D. maidis* ovariole. Tf, terminal filament; T, tropharium; F, follicles; P, pedicle. Vertical bar = 0.5 mm (adapted from Tsai and Perrier 1996); (**c**) schematic representation of a *Euscelidius variegatus* ovariole. C, trophic core; G, germarium; N, nutritive cord; O1, O7, oocytes 1 and 7; P, pedicel; T1, T2 and T3, tropharium regions 1, 2, and 3; TF, terminal filament, V, vitellarium (adapted from Cheung 1994); (**d**) schematic representation of the ovariole of the adult female of psyllids; Ao, arrested oocyte; Fc, follicular cells; Is, inner epithelial sheath; Nc, nutritive cord; Oc, oocyte; On, oocyte nucleus; P, pedicel; Tc, trophic core; Tf, terminal filament; Tn, trophocyte nucleus; Tr, tropharium; Vit, vitellarium (Adapted from Kot et al. 2016)

The mature oocyte is ovulated into the oviducts, fertilized, and oviposited (laid) (Nation 2001; Kot et al. 2016). The number and size of ovarioles vary depending on taxon. In Hemiptera generally the number of ovarioles per ovary ranges between 4 and 15, except for coccids and psyllids that represent special cases. In particular in psyllids there are up to 100 ovarioles and in particular there are 28–42 in *Cacopsylla melanoneura* (Förster) (Kot et al. 2016), thus 10 times the number found in typical hemipterans due probably to both ecological and ontogenetic reasons (Hodin 2009).

The mechanisms that allow the transovarial transmission of phytoplasmas are mostly unknown, while some more information is available concerning other plant pathogens and the symbionts of phytoplasma vectors. For instance, several studies were done on transovarial transmission of vector-borne plant viruses. In particular, since that viral pathogens themselves do not have the ability to enter the oocyte, but instead use the existing oocyte entry pathways in insects, Huo et al. (2014) suggested that the Tenuivirus *Rice stripe virus* (RSV) enters the oocyte of the planthopper vector *Laodelphax striatellus* (Fallén) along with vitellogenin, a protein synthesized by the fat body and transported into the growing oocytes via receptor-mediated endocytosis.

More recently Liao et al. (2017) demonstrated that the *Rice gall dwarf virus* (RGDV) initially enters the germ cells in the germarium, moves between follicular cells, then translocate across the microvilli to access the oocyte cytoplasm. They deduced that virus-induced tubule act as vehicle for spread of RGDV into ovary oocyte cells overcoming the transovarial transmission barrier of the insect vector. RGDV exploits virus-containing tubules composed of a viral non-structural protein Pns11 to pass through actin-based junctions between follicular cells or through actin-based microvilli from follicular cells into oocytes of the leafhopper vector *Recilia dorsalis* Motschulsky.

The examples given by symbionts are interesting to understand the transovarial transmission of phytoplasmas considering also their co-presence in the insect vectors. The same mechanism that allows the transovarial transmission of the symbionts likely provides a preadaptation for the transmission of phytoplasmas (Alma et al. 2008). Generally, in auchenorrynchans and psyllids, before the migration to the ovaries, symbiont bacteria are released from the bacteriocyte cytoplasm, change their shape to almost spherical, then gather around the posterior pole of the vitellogenic oocyte cells, and enter through the follicle cells and the pedicel (ovariolar stalk) of the ovariole. This type of symbiont mass entering an oocyte is often referred to as "symbiont ball", and appears to be a common feature of transovarial transmission of various intracellular microbes, including bacteria and yeast-like fungal symbionts (Fig. 5.2), in multiple insect lineages (reviewed in Szklarzewicz and Michalik 2017; Dan et al. 2017). As an example, the bacterium *Cardinium* sp. and some yeast-like symbionts (YLSs) were demonstrated to be vertically transmitted to the offspring of *Scaphoideus titanus* Ball, the leafhopper vector of the "flavescence dorée" phytoplasma. TEM examinations of the apical region of the ovary revealed the presence of *Cardinium* sp. close to the membrane of a bacteriocyte-like cell and protruding towards the oocyte cytoplasm. In particular a bacterium was observed within an invagination of the plasma membrane at the cell surface; then bacteria were observed inside the oogonia adjacent to the bacteriocyte-like cell. Symbiotic bacteria were also observed in the initial phases of embryo development, in the cytoplasm of the oogonia and in the apical region of the ovary in the third and fourth nymph instar respectively (Sacchi et al. 2008) (Fig. 5.2). In the same study, YLSs were observed in the fat body-ovary interface, enclosed by a thin layer of the fat body syncytium adhering to the ovarioles, in the cytoplasm of follicle cells and in the developing oocytes, providing a clear indication of the route followed by YLSs for vertical transmission from the thin layer of the fat body to the extracellular space and then to the follicle cells. Moreover the presence of an endocytoric envelope surrounding the YLSs within the oocytes proves an endocytotic process (Sacchi et al. 2008) (Fig. 5.3).

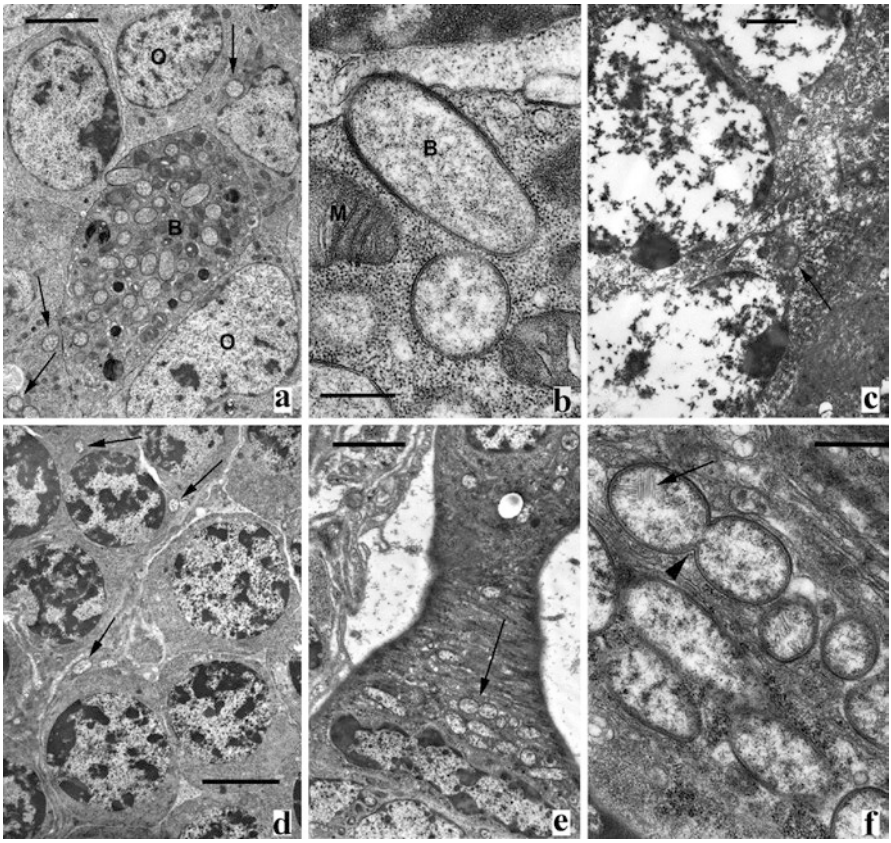

Fig. 5.2 TEM micrographs showing the distribution of *Cardinium* sp. in the ovaries and embryo of *Scaphoideus titanus*. (**a**) Apical portion of the ovary showing a bacteriocyte-like cell (B) with cytoplasm filled with numerous bacteria. This cell is encircled by oogonia (O) containing a few symbionts (arrows) in the cytoplasm. Bar = 3.4 μm. (**b**) Detail of panel (a) showing a bacterium (B) close to the bacteriocyte cell membrane protruding towards the oocyte cytoplasm. M: mitochondrium. Bar = 0.5 μm. (**c**) Embryo showing a symbiont (arrow) in the yolk. Bar = 1.1 μm. (**d**) Detail of the ovary of a third nymph stage showing symbiotic bacteria (arrows) in the cytoplasm of the oogonia Bar = 5 μm. (**e**) Cluster of bacteria (arrow) localized in the apical region of the ovary of a fourth nymph stage Bar = 3.4 μm. (**f**) Detail of the panel c showing a dividing symbiont (arrowhead) with, in evidence, the microtubule-like elements (arrow). Bar = 0.8 μm (Adapted from Sacchi et al. 2008)

5.4 Phytoplasma Transovarial Transmission

To assess phytoplasma vertical transmission, specific transmission trials should be carried out: the presence of the phytoplasmas in all the stages originating from the same infected female provides evidence of phytoplasma DNA inheritance to the progeny, while successful transmissions by the progeny adults is the final evidence of the vertical infectivity transmission (Bosco and Tedeschi 2013).

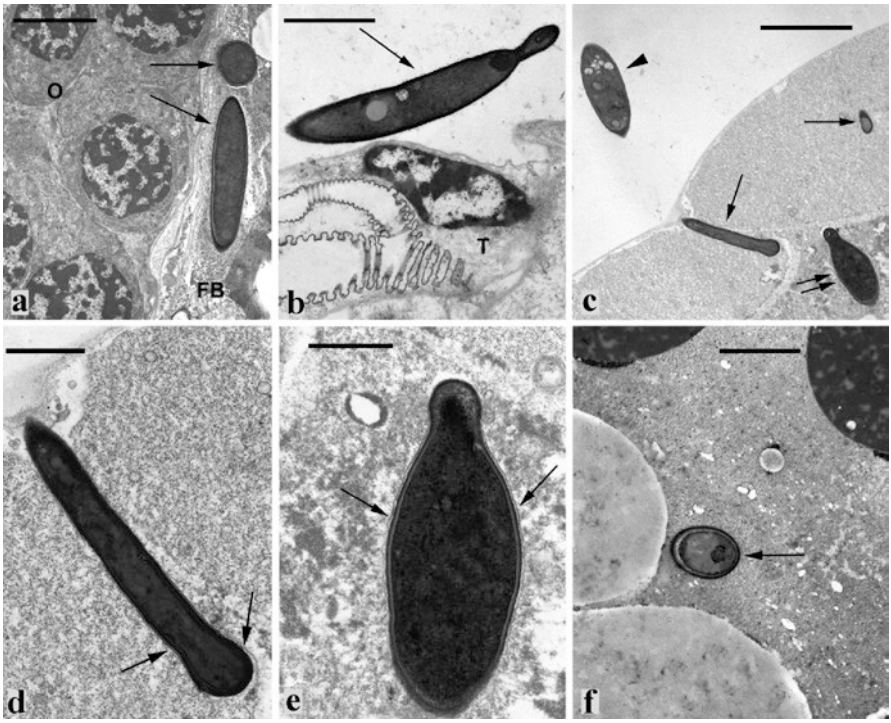

Fig. 5.3 Micrographs showing the distribution of YLSs in the fat body-ovary interface, ovaries and embryos of *Scaphoideus titanus*. (**a**) Detail of the interface fat body-ovary showing two YLSs (arrows) enclosed in the fat body layer (FB) adhering to the ovary (O). Bar = 5 µm. (**b**) A YLS (arrow) from the fat body was observed in the hemolymph near a tracheocyte (T) of the ovaric tissue. Bar = 3.4 µm. (**c**) Details of the ovaric region showing a YLS free in the hemolymph (arrowhead), two YLSs in the cytoplasm of the follicle cells (arrows), and a YLS (double arrow) in the ooplasma Bar = 5 µm. (**d**) and (**e**) Details of panel c showing the presence of an endocytotic envelope (arrows) around the YLSs. Bar = 1.2 µm. (**f**) Detail of a young embryo showing a YLS (arrow) in the yolk. Bar = 6.2 µm (Adapted from Sacchi et al. 2008)

The transovarial transmission was detected in *S. titanus,* the vector of "flavescence dorée" (FD) phytoplasmas, associated with one of the most dangerous grapevine disease in Europe. Dot hybridization testing showed that it was also infected by aster yellows-related phytoplasmas (Bertaccini et al. 1993); while it is not the vector of this phytoplasma under natural conditions, under experimental conditions it was later possible to obtain its aster yellows transmission (Alma et al. 2001). By applying nested-PCR assays with aster yellows specific primers this phytoplasma was detected in the different life stages of *S. titanus* reared on phytoplasma-free *Vicia faba* seedlings. Eggs (Fig. 5.4), newly hatched nymphs and adults resulted positive to aster yellows phytoplasma presence. The results on insects were confirmed by infection of a percentage of broad bean seedlings after rearing such nymphs. The proportion of infected plants started from 9% to 11% (for eggs and newly hatched

Fig. 5.4 *Scaphoideus titanus* eggs laid in the canes from grapevine pruning materials (DISAFA – Entomology unit, University of Torino, Italy)

nymphs that never fed) and reached 48–65% for nymphs and adults reared for a period of 30–40 days (Danielli et al. 1996; Alma et al. 1997).

Few years later Kawakita et al. (2000) found and identified mulberry dwarf (MD) phytoplasmas (16SrI-B group) in the genital organs and eggs of the leafhopper *Hishimonoides sellatiformis* (Ishihara) with electron microscope observations and performing PCR with aster yellows group specific primers, respectively. Direct and nested PCR revealed the presence of MD phytoplasmas in eggs, ovaries, testes and seminal receptacles. In particular in the eggs (tested in batches of five), the phytoplasma was detected only by nested-PCR suggesting a very low titer of the pathogen in this stage. Electron microscope observations confirmed the presence of MD phytoplasmas in ovaries, testes, and seminal receptacles, but not in the eggs, due to hard egg shells that made preparation of ultrathin sections very difficult. The authors could assess that the observed phytoplasmas were almost the same as those in salivary glands in their morphology (polymorphic within a diameter of 0.1–0.6 μm, lacking a cell wall, bounded by membrane). In this study, the presence of many MD phytoplasmas in the seminal receptacles of female adults and in the testes of male adults suggested also that MD phytoplasmas might be transferred into female adults from infected male adults through copulation. This is not uncommon for the closest relative of phytoplasmas, the mycoplasmas, that infect human and animal and for which the infection of urinary tracts and reproductive organs is quite common being in some cases also associated with miscarriage (Capoccia et al. 2013). The MD phytoplasmas was also found in newly hatched nymphs of *H. sellatiformis,* but since they could have fed on infected plants after hatching, although for a short time, so these data are not significant in proving transovarial transmission.

A vertical infectivity transmission was demonstrated in another leafhopper, *Matsumuratettix hiroglyphicus* (Matsumura), the vector of the sugarcane white leaf (SCWL) phytoplasma (16SrXI group) (Hanboonsong et al. 2002). After severe outbreaks of the disease in Thailand the phytoplasma reservoir was sought in the weeds that grow in and around sugarcane farming areas. Following the failure to identify evidence of such a reservoir in plants, the research was moved to verify the possible role of the insect vectors as reservoirs. Nested PCR analyses followed by sequencing

revealed the presence of the SCWL phytoplasma in eggs, nymphs from first to fifth instar and adults of two following generations reared on phytoplasma free sugarcanes grown from tissue culture. In this case, the pathogen was detected in eggs, nymphs and adults individually tested. Moreover, *in situ* PCR allowed to observe the phytoplasma in the female reproductive organs, supporting the hypothesis of its transovarial transmission. Moreover, plants on which transovarially infected insects had been reared tested positive only after the rearing, suggesting the ability of offspring generation to transmit, in their turn, the transovarially acquired phytoplasma and confirming previous results (Alma et al. 1997).

The vertical transmission of phytoplasmas through the eggs was reported also for psyllids of the genus *Cacopsylla*, the main vectors of fruit tree phytoplasmas in the Palaearctic region. Tedeschi et al. (2006) proved the passage of '*Candidatus* Phytoplasma prunorum' through eggs, nymphs and F1 adults of *Cacopsylla pruni* (Scopoli) as well as the infectivity of transovarially infected F1 individuals. Pairs of field collected adults were isolated in glass tubes containing a small plum twig from healthy plants (Fig. 5.5a, b). Then, after the eggs were laid, females were removed and singly PCR-tested. Only in the case of positive females, batches of 20 eggs were collected and PCR tested, while some others were kept to continue the rearing of the offspring (Fig. 5.5c) following the experimental design shown in Fig. 5.6. '*Ca.* P. prunorum' was found in sequences of eggs, nymphs and newly emerged adults.

Moreover this phytoplasma was also detected in plants where transovarially infected individuals of *C. pruni* were isolated. The PCR amplification signal observed in the newly emerged adults, stronger than that in eggs and nymphs, could be the result of multiplication of phytoplasma in the insect body.

Similarly, Mittelberger et al. (2017) proved transovarial transmission of '*Candidatus* Phytoplasma mali' in one of its psyllid vector *Cacopsylla picta* (Förster). Again field collected overwintered specimens were isolated in couples, on healthy apple plants. After egg laying parental insects were analyzed by quantitative

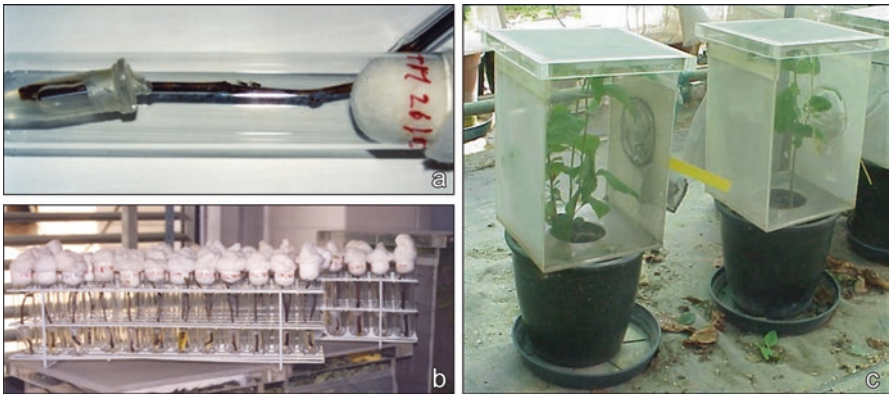

Fig. 5.5 Details of transovarial transmission trials carried out by Tedeschi et al. (2006). (**a** and **b**) Glass tubes containing pairs of field collected psyllid adults and a small plum twig for oviposition. (**c**) Cages for offspring rearing (DISAFA – Entomology unit, University of Torino, Italy)

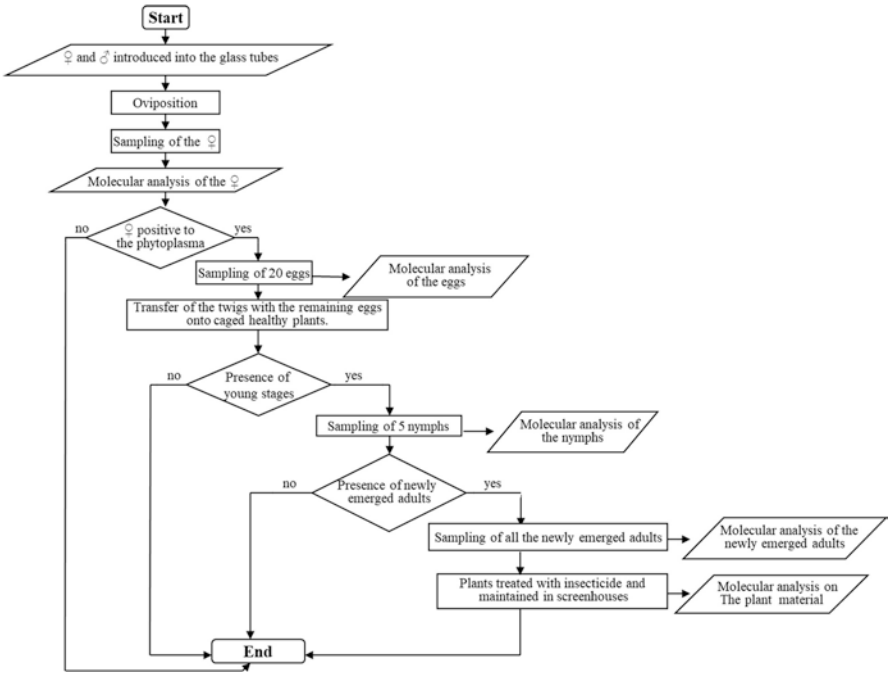

Fig. 5.6 Experimental design of '*Ca.* P. prunorum' transovarial transmission trials with *Cacopsylla pruni* (From Tedeschi et al. 2006)

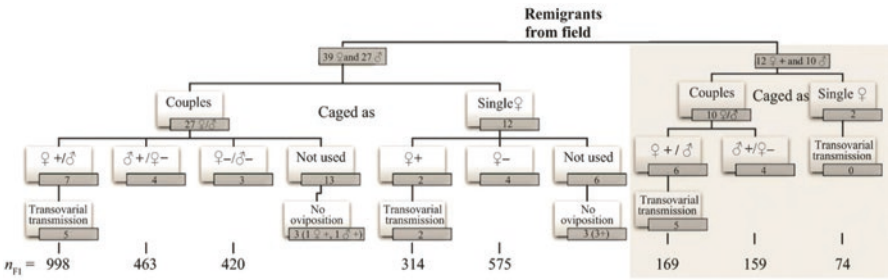

Fig. 5.7 Experimental design of '*Ca.* P. mali' transovarial transmission trials in *C. picta.* Field collected remigrants were caged as couples or single individuals and sorted according to their state of infection as follows: infected females (♀ +), infected males (♂ +), uninfected females (♀ −) and uninfected males (♂ −). nF1 = number of analyzed F1 individuals from the respective parental groups (Adapted from Mittelberger et al. 2017)

PCR and the plant material with the eggs was divided in: (i) progeny from an infected female, (ii) progeny from an infected male, and (iii) progeny from both uninfected female and male (Fig. 5.7). '*Ca.* P. mali' was detected in eggs, nymphs and offspring adults. The percentage of phytoplasma detected increased with each

developmental stage and the phytoplasma titer increased exponentially from the second instar nymph to the F1 adult stage. These authors observed also a higher percentage of transovarial transmission starting with infected females while only one individual out of 622 derived from uninfected female and infected male tested positive. Moreover parental females that did not transmit phytoplasma to their progeny had a significant lower phytoplasma titer, indicating a critical threshold of phytoplasma concentration for vertical transmission to occur in this phytoplasma-insect vector combination. The phytoplasma titer in F1 adults was similar to that in infected parental individuals, so transovarially infected F1 adults are as infective as remigrants and could act as efficient sources of inoculum in the spring in the apple orchards where the insect mates and lays eggs.

5.5 Transovarial Transmission and Phytoplasma Disease Epidemiology

Transovarial transmission of phytoplasmas has important implications in the epidemiology of diseases, as it can facilitate the persistence of the pathogens in the environment, especially during periods unfavorable for horizontal or normal transmission (Huo et al. 2014; Lequime and Lambrechts 2014). Indeed vertical transmission ensures the pathogen survival in cases where the host plant is absent, especially in crops where spatially and temporally varying rotations are being practiced or the environment becomes less favorable for the pathogen. In particular, whereas pathogens with wide host ranges have higher chances of survival in the absence of any given host, for pathogens with a specific host range transovarial transmission seems to act to ensure more survival chances. When the pathogen is transmitted to the eggs of the vector, the offspring becomes a key source of infection for crops in the absence of diseased plants. In such cases, depending on the population dynamics and mobility of the vector, the pathogen could persist locally, and/or migrate to areas/regions where the host plant is present (Hibino 1996; Jeger et al. 2009).

Actually phytoplasmas for which transovarial transmission in the insect vectors has been confirmed have a quite specific host range, supporting this theory. In this scenario, the insect is not only a vector, but also a reservoir of the phytoplasma and this has important implications for the disease management. For instance, in the case of apple psyllids, *Cacopsylla melanoneura* and *C. picta*, vectors of '*Ca.* P. mali', emphasis has always been put on remigrants because those specimens harbor, on average, a higher phytoplasma titer. For this reason, overwintered adults that recolonize the orchards at the end of the winter, are considered crucial in vectoring apple proliferation phytoplasmas and treatments are always concentrated against them, as soon as they arrive on apple plants. But, if the phytoplasma titer in newly emerged and transovarially infected F1 adults is similar to that of infected parental individuals, as demonstrated by Mittelberger et al. (2017) for *C. picta*, the input in the spreading of the pathogen should be completely revised and likewise, as a consequence, the control strategies as well. Moreover also the role of young stages in

the transmission should be reconsidered when transovarial transmission occurs. In many cases, nymphs of phytoplasma vectors are not efficient in the transmission, because of the low titer of the pathogen and because of the latency period that often, before adult emergence, is not completed. If the infection occurs in the ovary, nymphs should have higher infection rate than in absence of transovarial transmission and latency period could be completed before adult emergence. Mittelberger et al. (2017) suggested a critical threshold of phytoplasma concentration for vertical transmission to occur, but when it occurs there are high probabilities that this transmission will be successful also in the following generations.

Two factors may enlarge the impact of transovarial transmission in disease epidemiology, that are the vector turnover rate and the population sex ratio. If the phytoplasma vector has more than one generation per year, or if the population sex-ratio is female biased, the effects of transovarial transmission appear more worrisome.

5.6 Conclusions

The occurrence of transovarial transmission has important implications also in the pest risk analysis for quarantine pests as well as in the risk assessment for phytoplasma diseases. One of the easiest ways, for a pest, to be introduced in a new area is as eggs laid on propagation materials such as cuttings or young rooted plants. In the case of phytoplasma vectors, assuming that there is no transovarial transmission, the direct impact is not very high (EFSA PLH Panel et al. 2017).

Unfortunately very few studies have been carried out to ascertain the ability of phytoplasma vectors in transmitting the pathogen to the progeny through eggs, however only in a minimal part transovarial transmission was reported as not occurring (Tedeschi et al. 2006; Arismendi et al. 2015; Queiroz et al. 2016). One of the possible reason for the lack of transovarial transmission in the studied cases could be the short co-evolutionary history between the pathogen and the vector as suggested by Queiroz et al. (2016) for *Diaphorina citri* as vector of '*Ca.* P. aurantifolia' the agent of a devastating disease in lime, being *D. citri* present in Oman since only 2005. In conclusion, considering the important implications of phytoplasma transovarial transmission in the epidemiology of the diseases they are associated with, further researches are desirable in order to verify the possibility of transovarial transmission in many other phytoplasma-insect vector combinations. For this purpose, accurate protocols should be followed in order to avoid false results and to verify not only offspring infection, but also infectivity (Bosco and Tedeschi 2013).

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Chapter 6
Phytoplasma Transmission by Seed

Eleonora Satta, Samanta Paltrinieri, and Assunta Bertaccini

Abstract The transmission of phytoplasmas by seed was reported in several herbaceous and some woody plant species. The seedlings were usually analyzed for the presence of phytoplasmas in different stages of growth by nested-PCR/RFLP analysis and phytoplasmas belonging to different ribosomal groups according with species and geographical distribution were detected. The phytoplasma isolation from corn seedlings confirms the seed transmission of viable phytoplasma cells. The sudden epidemic events associated with the presence of phytoplasmas molecularly undistinguishable, in very distant geographic areas, on the same herbaceous species, provides further indications of transmission of these prokaryotes also by seeds. In all the analysed species, the phytoplasma detected in seedlings belong to different ribosomal groups, in some cases they are found also in mixed infection. Since phytoplasma presence is not contemplated in propagation material by the any of the worldwide plant protection quarantine protocols nor by seed producers, the movement of seeds from infected plants implies a wide dissemination of the pathogens and therefore of the associated diseases in still uncontaminated areas.

Keywords Molecular detection · Epidemiology · Culture · Isolation · Disease

6.1 Introduction

Phytoplasmas are cell wall lacking bacteria inhabiting the plant phloem that are transmitted by insect vectors, vegetative propagation methods such as grafting, cuttings, tubers or rhizomes, micropropagation (Bertaccini et al. 1992), and dodder (*Cuscuta* sp.). The phytoplasma transmission by seed was longtime considered a controversial issue and for the lack of a direct connection between the phloem system and the embryos it was considered not possible (Menon and Pandalai 1960). Moreover, the frequent association of floral abnormalities and fruit malformations

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_6

with phytoplasma presence, led to believe that the seeds originated from infected plants were not viable and germinating (McCoy et al. 1989). However, phytoplasmas are pleomorphic and have a small size that allow them to pass through the pores of the phloem, and to be transported by the flow of the assimilates; therefore, they are potentially able to reach other organs connected to the phloem. Moreover, the phytoplasmas were detected in different organs of the plants and are usually in high concentration in the floral structures of herbaceous host plants (Bertaccini and Marani 1982; Clark et al. 1989). The observation of phytoplasmas in inflorescences was also reported in stems, racemes, male and female flowers in palms infected by the Cape Saint Paul wilt disease (CSPWD) in Ghana (Nipah et al. 2007). European stone fruit yellows (ESFY) phytoplasmas were also detected in apricot flowers and fruits, but not in pollen samples (Nečas et al. 2008). In mulberry trees (*Morus* spp.) infected with mulberry dwarf (MD), phytoplasmas were found in the ovary, filaments, stigmas, sepals and anthers (Jiang et al. 2004). The demonstration of lethal yellowing phytoplasma presence in coconut palm (*Cocos nucifera* L.) fruits embryos using nested PCR (Harrison et al. 1994; Harrison and Oropeza 1997; Nipah et al. 2007) and *in situ* PCR (Cordova et al. 2003), led the national and international legislation to prohibit commercial movement of coconuts from areas where lethal yellowing is epidemic (Centre for Information on Coconut Lethal Yellowing, http://www.avxl82.dsl.pipex.com/CICLY/main.html). Phytoplasmas have been identified in seed tegument and kernels of apricot and mulberry infected respectively by ESFY (Nečas et al. 2008) and mulberry dwarf (Jiang et al. 2004) and in corn kernels infected by '*Candidatus* Phytoplasma asteris' (aster yellows: AY) (Nipah et al. 2007). AY has been detected also in *Brassica rapa* seeds (Olivier et al. 2006, 2010).

6.2 Phytoplasmas and Seed Production

The phloem is the ideal tissues for phytoplasma propagation and plant colonization. The sieve tubes consist of cells without nuclei with a reduced cytoplasm which offer low resistance to flow of assimilates, while the companion cells are very metabolically active (van Bel et al. 2002). Similar to oligopeptides, lipids and proteins, the phytoplasmas pass through sieve tubes by migration through the pores of sieve plates (Sugio and Hogenhout 2012), they were also found in the companion (Sears and Klomparens 1989) and parenchyma (Siller et al. 1987) cells, even if their dimensions are not suitable to pass through the plasmodesmata (Stadler et al. 2005). Ultrastructural changes in cytoskeleton were never observed in this type of cells in infected plants (Siller et al. 1987; Rudzinska-Langwald and Kaminska 1999): viruses, for example, can open passages, dilating plasmodesmata pores with the help of movement proteins (Oparka 2004), however in the genomes of phytoplasmas was not identified any gene encoding comparable proteins (Zambryski 2004). In general, many plant phytopathogenic bacteria have the type-III secretion system that injects bacterial proteins (effectors) into the host cytoplasm (Cornelis and van Gijsegem 2000; Büttner and Bonas 2003); some type-III effectors are virulence

factors that suppress plant defence responses (Jackson et al. 1999; Abramovitch et al. 2003; Hauck et al. 2003). However up today there are no descriptions of effectors able to modify the cytoskeleton in bacteria, except for *Xanthomonas campestris*, that produces a protein inducing mesophyll cell swelling (Marois et al. 2002), indicating a possible disruption to the plant microtubule cytoskeleton. *Pseudomonas syringae* pv. *tomato* DC3000 contains more than 36 type-III effectors whose function was not defined (Collmer et al. 2002), but the plant cytoskeleton could be a target for some of these proteins.

The production of seeds from infected plants is severely compromised by the phytoplasma presence in the mother plants both in quantity and quality, since they induce malformations, withering, reduced size and weight. A frequent symptom is the early budding of the seeds (i.e. pregermination). De La Rue et al. in 2002 conducted a 2 years' study on the effect of a little leaf disease on the production of *Stylosanthes scabra* seeds, and no significant reductions of seed was observed in plants in case of late infections. A decrease of 98.8% and 56.5% in seeed production has been observed in the plants showing symptoms respectively at 79 and 110 days after planting, indicating that it was closely linked to the precocity of the phytoplasma infection. Other observations in coconut palm suggest that, if the infection occurs at the early stages of development, the flowers became necrotic and the fruits are not able to ripe. The time between pollination and maturity of the fruit is around 12 months that is also the time of the disease incubation, thus only the fruits under development before the infection are able to complete the maturation (Cordova et al. 2003). Data on the germination percentages are variable and are depending on the species and the precocity of the infection. For instance, apricot seeds infected by ESFY showed a vitality 4.5 times lower (21.6%) and a germination 7 times less (9.4%) of the healthy control (Nečas et al. 2008); on the contrary, in coconuts, higher germination values (72.1%) were reported for seeds from diseased plants compared to healthy plants (57.6%) (Nipah et al. 2007) indicating that these differences could be linked to the diverse interaction of the different phytoplasmas involved in the two diseases.

Olivier and Galka in 2008, demonstrated the phytoplasma presence in seeds from plants of *Brassica napus* infected by aster yellows. The malformations observed in the mother plants were an increase in the number of trichomes, the lack of a point of growth, the enlargement of the stem, the leaf wilting and a generalized delay in growth. Malformed seeds, from symptomatic and asymptomatic plants were positive to phytoplasma presence respectively in the 25–80% and in 9–20% of the cases, while normal seeds were positive in the 20–60% and 2–10% of the cases. Malformed seeds, both from symptomatic and asymptomatic plants, had no germination, while normal seeds reached germination values of 50–90%. In all these studies phytoplasma DNAs were detected in plants by PCR methodologies, but it was not possible to confirm their presence by any kind of microscopy observation. The criticism that only phytoplasma DNA is present in these tissues and that it cannot be

associated with a living organism able to survive the process of budding was then raised.

Phytoplasmas infect the floral structures, the fruits, the seeds and even the embryos however the majority of the seeds produced by infected plants are generally viable and capable of germinating, although the production result qualitatively and quantitatively affected. Previous studies reported contrasting data on the germination percentages, for instance, Nečas et al. (2008) demonstrated that the apricot seeds infected by ESFY showed viability and germination lower than the healthy ones. On the contrary, Nipah et al. (2007) reported in coconut higher germination values for the seeds from the diseased plants than for those from the healthy plants, indicating that the germination is variable and depending on the species, and possibly also on the precocity of the infection. Recent data on the germination percentages in several herbaceous species such as sesame, tomato, brassica and corn infected by diverse phytoplasmas or by diverse phytoplasma mixtures indicate that there is not a decrease in germination percentage in seeds deriving from phytoplasma-infected mother-plants (Satta et al. 2016; Satta 2017).

6.3 Evidence for Phytoplasma Seed Transmission

In 2002 Khan and co-authors presented the first evidence of the presence of symptomatic and phytoplasma positive plants sowing *in vitro* seeds of alfalfa from phytoplasma infected mother plants (Fig. 6.1).

A similar research was carried out in carnation (*Dianthus* spp.) (Šeruga-Music et al. 2004), in tomato (*Solanum licopersicum* L.) and in *Citrus aurantifolia* (Christm.) Swingle (Botti and Bertaccini 2006) where a low percentage of plants

Fig. 6.1 *In vitro* germination of alfalfa seeds and plantlets produced (on the right) showing symptoms of witches' broom and stunting associated to the phytoplasma presence (From Khan et al. 2002)

derived from phytoplasma infected mother-plants resulted positive to phytoplasma presence (Fig. 6.2).

The identification of phytoplasmas in pea plants (*Pisum sativum* L.) germinated and grown in a protected environment and produced from seed derived from "stolbur" infected plants was also reported (Zwolińska et al. 2010). One hundred plants of *Sesamum indicum* L. (sesame) and the same number of *Cicer aretinum* L. (chickpea) plants obtained from seeds of plants infected by phytoplasmas belonging to the ribosomal group 16SrII-D were kept under observation in an insect-proof greenhouse until maturity without detecting any symptom referable to the presence of phytoplasmas (Akhtar et al. 2009a, 2009b), however they were not tested to verify the real absence of these prokaryotes. It was also not possible to identify phytoplasmas by nested PCR techniques in the plants of *C. aurantifolia* originated from the seeds of lime plants infected by witches' broom and cultivated for 2 years in an insect-proof greenhouse. The DNA of the '*Ca*. P. aurantifolia' was detected in the seed teguments, but not in the embryos and the analysis, carried out every 3 months, were negative for leaves, stems and roots. No symptoms were observed in individuals plants generated from seeds of symptomatic plants. However, the germination percentage of the seeds derived from symptomatic plants was higher when compared with the percentages of seeds resulting from healthy plants (Faghihi et al. 2011).

In the stems of young plants of *Brassica napus* L. deriving from the mother plants infected by aster yellows it was possible to identify DNA of phytoplasmas belonging to the 16SrI-B group, but it was surprising that, after the fourth leaf stage, the PCR results become negative (Olivier and Galka 2008). Lebsky et al. (2010) observed by scanning electron microscopy (SEM), but not supported by molecular

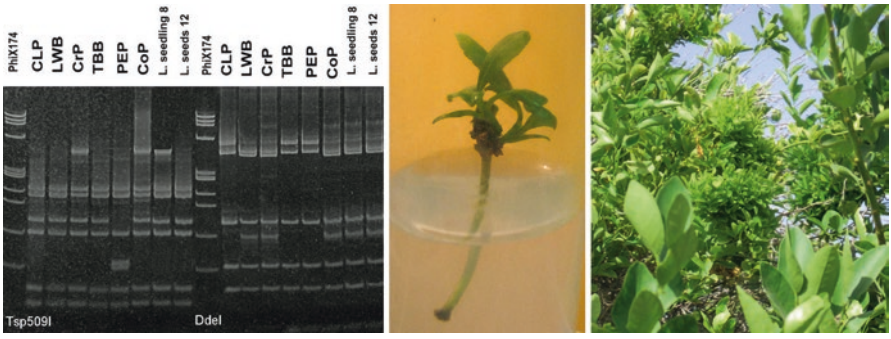

Fig. 6.2 From left: polyacrylamide gel showing results of RFLP analyses on R16F2n/R2 (16S ribosoaml gene) amplicons form seeds and *in vitro* germinated seedlings (centre) collected from lime tree infected by witches' broom disease (right) associated with the presence of '*Candidatus* Phytoplasma aurantifolia' (16SrII-B). Acronyms for the samples in the left: CLP, cleome phyllody (16SrII-A); LWB, lime witches' broom (16SrII-B), CrP, crotalaria phyllody (16SrII-C); TBB, tomato big bud (16SrII-D); PEP, *Pichris echoides* phyllody (16SrII-E); CoP, cotton phyllody (16SrII-F). PhiX174, marker DNA digested with *Hae*III, length from top to bottom of the fragments in bp: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72

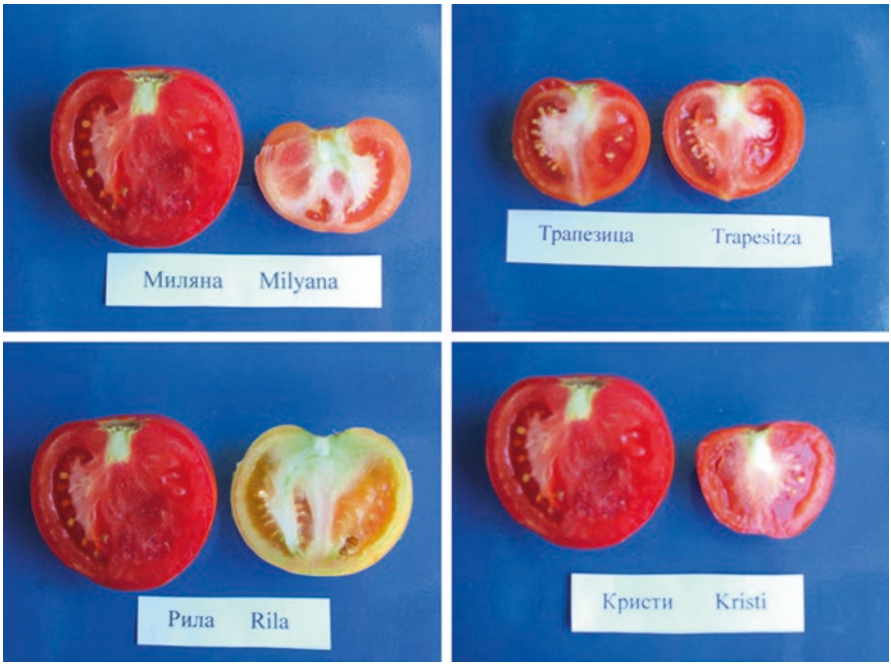

Fig. 6.3 In each picture: healthy (left) and "stolbur" infected (right) tomatoes (From Calari et al. 2011)

analysis, the presence of structures related to phytoplasmas in different phloem tissues and organs, including flowers, mature seeds and germinated seeds within the fruit in papaya plants (*Carica papaya* L.) showing symptoms referable to phytoplasmas.

The seed transmission of phytoplasmas was evaluated in winter oilseed rape, tomato and corn from different geographical origin, but all collected from infected or symptomatic mother plants. Almost 1,000 seeds were germinated under controlled conditions and 652 seedlings were obtained. Samples were grouped in 214 batches and 74 of them resulted positive to phytoplasma presence after three to 90 days from germination by nested PCR assays on 16S ribosomal gene. All the tested species resulted carrying phytoplasmas belonging to the ribosomal groups retrieved in the infected mother plants, in particular in tomato 23 batches from six variety were tested (Fig. 6.3) and only Rila and Milyana resulted negative to phytoplasmas presence, two batches were positive to 16SrXII-A ("stolbur") and 5 to 16SrI-B (aster yellows) phytoplasmas (Calari et al. 2011). In another experiment 250 seeds deriving from infected plants of the same field (Fig. 6.4) were sown in Murashige and Skoog (1962) (MS) medium. The total germination was 78.4% with 196 germinated seeds and all were tested by PCR/RFLP analysis grouped in 28 batches (7 seedlings/batch) at 30 days after germination: 15 of these samples resulted positive for phytoplasma presence. Moreover, the seedlings positive to phytoplasmas at 150 days after the germination were tested again and positive plants,

Fig. 6.4 Tomato crop infected by "stolbur" phytoplasmas in Southern Italy: the symptomatic plants only produces very few fruits but the seeds are able to germinate

even if with a lower number respect the first testing, were again detected (Satta 2017).

Phytoplasma seed transmission was recently investigated also to verify both phytoplasma presence by molecular analysis and phytoplasma viability by culturing (Satta et al. 2016, 2017; Satta 2017) in some selected horticultural crops.

Sesame (*Sesamum indicum* L.). Phytoplasma associated with sesame induce phyllody, witches' broom, virescence, yellowing, floral sterility (Akhtar et al. 2008). Sesame phytoplasmas are transmitted by leafhopper (Vasudeva and Sahambi 1955) and the disease causes significant economic losses mainly because it affects the seeds production (Ikten et al. 2014). So far diverse phytoplasmas were detected mainly according with geographical distribution: 16SrI-B in Myanmar, 16SrII-A in Thailand and Taiwan, 16SrII-D in Oman and India, 16SrIX-C in Iran, and 16SrVI-A and 16SrIX in Turkey (Catal et al. 2013). Seeds deriving from infected mother plants provided by B. Uzun, Turkey were sown under greenhouse in insect proof conditions to verify phytoplasma presence in seedling. Seeds from 5 plant genotypes were sown in sterile soil. The total germination was 95% with 190 germinated seeds. In total 190 samples grouped in batches of 10 seedlings per sample were tested by PCR/RFLP analysis at 30 days after germination. Fifteen batches out of the 19 tested resulted positive for phytoplasmas belonging to ribosomal groups 16SrI, 16SrII and 16SrXII-A. Twenty plants were also individually tested at 80 days from germination: 19 resulted positive for phytoplasmas that resulted of the same groups in some cases in mixed infection (Satta 2017).

Brassica napus phytoplasma infected plants show stunted growth, leaf reddening, virescence and floral malformation. Only a few flowers are able to complete the maturation; a limited number of seeds that are small, shrivelled and malformed is

produced. In different parts of the world these symptoms are associated with phytoplasma presence, in particular phytoplasmas belonging to ribosomal subgroup 16SrI-B ('*Ca.* P. asteris') were identified only in symptomatic samples (Bertaccini et al. 1998; Olivier and Galka 2008; Maliogka et al. 2009; Olivier et al. 2010). After sterilization, 133 seeds deriving from three plants were sown in sterile soil. The total germination was 97.7%. The seedlings were grouped in batches of 10 and tested at 30 days after germination by PCR/RFLP analysis: 7 resulted positive for phytoplasmas of 16SrI and 16SrXII-A ribosomal groups. Twenty seedlings were also singly tested at 80 days from germination and 7 resulted positive for phytoplasmas belonging to ribosomal groups 16SrI, 16SrVI and 16SrXII-A (Satta 2017).

Triticum aestivum L. is a very important crop for human food and phytoplasma diseases have been reported in different cultivation areas in the world. The wheat blue dwarf disease (WBD) was firstly reported in 1960 in China where a 16SrI-C phytoplasma transmitted by *Psammotettix striatus* was identified (Wu et al. 2010). Symptomatic plants show stunted growth, culm's proliferation, rolled and chlorotic internal leaves, basal leaves with typical blue-green color and roots necrosis. These symptoms were also reported in the United States of America in *T. aestivum* and *Hordeum vulgare* (Hollingsworth et al. 2008). In Lithuania 16SrI-B phytoplasmas were associated with a barley deformation and a triticosecale stunt (Urbanavičienė et al. 2004). Wheat seeds are often subject to the pregermination phenomenon that is known for a long time especially in areas with abundant precipitation and high temperatures and humidity during seeds maturation period (Nielsen et al. 1984). The pregermination resistance of varieties depends from a number of genes that influence the assimilation, the seeds drying time and their dormancy. A recent study tried to verify the possible involvement of phytoplasmas in wheat pregermination: the seedlings obtained from 18 samples belonging to 6 varieties were tested, after removing the seed residues, for phytoplasma presence by PCR/RFLP analysis and the 17% of them resulted positive for phytoplasmas that were identified as belonging to ribosomal groups 16SrI-B (A. Bertaccini et al. unpublished). Further research should be carried out to verify the environmental conditions favouring the phytoplasma transmission in these crops and the possible link of the pregermination phenomenon to the phytoplasma presence.

Petunia hybrida L. Commercial seeds of spreading type petunia cultivar Shock Wave Denim, Easy Wave Red, Wave Purple Classic were tested in Korea to verify seed transmission after sowing them in sterilized compost and grown in insect proof glasshouse. About 1 month after sowing the seedlings tested allowed the "stolbur" (16SrXII-A) phytoplasma detection in the two cultivar Shock Wave Denim and Easy Wave Red with percentages of 15.4% and 25% respectively. High sequence similarities between the "stolbur" (16SrXII-A) strains originated from seedlings and those from farm grown plants lead to the conclusion that phytoplasmas detected in commercial farms (Chung et al. 2013) might be transmitted through seeds. This conclusion explained why such a large proportion of the petunia plants collected from commercial farms were infected with phytoplasmas, although most of them were symptomless. Petunia plants infected with "stolbur" phytoplasma should not

be used as parent in breeding program, however the verification if both pollen and mother plant are involved in the phytoplasma seed transmission should be also evaluated (Chung and Jeong 2014).

Celosia argentea L. The detection of '*Ca*. P. asteris' phytoplasma was reported in seeds of *Celosia argentea* by nested PCR assays; however, no evidence of phytoplasma presence was detected in the seedlings raised from these seeds (Madhupriya et al. 2017).

6.4 Transmission Trough Second Generation Seedlings in Tomato

Phytoplasma infections in tomato have been described in different parts of the world. The symptoms associated with the presence of these bacteria in tomato generally consists of stunted growth, leaf yellowing or reddening, proliferation of lateral buds and emission of adventitious roots, virescence, phyllody, malformations and abortion of the floral organs (Fig. 6.5). The plant also produces a few fruits, of small size and only on older branches in case of late infections. The tomato fruits ripen early and have inappropriate texture and flavour.

The phytoplasmas most frequently identified in tomato belong to the ribosomal subgroups 16SrXII-A ("stolbur") and 16SrI-B (aster yellows). The tomato big bud disease has been described for the first time in the United States of America (Dana 1940) then in Israel (Zimmermann-Gries and Klein 1978) and India (Varma 1979) and the phytoplasma associated were enclosed in the ribosomal groups 16SrI, 16SrII and 16SrVI. The disease was also reported in China, Saudi Arabia (Xu et al. 2013), Iran, Turkey (Özdemir et al. 2009), and Australia (Samuel et al. 1933). In

Fig. 6.5 Symptoms of early "stolbur" phytoplasma infection consisting in malformation and virescence in tomato flowers (Courtesy of B. Duduk)

Greece, Poland and Italy phytoplasmas belonging to groups 16SrI, 16SrIII, 16SrV and 16SrXIII (Alivizatos 1989, 1993; Del Serrone et al. 2001; Anfoka et al. 2003; Vibio et al. 1996) were identified. In Italy, the disease is mainly localized in the centre and in the south regions (Martelli et al. 1969; Giuliani et al. 2010; Albanese et al. 1998; Marcone and Ragozzino 1995; Lisa et al. 1983; Minucci and Boccardo 1997), but some cases it was also reported in northern regions (Marzachì et al. 2000; Favali et al. 2000; Terlizzi et al. 2010).

Tomato seeds from symptomatic plants were collected and assayed for the presence of phytoplasmas. After sterilization, 68 seeds deriving from a pool of plants of the same field were sown in MS agar medium. The total germination was 64.7% with 44 germinated seeds and all were tested by PCR/RFLP analysis at 30 days after germination. Among the nine positive samples 8 resulted positive for 16SrI phytoplasmas while one was positive for 16SrXII-A phytoplasmas. One sample resulted 16SrI positive also at 150 days after germination. The seeds produced from *S. lycopersicum* plants positive to phytoplasmas were employed for further testing and 85 seeds from 5 plants were sown in MS agar medium and transplanted in sterile soil producing 58 plants of second generation (germination percentage 68.2%). Seven among these seedlings resulted positive for 16SrI and 16SrXII phytoplasmas. Phytoplasma presence was therefore detected until the second generation of seedlings indirectly proving the pathogens transmission (Satta 2017).

6.5 Coconut Lethal Yellowing Infected Plumules

Oropeza and co-workers in 2017 demonstrated the phytoplasma 16SrIV transmission by seed in coconut from embryos to plantlets. Lethal yellowing (LY) associated with 16SrIV phytoplasmas is a disease that has devastated coconut plantations in the Americas (Oropeza et al. 2011; Vazquez-Euan et al. 2011; Myrie et al. 2012, 2014; Ntushelo et al. 2013). Plumules from the embryos of LY-diseased coconut palms were cultured *in vitro* to determine the presence of phytoplasmas in the plantlets. The results showed detection of these prokaryotes in 20 samples out of the 185 embryos tested (11%) in nested PCR and in 59 samples (32%) with quantitative PCR (qPCR). The plumules of their respective embryos and the haustorial tissues were also tested and among 124 embryos no positive nested PCR results were obtained; however 42 haustorial tissue samples (32%) were positive in qPCR assays. The 124 plumules isolated from the embryos were grown under *in vitro* conditions; some of them were followed for shoot formation, while others were followed to the plantlet stage. After 3 months, 33 cultures (50%) within the first group were analysed for LY phytoplasma DNA presence by qPCR assay and 14 (42%) tested positive. After 18 months, 20 plantlets (34%) became necrotic. The remaining plantlets were analysed for the detection of the LY phytoplasma DNA, and 15 and 11 (39% and 29%) of the samples tested positive with qPCR and nested PCR assays, respectively.

6.6 Seed Transmission in *Zea mays* and Phytoplasma Cultivation

The most well-known and widespread diseases due to *Mollicutes* in maize plants are the bushy stunt (MBS) and the corn stunt (CS), both present in the American continent. MBS is associated with a phytoplasma belonging to the ribosomal group 16SrI-B (aster yellows) and is characterized by symptoms of marginal chlorosis of young leaves, gradual reddening of the base and proliferation of axillary buds, which are also subject to chlorosis and redness. In the case of early infection, the plant has very short internodes and numerous small cobs with a limited number of seeds (Ebbert et al. 2001; Harrison et al. 1996; Lee et al. 2004). CS is a disease caused by *Spiroplasma kunkelii* and characterized by symptoms similar to that of MBS (Chen and Liao 1975; Williamson and Whitcomb 1975). Both pathogens are transmitted by leafhoppers belonging to the genus *Dalbulus*. In Europe, a disease named redness was reported since 1957 in Serbia (Marić and Savić 1965), it became epidemic in the early 1960s and in the late 1990s (Šutić et al. 2004). During these epidemic stages, symptoms can be present in more than 90% of the plants and these can cause crop losses of more than 50% (Blaženčić 1982; Starović et al. 2004). The dimensions of the symptomatic plants are similar to those of asymptomatic, but the kernels are dry, and their weight is severely reduced, in particular, the few seeds produced are malformed and ripen earlier (Fig. 6.6).

Duduk and Bertaccini in 2006 detected phytoplasmas belonging to ribosomal subgroup 16SrXII-A ("stolbur") only in symptomatic samples and suggested their association with the redness disease. In most cases the corn infected fields have the most severely infected plants along the edges suggesting that the infection could come, through insect vectors, from the wild or cultivated plants nearby.

Fig. 6.6 Left : corn leaf with reddening symptoms in Serbia; right: corn cobs of which the three on the left show reduced and dry kernels associated to "stolbur" phytoplasma presence, while the one on the right is from an asymptomatic corn plant (Courtesy of B. Duduk)

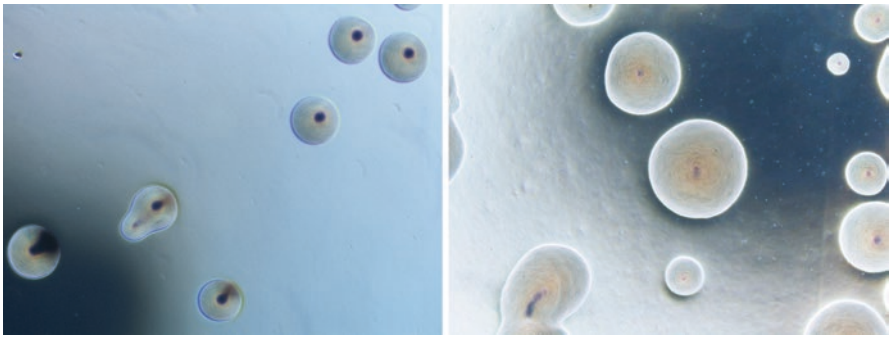

Fig. 6.7 Phytoplasma colonies from corn seedlings derived from corn cobs infected with the reddening disease

Corn cobs were collected from some symptomatic plants in Serbia and tested for phytoplasma presence (Fig. 6.6). The seeds were sown in soil in greenhouse under insect-proof cages. After 40 and 90 days from the germination (83%) 79 plantlets were tested by PCR/RFLP analysis and 17 resulted positive for 16SrI and 16SrXII-A phytoplasmas. Among these plants, 6 were still positive at 90 days after germination and two produced seeds allowing the testing of the second generation seedlings that resulted however negative for phytoplasma presence (Satta 2017).

The corn seedlings resulted positive by molecular analyses were also tested for phytoplasma viability with isolation and growth in artificial media (Contaldo et al. 2012, 2016). Seventeen positive samples at 40 days from sowing and 6 positive samples at 90 days after germination were obtained and used for isolation trials in CB liquid medium. After purification steps, 52 tubes from the 40-day-old samples and 67 tubes from the 90-day-old samples showed the expected colour change due to pH acidification in the medium. After DNA extraction and PCR amplification three tubes resulted positive to phytoplasma DNA presence. Moreover from seedlings isolated at 90 days after germination, it was possible to obtain colonies of different sizes and shapes (Fig. 6.7) resulting positive to phytoplasma presence. The viability of phytoplasmas isolated from corn seedlings grown in insect-proof environment and obtained from phytoplasma-infected mother plants confirms the phytoplasma seed transmission in corn (Satta et al. 2016).

6.7 Conclusions

Generally, phytoplasmas spreading is mainly operated by propagation/movement of infected plant materials and insect vectors. However, the sudden epidemic events associated with the presence of phytoplasmas molecularly indistinguishable, in very distant geographical areas, on the same herbaceous crops, strongly suggest the transmission of these prokaryotes also by seeds. In all the analysed species, the

phytoplasma detected in seedlings belong to different ribosomal groups, in some cases they also resulted in mixed infection, however the phytoplasma detected in seedlings were in general also detected in the mother plant of the same species.

The spread by seeds of phytoplasma associated diseases is of relevant importance mostly when the infected crops (such as corn in some areas of the world) undergoes two consecutive sowing in the same fields. In these cases seeds deriving from the first cycle that can be affected by phytoplasmas remain as disease source allowing the phytoplasmas spread through the vectors to the second sowing cycle plants. However several issues still deserve further studies and experimental demonstration such as obtaining progeny in which the phytoplasma is maintained over the time and induce symptoms, the ability of seed phytoplasmas to transmit the disease to healthy plants by grafting or insect vectors. Furthermore, since phytoplasmas are not yet contemplate in the propagation material sanitary issues neither by the plant protection quarantine protocols nor by seed producers, the movement of seeds from infected plants imply the geographic dissemination of these pathogens and therefore of the associated diseases to still uncontaminated areas.

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Chapter 7
Transmission of Phytoplasmas by Agronomic Practices

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Abstract The propagation materials such as rootstocks, cuttings and other types of grafting materials used as scions play a relevant role in the dissemination of phytoplasma-associated diseases. In particular in the woody plants the propagation material sanitary status plays an important role for both long-distance transmission and disease introduction in the new areas. Since the phytoplasma infection is systemic in the plants, the vegetative propagation of many horticultural crops allows their spread through cuttings, bud wood, tubers, runners and bulbs. It is, therefore, an efficient method of phytoplasma spreading and establishing infection in new plants. Although the phytoplasma spread through vegetative plant propagation occur over short distances by the use of infected propagation materials such as tubers, the worldwide movement of phytoplasmas should be mainly attributed to the man distributing infected propagation materials. The possibility for the phytoplasma vegetative propagation is present in all the shoots and roots comprizing basal shoots, stems, rhizomes, tubers, stolons, corms, buds and bulbs. Some crops like potato, sweet potato, cassava, carrot, onion, garlic, ginger, sugarcane, banana, pineapple, strawberry and many ornamentals like carnations and *Chrysanthemum* are only vegetatively propagated and hence they have the maximum chances of phytoplasma spread. The fruit tree propagation is usually achieved by grafting or budding of the selected variety onto a suitable rootstock, and this is the main propagation method for the stone and pome fruit trees, grapevine and other fruit trees and shrubs. Also the shoot micropropagation together with grafting, cutting, and other systems to propagate plant germplasm that avoid sexual reproduction is an efficient manner to maintain and transmit the phytoplasma diseases. The importance of phytoplasma infection spread by the vegetatively propagated plants is discussed in this chapter.

Keywords '*Candidatus* Phytoplasma' · Transmission · Vegetative propagation · *In vitro* propagation

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_7

7.1 Grafting

Grafting is a vegetative, asexual plant propagation technique known and applied since very long time. This practice was first applied 4,000 years ago in the ancient China and Mesopotamia. A number of diverse grafting methods, linked to the characteristics of the scions and the rootstocks, can be employed to verify the presence of phytoplasma infection on woody or herbaceous plants. Many plants carry viruses and phytoplasmas without visible symptoms, and the presence of these pathogens in the plants can be verified by grafting the suspicious scions onto another highly susceptible plant that will then display specific symptoms. There are several grafting methods (e.g. bark or side grafting, whip, chip budding and budding) differing in the procedure technical details including the types of scion and rootstock. Sometimes one method is selected for some particular species or scion-rootstock combination. Nevertheless, regardless of the method used, the principle involved remains the same; the vascular tissues of rootstock and scion have to be joined in a way they can continue to live and grow together as one grafted plant.

Woody Plants The understanding of a few basic points about the plant anatomy is important for the successful grafting. In the woody plants, the vascular (main or wood) cambium is a thin cell layer located between the bark and the wood. In the spring, by peeling off the bark from a twig or a tree trunk, the cambium is exposed as the slippery layer that separates it from the wood. This is the layer of actively growing cells of the tree that should receive the graft (rootstock) and must be put in contact with the same cell layer of the scion that is the plant part to be grafted. Grafting seals (tapes, rubber budding strips, waxes) should be used to prevent the drying out of the graft union since the cambium layer and the resulting callus growth are easily drying. However, the transmission of pathogens do not require a graft union that is compatible or physiologically functional. It was demonstrated that even short-term mechanical contact is sufficient to disseminate viruses and walled bacteria (Barbosa et al. 2005; Bausher 2013). A successful graft, at least for a few hours is, however, necessary for phytoplasma transmission since it is not possible to transmit them by sap mechanical inoculation (Lee et al. 2000). There is a relevant risk when using infected scions since they are, in several cases, collected at the plant dormant stage when the symptoms are difficult or impossible to observe. *Prunus cerasifera* (myrabolan) and the cultivar of *P. domestica* (European plum) are very tolerant to the European stone fruit yellows (ESFY) and are very often asymptomatic, when infected. Therefore, they are a very important a source of inoculum as both scions or rootstocks for the susceptible stone fruit species (Kison and Seemüller 2001; Paltrinieri et al. 2004). The apple proliferation phytoplasma (AP) was reported as not transmitted by scions in central European climate, for the low phytoplasma concentration during winter in the branches of infected trees (Seemüller et al. 1984). The '*Candidatus* Phytoplasma pyri', agent of the pear decline, was detected in the tree aerial parts for longer periods in the Mediterranean areas, than in the central Europe orchards and, although it was shown that it is not transmissible at winter time in central Europe, there are confirmations of its transmission by infected

scions in the Mediterranean areas (Schaper and Seemüller 1982; Garcia-Chapa et al. 2003; Yavuz et al. 2011). This variability of phytoplasma persistence according to the climatic conditions and to the plants species should be taken into consideration when the material is collected for the vegetative multiplication of plants.

Using phytoplasma-infected planting material, regardless of the source of infection being either from rootstock or scion, the symptom manifestation may be delayed a few months until several years, according to the plant species and the variety or cultivar. Because of the latent period, apparently healthy grafted plants could carry phytoplasmas and this is also a problem when trying to find out the source of infection. Although the phytoplasma detection molecular techniques are very sensitive, there are still difficulties due to seasonal variation of pathogen concentration, uneven distribution and low concentrations of phytoplasma cells in the woody plants particularly in young, grafted materials (Seemüller et al. 1984; Garcia-Chapa et al. 2003). After the grafting from the mother plants surviving from a severe phytoplasma outbreak the apricot and Japanese plum varieties obtained were used to verify the percentage of the phytoplasma graft transmission efficacy under experimental conditions. Furthermore three-year-old apricot plants were patch grafted with apricot tissues infected by ESFY and kept in a screen-house. Between 1 and 6 months after grafting, they were analyzed by PCR/RFLP analyses to verify the phytoplasma presence. The phytoplasma transmission by patch grafting was demonstrated in five out of ten apricot varieties, and in all of the Japanese plum plants tested. Symptomatology in some of the apricot varieties were represented by little leaves growing at the bottom of those fully developed and the young branches dies-off. However, just in one Japanese plum variety the witches' broom symptoms were observed (Pastore et al. 2001).

The phytoplasma persistence in the crown of the woody plants is linked to the seasonal conditions of the phloem (Braun and Sinclair 1976). In the majority of the cases, the stem scions grafting and budding allows the disease transmission in summer or autumn (Schneider 1970; Schaper and Seemüller 1982), and not in winter (Blodgett et al. 1962; Shalla et al. 1964; Seidl and Komarkova 1973) due to sieve tubes degeneration in the latter season. Conversely, the root grafting transmission in the winter could allow the transmission of the disease (Seidl 1965; Kunze 1972). Considering the disease graft transmission results, it was accepted that phytoplasma overwintering in the roots is possible. Nevertheless, it was shown that the phytoplasma was present and transmissible from aerial parts of the European stone fruit yellows infected *Prunus* in the winter (Seemüller et al. 1998). In Germany, the pear decline (PD) phytoplasma detection throughout the year has been observed by optical microscopy with fluorochrome 4,6-diamidino-2-phenyl-indole (DAPI) (Seemüller et al. 1984): the phytoplasma concentration peak was detected in summer period, the titer resulted constant in autumn but decreased in the winter. Pear decline is efficiently transmitted by grafting tissue from a suitable host such as *Pyrus communis* to a recipient tree. Usually, better results are obtained by scion grafting than by budding or chipping. Root tissue is often a better inoculum than the stem tissue, in particular when the phytoplasma titer in the roots is higher than in the stem, and also when the donor tree is asymptomatic. Also, unlike stems, roots are

not subjected to seasonal fluctuation of the phytoplasma colonization. Grafting tissue from a low-titer host such as quince usually results in low transmission rates (Poggi Pollini et al. 1995; Seemüller et al. 1986). To determine pear decline transmission rates by grafting using dormant buds from naturally infected plants, a study has been performed to verify influence of sieve tubes degeneration during the winter in the aerial part of the pear trees. A quantitative PCR assay for pear decline phytoplasma detection was carried out using 92 seedlings of *P. communis* testing them for one year after they were grafted using dormant buds. Buds were taken from pear decline-positive cultivars Abate Fetel, Conference and Williams. No phytoplasma presence was detected in any of the 92 grafted seedlings, while, in a parallel experiment with only ten seedlings root grafted with the above mentioned cultivars as a source material, six out of ten plants were positive to the molecular testing. The results suggested that the agent of pear decline was not transmissible with dormant buds to healthy trees (Babini et al. 2008).

The crown colonization of '*Ca*. P. mali' of apple showed differences according to season. The higher phytoplasma titer was recorded from the summer to the winter ranging from a very low numbers of phytoplasma cells to absence during spring. The evaluation of the transmission efficiency of phytoplasmas by grafting in various periods of the year is therefore of great importance. Pedrazzoli et al. (2008) studied the most suitable period for graft transmission of '*Ca*. P. mali'in Italy from March to August 2003. Batches of 25 apple plants cultivar Golden Delicious were grafted each month by double chip budding using scions randomly collected from two apple proliferation phytoplasma infected trees of the same cultivar. This grafting was carried out in 5 repetitions per branch for a total of 350 plants. A symptom inspection followed by ELISA testing were done in November 2003 and 2004. Obtained data indicated that the pathogen take time to become distributed within the plant. The infected plant percentage increased during the second year of the experiment. The success of disease transmission differed according to the period of the scion harvesting. No apple proliferation transmission or low transmission rates were recorded in March and May, while in June and August, the transmission rates were epidemiologically relevant. These results confirm the distribution of this phytoplasma according to the season as established by DAPI staining followed by epifluorescence microscopy observations. The suitable period for the apple scions collection was the spring time, when the apple proliferation transmission probability by grafting was the lowest. Yavuz et al. (2011) and Çağlayan et al. (2014), reported the transmission efficiency of '*Ca*. P. pruni' and '*Ca*. P. pyri' by grafting when wild apricot cv. Zerdali and B29 as rootstocks, which are the most common rootstocks for apricot and pear in Turkey, respectively were employed. As inoculum source, phytoplasma infected buds obtained from apricot cv. Tyrinthe and local pear cv. Deveci growing in open field were used. Fifty rootstocks form each cultivar were grafted by infected buds for each phytoplasma in August 2009. All grafted plants were kept in a screen-house in open field and besides symptom observations they were tested every 3 months by nested PCR assays. During symptom observations dwarfing and weaker shoot growth was recorded in inoculated plants as compared to the healthy ones (Fig. 7.1). The first positive results of PCR analysis for both

Fig. 7.1 Left: one-year-old Deveci (Turkish cultivar) pear trees infected by '*Ca.* P. pyri' on *Pyrus communis* rootstocks 1 year after inoculation kept in a screenhouse (on left), non-inoculated plant (on the right). Right: inoculated trees with typical symptoms of decline showed 2 years after inoculation and healthy pear tree cv. Deveci in the middle

phytoplasmas were obtained approximately 1 year after inoculation; the transmission rates by grafting for '*Ca.* P. pyri' and '*Ca*. P. pruni' were 6% and 18%, respectively. All inoculated plants died two years after inoculation. These data confirm the high percentage of phytoplasma transmission when the grafting was performed in the late summer when the phytoplasma concentration is high (Gazel et al. 2012). The correct choice of cultivar/rootstock combination can also affect the success of the graft transmissibility (Landi et al. 2010). Different pear cultivars and rootstock–scion combinations to pear decline phytoplasma were compared for their growth responses in experiments carried out under a screen house in which each measurement was expressed as a proportion of the total average value of the variable observed in the not inoculated control plants (K. Çağlayan, unpublished data; Gazel et al. 2012). The Turkish local cultivar Deveci resulted the most susceptible as compared to the other local cultivars Ankara and to well known pear cultivars, Williams and Santa Maria, on Quince rootstock (BA29) than on *P. communis* seedlings. Most of the plants belonging to the Deveci x *P. communis* combinations died 1 year after inoculation. The pear decline disease was reported to impair the carbohydrate translocation from stem to roots and this is believed to be the reason of decline of trees on susceptible rootstocks (Batjer and Schneider 1960; Blodgett et al. 1962).

While the relevance of the planting material health status in case of long-distance phytoplasma transmission is well known, in some cases the influence of planting in areas with high disease pressure is under controversial discussions. In vineyards affected by "bois noir" (BN) epidemic outbreaks the planting of a low number of infected cuttings result to be a minor problem, while the presence of just a single

grapevine plant "flavescence dorée" (FD) infected implies relevant risks in the areas where the main FD vector *Scaphoideus titanus* is present (Maixner 2006). Also, the rootstocks *Vitis rupestris* and *V. riparia* resulted tolerant to both diseases, therefore they could carry latent infections. Recently, grapevine yellows symptoms were reported in the rootstocks where the FD associated phytoplasmas were identified (Borgo et al. 2009). Another study confirmed that the Kober 5BB, 420A and SO4 rootstocks can be the source of BN phytoplasmas to healthy grafted *V. vinifera* plants. In this BN study, the highest number of infections was detected in grapevines grafted on 420A and SO4 rootstocks (Ermacora et al. 2011). These results indicated both FD (Borgo et al. 2009) and BN phytoplasmas as being transmissible by various rootstocks, therefore the epidemiological relevance of propagation material should be reconsidered. These findings also imply the need for new management guidelines of nurseries against the spreading of economically important phytoplasma diseases.

Herbaceous Plants In the early twentieth century the grafting practices were introduced also in vegetable crop production. This is mainly done with *Cucurbitaceae* and *Solanaceae* species. There is a number of phytoplasmas reported as graft transmissible in different vegetable crops. Eggplant is affected by little leaf phytoplasma disease (brinjal little leaf) causing considerable economic losses (Mitra 1993). In the infected plants severe stunting, short internodes, shoot proliferation, leaf size reduction and phyllody are visible. Phylogenetic and restriction fragment length polymorphism analyses based on 16S rDNA sequences revealed that brinjal little leaf phytoplasma in India was mainly classified in the clover proliferation group (16SrVI), '*Ca.* P. trifolii'-related (Rao and Kumar 2017). The studies on graft transmission indicated that this phytoplasma could be successfully transmitted by wedge grafting from eggplant to eggplant. The symptom expression was recorded 20–25 days after grafting and included typical leaf size reduction, virescence, axillary shoot proliferation, phyllody and diminished plant development. The phytoplasma presence was tested in grafted eggplants by direct PCR using P1/P7 primers and confirmed only in the samples from symptomatic plants.

Phytoplasmas associated with chrysanthemum yellows, *Crotalaria saltiana* phyllody, strawberry green petal, coconut phyllody, sweet potato little leaf, elm yellows, plum leptonecrosis, apple proliferation and potato witches' broom maintained in periwinkle have been also successfully graft transmitted in periwinkle. Typical disease symptoms were observed after 6–8 weeks from grafting and nested PCR and *secA* gene amplification and sequencing demonstrated the transmission of the phytoplasma (Kawicha et al. 2012). Toria phyllody phytoplasma has been graft transmitted to healthy toria as well as to other brassicaceous plants. This procedure allow to induce typical phytoplasma symptoms such as phyllody, virescence, silique malformation, sterility of the flowers and little leafs in yellow and brown sarson, toria, *Eruca sativa* and *Brassica napus* plants 2 months after grafting (Azadvar et al. 2011). The healthy sugarcane or periwinkle seedling grafting using scions from diseased plantlets was also able to transmit the disease. The symptoms induced on periwinkle plants grafted using symptomatic sugarcane plantlets in 3–4 weeks were

a leaf chlorosis and the proliferation of short branches on all the 250 plants grafted, while all the 50 control periwinkle plants grafted with healthy scions remained symptomless. The identity of sugarcane white leaf phytoplasma was confirmed by using both 16S and 16S–23S rDNA primers in both graft recipient sugarcane and periwinkle plants (Wongkaew and Fletcher 2004). The top grafting was used to transmit a phytoplasma from symptomatic lily plants and an experimentally infected *Alstroemeria* plants to periwinkles (Kaminska and Korbin 1999). It induced yellowing and malformation of the leaves and stunted growth, after 4–6 weeks. A few infected plants did not produce flowers remaining always in vegetative stage. Damam (2012) reported that sesame phyllody phytoplasma can be successfully transmitted from infected to healthy sesame and produced typical phyllody symptoms within 25–35 days by side grafting. '*Ca.* P. asteris' was also successfully transmitted through wedge grafting from the periwinkle plants showing phytoplasma symptoms under natural conditions to healthy periwinkle plants and produced typical phyllody and virescence symptoms within 45 days (Kumar 2010).

The periwinkle [*Catharanthus roseus* (G) Don] is a unique plant in its ability to form distant interspecific grafts even though woody buds when used as inoculum source. The transmission of the apple proliferation phytoplasma was achieved from infected scions to periwinkle (Aldaghi et al. 2007). On the other hand, it was not shown that this grafts were resulting in a true and functional union of the two plants (Goldschmidt 2014). The grapevine phytoplasma agents of diseases have been also successfully transmitted from grapevine to periwinkle via grafting (Tanne and Orenstein 1997). The transmission of grapevine phytoplasmas by shoot tip grafting onto periwinkle was shown as efficient. The grafting of grapevine shoot tips onto periwinkle is relatively easy and symptom development and PCR analysis with universal primers confirmed the presence of phytoplasma sequences in the diseased periwinkle. Group-specific nested PCR and amplicon RFLP analysis indicated a matching between the phytoplasma detected in the donor grapevine and that in the recipient periwinkle. Phytoplasma titers in periwinkle were higher than in grapevine making their isolation, as well as the extraction of their nucleic acids, easier in the former than in the latter. The transmission to periwinkle may facilitate the study of phytoplasmas until a better source for further studies on phytoplasma becomes available.

7.2 Cuttings, Tubers and Root Bridges

Despite the fact that cuttings are used as a suitable propagation material for some fruit trees such as figs and olives, this method has a very low success rate for most of the fruit tree cultivars. In theory, phytoplasma diseases may be transmitted via cuttings taken from infected plants, but in practice, it is not one of the most common ways of phytoplasma transmission. Sugarcane white leaf, sugarcane grassy shoots and sugarcane leaf yellows cause significant economic losses to sugarcane industry in Asian countries (Rao et al. 2018; Parmessur et al. 2002). They spread rapidly to

new locations mainly by the use of infected propagation material. If diseased setts are used for planting, the germination percentage is reduced 30–60% (Dhumal 1983). Bachchav et al. (1979) have reported 40–90% losses for these diseases. There are a few reports of phytoplasma diseases transmitted by cuttings like the blueberry stunt disease (BBSD). The blueberry propagation is usually carried out from softwood or using selected twigs from vigorous disease-free mother plants. Therefore, BBSD's major means of transmission in nature is not vegetative propagation unlikely by the sharp nosed leafhopper (Maeso Tozzi et al. 1993). The phytoplasmas associated with napier grass stunt disease (NSD) in some of the African countries can spread by the farmer usual practice of sharing cuttings and root splits of napier grass as planting materials. This disease originated until the 70% of biomass loss for each infected plant. This severely affects the life of small farm holders relying on Napier grass for feeding their animals and generating most of their food and income. NSD has been reported in Kenya since 2004 (Jones et al. 2004), and later it was again found in Ethiopia (Jones et al. 2007), Uganda and Tanzania (Nielsen et al. 2007). In Kenya, Uganda and Tanzania, the phytoplasmas associated with NSD are related to '*Ca.* P. oryzae' (group 16SrXI), while the pathogen detected in Ethiopia is related to '*Ca.* P. pruni' (group 16SrIII).

Phytoplasmas are believed not to be able to survive in tubers, taking into account also the fact that infected tubers normally fail to sprout or made nonviable sprouts (Banttari et al. 1993; Slack 2001). Although this appears to be a common problem for potatoes, there is little information about phytoplasma transmission by tubers when there is a primary infections in mother tubers. This is particularly relevant for the possibility that daughter plants (secondary infections) of commercially important potato cultivars could result infected as well. Recent studies clearly showed that the potato purple top disease was transmitted in some cases at rates higher than 50% to daughter tubers, moreover these can result also in producing infected plants (Crosslin et al. 2011). Even though the tuber transmission of phytoplasmas was found in high level in that study, it is usually considered to be of little importance in field conditions. Norris (1954) found a little "carryover" of the purple-top wilt disease in a number of potato cultivars grown in Australia due to the reduced tuber emergence from the infected plants and the production of little numbers of symptomatic infected plants from infected tubers. The phytoplasma infected plants growing from those tubers showed a weak growth and clear symptoms under greenhouse conditions. If this had occurred in the seed production field, these plants would have been eliminated and only a few infected tubers would have been still present in those seed lots. Using high starch content potato varieties collected in fields in which there was an high infection pressure, a study was carried out to investigate the "stolbur" phytoplasma tuber transmission rate employing PCR-RFLP and quantitative PCR (Ember et al. 2011); among 702 tubers tested, 83.8% was positive for "stolbur" phytoplasma. In a three-year survey, this phytoplasma was detected in the 0.5% of daughter plants. These results indicate that the "stolbur" phytoplasma tuber transmission also occurred in these varieties in a low percentage. It is very likely that in the tested varieties the phytoplasma tuber transmission have a low impact at

the epidemiological level. However, in the case of other varieties, the impact of "stolbur" tuber transmission should be also taken into consideration.

In the past, many researchers discussed the root transmission as a possible way of disease transmission, but only recently the root transmission has become a scientific investigation topic. In orchards with high frequency of apple proliferation infected trees, the disease usually appeared in patches and several neighboring infected trees followed one after the other in a row which suggested the possibility of phytoplasma transmission via root bridges. The apple tree roots sometimes naturally "graft" (i.e. produce root anastomoses) which could allow the transmission of plant pathogens. '*Ca.* P. mali' transmission by root bridges was demonstrated by Ciccotti et al. (2007) who showed a 16% root bridge transmission in apple Golden Delicious seedlings. The noteable presence of this phenomenon suggests that in this case the natural apple root anastomoses had a role in the short distance disease spreading in the orchards.

7.3 Micropropagation

The maintenance of the phytoplasmas in living shoots allowed the setup of strain collections in selected or experimental plant species and this has been achieved by using micropropagation techniques (Bertaccini et al. 1992; Jarausch et al. 1996). The same methodology is also employed for elimination of phytoplasma from diseased plants as described in Chapter 9. However, Petrovic et al. (2000) used the tissue culture to allow an increase in the GY phytoplasma concentration in the tissues of infected grapevine to improve its detection; while it was shown that the FD phytoplasma is not able to efficiently infect the *in vitro* cultured grapevine shoots (Gribaudo et al. 2007). The risk of GY dissemination by propagation material is very much linked to its graft transmissibility during the vegetative propagation.

Although the *in vitro* grafting has been used to eliminate pathogens from diseased plants, a few reports indicate the *in vitro* grafting as a screening or indexing method for the pathogen identification. However, graft inoculation is used *in vivo* (Jarausch et al. 1999) and a similar methods was employed in a combination in which apple proliferation and European stone fruit yellows infected plants were grafted on healthy rootstocks using developed shoot tips. This system was used to study the phytoplasma movement in the phloem from top to bottom of the plant. As phytoplasmas are limited to the phloem tissues, attention must be provided to the graft union since transmission can only happen when sieve elements are correctly connected. In that case, graft transmission rates from 90 to 94% were obtained for apple proliferation and European stone fruit yellows phytoplasmas 3 months after grafting, respectively. The phytoplasma transmission rate was very efficient and the resulting values were higher than those obtained by *in vivo* inoculations. No differences have been found in healthy and phytoplasma infected plant material or using *Malus* and *Prunus* tissues. The higher transmission rate for the apple proliferation phytoplasma has been detected 1 month after grafting, while the European stone

fruit yellows transmission increased with the duration of a viable graft contact. A difference in phytoplasma concentration in the graft tip that is usually higher in the apple proliferation phytoplasma infected shoots could explaine these differences (Jarausch et al. 1994, 1996). These results demonstrated that the *in vitro* grafting pathogen inoculation is a valid tool to carry out a screening for the preliminary assessment of phytoplasma resistance in *Malus* and *Prunus* genotypes. Its advantages compared to usual procedures are that it is a rapid method, requiring little space, not restricted by the environmental conditions or by the presence of other pathogens and easy to standardize. Biological studies on interactions of fruit tree phytoplasmas with their plant host are usually carried out for field grown plants having a vegetative cycle. Due to the long duration of these experiments, attempts have been made to maintain the woody plant phytoplasmas in micropropagated shoots. By micropropagation, it was possible to maintain the phytoplasma strains transmitted to periwinkle but also in other naturally infected plant species. To this purpose the shoots should be 1–3 cm in length, and after sterilization should be grown in a Murashige and Skoog (MS) medium containing micro- and macroelements and 0.12 mg/L benzylaminopurine (BAP). The phytoplasma detection in these periwinkle shoots micropropagated for up to 20 years was shown by nested PCR of the 16S rRNA gene (Bertaccini et al. 2012). There are studies on different plants indicating the possibility to maintain the aster yellows (AY) phytoplasma in micropropagated strawflower, annual statice (*Limonium sinuatum*) and gladiolus for periods longer than 12 months. Micropropagation of annual statice has been adapted to produce the plant materials throughout the year (Harazy et al. 1985; Gabryszewska and Podwyszynska 1992) and then Gabryszewska and collaborators (2000) showed the possibility to maintain AY phytoplasma in micropropagated statice shoots. Later on, Kaminska et al. (2002) reported that in 24 months of tissue culture at constant temperature of 20°C phytoplasmas were found in symptomatic shoots of annual statice grown on media with and without plant hormones. The possibility of phytoplasma detection in infected gladiolus and strawflower was lower than in the annual statice. In particular in the strawflower the phytoplasma detection was only achieved in the shoots growing on media in which the BAP was added or without hormones. In the plantlets derived from diseased or healthy plants, no symptoms or phytoplasmas were detected by electron microscopy after 24 months *in vitro*. Phytoplasma diseased micropropagated gladiolus shoots growing on diverse media and at temperatures between 4°C and 13°C were tested to verify whether phytoplasmas are sensitive to temperature (Kaminska et al. 2000). The PCR tests indicated that the phytoplasma detection rate was higher in the shoots cultured at lower temperatures. The phytoplasma concentration in shoots kept for one-three months at low temperatures was higher than the one detected in the plants kept at the standard temperature (20°C). The higher phytoplasma detectability has been observed in shoots grown on a medium supplemented with BAP and 1-naphthaleneacetic acid (NAA). Perhaps the use of culture media with different hormone combinations holds more promise for phytoplasma elimination from plants as suggested from the study of Ćurković Perica et al. (2007) where the influence of indole-3-butyric acid (IBA) in periwinkle shoots infected separately with strains EY-C (elm yellows; 16SrV-A subgroup),

HYDB (aster yellows; 16SrI-B subgroup) and SA-1 ("stolbur"; 16SrXII-A subgroup) was investigated. The plant growth, photosynthesis and remission of symptoms was supported by the IBA supplemented to the medium for all phytoplasma-infected shoots, but affected the presence of only HYDB phytoplasma in approximately half of the tested shoots. When some of HYDB phytoplasma containing periwinkle shoots were transferred to the medium containing BA after their propagation for 1 year in an IBA containing medium, the shoots remained symptomless and negative when tested for phytoplasma presence.

Experiments carried out by Jarausch et al. (1998) show that the *in vitro* culture of axillary buds is not sufficient to eliminate the European stone fruit yellows phytoplasma. Plantlets regenerated from buds of 2 mm in size did not show '*Ca.* P. prunorum' elimination after testing by PCR. The experimental plant hosts *Pyronia veitchii* and *Prunus* 'Marianna' were inoculated with micropropagated shoots of *Pyrus communis* infected by pear decline and *Malus pumila* infected with apple proliferation phytoplasmas, respectively. The transmission rate of pear decline phytoplasmas from *P. communis* to *P. veitchii* was 35%, whereas it was 75% from *P. communis* to *P. communis* (Davies and Clark 1994). Similarly, for apple proliferation phytoplasmas, the transmission rates were 52% and 90% from *Malus* to *Pyronia* and *Malus* to *Malus*, respectively. No transmission of pear decline or apple proliferation phytoplasmas to *Prunus* was achieved (Jarausch et al. 2000).

7.4 Conclusions

Phytoplasmas that systemically colonize their host tissues can spread by vegetative propagation methods such as grafting to healthy plants, cuttings, micropropagation and other methods used to produce plant material avoiding genetic crossing. Grafting branches or buds from diseased plants on healthy plants is one of the transmission ways of phytoplasmas. Natural grafting is also possible by roots and this is a transmission that mostly take place in forest, tropical environments and some fruit trees. The grafting for the plant disease is a "double-edged sword": it played an important role in spreading many devastating plant pathogens, including phytoplasmas, however it has also become a very relevant tool to overcome the most hazardous plant epidemics and pests. Phytoplasma graft transmission is of relevance in both the disease management and the phytosanitary certification. Considering that phytoplasmas are phloem-limited pathogens, a carefull attention must be provided to the graft union quality since the pathogen transfer could only be achieved when the sieve tubes are connected in the appropriate manner. The woody plant transmission of phytoplasmas is not easy due to both low titre and its seasonal changing. Some problems can be overcome by the phytoplasma transmission to periwinkle (*C. roseus*), commonly employed as experimental plant to propagate the selected strains since it harbours higher titre of phytoplasmas. The success of fruit trees grafting for phytoplasma transmission is not very efficient since it also depends on the technique used. Grafting may succeed in transmitting phytoplasmas where other methods fail,

however it is not always an efficient process. In theory, phytoplasma diseases may also be transmitted via cuttings taken from infected plants, but in practice it is not one of the most common ways of phytoplasma transmission. The phytoplasma transmission by potato tubers was described only in a few cases, nevertheless the importance of grafting, cuttings and tuber transmission for phytoplasmas must not be underestimated and should be taken in the correct consideration.

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Chapter 8
Control of Phytoplasma Diseases Through Resistant Plants

Carmine Marcone and Govind Pratap Rao

Abstract Phytoplasma diseases are difficult to control and one of the most promising approaches is through the use of resistant plants. Intra- and interspecific differences in the response of plants to various phytoplasma diseases have been observed over the last decades under both experimental and natural infection conditions. This chapter summarizes information on identification of resistant genotypes to a number of major phytoplasma diseases of temperate fruit trees, sesame, brinjal, coconut, jujube and forest trees and shrubs, for which the current knowledge is more advanced than that of other phytoplasma diseases. The resistant genotypes are suitable for disease management and should be regarded as a safe, effective and environmentally friendly control measure. Also, although some genotypes so far identified are not entirely satisfactory for agronomical purposes, they can be further exploited either in conventional breeding programs or through biotechnological approaches aimed at developing plants with suitable agronomic properties in which resistance to phytoplasma diseases is stably inserted.

Keywords '*Candidatus* Phytoplasma' species · Disease management · Stone fruits · Pome fruits · Forest trees · Sesame · Brinjal · Coconut · Jujube

8.1 Introduction

Phytoplasma diseases are difficult to control. As they are spread by insect vectors, measures against the vectors could reduce disease incidence. However, satisfactory vector control is often not possible because the vectors and their biology are unknown in many instances and the insecticide treatments cannot always be safely

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_8

applied, for example during flowering and harvesting. Also, insecticide resistance of insects may be a problem. Other preventive and phytosanitary measures including healthy plant material use and removal of symptomatic plants are not fully effective as further infections may occur. The same applies for treatments with tetracycline antibiotics, which are also not allowed in many countries. The best method to prevent phytoplasma disease is the use of plants resistant to these pathogens. Intra- and interspecific differences in the response of plants to various phytoplasma diseases have been observed under both experimental and natural infection conditions (Sinclair 2000; Sinclair et al. 2000a, 2000b; Seemüller and Harries 2010). Both resistance and tolerance have been identified in the genotypes of some plants which may be suitable for disease management. This chapter summarizes information about resistance to a number of major phytoplasma diseases of temperate fruit trees, important economic crops and forest trees and shrubs.

8.2 Temperate Fruit Trees

European Stone Fruit Yellows (ESFY) This is the common name of several economically relevant diseases of *Prunus* species in Europe described under different names according to different species such as apricot chlorotic leaf roll of apricot (*Prunus armeniaca*), leptonecrosis of Japanese plum (*P. salicina*) and decline disorders of peach (*P. persica*), European plum (*P. domestica*), almond (*P. dulcis*), ornamental cherry (*P. serrulata*) and various rootstocks used for stone fruits. It is associated with '*Candidatus* Phytoplasma prunorum' presence (Seemüller and Schneider 2004; Marcone et al. 2010, 2014). Stone fruit trees differ in their susceptibility to ESFY phytoplasma infections. Most affected are apricot, Japanese plum, peach, almond and flowering cherry. Several other *Prunus* taxa which are known to be host of '*Ca*. P. prunorum' including European plum, sour and sweet cherry (*P. avium* and *P. cerasus*) are less affected showing less pronounced symptoms under both experimental and natural infection conditions. Infected trees of these taxa never develop clear-cut symptoms and only rarely or temporarily show mild symptoms such as slight yellowing and slightly reduced vigor and terminal growth. In some instances, they are also tolerant (Marcone et al. 2011). Also, rootstocks greatly affect the response of grafted trees to ESFY phytoplasma presence. For instance, diseased apricot, Japanese plum and peach trees grown on plum rootstocks, e.g., *Prunus* Marianna GF8/1 (*P. cerasifera* × *P. munsoniana*), myrobalan (*P. cerasifera*) seedling, *P. insititia* and *P. domestica* stocks gradually decline and die over a period of 1–4 years after the appearance of the first symptoms. These rootstocks, depending on the aggressiveness of phytoplasma strains, may survive the death of the scion (Seemüller and Foster 1995; Kison and Seemüller 2001). Trees on peach and apricot rootstocks are more severely affected. They show yellowing and rolling of leaves, defoliation in summer or early fall and usually die within a few months, or one or 2 years after the disease appearance. Therefore, the choice of an appropriate rootstock may extend productivity of affected trees. '*Ca*. P. prunorum' is detectable

during the winter in the above-ground organs of affected trees, but it is present in the root apparatus all the year round (Seemüller et al. 1998a). Resistance of both rootstock and scion cultivars is necessary for a successful disease control. In a previous work the resistance of 23 rootstoks of *Prunus* widely employed in Europe was examined by graft-inoculation, PCR assays and DAPI (4′-6-diamidino-2-phenylindole) fluorescence method (Kison and Seemüller 2001). Several different '*Ca*. P. prunorum' virulent strains from various ESFY-affected apricot, almond, ornamental cherry, peach and Japanese plum trees, were tested. The inoculations were performed grafting the tissue from symptomatic plants in healthy rootstocks and in the grafted plants the shoot arising from the inoculum was grown as a scion. The symptom presence was assessed for a period of 5 to 8-years in the rootstocks and relevant differences to '*Ca*. P. prunorum' infection were observed. These were mainly represented by reddening, yellowing, rolling, and casting of leaves, phloem necrosis, off-season growth, reduced vigor, and death. The mortality in the same rootstock and scion combination could also greatly vary, according to virulence of involved strains. The scions grafted on *P. domestica* stocks Achermann's, Brompton and *P.* 2175 and *P. cerasifera* stock myrabi (P 2032) showed very little symptoms. The majority did not show dead branches nor leaf symptoms nor reduction of vigor. Also the phloem necrosis was rarely observed and, even in presence of severe strains, the scion resulted usually less affected than on other rootstocks. A little more damage was observed in varieties grafted on rootstocks GF 677 (*P. dulcis* × *P. persica*), *P. insititia* stocks St Julien A and St Julien GF 655/2, and *P*. Marianna GF 8/1. Some genotypes grafted on these rootstocks were strongly symptomatic, showing leaf symptoms, phloem necrosis and mortality. Myrobalan, peach rootstocks Higama and GF 305 and Ishtara [(*P. cerasifera* × *P. persica*) × *P. salicina*] resulted with susceptibility ranging from moderate to high levels. These rootstocks were only mildly affected by the mild strains and also by some of the severe strains, but they were strongly affected by different strongly aggressive strains. The rootstocks of peach Rutgers Red Leaf, Montclair, Rubira, peach and apricot seedlings, and *P. insititia* stock St Julien 2 were among those more susceptible to ESFY agent. Up to 100% of losses were observed in all the most susceptible peach rootstocks and in apricot. The genotypes grafted on apricot rootstocks were most severely damaged when scion was apricot and the infecting strain was from apricot, while the susceptibility of genotypes on peach rootstocks was not related to scion nor inoculum source. The presence of off-season growth usually depends on both host and pathogen strain: it occurs in the presence of the most virulent strains and of the most susceptible host plants. Among the ornamental cherry trees grafted on different rootstocks, those on F 12/1 (*P. avium*) and Gisela 3 (*P. fruticosa* × *P. avium*) resulted the less susceptible, whereas those on Weihroot 158 (*P. cerasus*), Gisela 1 (*P. cerasus* × *P. canescens*) and Gisela 5 (*P. fruticosa* × *P. cerasus*) were the most affected. The ornamental cherry scions resulted often more prone to dye than the rootstocks. DAPI test or PCR assays used to detect phytoplasma presence indicated that the colonization was very reduced or not detectable in the resistant rootstocks than in those susceptible. Differences were also observed between the root and the above-ground parts colonization of the rootstocks. In the same genotype the phytoplasma

concentration was usually higher in the roots than in the trunk. In some of the rootstocks the phytoplasmas were only present in the roots and were not detected in the trunk. A persistent colonization during the 5–8 years of observation was however observed even in the less affected rootstocks such as *P. cerasifera* stock myrabi, *P. domestica* stocks Achermann's, and Brompton and P 2175. At the end none of the examined genotypes could be evaluated as resistant since trees on these rootstocks were affected even if with light symptoms. Using a French ESFY strain Jarausch et al. (2000) verified the reaction of sixteen *P. domestica* genotypes grown on *P.* Marianna GF 8/1. The plants were monitored for 6 years and Prune d'Ente genotypes were found to be moderately to highly susceptible. The scion cultivars Lorida, Primacotes, Tardicotes and Spurdente showed severe symptoms such as leaf roll, reduced vigor and productivity, off-season growth and plant death. Several γ-ray mutants and hybrids of Reine Claude (greengage) cultivars resulted little or not affected and did not show mortality while only a few Fermareine and P9184 trees showed the off-season growth. Phytoplasma presence could be detected by PCR assays in all tested genotypes, irrespective of symptom expression. However, frequency and titer of the colonizing ESFY agents were lower in the tolerant than in the susceptible genotypes. In genotypes P1771 and P1119 of Reine Claude, phytoplasma presence was only detectable in the rootstocks. A high level of resistance to '*Ca*. P. prunorum' was detected in 13 graft-inoculated sweet cherry cultivars (Jarausch et al. 1999). Audergon et al. (1991) reported that 155 cultivars and selections of apricot grown on A1236 Manicot apricot seedling, had a considerable susceptibility difference to the strain G32 Noves of the ESFY agent, following graft inoculation. Tardif de Bordaneuil, Bebeco LA 2-A and Caid Azdz 1 were very susceptible and, thus suitable as indicators whereas other, *e.g*., Jaubert Foulon, Marculesti 23/4, Max Gold, Cot 1071 and Chastemi were tolerant. Significant differences in susceptibility of apricot cultivars to ESFY infections were also identified by Morvan (1977). The less susceptible cultivars including Hungarian Best showed a slower decline than the very susceptible cultivars as Canino under experimental inoculation conditions in the areas of Lyons (France). Studies by Osler et al. (2014, 2016) showed the occurrence of a durable tolerance to ESFY in stably recovered Bulida apricot trees. This tolerance was graft-transmissible with a very high efficiency from recovered mother plants to vegetatively propagated progeny plants in which continued to be stably maintained. The transmitted tolerance proved to be not associated with a cross-protection phenomenon in which avirulent strains seems to be able to contain the aggressive strains (Osler et al. 2014, 2016). These findings provided some preliminary evidence that induction of the observed durable tolerance is a plant-mediated process.

Pear Decline (PD) It is a relevant pear (*Pyrus communis* L.) diseases that is widespread in America and Europe associated with the presence of '*Ca.* P. pyri' (Seemüller and Schneider 2004; Seemüller et al. 2011a). The expression of symptoms largely depends on the rootstock; three syndroms can be distinguished such as quick and slow decline, and reddening of the foliage, which is often associated with leaf curl. Quick decline is prevalent in trees grafted on oriental rootstocks *P. pyrifo-*

lia and *P. ussuriensis*, which, because of their susceptibility, are no longer used in orchards. Quick decline may also occur in trees grafted on *P. communis*, but it shows a considerable variability within this species occurring mainly in some oriental and less tolerant *P. communis* rootstocks. Reddening of the foliage in late summer and fall, which is a mild form of slow decline, occurs in trees grafted on *P. betulifolia*, *P. calleryana*, and less susceptible clones and seedlings of *P. communis*. '*Ca.* P. pyri' is not detected in the branches of diseased pear trees during winter due to degeneration of sieve tubes on which phytoplasmas are located. In contrast, it overwinters at root level where intact sieve tubes are always present. The PD agent in the spring may recolonize from the roots the above-ground organs of the tree when sieve tubes new and functional are being developed in case of tolerant rootstocks. A similar colonization pattern is also known for apple (*Malus* spp.) trees affected by AP disease. Therefore, growing scion cultivars on resistant rootstocks can be used to manage both diseases (Seemüller et al. 1984a, b). An extensive screening of *P. communis* genotypes, other *Pyrus* species and quince (*Cydonia oblonga*) genotypes for PD resistance has been performed in North America (Seemüller 1992; Seemüller et al. 2011a). In this work, open-pollinated seedlings of *P. betulifolia*, *P. calleryana*, *P. nivalis*, *P. elaeagrifolia*, *P. syriaca*, *P. pashia*, *P. dimorphophylla*, and *P. communis* Kirkensaller, Bartlett, and Winter Nelis, clonal Quince A and C, own-rooted *P. communis* Old Home, Anjou, Bartlett and Winter Nelis, and several clonal OH × F rootstocks deriving from the *P. communis* Old Home × Farmingdale crosses were assessed as resistant. Seedlings of *P. caucasica*, *P. amygdaliformis*, *P. cordata*, *P. fauriei*, *P. pyrifolia*, *P. communis,* and *P. ussuriensis*, grown from imported French seeds were rated as susceptible or very susceptible. Some of the results obtained in North America in assessing PD resistance were confirmed in Europe. Work in Italy showed that own-rooted trees of *P. communis* Conference were little affected, own-rooted trees of *P. communis* William, Abate Fetel and Kaiser were moderately affected, while own-rooted Comice trees were severely affected under the same conditions (Poggi Pollini et al. 1994; Giunchedi et al. 1995). Also Conference trees grafted on Quince and *P. communis* seedling rootstocks showed mild symptoms, whereas Comice trees showed mild symptoms when grafted on quince rootstocks, but had severe symptoms when grafted on *P. communis* seedling rootstocks. William, Abate Fetel and Kaiser trees were moderately affected when grafted on *P. communis* seedling rootstocks, but were severely damaged when grafted on Quince rootstocks. These studies also showed that trees on Quince BA29, Quince CTS212 and French seedling rootstocks suffered less from PD phytoplasma infections than those on Quince A, Quince C and OH × F 333 rootstocks (Giunchedi et al. 1994, 1995). Studies by Pastore et al. (1998) showed that pear trees grown on *P. communis* seedling rootstocks in an experimental field of southern Italy were less affected than those on quince rootstocks, and that Quince C proved to be less affected than Quince A during a three-year observation period. In England own-rooted Conference trees remained entirely asymptomatic over a two-year observation period despite their extensive colonization by the PD phytoplasma whereas those grafted on Quince rootstocks showed only mild symptoms or were tolerant (Davies et al. 1992). Studies carried out in Germany have shown that trees on Quince A rootstocks were

significantly less affected by PD phytoplasma than those on *P. communis* Kirkensaller seedlings, owing the poor host properties of Quince (Seemüller et al. 1986). In Quince A rootstocks, the PD phytoplasma occurred in a very low titer which adversely affects the recolonization of the above-ground parts in spring, resulting in the lack of symptoms that, if present, are quite mild (Seemüller et al. 1986). The greater severity of PD affecting pear trees on Quince A rootstocks in Italy, as mentioned above, may be related to the fact that psyllid infestation is heavier in Italy than in Germany (Poggi Pollini et al. 2001; Seemüller and Harries 2010). Studies in Germany also revealed a considerable variation in PD resistance between and within progenies of *Pyrus* taxa (Seemüller et al. 1998b, 2009). Of the OH × F clonal rootstocks tested, only clone 87 showed satisfactory resistance, whereas the clones 267, 18 and 333 were susceptible and the 69 and 217 were very susceptible (Seemüller et al. 1998b). Similar differences were observed among seedling progenies. After graft inoculation and monitoring the disease development for over 18 years, all the progenies of 39 open-pollinated genotypes in 26 *Pyrus* taxa resulted not affected or little/moderately to severely affected (Seemüller et al. 2009). In about one third of the genotypes, most of the seedlings demonstrated a high level of resistance, as evidenced by the low values of the cumulative disease index (CDI) (less than 10), they also show above 50% of not or slightly affected trees and low rates of death plants. Trees on progenies of *P. communis* originating from Moskow, Russia, *P. calleryana* Bradford and *P. betulifolia* originating from Ibaraki, Japan, were most resistant. Considerably different was another third of the genotypes, on which the majority of the grafted trees proved to be susceptible, as confirmed by CDI results that were above 13.5, they also showed a low percentage of tree that were not or little affected and high number of dead plants. Between these two groups there were progenies which were rated as tolerant. Significant differences in resistance to PD were also reported among progenies derived from different genotypes of the same species. For instance, within the *P. communis* genotypes examined, seedlings from Moscow were the most resistant ones (CDI = 4.6; not or slightly affected trees = 88; mortality rates = low), whereas those of the French cultivar Feudière were found to be highly susceptible (CDI = 14.5; not or slightly affected trees = 31; mortality rates = high) (Seemüller et al. 2009). These findings indicate that resistance is a segregating feature and that seedling progenies are unsuitable as rootstocks. Resistant genotypes have to be carefully selected and propagated vegetatively.

Apple Proliferation (AP) It is a major infectious disease of cultivated apple (*Malus* × *domestica*) which is known to occur in Europe. It is associated with the presence of '*Ca.* P. mali' (Seemüller and Schneider 2004; Seemüller et al. 2011b). This pathogen is associated with a variety of symptoms including witches' broom, rosettes, enlarged stipules, reddening and yellowing of leaves, growth suppression, and undersized fruits. As mentioned above, due to its colonization behavior, the AP agent can be managed using resistant rootstocks. A large screening carried out on numerous established and experimental rootstocks, mainly based on *Malus* × *domes-*

tica, revealed the absence of suitable resistance (Seemüller and Harries 2010; Seemüller et al. 2011b). However in some experimental rootstock selections derived from crossing the genotypes of the non-apomictic species *M.* × *domestica* and *M. purpurea* with the apomictic *M. sieboldii* it was possible to detect resistance (Kartte and Seemüller 1991; Seemüller et al. 1992). Further long-term investigations in which open-pollinated seedlings of *M. sargentii* and *M. sieboldii* and several apomictic rootstocks selections with either *M. hupehensis*, *M. sieboldii* or *M. sargentii* in their ancestors had been checked for the presence of resistance to AP, were compared to clonal *M.* × *domestica*-based rootstocks M9, M11, M13, to the Budagovsky B, *M. robusta* and Polish P series seedlings, showed a the presence of high resistance in *M. sieboldii* progenies and in the majority of the selections having *M. sieboldii* in their ancestors. These selections included 3432, 4551, 4556, 4637, C1907, 4608, D1131, Gi477/4, H0801, D2212 and H0909 (Seemüller et al. 2008). Diseased apple trees grown on *M. sieboldii*-based AP-resistant apomictic rootstocks never showed symptoms or, only rarely, temporary mild symptoms. Such trees were not colonized in the aerial parts and harboured extremely low phytoplasma numbers in the roots. In these rootstocks, the AP phytoplasma concentration was proved to be 100 to 5,000 times lower than that occurring in susceptible *M.* × *domestica*-based rootstocks. Thus, the low starting phytoplasma population in the roots and the unsuitable host properties of *M. sieboldii*-based genotypes may impair the colonization of the aerial parts in spring starting from the roots (Kartte and Seemüller 1991; Bisognin et al. 2008; Seemüller et al. 2008). The apple trees on *M. sieboldii*-based AP-resistant apomictic rootstocks were however more vigorous and less productive than those on M9, the major rootstock for commercial apple growing in Europe. A breeding program was therefore carried out to obtain plants with reduced vigor and enhanced productivity by crossing and backcrossing *M. sieboldii* and its apomictic hybrids with M9 and other dwarfing rootstocks (Seemüller et al. 2010). Out of these crosses 23 genotypes produced a considerable number of seedlings while due to unsuitable pollen characteristics the other crosses failed to produce progenies. More than 3,000 seedlings obtained have been genetically examined using simple sequence repeat (SSR) analysis supported by flow cytometry to distinguish recombinants, *i.e.* sexually derived seedlings from apomicts in the progenies (Bisognin et al. 2008, 2009). All recombinants for a total of 1,800 seedlings were evaluated for AP resistance by graft inoculation followed by symptom evaluation in the nursery and in open field growing conditions. Several genotypes showed to have inherited good resistance such as crosses 4608 × M9 and D2212 × M9, which had a high percentage of plants that never showed symptoms or only temporarily mild symptoms accounting for CDI values of 1.1 and 1.3, respectively, compared with an average CDI of 4.1 of all progenies. Also, the AP phytoplasma concentration in the roots of trees showing no or mild symptoms was 15–30 times higher than in the roots of moderately to severely affected trees (Seemüller et al. 2010).

8.3 Other Economic Important Crops

Sesame Phyllody *Sesamum indicum* L. is one of the most relevant crop for oil production that has been domesticated since 3000 years and belongs to Pedaliaceae family; its cultivation extends from 40°N to 40°S latitude, covering the tropic and subtropic areas of Asia and Africa that is cultivated worldwide for 6.5 million hectares, and has a seed production of above three million tons. China, Myanmar, India and Sudan have the major cultivation in the world with 68% of production (Chattopadhay et al. 2015). The sesame phyllody associated with the presence of phytoplasmas is the most destructive disease of this crop reported in more than 15 countries of the world (Akhtar et al. 2009; Rao et al. 2015). The disease is also reported in wild species of *Sesamum*, *e.g*. *S. indicatum* and *S. alatýun* (Ramanujam 1944), *S. radiatum* and *S. occidentale* (Mazzani and Malaguti 1952). In the diseased plants the flowers are transformed into leaf-like structures and the entire plants is stunted resulting, in the cases with severe incidence, in a yield loss up to 100% (Abraham et al. 1977; Sarwar and Haq 2006). The most important symptoms are shoot proliferation, deformation in flowers and capsules along with leaf curling. Considering its economic importance, the research aimed to the selection of phyllody resistant genotypes are not numerous. Field-based selection and identification of resistant and moderately resistant sesame genotypes was described by Singh et al. (2007) and Akhtar et al. (2013). Inheritance of phyllody resistance was reported (Singh et al. 2007; Shindle et al. 2011), however, for understanding the genetic bases of this resistance, it is necessary to develop appropriate and improved diagnostic tools to verify the phyllody resistance since there is a certain number of symptomless infection cases in some cultivars at early stage of infection (Nejat and Vadamalai 2013; Pervaiz et al. 2013; Yankey et al. 2014). Moreover genotypes selected as disease resistant under field environments showed diverse response of disease resistance under various environmental conditions (Atkinson and Urwin 2012). Recently, Ikten et al. (2016) and Ustun et al. (2017) utilized quantitative PCR technologies to detect and quantify specific phytoplasmas infecting sesame and to identify sesame genotypes as tolerant or resistant to phyllody symptoms in Turkey. The identified potential resistant genotypes were further screened in greenhouse conditions using phytoplasma-infected insects. The sesame accessions ACS102 and ACS38 resulted disease resistant after field, and greenhouse evaluations, and qPCR testings. Quantification of the phytoplasma is important for the kinetic monitoring in order to verify the infection speed and the number of phytoplasma copies in the colonized tissues (Torres et al. 2005). In order to improve the selection efficiency for evaluating resistance in sesame genotypes also the size of the collection is relevant. Development of cultivars with durable resistance to phyllody would be the best management tool and should be a basic element of the program devised for sesame breeding. Considering that most of the sesame genotypes under cultivation are susceptible, wild sesame relatives as sources of genes linked to resistance are a promising option. Tandon and Banerjee (1968) evaluated several sesame genotypes in India and identified some cultivars with moderate resistance to phyllody. Beech

(1981) also suggested that varietal resistance against the insect vector and the disease is the best way for sesame phyllody management. The wild species *S. alatum* was identified as a potential source of resistance to phyllody (Srinivasulu and Narayanaswamy 1995; Singh et al. 2007), however, the presence of an high degree of barriers in the crossing made the transfer of this trait from wild to the cultivated varieties mostly unsuccessful (Kedharnath et al. 1959). Later, Ramalingam et al. (1992) was successfully producing an interspecific hybrid (*S. alatum* × *S. indicum*), having very low (0.04%) crossability. Singh et al. (2007) detected seven sesame genotypes and two wild species, *S. mulayanum* and *S. alatum* being resistant by visual rating under field conditions. He also discovered in *S. alatum* a dominant gene linked to the phyllody resistance. Shindle et al. (2011) conducted another study for phyllody resistance inheritance using crosses between resistant landrace and susceptible varieties. They evaluated 149 lines in F3 generation and found two dominant genes with complementary action related to the phyllody resistance. The studies about inheritance of the genetic resistance to sesame phyllody needs further evaluation. Akhtar et al. (2013) evaluated 133 sesame genotypes and found that some were tolerant to the phyllody, but none of them showed resistance. The presence of recessive resistance genes in two independent non-allelic genes exhibiting duplicate dominance in the cultivated varieties, was discovered by carrying out allelic test on intraspecific crosses; the dominance of resistance with the involvement of one dominant and one recessive gene was however obtained by interspecific crosses of wild species. These resistant genotypes can be used as sources of resistance in breeding programs for the development of elite phyllody resistant sesame lines. The most effective method to manage plant diseases in the fields is considered to be the selective breeding for detecting the resistance traits (Keller et al. 2000). To sesame phyllody effective management, it is necessary to find new sources of resistance that could be incorporated in the commercial varieties. The availability of qPCR assays for 16SrII and 16SrIX phytoplasma quantification will be an important tool to verify the visual observations for a reliable and quick evaluation of new resistant sesame varieties (Ikten et al. 2016).

Brinjal Little Leaf *Solanum melongena* L. is a vegetable crop cultivated and employed in both subtropic and tropic areas. The phytoplasma-associated brinjal little leaf disease is quite severe and induces yield losses that are over 40% (Mitra 1993; Rao et al. 2017). Almost all the varieties of eggplants under cultivation are susceptible to brinjal littler leaf (BLL) disease. The infected plants are characterized by little leaf, phyllody and severe stunting (Rao and Kumar 2017). The losses are more severe in the case of early infection, however, in the late infection, the fruits appears shriveled and malformed and in the severe epidemic the losses reach 100%. Limited information is available about the genetic sources of eggplant cultivar resistance against BLL and the selection and screening of resistant brinjal genotypes is based on intensity/severity of symptoms induced by the phytoplasma presence. Chakrabarti and Choudhury (1975) reported *Solanum gilo* and *S. integrifolium,* wild relatives of the brinjal, as sources of resistance against BLL and developed the S.212–1 genotype as a field resistant variety.

Coconut Lethal Yellowing Coconut palm (*Cocos nucifera* L.), is widely cultivated in 12 million hectares mostly in tropical areas, with the production in Asian continent that overcome the 80% (Adkins et al. 2006; FAO 2014). A recent outbreak of the lethal yellowing (LY) disease in Côte d'Ivoire destroyed over 350 ha of plantations (Arocha-Rosete et al. 2014). In the last period there was the first report of a LY disease in Oceania where is was known as "Bogia" coconut syndrome (BCS) in Papua New Guinea (Lu et al. 2016). Numerous outbreaks of LY have been recorded during the late nineteenth century in the Caribbean and Africa and were associated with the millions of palms death during the twentieth century in several continents (Mora-Aguillera 2002; Dollet et al. 2009; Harrison et al. 2014). Some coconut palm varieties and hybrids are known to be less susceptible to LY, however, no genotype of coconut was declared as resistant to LY (Baudouin et al. 2009). Genetic improvement of coconut is quite difficult and time taking also due to the relatively scarce seeds production from each palm (Cardeña et al. 2003). Considering the long time that usually pass between the infection and the symptoms expression the existing genotype screening for LY resistance remains difficult even if the PCR method application allows fast and accurate detection of infected palms. In some instances, it was speculated that genetics should not linked necessarily to resistance because the palms succumbed to LY exposed to different geographical and environmental conditions (Mpunami et al. 2002; Baudouin et al. 2009). Besides environmental conditions, it is also possible that a coconut resistant variety will reduce the level of resistance when encounter a new strain of phytoplasma and a new insect vector species. This requires further studies on taxonomical identification of LY associated phytoplasmas and their insect vectors (Mpunami et al. 2002; Baudouin et al. 2009; Odewale et al. 2012). The transmission experiments required for the evaluation of the level of resistance could be carried out by graft methods (Carraro et al. 1998), however these are not easily applicable to coconut (Wallace 2002). Testing for resistance in coconut palms is usually performed by planting different varieties under natural infection conditions (Baudouin et al. 2009; Odewale and Okoye 2013). In Papua New Guinea, the BCS disease is widespread in the area where the coconut national germplasm collection is located. This offers the testing of resistant or tolerant coconut varieties within this collection. The strategy that is among those commonly recommended is that a range of resistant varieties is planted in a given area in order to reduce the widespread plant death risk and to avoid that resistance will be eroded by adaptation to either the pathogen or the insect vector (s). A resistance breakdown against LY disease is becoming a serious problem where the resistant varieties have shown symptoms again (Broschat et al. 2002; Been and Myrie 2005; Quaicoe et al. 2009). Cultivar screening for resistance should be arranged at local level because resistance may be influenced by a degree of site specificity, genetic diverse phytoplasma populations and factors linked to the environment (Quaicoe et al. 2009; Odewale et al. 2012, 2013). The small and instable phytoplasma genomes greatly help their adaptation possibilities. To reduce this problem, the use of a resistance gene stacking strategy was suggested to prevent its quick breakdown (Gurr et al. 2016). The use of CRISPR-based tools for gene editing allows progress in verifying and exploiting the genetic bases of the resistance to phytoplasma diseases (Belhaj et al. 2013; Baker 2014). This new technology is attractive and

should be applicable in context of LY. Finally, the large scale production of seedlings is difficult and slow, also when this resistance is identified. This could be alleviated by the improvements of the tissue culture methods (Nguyen et al. 2015). A more general problem with the use of resistant plants to prevent LY could also be the lack of resistance for other serious biotic threats which might geographically constrain their value. In Jamaica, because of severe losses due to LY, the original Jamaican tall coconut cultivar was replaced to a wide extent with the Malayan dwarf and MayPan and varieties, that played a major role in the recuperation and survival of the industry linked to coconut cultivation (Been 1995; Harrison et al. 2002). Further, a new LY outbreak was reported in Jamaica killing up to two thirds of the MayPan and Malayan varieties; this pushed research studies aimed at verifying the diffusion of the new phytoplasma strain(s) and insect vector or verify the presence of changes in the virulence of the existing strain. The replanting of coconut trees in old palm plantations in several parts of tropics must be carefully designed to avoid the loss of erosion control, and maintain the shading of other crops and facilities (Snaddon et al. 2013). Currently, the management of LY is suggested through early diagnosis, replantation using alternative crop species or less susceptible palm genotypes (Baudouin et al. 2009; Myrie et al. 2011).

Jujube Witches' Broom Chinese jujube (*Ziziphus jujuba* L.) is a multi-purpose fruit tree that has great economic relevance especially in Asia. Jujube witches' broom (JWB) is a phytoplasma disease quite destructive and associated with the presence of '*Ca*. P. ziziphi' subgroup 16SrV-B. This disease kills 3–5% or even more jujube trees per year in many Chinese and Korean orchards (Jung et al. 2003). Liu et al. (2004) tested 30 jujube accessions free of JWB for settling a gene bank. They developed a protocol for disease transmission through grafting healthy germplasm onto seriously diseased plants and found it as much more effective for detecting the highly-resistant accessions selection compared to the traditional grafting of diseased bark onto healthy genotypes. A JWB resistant genotype named Xingguang (clone of the cultivar Junzao) was crossed and released in 2005 in China (Zhao et al. 2009; Liu et al. 2004). In these very resistant genotype it was found a low disease rate and a late symptom appearance. It was also discovered that the transgrafting could significantly enhance the resistance: the disease index of the accessions O, U, X and E resulted 0 after 3 years of transgrafting, while it was 12.5–50% at the start of grafting. This system was then used to establish orchards free from this phytoplasma disease. Based on this study, a control strategy for JWB has been suggested. Very recently the gene expression and metabolism regulation were studied with new approaches that allow to verify the jujube resistance pathways in healthy and diseased plants of jujube Lanzao. The two libraries from leaves, showed the presence of 4,266 genes differentially expressed (DEGs) including 2,070 that were upregulated and 2,196 that were downregulated. The libraries from jujube flower comparison showed the presence of 3,800 DEGs, of which 1,965 were upregulated and 1,835 were down-regulated. Some specific genes appeared to be functionally linked to the metabolism of aminoacids and to the pathway of the carotenoids, these were hypotized to be linked to the lack of appropriate nutrition and therefore to the symptoms expression (Fan et al. 2017).

8.4 Forest Trees and Shrubs

Elm yellows (EY) is a phytoplasma disease affecting several *Ulmus* (elm) genotypes in North America and Europe (Marcone 2017). EY is associated with the presence of '*Ca.* P. ulmi', subgroup 16SrV-A (Lee et al. 2004). Elm species differ greatly in their responses to EY infections. North American species such as *U. rubra*, *U. americana*, *U. alata*, *U. crassifolia* and *U. serotina* are highly susceptible. In affected trees symptoms of foliar yellowing, extensive phloem necrosis, and death within one or a few years after foliar symptom appearance were present. In contrast, European and Asian species including *U. laevis*, *U. wilsoniana*, *U. minor*, *U. glabra* and *U. pumila* are less damaged displaying tolerance (Mittempergher 2000; Sinclair 2000; Marcone 2017). Affected trees of Eurasian elm genotypes show mainly witches' brooms as a specific symptom but not phloem necrosis and are less prone to decline (Braun and Sinclair 1979; Mittempergher 2000; Sinclair 2000). In a previous work, several American and Eurasian elm genotypes resistant to the Dutch elm disease were checked for resistance to EY (Sinclair et al. 2000a). After graft inoculation with a North American strain of the EY agent and monitoring symptom expression over a 3-year period, the inoculated trees of the Eurasian cultivars Frontier (*U. minor* × *U. parvifolia*), Pioneer (*U. glabra* × *U. minor*), Prospector (*U. wilsoniana*), Pathfinder (*U. parvifolia*), and the complex hybrids Patriot and Homestead showed considerable differences to EY infection (Sinclair et al. 2000a). These responses ranged from no symptoms to phloem necrosis and tree death. Diseased Frontier, Patriot and Pathfinder trees showed yellowing and reddening of leaves, reduced terminal growth, stunting and witches' broom. However the symptoms occurring in *U. rubra* and *U. americana* and consisting in lethal decline and phloem discoloration or necrosis were not observed therefore, these genotypes may be considered as tolerant. The Pioneer trees showed a relevant greated susceptibility since they were more symptomatic, the majority of them (13 out of 16) became infected, as shown by PCR and DAPI fluorescence assays, and died (12 trees) in the 3 years of the observation period. Only two out of the 20 inoculated Prospector trees showed symptoms and only one died. The 14 Homestead experimentally inoculated plants resulted all negative to the phytoplasma presence however they showed in stems below the inserted grafts a localized phloem necrosis very likely resulting of the defense reaction that prevented systemic movement of the pathogen, indicating thus, a possible resistance of this cultivar (Sinclair et al. 2000a). Tolerance to EY phytoplasma infections was observed in (*U. glabra* × *U. wallichiana*) × *U. minor* open-pollinated (= clone 808) and (*U. glabra* Exoniensis × *U. wallichiana*) × *U.* × *hollandica* Bea Schwarz selfed (= clone Lobel) on the basis of field observations and PCR assays in three experimental fields established to evaluate the adaptability of elm species and 33 hybrid clones to environmental conditions of northern and central Italy (Lee et al. 1995; Mittempergher 2000). Clone Lobel and clone 808 showed very high infection rates, irrespective of symptom expression. Thus, they were rated as tolerant (Mittempergher 2000). It was suggested to employ tolerant taxa in locations where the presence of EY is endemic, since EY

reservoirs are already present in the environment, while the varieties that are resistant to the Dutch elm disease and exhibit severe symptoms of EY should be planted in locations where EY was not found (Sinclair 2000).

Lilac witches' broom (LWB) and ash yellows (AshY) are phytoplasma diseases of *Syringa* (lilac) and *Fraxinus* (ash) species, respectively, which are present in North America (Sinclair et al. 1996). These diseases are both associated with '*Ca*. P. fraxini', subgroup 16SrVII-A (Griffiths et al. 1999; Davis et al. 2013). AshY and LWB cause in highly susceptible taxa reduced apical and radial growth, progressive loss of vitality, dieback of branches and premature death. *Fraxinus* and *Syringa* are closely related genera in the family Oleaceae and, as they are graft-compatible, some lilac cultivars have been grown on ash rootstocks. The natural host plants of '*Ca*. P. fraxini' encloses 19 lilac and 12 ash species and numerous infraspecific taxa and interspecific hybrids (Sinclair et al. 1996). Several studies based on field observations and graft-inoculation experiments have shown that *F. pennsylvanica* (green ash) and *F. velutina* (velvet ash) show higher level of tolerance than *F. americana* (white ash) to '*Ca*. P. fraxini' infections and that heritable intraspecific tolerance variation occur in green ash and very likely in other species of ash (Sinclair et al. 1994, 1997a, b). Sinclair et al. (1997b) reported that in graft-inoculated plants '*Ca*. P. fraxini' suppressed the shoot growth of white ash and green ash at the onset of bud break, but in velvet ash this happens only 60 days after the inoculation. Growth losses in height, stem diameter, and root volume were 80%, 93% and 98% in the white ash; 60%, 57% and 79% in the green ash; and 23%, none, and 12% in the velvet ash. The growth of affected velvet ash grafted on white ash rootstock was severely reduced when it was compared to the one of the infected self-rooted velvet ash, while the infected white ash scions grafted on velvet ash rootstocks registered a relevant growth reduction when compared to the one of infected self-rooted white ash plants. White ash witches' brooms grafted on velvet ash rootstocks continued to retain their original form, but did not produce vigorous shoots. These findings indicate that although tolerant rootstocks mitigate the impact of '*Ca*. P. fraxini' infections on scions, the management of this disease based on tolerant plants may require tolerance in both scions and rootstocks (Sinclair et al. 1997b). The responses of *Fraxinus* cultivars to '*Ca*. P. fraxini' infections were evaluated under natural infection conditions in which six cultivars of green ash and five cultivars of white ash, all grown on green ash seedling rootstocks, were graft-inoculated with six distinctly different strains at two different locations of Iowa and New York, where the inoculated plants were maintained under observations for 3 years. Green ash cultivars Dakota Centennial, Bergeson and Patmore and the white ash Autumn Applause showed to be among those less affected at both locations (Sinclair et al. 2000b). Several LWB-tolerant lilac taxa have also been identified (Hibben and Franzen 1989). *Syringa vulgaris* cultivars, including Aurea, Boule Azurée, Bleuatre, Capitaine Perrault, Charles Joly, Carmine, Colbert, Dr. Charles Jacobs, Col. Wm. R. Plum, Edith Cavell, Frank Patterson, Fountain, Gaudichaud, Grand-Duc Constantin, Geheimrat Heyder, Hunting Tower, Hugo Koster, Joan Dunbar, Jessie Gardner, Kim, Lucie Baltet, Le Gaulois, Maurice de Vilmorin, Miss Ellen Willmott,

Mauve Mist, Madame Florent Stepman, Mademoiselle Fernande Viger, Montaigne, Nana, Nadezda, Patrick Henry, Petersons, Paul Hariot, Pinkie, President Viger, President Poincare, Prof. E.H. Wilson, Princess Camille de Rohan, Sarah Sands, Souvenir de Henri Simon, Souvenir de Claudius Graindorge, Sulte, Triste Barbaro, Vestale, Victor Lemoine and Verschaffeltii were more tolerant to '*Ca*. P. fraxini' infections than non-*vulgaris* lilacs such as *S.* × *diversifolia* Noveau, *S.* × *henryi* Lutece, *S.* × *josiflexa* Anna Amhoff, Elaine, Enid, Royalty, *S. josikaea* Eximia, *S. komarowii*, *S. julianae*, *S. laciniata*, *S. microphylla* Superba, *S. meyeri*, *S.* × *nanceiana* Floreal, Rutilant, *S. oblata* Dilatata, *S.* × *persica*, *S.* × *prestoniae* Alexander's Aristocrat, Alice, Calpurnia, Constance, Coral, Francisca, Isabella, Miranda, Ursula, Virginia, *S. sweginzowii*, *S. tomentella*, *S. villosa*, *S. villosa* × *sweginzowii* Hedin, and *S. yunnanensis*. Witches' broom symptoms were not found in any *S. vulgaris* cultivar, also when they were interplanted with non-*vulgaris* lilac plants exhibiting very strong broomings, whereas latent infections were also detected by DAPI and Dienes'stain tests and transmission electron microscopy in some *S. vulgaris* lilacs (Hibben et al. 1986; Hibben and Franzen 1989).

8.5 Conclusions

The most important measure to reduce losses due to phytoplasma diseases is the use of resistant plants. This measure is regarded as a safe, effective and environmentally friendly crop management procedure. Over the last decades, the improved understanding of phytoplasma diseases and the availability of DNA-based methods enabled identification of a considerable level of resistance to a number of phytoplasma diseases in various genotypes. However, the molecular, anatomical, and physiological basis of phytoplasma resistance is still poorly understood. In some instances only tolerance was present associated with poor host properties and low phytoplasma concentration in the colonized tissues, as has been shown for apple trees grown on *M. sieboldii*-based AP-resistant apomictic rootstocks and pear trees on clonal Quince rootstocks. Also, a few breeding programs have been carried out that clearly showed the inheritance of the resistance to phytoplasma diseases. Although some resistant genotypes so far identified are not entirely satisfactory for agronomical purposes, they can be further exploited either in conventional breeding programs or through biotechnological approaches aimed at developing plants with suitable agronomic properties in which the resistance to phytoplasma diseases is stably perpetuated. The current knowledge on the resistant genotypes to phytoplasma diseases not covered in this chapter is less advanced. However, differences in susceptibility of genotypes to paulownia witches' broom, mulberry dwarf, X-diseases of stone fruits, aster yellows, sugarcane white leaf, and rice yellows, are known. For phytoplasma diseases of grafted trees such as AP and PD, due to the colonization behavior a satisfactory control can be achieved by the use of resistants rootstocks. However on other cases both rootstocks and scion cultivars need to be resistant for a successful control. A drawback of the use of resistant genotypes in

controlling phytoplasma diseases of woody plants is the difficulty of a fast replacing of the susceptible genotypes with the resistant genotypes and the maintenance of the pathogen-free situation in the resistant ones since new strains of a given taxon are developing during the life span of both the forest and fruit trees.

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Chapter 9
Phytoplasma Elimination from Perennial Horticultural Crops

Margit Laimer and Assunta Bertaccini

Abstract The presence of phytoplasmas is a major threat for plant survival and production, especially in perennial crop species. The fact that infected plants cannot be healed emphasizes the importance of strategies for their elimination. Several methods were exploited and applied to verify their disappearance from plant tissues *in vivo,* such as thermotherapy by hot water or hot air of propagation material and chemotherapy of both propagation material and plants in the field. Numerous *in vitro* methods were also tested from meristem tip culture to chemotherapy with antibiotics or other antimicrobial molecules as well as cryotherapy that showed some level of phytoplasma elimination. The most promising approach to plant sanitation from phytoplasma infection still appears to be the combination of *in vitro* thermotherapy and shoot-tip culture.

Keywords Thermotherapy · Meristem-tip culture · Chemotherapy · Cryotherapy · Plasma activated water

9.1 Introduction

The plant-pathogenic bacteria lacking cell wall know as phytoplasmas which infect about hundreds of plant species, transmitted by insects, are causing very serious crop losses worldwide. The infected plants exhibit a range of symptoms, e.g. dwarfing, witches' broom, yellowing, purple top and phyllody (Hogenhout et al. 2008; Bertaccini and Duduk 2009). Inspite of their agricultural relevance and unique biological aspects, the phytoplasmas are among the less characterized plant pathogens. To date, several hundred of phytoplasma molecularly different strains were reported worldwide, and classified into 43 '*Candidatus* phytoplasma' species (IRPCM 2004;

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_9

Bertaccini and Lee 2018). Kakizawa and Yoneda (2015) listed five complete and several draft genome sequences for phytoplasmas. While in the early days, optical and electron microscopy were the common methods used to detect the phytoplasma presence the same methods were also employed to evaluate the success rate in their elimination. However, today a number of general and ribosomal group specific primers are available for polymerase chain reaction (PCR)-based analyses. In the 1990s, 16S rDNA sequences of phytoplasmas amplified by PCR were used for phytoplasma detection and classification (Lee et al. 1998). In fact, this is a very powerful tool that allow to detect phytoplasmas in plants and insect vectors because its highly sensitive and easy handling. In addition, 16S rDNA – being a conserved gene among bacteria - is very useful to classify phytoplasmas (IRPCM 2004). Thus, PCR amplification of 16S rDNA has been applied to phytoplasma strains worldwide, becoming the standard method to their detection and classification.

Currently applied sanitation techniques are based on hypotheses and approaches formulated several decades ago. The present chapter covers an overview of historic elimination methods beginning with *in vivo* methods (hot water treatment and chemotherapy on trees and on budwood) and moving to *in vitro* methods (*in vitro* thermotherapy, meristem tip culture, chemotherapy *in vitro*) with their obvious advantages of reduced space and time requirements and higher elimination rates.

9.2 *In Vivo* Methods – Thermotherapy by Hot Water or Hot Air

Hot water therapy (HWT) has been proposed and used in the past to obtain healthy plant material from plants infected with diseases now known to be associated with phytoplasma presence like peach yellows (Kunkel 1936), rubus stunt (Thung 1952), sugarcane white leaf and sugarcane grassy shoot (Liu 1963; Viswanathan and Rao 2011) and grapevine phytoplasmas (Caudwell 1966; Caudwell et al. 1990; Tassart-Subirats et al. 2003).

It is astonishing that - without knowing the nature of the causal agents – Kunkel (1936) found a cure for dormant peach trees affected by yellows. He then compared a treatment of dormant budwood for initially 10 minutes in water at 50°C, where the "virus" was inactivated without injury to the buds, to an immersion of the bud sticks for 4–5 days at 34°C to 35°C, for 11 hours at 38°C, for 40 minutes at 42°C, for 15 minutes at 46°C, for 14 minutes at 48°C, for 3 to 4 minutes at 50°C, for 1.5 minutes at 54°C with finally 15 seconds at 56°C. Little peach and red suture diseases proved amenable to treatments effective against yellows, but rosette was more refractory, since the symptoms were surviving to two hours exposure to a temperature of 40°C and were only eliminated after 8 to 10 minutes at 50°C.

As observed also for viruses, effective treatments have been described to vary widely in temperature and duration, *e.g.* from 3 days immersion at 32°C for "flavescence dorée" of grapevine (Caudwell 1966) to 10 minutes at 50°C for peach

rosette (Kunkel 1936). In grapevine the "stolbur" phytoplasmas ("bois noir" disease, '*Candidatus* Phytoplasma solani') resulted more difficult to eradicate using HWT than the phytoplasmas associated with "flavescence dorée" (Mannini and Marzachì 2007).

Hot water treatments (HTW) were employed as prevention and quarantine measures when applied to the grapevine propagation material. The technique involves a long-duration treatment to control exogenous and endogenous pests and pathogens as listed in the EPPO Standard PM 4/8 pathogen-tested material from grapevine varieties and rootstocks (EPPO 2012) as a recommended method. The application of HWT however, must be careful since it may affect the vitality of the propagation material (Mannini 2007). When elm plants infected with elm yellows disease ('*Ca.* P. ulmi') were subjected to HWT in autumn and then forced to sprout, the technique was a quite easy and reliable manner to produce phytoplasma-free planting material that could be used also in long distance trade circulation (Boudon-Padieu et al. 2004). However, HWT treatment in spring followed by harsh planting conditions in the forest led to a decreased plant survival. Similar observations were made in grapevine plants, where HWT treatment must be applied in the start or at the end of the conservation in cold environment to guarantie the best survival of the material (Boudon-Padieu and Grenan 2002; Tassart-Subirats et al. 2003). The first experiences on the use of HWTs on dormant wood from grapevines, have been carried out for the elimination of pathogens such as the agent of Pierce's disease, a dangerous bacteriosis present in California, by Goheen et al. (1973) by immersion in water at 45–55°C for 10–150 minutes. Treatments of this type were also useful for eliminating *Phytophthora cinnamoni*, *Agrobacterium tumefaciens*, *Xanthomonas ampelina* and the nematodes *Xiphinema index* and *Melidogyne* (Goussard 1977; Offer and Goussard 1980; Burr e Katz 1989). In the 1960s, the first treatments against "flavescence dorée" were carried out in France. According to these studies, the HWT would reduce the appearance of symptoms in the cuttings obtained by 80% (Caudwell 1966). These data have been confirmed by further indication that thermotherapy does not damage the cuttings treated when they are asymptomatic and for the cuttings from symptomatic canes the treatment increases the vitality thanks to the elimination of the pathogen (Caudwell et al. 1997). In Italy experiments have been carried out using molecular tests to verify phytoplasma elimination after thermotherapy (Murari et al. 1999; Borgo et al. 1999; Bertaccini et al. 2000) and confirmed the efficacy of the method; all the plants produced were asymptomatic (Fig. 9.1) and it was observed the elimination of "flavescence dorée" phytoplasmas and to some extent also of "stolbur" phytoplasmas; however the produced surviving plants show some relevant rate of aster yellows phytoplasma presence. Moreover for the plant fitness the only method resulting practically suitable was the HWT of grafted canes since they guarantee the best survival rate after the treatment.

Fig. 9.1 Rooted cuttings of grapevine after HWT maintained in an insect proof screenhouse during the testing for phytoplasma elimination

Pear decline ('*Ca.* P. pyri') phytoplasmas were eliminated successfully from budwood by hot water treatment at 47.5°C for 0.5 hour or 45.0°C for 1 hour. Budsticks of approximately 30 cm were immersed in a laboratory water bath and held in place between wire meshes (Adams and Davies 1992). The authors suggested that - if HWT was to be used - it would be prudent to use the longer time and higher temperature allowing the survival of buds.

***In vivo* thermotherapy by hot air** of most fruit tree species involves 2 year old plants treated for several weeks and grafting on pathogen-free rootstocks, a method optimized for virus elimination initially (Mink et al. 1998).

9.3 *In Vivo* Methods – Chemotherapy

Since the very first days of discovery of phytoplasmas, at that time named mycoplasma like organisms (MLOs) by Doi et al. (1967), attempts were carried out to set chemical control of their systemic spread in the host plants. The treatments to eliminate phytoplasmas were focused on molecules having an antibiotic activity. Ishiie et al. (1967) reported the first clear results of the therapeutic effects of tetracycline antibiotics. Among the many antibiotics tested, only tetracycline and to a lesser extent chloramphenicol reduced the disease symptoms. Tetracycline therapy became an essential step in proving the association between phytoplasmas and diseases. Typical methods of application included foliar sprays, root immersion, soil drench and trunk injection, but in all cases symptoms reoccurred once the antibiotic treatments were suspended. Today, antibiotic therapy is rarely used as a crop protection method, although it has been used in the case of palms of a high landscape value in the urban areas of Southern Florida (McCoy 1982).

Nyland and Moller (1973) first reported that tree decline and leaf curl as typical symptoms induced by pear decline, could be prevented by injecting solubilized oxytetracycline hydrochloride in diseased trees from plastic reservoirs through plastic tubes connected to 6–8 small holes drilled in the trunks. The application of tetracycline antibiotics was successfully used in the United States of America for pear decline control over many years (McCoy 1982). However, this laborious treatment has been discontinued because rootstocks less susceptible than oriental ones are available and the psyllid problem can be better managed. Attempts to cure peach rosette phytoplasma and X-disease affected trees by chemotherapy in the California used tetracycline injections into the trunk (Kirkpatrick et al. 1975). Besides the phytotoxic effects and the potential environmental risks, a remission of symptoms occurred in a number of treated trees and generally, the symptoms reappeared after 1–2 years. In most countries, this approach is forbidden as standard agricultural practice (Ragozzino 2011), in particular in Europe the use of antibiotics in agricultural crops is forbidden for safety reasons and to avoid raising of microbial strains that are resistant to them.

Paulownia witches' broom (PaWB) is among the most serious diseases affecting paulownia trees and it is associated with phytoplasma presence. No effective *in vivo* chemotherapy is available to cure PaWB disease, although tetracyclines suppress the development of symptoms and even temporarily eliminate them, the pathogen remains in the treated trees, and the symptoms reappear later after the chemical treatments are discontinued (Jin 1982). To maintain their suppressive effects, the chemical treatments must be repeated at least once a year and must be accompanied by thorough vector control (Raju and Nyland 1988).

The effect of tetracycline is a temporary remission of symptoms also reported in brinjal infected with little leaf disease but could not eliminate the pathogen completely in the host plant (Bindra et al. 1972; Anjaneyulu and Ramakrishnan 1973). Root dip treatment of infected onion seedling in tetracycline solution for 15 weeks at 7 days interval allows to detect the presence of phytoplasmas only in non- treated infected plants (Tanaka and Nonaka 1984). Furthermore, the effects of different molecules such as biophenicol, chlorophenicol, enteromycelin, lycercelin, paraxin, roscillin, camphicillin, oxytetracycine, chlorotetracycline, rose oil, clove oil, eucalyptus oil etc. on brinjal cultivar infected with phytoplasma were studied and found that application of antibiotics are quite ineffective and did not show any significant effect in controlling brinjal little leaf disease. Moreover, no flowers and fruits could be observed in any of the brinjal cultivars treated with antibiotics (Upadhyay 2016).

An attempt has been made in India for elimination of '*Candidatus* Phytoplasma australasia' (16SrII-D) from *Chrysanthemum morifolium* cv. Ajay Orange by application of oxytetracycline *in vivo* treatments. The chrysanthemum cuttings from six months old mother plants were treated with different concentrations of oxytetracycline (20, 40, 60, 80, 100 g/l) for *in vivo* screening with two sets of experiments. In the dip method, the cuttings were dipped in different concentrations of oxytetracycline treatments for 16 hours. The foliar spray method was carried out with different concentrations of oxytetracycline on plants raised from the infected cuttings. The oxytetracycline concentration of 80 mg/l was found optimal for the foliar spray in

Fig. 9.2 On the left illustration of the PAW production and the right PAW injection system in grapevine trunk in the vineyard (Courtesy by A. Canel)

in vivo method with phytoplasma elimination of 80%, whereas in the dip method, both 80 and 100 mg/l were found to be optimal for phytoplasma elimination of 80 and 100%, respectively.

Chemotherapy of Budwood Several methods were used to apply antibiotics to cure mulberry plants phytoplasma infected: foliar spray, root immersion, cutting treatment prior to the planting on stored and unsprouted shoots, and finally grafting treated budwoods (Asuyama and Iida 1973). In Europe, Seidl (1980) reported that apple proliferation ('*Ca*. P. mali') phytoplasma can be eliminated from budwood during summer, if foliated apple budsticks are exposed for 24–48 hours to a oxytetracycline or chlortetracycline solution (100–200 ppm) immediately before using the buds for grafting.

The use of an atmospheric non-equilibrium plasma, able to modify the chemical composition of water (plasma activated water, PAW) (Fig. 9.2), with a consequent increase in the quantity of nitrites, nitrates and peroxides and a decrease in pH was tested under field condition for verification about possible application to reduce the impact of grapevine yellows diseases (Laurita et al. 2015).

PAW is a very reactive solution, therefore it has been applied by endotherapy directly into the vascular system of grapevines, to avoid interference with the external environment and make the solution readily available to the plant. The field trials were performed on 120 plants selected from 17 vineyards with treatments performed in April, June and July for 3 years. It was possible to verify that the coolest hours of the day represent the most suitable time span to perform the injections and comparing the three treatments the one done in April was the most efficient (Zambon et al. 2017). The treated plants showed a light reduction of the symptoms and a

delay in their appearance (from end of August/beginning of September to the beginning of October), which allowed the plants to carry on their own productive load. The analyses carried out to detect the presence of phytoplasmas allowed to verify that the treatment reduced the number of infected plants. Considering the multiple variables present in the field, it is not yet possible to state the impact of this containment strategy, but certainly it represents a completely new and eco-sustainable management of phytoplasma associated diseases in grapevine. Studies on different species and cultivation systems are demonstrating how treatment with PAW increases the main genes involved in the defense mechanisms of plants towards pathogens expression, confirming the results found so far in the laboratory in *Catharanthus roseus* and in the field in grapevines (Zambon et al. 2018).

9.4 *In Vitro* Culture

In the early days, *in vivo* thermotherapy and subsequent tissue culture steps were combined, e.g. to cure Cabot highbush blueberry (*Vaccinium corymbosum*) from a phytoplasma infection (Converse and George 1987). By fluorescent staining with the DNA-specific dye diamidino phenylindole (DAPI), sieve tube fluorescence due to phytoplasma presence was observed in the longitudinal sections of roots of *V. corymbosum*. The trials to eliminate these pathogens by conventional heat therapy of whole plants in a growth chamber were not successful and the plants did not produce new growth and died within 4 weeks when grown at 38°C with CO_2 at the ambient concentration. However, when 1,200 ppm of CO_2 were used at 38°C, the Cabot plants survived and produced new shoot growth for 6 weeks, enabling the collection of softwood cuttings. The plants resulting positive in DAPI staining and derived from cuttings treated with heat therapy decreased in number according to the time for which the original plants were kept at 38°C, and were zero after 5 and 6 weeks. Shoot apices of 10–20 mm were excised from newly grown shoots after heat therapy periods of increasing duration and established *in vitro*. After a propagation phase, plantlets were rooted and acclimatized to greenhouse conditions.

Tissue culture was successfully utilized for the elimination of phytoplasmas. Symptomless, phytoplasma-free plants were regenerated using the callus culture of *Catharanthus roseus* and sugarcane (Möllers and Sarkar 1989, Parmessur et al. 2002), meristem-tip culture of sugarcane (Wongkaew and Fletcher 2004), and stem culture of mulberry (Dai et al. 1997). Regeneration *via* callus culture is not feasible for many perennial crop plants, and potentially also carries some risks through spontaneous mutations. The fact that real meristem tip (dome) that lack any leaf primordia, are mostly free of pathogens (Pierik 1987) facilitates the production of phytoplasma-free tissue cultures. In addition, the absence of vascular elements in the meristem may effectively hinder the transport of phytoplasma within a developing plant (Kartha and Gamborg 1975). Meristem culture is applied to eliminate the phytoplasmas from the infected *in vitro* shoots, even though it is a technique that is size-dependent. The smaller meristems do not regenerate plants, while those that are

larger can do it however in the latter case the plantlets obtained resulted not phytoplasma-free (Parmessur et al. 2002).

Stem culture *in vitro* was attempted for elimination of the mulberry dwarf phytoplasma from mulberry plants (*Morus alba*) (Dai et al. 1997). Stem segments of heavily phytoplasma-infected shoots were cultured on solid Murashige and Skoog nutrient medium (1962) without growth regulators during 2–3 months. The tissue cultures, and the regenerated plants grown under greenhouse conditions for 3 years, were verified by PCR and DAPI staining to asses phytoplasma presence, and also observed to detect mulberry dwarf symptoms. About 70% to 90% of the regenerated plants were apparently freed from the phytoplasma since they remained PCR-negative, DAPI-negative and symptom-free for the next 3 years (Dai et al. 1997).

Almond witches' broom ('*Ca.* P. phoenicium') was eliminated from two Lebanese almond varieties by different tissue culture methods (Chalak et al. 2005). The oxytetracycline treatment completely stop the explant growth, but the cultures from stem cutting treated hot therapy, the shoot tip cultures subjected or not to thermotherapy, and the micrograft using shoot tips resulted all good methods allowing the correct regeneration of shoots or the phytoplasma elimination. Morevoer, the stem cutting technique added to thermotherapy resulted a very effective method in the regeneration of almond plantlets free from phytoplasmas.

To verify the persistence of "flavescence dorée" and "stolbur" phytoplasmas in grapevine shoots in micropropagation, rooted cuttings of field-infected grape varieties Lambrusco and Ancellotta and cuttings of the Serbian variety Plovdina grafted with symptomatic material were employed. Single node explants (2–3 cm) were excised from April to June from greenhouse grown cuttings established from infected budwood. Regular subcultures were performed every 2 months using the Murashige and Skoog medium additioned with 0.5 mg/l of indol-3-acetic acid (IAA) (Fig. 9.3).

Seventeen months after culture initiation grapevine shoots were tested individually by nested PCR/RFLP analyses. After 60 days from testing 70% of "stolbur" infected shoots did not survive, while only 10% of phytoplasma-free shoots died. The 31.4% of *in vitro* shoots resulted positive to "stolbur", but none to "flavescence dorée" phytoplasmas. This confirms the ability of "stolbur" phytoplasmas associated with the "bois noir" disease to survive in micropropagated material (Calari et al. 2006).

9.5 *In Vitro* Meristem Preparation in Combination with *in Vitro* Thermotherapy

Heat-treatment is an effective way to eliminate both plant viruses and phytoplasmas of many plant species including flowers (Morel and Martin 1952), sugarcane (Quak 1977), and cassava (Kartha and Garnborg 1975). In the tissue-cultured meristem, where phytoplasma occurs at low concentrations (Caudwell et al. 1990), the f heat-treatment combined with meristem culture was found to increase the possibility of

Fig. 9.3 Grapevine shoots infected by phytoplasmas in micropropagation

obtaining phytoplasma-free plants (Pierik 1987). *In vitro* methods take advantage of small-scale explants for *in vitro* thermotherapy, which involves a few months old *in vitro* cultures treated for several days, followed by meristem preparation and plant regeneration (Laimer and Barba 2011) (Fig. 9.4).

The *in vitro* thermotherapy followed by the meristem tip culture techniques employed for the '*Ca*. P. prunorum' elimination from diseased *Prunus* plants resulted successful and the '*Ca*. P. mali' (agent of apple proliferation, AP), '*Ca*. P. pyri' (the agent of pear decline, PD) and '*Ca*. P. prunorum' (agent of European stone fruit yellows) were eliminated by *in vitro* thermotherapy combined with meristem preparation from several cultivars of apple, pear, apricot and peach (Laimer 2002; Laimer and Bertaccini 2006), and more recently of '*Ca*. P. rubi' from *Rubus idaeus* (Ramkat et al. 2014) and *Vaccinium myrtillus* (M. Laimer et al. unpublished).

Six different protocols were tried on Chardonnay grapevine plants affected by "bois noir". A comparative trial was carried out for a period of 40 days after stem cuttings and shoot tips preparation between plants heat treated at 38 ± 1°C and untreated ones and stem cuttings combined with a hot water treatment at 50°C for 15 and 30 minutes before the culture starting. Both protocols resulted suitable for shoot regeneration and elimination of phytoplasmas. However the stem cutting technique coupled with heat or hot water treatments resulted the most effective in phytoplasma elimination (Chalak et al. 2013). Wang and Hiruki (1996) exposed paulownia witches' broom (PaWB) – infected *Paulownia* tissue culture shoots with two pairs

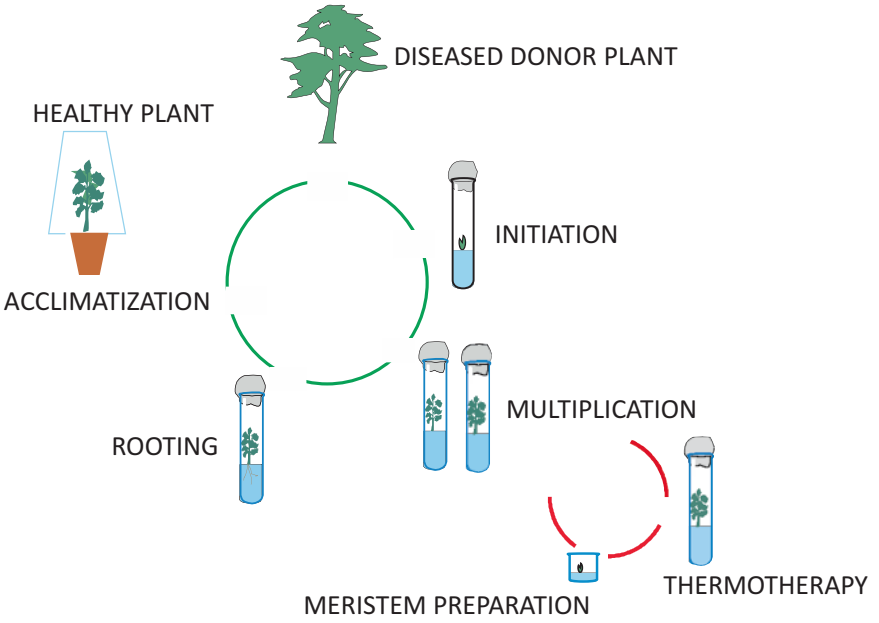

Fig. 9.4 The combination of *in vitro* thermatherapy and the meristem technique has been devised and successfully applied at PBU, Vienna in the late 1980s

of axillary buds to 35°C at a 16-hours day length under 30 $\mu mol \cdot m^{-2}s^{-1}$ of light for 5 weeks. Meristems of shoots were taken from treated tissue cultures. The obtained plantlets appeared healthy with newly enlarged leaves and elongated internodes. No symptoms of witches' broom re-appeared in the clonal plantlets produced.

9.6 *In Vitro* Chemotherapy

Additionally the already mentioned chemical compounds were applied to tissue cultures for *in vitro*- chemotherapy. It is interesting that with the oxytetracycline treatment the phytoplasma elimination resulted to be independent from the size of the shoot tip and can help overcoming the complications in excising the meristem of reduced dimension and the consequent difficult regeneration. Tetracyclines in several cases appear to have just a bacteriostatic effect on phytoplasmas, since in treated plants the symptoms may reappeared after the transfer of the shoots to antibiotic-free media (Davies and Clark 1994; Wongkaew and Fletcher 2004).

Phytoplasma infected pear shoots (*Pyrus communis* cultivar Pine) were maintained on Murashige and Skoog medium additioned of gibberellic acid, indole butyric acid and benzyl amino purine for more than 3 years. Phytoplasma

concentrations in micropropagated shoots resulted higher than the one in samples from field-grown plants, although the first remained symptomless. Phytoplasmas were eliminated by the addition of 100 g/ml oxytetracycline to the growth medium and by the maintenance of shoots in this medium for a period of 4 weeks. These results must be taken into consideration when plant propagation schemes are enclosing symptomless phytoplasma-infected shoots obtained by micropropagation.

The growth and shoot multiplication rate of potato *in vitro* cultures in increasing concentrations (0, 32, 64, 128, 256, 512, and 1,024 mg/l) of chloramphenicol, streptomycin and tetracycline in the media (Pereira and Fortes 2003) were inducing severe phytotoxic effects. In the attempt to eliminate almond witches' broom from different almond varieties, no plant regeneration occurred from 1 cm cuttings subjected to 50, 100, and 150 μg/mL of oxytetracycline (Chalak et al. 2005). Phytotoxic effects of oxytetracycline were also observed on grapevine, i.e. axillary buds were very much affected, when a culture medium with 100 mg/l oxytetracycline was used (Gribaudo et al. 2007).

Carvalho et al. (2017) developed a methodology for cassava frog skin disease (CFSD) elimination by shoot tip culture of cassava combining thermotherapy and tetracycline treatments. Cuttings from various genotypes were exposed for 3 minutes to different tetracycline concentrations, and then to thermotherapy at increasing temperatures (35°C, 38°C, 40°C, 45°C and 55°C). Shoots of 2 cm were collected, surface-sterilized and tips having different decreasing were excised (0.2, 0.4, 0.5, and 1.0 mm) for growth in a medium containing 0, 5, 10, and 15 mg/l tetracycline. The shoots were transferred after 60 days in a prolifaration medium without antibiotics. The viability of the regenerated plants was verified after 30 days. Subsequently plantlets were grown for 70 days under greenhouse conditions and then moved to the field. A root inspection and PCR testing were carried out after 7 months to verify the elimination of CFSD from the accessions. The majority of the treatments allowed to obtain 100% of plants that were free of CFSD.

The susceptibility of '*Ca*. P. mali' to antimicrobial agents having different target activities against micro-organisms, e.g. nisin, esculetin, pyrithione and chloramphenicol was evaluated. Their activity was compared with the one of tetracycline and enrofloxacin antibiotics used as controls. These substances were used in micropropagated '*Ca*. P. mali'-infected apple shoots added to the proliferation medium at concentrations of 100, 500, 1,000 ppm, while nisin and pyrithione were employed at 10, 100 and 500 ppm. Phytoplasma presence was quantified by qPCR that showed the phytoplasma not detectability after 1 and 2 months in the presence of 10 and 100 ppm of pyrithione. Also some of the other substances were able to lower the phytoplasma concentration but only 2 months after the treatment. The micropropagated shoots grown in media additioned with essential oils died or withered (Aldaghi et al. 2008).

Plant growth regulators like kinetin show effects on cytokinesis and cell differentiation, induction of organogenesis and reduction of leaf senescence (Skoog and Miller 1957; Osborne 1962). At high temperatures, cytokinins showed to have a protective role in plants. However, experiments performed with kinetins were ineffective in phytoplasma elimination (Plavsic et al. 1986). Also a treatment with

b-aminobutyric acid on phytoplasma-infected periwinkle shoots (Curkovic-Perica et al. 2007) proved to be ineffective, while the use of putrescine, spermidine or spermine caused various modifications of the phytoplasma ultrastructure, that could be related to a low multiplication rate and uneven distribution of the pathogens. A slow appearance of symptoms has been also registered in shoots treated with polyamine compared to untreated healthy shoots (Musetti et al. 1999). The effect of external application of auxins to healthy and phytoplasma-infected periwinkles was studied in greenhouse-grown and shoot-tip cultures of *C. roseus* infected with *Spiroplasma citri* and phytoplasmas by Chang (1998) and in *in vitro*-grown '*Ca*. P. trifolii'-infected *C. roseus* plants by Pertot et al. (1998). Chang (1998) observed that various concentrations of plant growth hormones are necessary to guarantie a functional rooting of healthy *versus* infected plantlets and hypothesized that a block of the auxin transport affecting also endogenous auxin concentration was induced in infected periwinkles (Leljak-Levanic et al. 2010). Pertot et al. (1998) described increased endogenous levels of indole-3-acetic acid (IAA) in phytoplasma-infected plants and observed a reduced number of phytoplasmas in infected *C. roseus* to which high quantity of exogenous auxins were applied. On the other hand, when i*n vitro* shoots of periwinkle infected with different '*Ca.* P. pruni' (strain KVI, clover phyllody from Italy) and '*Ca.* P. asteris' (strain HYDB, hydrangea phyllody) have been exposed to IAA or indole-3-butyric acid (IBA), the symptom disappearance from shoots was observed, and IBA resulted the most effective in symptom elimination (Curkovic-Perica et al. 2007). It could be observed that this recovery was linked to '*Ca*. Phytoplasma' species, duration of the treatment and concentration and the type of auxin. On the contrary, '*Ca.* P. ulmi' (strain EY-C) and '*Ca*. P. solani' (strain SA-1) was still present in the plant tissue, even if with lack of symptomatology the phytoplasmas were always detected by PCR (Curkovic-Perica 2008).

An interesting approach applying Pap-II, that is a protein inactivating the ribosom activity extracted from the leaves of *Phytolacca americana* has been presented by Veronesi et al. (2000). It appears that PAP–II could interact with periwinkle tissue cultures and eliminating hydrangea virescence phytoplasma (HyV) (aster yellows, 16SrI-B). The elimination rate detected appears to be related to both PAP-II concentration and exposure time showing an optimum at 1: 1000 dilution for approximately 3 months. Micropropagated periwinkle shoots were immersed in decreasing amounts of PAP-II, for various periods showing an increasing in the number of necrotic shoots after 48 hours. However, no phytotoxicity was detectable, when the PAP-II was supplemented to the medium by percolation. Groups of shoots 1–3 cm long were grown in micropropagation and treated with purified PAP-II for 15 to 150 days. Nested-PCR employing universal and group-specific phytoplasma primers was applied to verify phytoplasma elimination. The resulting percentage of phytoplasma-free shoots varied from 40% to 50% in the concentrations from 1:10 to 1:1000 and for times varying from 50 and 150 days (Fig. 9.5).

Recently shoot tip cryotherapy, as a variant of the cryopreservation technique, has been described as a tool for plant pathogen eradication (Yin et al. 2011, Feng et al. 2013). It was successfully eliminating several graft-transmissible pathogens, mainly viruses, from plants. However, also the elimination of phytoplasmas, *e.g.*

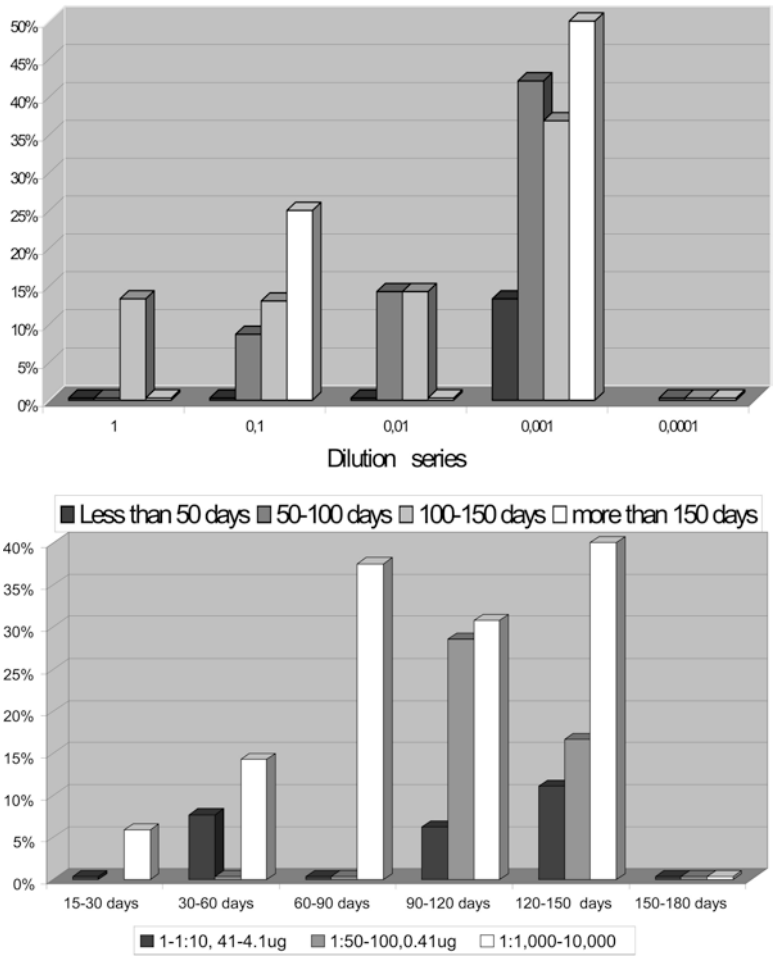

Fig. 9.5 Percentage of phytoplasma elimination by using the PAP-II in the culture medium (From Veronesi et al. 2000)

sweet potato little leaf phytoplasma (SPLL, 16SrII-D) from sweet potato (Wang and Valkonen 2008) and jujube witches' broom phytoplasma ('*Ca*. P. ziziphi', 16SrV-B) from Chinese jujube (*Ziziphus jujuba*) (Wang et al. 2015) has been reported. Independently from shoot tip size and cryogenic methods the shoot tips treatment with cryotherapy produces pathogen-free plants at high frequency. The production of pathogen-free plants by cryotherapy of shoot tips involves six major steps: (i) introduction of materials into *in vitro* cultures; (ii) shoot tip excision; (iii) cryotherapy; (iv) plant regeneration using specific media; (v) molecular indexing to verify pathogen presence after cryotherapy; and (vi) production and maintenance of pathogen-free plant materials as nuclear stock. The steps (ii), (iii), and (iv) are similar to those employed for the cryopreservation process, but had a fundamental

Fig. 9.6 Original appearance of recovered shoots (7a) in phytoplasma infected cultures (7b) of *Vaccinium myrtillus* and obtention of asymptomatic shoots after several cycles of subculture

importance to produce the pathogen eradication from high number of shoots (Wang and Valkonen 2009).

Vaccinium myrtillus in vitro shoots infected with a phytoplasma of the 16SrVI ribosomal group (clover proliferation) (Borroto-Fernandez et al. 2007) spontaneously developed recovered shoots. These were subcultured carefully for several passages according to the method developed for *Vaccinium cylindraceum* by Steniczka et al. (2006), until totally recovered shoots were obtained (Fig. 9.6) (M. Laimer et al. unpublished).

Phytoplasmas occur only in the phloem tissues of infected plant hosts, which render detection rather tedious. The total DNA from apple proliferation-diseased apple phloem contained about 2% phytoplasma DNA (Kollar et al. 1990). Since phytoplasma DNA is less than 0.1% (w/v) of the total DNA in the extracts from woody hosts exhibiting disease symptoms results (Kirkpatrick 1989) the phytoplasma titer in asymptomatic infected plants is usually very low. PCR serves as the most sensitive method for the detection of phytoplasmas, since as little as 16 pg total DNA can be detected (Lee et al. 1998). Therefore, through screening by PCR, the reliability of establishing phytoplasma-free tissue cultures by heat treatment and meristem culture is strengthened.

In woody plants, grapevine, apple and peach species are those that result frequent targets for sanitation by thermotherapy, chemotherapy and tissue culture trials, since their health status is strictly regulated by international rules (Panattoni et al. 2013). Novel insights generated on host pathogen-interactions will also allow to design not only resistance breeding strategies (Hogenhout et al. 2008), but also to improve our understanding of the mechanism underlying the different elimination strategies. As described manifold for the elimination of viruses, e.g. *Rubus* and ASGV (Knapp et al. 1995; Laimer 2002), which correlates with the different

genome organization, major differences in the ease of elimination also exist for phytoplasmas such as for "flavescence dorée" *versus* "bois noir" (Mannini 2007). For phytoplasmas increasing evidence and data become available. As obligate parasites, phytoplasmas have small genomes, since they have lost important metabolic genes in their host-dependent life cycles, thus lacking essential biosynthetic pathways (Oshima et al. 2013).

The mycoplasmas that are pylogenetically close relative of phytoplasmas, do not possess the genes for the sterol and fatty acid biosynthesis, tricarboxylicacid cycle, *de novo* nucleotide synthesis, and biosynthesis of most amino acids. These prokaryotes appear to entirely depend on their host that provide them the molecules produced by these pathways (Razin et al. 1998). However, the phytoplasmas appears to have lost an high number of metabolic genes than mycoplasmas (Oshima et al. 2004; Bai et al. 2009), including those of pentose phosphate pathway. Phytoplasmas however seems to possess multiple copies of genes related to transporter functions not identified in the mycoplasmas (Oshima et al. 2004). These features suggest that phytoplasmas should be very much dependent on the metabolic compounds provided by their host. However the relevant glycolysis metabolic pathway is activated inspite of the fact that the genes for this reactions result completely absent in '*Ca*. P. mali' (Kube et al. 2008), in which the gene for the 2-dehydro-3-deoxyphosphogluconatealdolase (*eda*) was found (Kube et al. 2012). The alternative pathway hypothesized is that in '*Ca*. P. mali' the pyruvate could be formed with reactions not related to the glycolysis (Kube et al. 2012).

A number of membrane and secreted proteins are reported as possible virulence factors and phytoplasma genomes have been accurately mined to verify the presence of genes coding them. In '*Ca*. P. asteris' OY-M strain, TENGU transgenic plants show symptoms referable to those of phytoplasma infection, including witches' broom and dwarfing (Hoshi et al. 2009; Sugawara et al. 2013). The expression of many auxin-related genes was significantly downregulated in these tengu-transgenic plants, as indicated by a microarray analysis, suggesting a role of TENGU as suppressor of the auxin signaling or biosynthesis pathways (Hoshi et al. 2009; Denancé et al. 2013).

In the '*Ca*. P. asteris'AY-WB strain genome, it was possible to identify about 56 genes encoding predicted secreted proteins. The most studied of these is the SAP11 that was shown to have an eukaryotic nuclear localization (Bai et al. 2009). The transegenic plants expressing SAP11 have crinkled leaves and produce several stems; also the insect vector fecundity of this phytoplasma resulted increased in SAP11 transgenic plants (Sugio et al. 2011). The identification of TENGU, SAP11, and SAP54 (MacLean et al. 2011) provide indication about effector protein production and modification of plant-gene activity operated very likely by the phytoplasma presence (Hoshi et al. 2009; Sugio et al. 2011; Himeno et al. 2011). Several experimental evidences show that the presence of phytoplasmas induce to developmental modifications in infected plants by deregulation of genes that are relevant in the development. Infected grapevine plants resulted to have a carbohydrate metabolism modified very likely related to the significant upregulation of genes encoding sucrose synthase and alcohol dehydrogenase I (Hren et al. 2009). Jagoueix-Eveillard

et al. (2001) showed that gene encoding putative sterol-C-methyltransferase is down regulated in periwinkle plants with leaf yellows and stunting due to "stolbur" phytoplasma, but the same gene shows no deregulation in periwinkles infected with other phytoplasmas. Pracros et al. (2006) reported that a strain of the "stolbur" phytoplasma induces flower malformations and changes in the floral development genes expression related with the inhibition of a gene-specific demethylation expressed at the floral development time. Aldaghi et al. (2012) in apple plants with apple proliferation disease reported an auxin efflux carrier as downregulated. Pertot et al. (1998) describes the increased concentrations of indole-3-acetic acid (IAA) in micropropagated '*Ca.* P. trifolii'-infected plants and a reduced quantity of the phytoplasmas present in cells of infected *C. roseus* treated using an high quantity of exogenous auxins. These results suggest that the addition of exogenous auxins could interfere with the phytoplasma growth in the plant. On the other side, auxins might be involved also in disease susceptibility since a miRNA-mediated suppression of auxin signaling lead to obtain some resistance (Navarro et al. 2016).

The testing of different techniques and treatments against phytoplasmas was partly abandoned when the recovery, described as disappearance of symptoms previously present in infected plants, was studied. This phenomenon is both involving or not involving the elimination of the pathogen from the host, and it was described in apple, apricot and grapevine (Carraro et al. 2004). In apple trees the recovery was also characterized by the disappearance of '*Ca.* P. mali' from the aerial parts of the plants, but not from the undergroup apparatus (Musetti et al. 2004). The grapevine "flavescence dorée" phytoplasma also was not detectable in recovered plants leaves (Musetti et al. 2006, 2007), while '*Ca.* P. prunorum' was always detected in the leaves of asymptomatic apricot trees subjected to recovery (Musetti et al. 2005). The factors that are involved in recovery from symptoms in vineyards and orchards plants are not yet fully understood despite several studies on the topic. Musetti et al. (2004, 2005, 2006, 2007) suggested that H_2O_2 and its produced molecules together with the enzymes involved in its metabolization might be the factors that are reducing the virulence of the pathogen and its symptom expression suggesting that a kind of systemic acquired resistance (SAR) is linked to the process.

The results of *in vitro* oxytetracycline treated *Chrysanthemum morifolium* cv. Ajay Orange plants showed 91.3% elimination at 80 mg/l of oxytetracycline concentration. In the *in vitro* method nodal segments of *C. morifolium* maintained on MS medium, were transferred to media with the different concentrations of oxytetracycline (20, 40, 60, 80, 100 g/l). The results of the present study revealed that *in vitro* method of using oxytetracycline at 80 mg/l was the most efficient in elimination of phytoplasmas from infected *C. morifolium* plants (Taloh et al. 2018).

9.7 Conclusions

Phytoplasmas are known to be maintained by multiplication of scions and/or cuttings taken from diseased plants especially when symptoms are not present (latency period). Therefore the source of mother plants necessary to establish commercial nursery and to provide the appropriate conservation conditions for the maintenance of pathogen-free mother plants, remains as outmost importance for a thriving horticultural and agricultural sector. The most promising approach to plant sanitation from phytoplasma infection remains the combination of *in vitro* thermotherapy and shoot-tip culture. In this approach, the major bottleneck remains the skilled excision of meristems of sufficiently small size and the availability of a proper meristem regeneration medium (Laimer 2002, Howell et al. 1998) in combination with a reliable testing for verifying phytoplasma elimination (Heinrich et al. 2001).

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Chapter 10
Microbe Relationships with Phytoplasmas in Plants and Insects

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Abstract The hosts of phytoplasmas, i.e. plants and insect vectors, are inhabited by diverse microorganisms having interactions spanning from mutualism to parasitism. When the pathogens colonize a host, they may thus be exposed to diverse interactions with complex microbial communities. These relations are still poorly recognized for phytoplasmas, even though many beneficial or harmful interactions have been described for other plant pathogens. The knowledge on traits of microbial relations involving phytoplasmas in insects and plants is regarded as a valuable tool for designing new control methods against the diseases associated with these pathogens, by displaying direct antagonistic activities, altering the vector fitness or competence for transmission, or promoting plant immune response or growth. In insect vectors, which mainly host bacterial associates, with few yeast-like symbionts, direct interactions with phytoplasmas were described for bacteria of the genera *Frauteria* in *Hyalesthes obsoletus* and *Asaia* in *Euscelidius variegatus*. In plants, the most studied systems are grapevine, apple and coconut palm, along with model organisms such as *Catharanthus roseus* and *in vitro* micropropagated plants. Here, many bacteria, mainly of the genera *Pseudomonas*, *Burkholderia* and *Paenibacillus*, as well as the fungal endophyte *Epicoccum nigrum,* were shown to inhibit phytoplasma growth and related symptoms in the plant hosts. Overall, the recent advances

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A. Bertaccini et al. (eds.), *Phytoplasmas: Plant Pathogenic Bacteria - II*,
https://doi.org/10.1007/978-981-13-2832-9_10

concerning the knowledge on microbial symbioses in phytoplasma plant and insect hosts can consistently support future research regarding the phytoplasma infection process, and eventually drive new control strategies against phytoplasma-associated diseases.

Keywords Symbionts · Endophytes · Symbiotic control · Pathogen suppression

10.1 Introduction

The phytoplasmas associated with severe diseases in both perennial woody (such as pome and stone fruit trees, grapevine, and coconut palm) and annual herbaceous species (including Solanaceae and Brassicaceae) (Bertaccini 2007), typically display tripartite relationships with plants and insect vectors. These associations have several peculiar traits, mainly related to the fact that phytoplasmas are intracellular bacteria restricted to hosts belonging to different kingdoms. A diversity of specificity levels is shown in interactions involving different phytoplasmas, considering both plant and insect hosts: generalist phytoplasmas, which infect a wide array of plants and are vectored by many leafhopper species, may be found along with specific phytoplasmas transmitted by a limited number of insect vectors to a few plant species (Alma et al. 2015). Phytoplasma genomes, which are the most reduced among plant pathogenic bacteria, still retain a gene repertoire that allows interfacing with such different hosts. They encode several secreted proteins that are thought to alter host cell physiology, *e.g.* by interfering with hormone-related pathways (Perilla-Henao and Casteel 2016). Moreover, immunodominant membrane proteins (IDPs) have been shown to interact with both plants and insects, possibly being involved in the transmission process (Konnerth et al. 2016). However, the molecular machineries governing the interaction between phytoplasmas and their hosts are still poorly understood. Recently, research focus has been given to the notion that phytoplasmas are not the only microorganisms relating with their hosts, but are included in complex microbiomes enclosing bacteria, fungi, and viruses which could in turn influence such relationships. Insect symbionts (bacteria, fungi, and viruses) are well-known influencers of life history, ecology and evolution of their hosts. Like all sap feeders, phytoplasma vectors, namely leafhoppers, planthoppers and psyllids, rely on bacterial symbionts that supplement their unbalanced diet (Zchori-Fein and Bourtzis 2011). Along with supplying the host with nutrients, insect microbial symbionts provide protection against pathogens or other stress factors or manipulate the reproduction.

Plants are commonly inhabited by a plethora of endophytic microorganisms, which reside in their internal tissues without being harmful (Petrini 1991). Endophytic bacteria include both Gram-positive and Gram-negative bacteria (Gouda et al. 2016); the studies carried out this far managed to identify over 200 bacterial genera, both in wild and cultivated plants (Arora and Ramawat 2017). Most of the known endophytic bacteria belong to four phyla: Proteobacteria, Actinobacteria, Firmicutes, and

Bacteroidetes (Hardoim et al. 2015), which include genera such as *Acetobacter*, *Bacillus*, *Enterobacter*, *Burkholderia*, *Pseudomonas*, *Serratia*, and more (Hardoim et al. 2015; Arora and Ramawat 2017). Endophytic fungi are ubiquitous in nature, as they have been found within plants adapted to a wide range of ecosystems and are present in all the major lineages of land plants (Lugtenberg et al. 2016). Rodriguez et al. (2009) described different functional groups of endophytic fungi based on their phylogeny and life history traits. Class 1 endophytes are defined as the clavicipitaceous endophytes (including *Balansia* spp., *Epichloë* spp. and *Claviceps* spp.); they form systemic associations with the aboveground tissues of grass, rush and sledge hosts. The non-clavicipitaceous types are further separated into classes 2, 3 and 4 based on host colonization and transmission, *in planta* biodiversity and fitness benefits conferred to hosts. The diverse class 2 endophytes encompass both Ascomycota and a few Basidiomycota (the Dikarya), whose most distinctive feature is their ability to colonize roots, stems and leaves and the formation of extensive plant infections. Endophytes from class 3 are extremely diverse and form highly localized infections in aboveground tissues, such as in the leaves of tropical trees and non-vascular and vascular plants (Rodriguez et al. 2009). Class 4 endophytes are also referred to as the dark-septate endophytes (DSE) (Rodriguez et al. 2009); these facultative biotrophic fungi colonize plant roots and as distinguishing feature they have melanized dark septate hyphae (Jumpponen and Trappe 1998; Jumpponen 2001). Lugtenberg et al. (2016) recommended an additional class, which is needed to recognize the endophytic entomopathogenic fungi as symptomless endophytes of plants that have the unique ability to infect and colonize also insects.

Both endophytes and insect symbionts may depend on their host metabolism for survival (obligate life style) or have the ability to live inside and outside plant/insect body (facultative life style). Furthermore, plant and insect associates can be vertically transmitted (through the seed or the egg) or horizontally transferred. The latter transmission route may take place through air or soil dispersal, or by insect feeding or mating. In the case of the plant associates, transmission of class 1 fungal endophytes is primarily vertical via host seed infections. Transmission of class 2, 3 and 4 fungal endophytes is primarily horizontal, although class 2 endophytes are also transmitted vertically via seed coats, seeds or rhizomes. In the case of horizontal transmission, propagation is usually dependent on the reproductive structures of the endophyte, such as spores, that move by wind or rain dispersal, or are moved by a vector, from plant to plant. Root fungal endophytes are likely to derive from the soil, whereas fungal endophytes colonizing above-ground tissues are transmitted via spores in the air.

Different insect symbiont types have been recognized, and generally divided based on their role and mode of association (Moran et al. 2008; Prosdocimi et al. 2015). Obligate symbionts, strictly required by the host for its survival, typically reside intracellularly in specific organs called bacteriomes, they are vertically transmitted and generally referred to as primary symbionts. Facultative symbionts can occupy different insect organs intra- and extra-cellularly, they are not necessary for insect survival and are named secondary symbionts. Finally, bacteria that exploit the host's reproduction for their spread are called reproductive manipulators.

Endophytes as well may play many different roles in the plant, ranging from protection from biotic and abiotic stresses – through the production of bioactive

compounds – to nutritional support (Bacon and White 2016). Biotic stresses include plant pathogens, insects and nematodes; whereas abiotic stresses include nutrient limitation, drought, salinity and extreme pH values and temperatures (Lugtenberg et al. 2016). Different endophytic bacteria are associated with different organs of the plant, such as roots, leaves, or trunk (Mocali et al. 2003; Compant et al. 2011), and can be influenced by the processes employed for the management of crops (Campisano et al. 2014). Also the sanitary status of the plant can influence the composition of the bacterial community present in a plant (Trivedi et al. 2010; Bulgari et al. 2011; Morales-Lizcano et al. 2017). Fungal endophytes play key roles in ecosystems by protecting plants against many biotic and abiotic stresses, increasing their resilience, and helping plants to adapt to new habitats. Plant protection against invading pathogens and (arthropod) herbivores, is conferred either via antibiosis or via induced resistance. Fungal endophytes protect plants especially through the production of secondary metabolites that have growth-inhibitory activities toward plant pathogens and pests. Several examples have been published on the production of antiviral, antibacterial, antifungal, and insecticidal compounds by fungal endophytes (Hardoim et al. 2015; Lugtenberg et al. 2016).

Phytoplasmas are exposed to (and included in) such complex microbiomes when colonizing their hosts, and they are even thought to originate from insect symbionts (Chuche et al. 2017). Direct relationships have been described between symbionts and plant pathogens. For example, in *Nephotettix cincticeps* (Uhler) '*Candidatus* Sulcia muelleri', the primary symbiont of Auchenorrhyncha, was found to mediate the transovarial transmission of the plant pathogenic rice dwarf virus (Jia et al. 2017). Beside potential direct interactions, the microbiome influence on vectors and plant hosts (e.g. plant growth promotion or defence of insect from natural enemies) may also affect community structure and the whole food web (McLean et al. 2016). On the other hand, even in the absence of direct effects associated with phytoplasmas (e.g. in asymptomatic plant hosts or in vectors), the pathogen presence could lead to indirect host injuries due to immune stimulation, which may in turn alter the microbiome composition. Similar indirect interactions were reported for insect-vectored human pathogens (Saldaña et al. 2017).

Such complex interaction could be exploited to develop microbe-based control strategies against phytoplasma-borne diseases, by applying the general concept of microbial resource management (MRM) which is defined as the resolution of practical problems by managing complex microbial systems and their associated metabolic capabilities (Crotti et al. 2012) to insect symbionts and endophytes. Many symbiotic traits could be exploited for control purposes. For example, in insects, the defensive role of microbial associates against pathogens is regarded to as a promising phenotype leading to vector competence reduction (Saldaña et al. 2017). In plants, direct antagonism against pathogens, e.g. by production of antibiotics, biocide compounds or lytic enzymes (Strobel et al. 2004; Toklikishvili et al. 2010) has been recorded. Furthermore, indirect antagonism may be displayed, such as interference with the *quorum sensing* of the pathogen or activation of defence mechanisms in the host (ISR, SAR), as well as influence on the methylation of DNA in the plant host, and therefore the expression of genes (Lugtenberg and Kamilova 2009;

Forchetti et al. 2010; Mauch-Mani et al. 2017). Moreover, the positive influence on the fitness of the host plant, promoting its growth, may provide further indirect effects on pathogen infection.

Examples of interactions that could potentially be manipulated include a diverse array of host-microbiome systems. An endophytic strain of *Bacillus subtilis* was shown to have antagonistic effects on symbionts of the Colorado potato beetle *Leptinotarsa decemlineata* Say, namely *Enterobacter* ssp. and *Acinetobacter* ssp., resulting in increased insect mortality (Sorokan et al. 2016). In the brown planthopper *Nilaparvata lugens* (Stål), a symbiotic strain in the genus *Enterobacter* inhibits the rice pathogen *Xanthomonas campestris* pv. *oryzae* by producing the antimicrobial compound andrimid (Fredenhagen et al. 1987). In tsetse flies *Glossina morsitans morsitans* Westwood, the primary symbiont *Wigglesworthia glossinidiae* triggers host expression of peptidoglycan recognition protein, with a consequent anti-trypanosome activity that confers resistance against the agent of the sleeping sickness (Wang et al. 2009). Finally, a mutual exclusion was reported between the endophyte *Methylobacterium mesophilicum* and the pathogen *Xylella fastidiosa* in citrus trees (Azevedo et al. 2016). In cases where pest control strategies are already available, the knowledge of symbiotic relationships may offer additional advantages. As an example, the well-known entomopathogenic fungi of the genera *Metarhizium* and *Beauveria* are endophytes colonizing the roots of many plants. This dual life style could provide advantages for control applications (Barelli et al. 2016).

A field where the use of symbionts for control purposes have been already developed is that of vector-borne human diseases. Symbiotic control strategies were proposed against protozoan and viral pathogens, based on the use of both wild type and engineered microorganisms. The latter case falls in an approach known as paratransgenesis, which is the genetic manipulation of symbionts to produce anti-pathogen factors. Alternatively, symbiotic microbes may be transformed to express RNA interference for silencing of essential genes, inducing insect mortality (Saldaña et al. 2017). The first symbiotic control efforts were made for the control of Chagas disease, by manipulating a symbiont of *Rhodnius prolixus* (Stål), vector of the pathogen *Trypanosoma cruzi*, to produce anti-trypanosomal molecules (Durvasula et al. 1997). Another extensively developed approach takes advantage of the reproductive manipulator *Wolbachia*, one of the most widespread microbial-associates among arthropods. Strains with antiviral activity have been successfully introduced into the mosquito *Aedes aegypti* L., resulting in vector competence reduction to arboviruses such as *Dengue Virus* (DNV), *Yellow Fever Virus* (YFV), *Chikungunya Virus* (CHIKV), and *Zika Virus* (ZIKV) (Saldaña et al. 2017). Another *Wolbachia*-based strategy is the incompatible insect technique, which exploits the phenomenon of cytoplasmic incompatibility (CI), occurring when crosses between symbiont-infected males and uninfected females fail to produce progeny. Transinfection of the *Wolbachia*-free Mediterranean fruit flies *Ceratitis capitata* (Wiedemann) with a CI inducing strain caused a significant reduction of uninfected populations (Zabalou et al. 2004).

The development of symbiont-based control strategies requires addressing several issues. First, a candidate agent must be selected among those showing relevant

association with the pathosystem, occupying the same niche as the pathogen. Moreover, the microbe must be easily manipulated *in vitro*, and should be efficiently spread among hosts, by vertical and/or horizontal transmission. Additionally, potential control agents must be able to pass subsequent regulatory scrutiny, taking into account their possible impact on vectors and plant hosts, as well as their microbiomes and the ecosystem in which they are included (Alma et al. 2010; Bourtzis et al. 2012).

10.2 Exploiting the Host Microbiome Interference with Phytoplasma Transmission

In light of the diversity of interactions occurring in insects and plants, different strategies may be attempted to design new phytoplasma control methods using microbial symbionts. These efforts should take into consideration different modes of action, including direct toxic effect against the pathogen inside the insect/plant host, vector suppression, unbalancing of vector populations, competitive exclusion, and elicitation of host immunity. Hence, possible approaches should involve: (i) identifying direct anti-phytoplasma or anti-vector activity expressed by endophytes in the plant; (ii) altering the mutualistic exchange between vectors and their primary symbionts, resulting in vector eradication; (iii) exploiting facultative plant or insect symbionts traits to affect phytoplasma vector competence. For *in planta* approaches, both direct anti-phytoplasma effects, related to the production of secondary metabolites, and indirect effects could be considered (Gonzales et al. 2016). For insect-targeted strategies, many aspects should be taken into account to reduce transmission. Vectorial capacity has been reported to be associated with vector density, probability of the vector feeding on a host plant, vector survival and longevity, duration of latent period, and vector competence (Chuche et al. 2017). This means that generalist, very mobile and long-lived insects are theoretically more efficient vectors, especially in case of ancient relationships with vectored phytoplasmas, which can be expected to have led to high vector competence. Thus, any symbiont that may negatively alter insect longevity, mobility or host range may serve as a direct antagonist. Primary and secondary symbionts, as well as reproductive manipulators, could be exploited for this purpose. Primary symbionts are essential for the insect vector to complete its life cycle (Moran et al. 2008) surviving long enough to overcome the required latent period. Secondary symbionts have been reported to influence insect ecology by driving plant choice and governing interactions with stresses (Zhu et al. 2014). Reproductive manipulators may use CI induction, which provides infected females with a fitness advantage, to drive genetic elements associated with disease control into populations (Turelli and Hoffmann 1999).

In this chapter, an overview of the microbial associates of phytoplasma vectors (leafhoppers, planthoppers, and psyllids) and host plants will be provided, and the potential role of microbes for developing new disease control strategies will be

addressed. Focus will be given to symbiotic bacteria and fungi, as very little information is available concerning beneficial interaction between viruses and phytoplasmas hosts/vectors, even though they deserve attention (Roossinck 2011, 2015), being a possible alternative source for symbiont-based control strategies.

10.3 Symbiotic Microbes in Insect Vectors of Phytoplasmas

All phytoplasma insects vectors belong to the Hemiptera. Members of this order host a relatively low microbial diversity, compared to that of other insects (Jing et al. 2014). Moreover, since the plant saps they feed on lack necessary nutrients such as essential amino acids, they rely on primary symbionts to complement their diets (Baumann 2005). Due to this mutual interdependence, host and symbiont(s) underwent a strict coevolution, resulting in a marked gene loss in the latter. Primary endosymbionts of Hemiptera, especially in Auchenorrhyncha and Sternorrhyncha, are the organisms with the most reduced genomes (Latorre and Manzano-Marín 2017), which had often lost genes that are considered to be essential for basic cellular functions (Sloan and Moran 2012). Additionally, both Auchenorrhyncha and Sternorrhyncha have facultative associations with secondary symbionts, which have been reported not only to provide additional fitness benefits, but also, in some cases, to complement the nutrient supply of primary symbionts, where some metabolic pathways have been lost. For this reason, secondary symbionts often display typical traits of obligate mutualism, like intracellular life style or high levels of vertical transmission, while their horizontal transmission rates are low. On the other hand, Hemiptera are generally inhabited by a limited number of gut bacteria, reflecting the relatively low microbial diversity of their feeding substrates, i.e. plant saps. Among phloem feeders, Sternorrhyncha host less gut bacteria than Auchenorrhyncha, because of their feeding mode and gut anatomy (Jing et al. 2014; Morrow et al. 2017). Despite the reduced incidence of those microorganisms that are ingested by hemipteran hosts during the feeding process, in phytoplasma vectors a certain level of microbial exchange between insect and plant occurs, as suggested by their vector role itself. Single endophytic species or even whole endophytic bacterial communities can be transferred from plant to plant by vectors (Raddadi et al. 2011; Lòpez-Fernàndez et al. 2017; Iasur-Kruh et al. 2017); nevertheless vectors are not able to indistinctively host and transmit all bacterial endophytes, resulting in a transmission selectivity which may shape the plant microbiome structure (Lòpez-Fernandez et al. 2017). Beside mutualists and commensals, hemipteran insects are colonized by well-known reproductive manipulators, such as *Wolbachia*, *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Arsenophonus*; however to date it is still unknown whether one or more of these bacteria manipulates their host's reproduction (Marzorati et al. 2006; Gonella et al. 2011; Jing et al. 2014; Morrow et al. 2017; Iasur-Kruh et al. 2017). The microbial communities affiliated with hemipteran phytoplasma vectors, namely cicadellids, cixiids, and psyllids are described below.

10.4 Bacteria

Bacteria in Auchennorrhyncha All Auchenorrhyncha share a Bacteroidetes primary symbiont, '*Candidatus* Sulcia muelleri' (hereafter *Sulcia*), strictly co-evolved since their separation from the other Hemiptera (Moran et al. 2005). Due to the long co-speciation, the evolution pressure on *Sulcia* led to a considerable genome reduction, which resulted in functional modifications within the host. Indeed, even though *Sulcia* are able to provide their hosts with most of the essential amino acids, they lost the genetic machinery for histidine and methionine synthesis. The latter are produced by an array of other obligate symbionts, named co-primary symbionts, residing in bacteriocytes other than those occupied by *Sulcia*, which complement the metabolic pathways missing in this bacterium (McCutcheon and Moran 2010). All co-primary symbionts are thought to derive from a common β-proteobacterial ancestor (named beta-symb) (Koga et al. 2013), which have been replaced by different symbionts multiple times (Bennet and Moran 2013). Described co-primary symbionts of phytoplasma vectors include the β-proteobacteria '*Candidatus* Nasuia deltocephalinicola' (hereafter nasuia) in the subfamily Deltocephalinae of Cicadellidae, and '*Candidatus* Vidania fulgoroideae' (hereafter vidania) in Cixiidae and other fulgorid planthoppers (Bennet and Moran 2013). It has been suggested that vidania and nasuia, as well as their relative '*Candidatus* Zinderia insecticola', the co-primary symbiont of spittlebugs, are subclades of beta-symbiont co-diversified for more than 200 million years with their hosts, contrarily to other hosts, where beta-symbiont was replaced by α- or γ- proteobacteria (Bennet and Moran 2013). Very little information is available concerning vidania symbionts, because their genomes have not been sequenced yet; conversely, nasuia was reported as the organism with the smallest bacterial sequenced genome (Bennet and Moran 2013). The first studied co-primary symbionts of leafhoppers were those associated with *N. cincticeps*, the vector of rice yellow dwarf phytoplasma, and *Matsumuratettix hiroglyphicus* (Matsumura), transmitting sugarcane white leaf phytoplasma (Noda et al. 2012; Wangkeeree et al. 2011, 2012). However, most subsequent work was focused on the genus *Macrosteles*. In *Macrosteles striifrons* Fieber, *M. sexnotatus* (Fallen), *M. laevis* (Ribaut), and *M. quadrilineatus* DeLong & Caldwell, sulcia and nasuia were found in host bacteriomes with close to 100% prevalence, and were transovarially transmitted (Ishii et al. 2013; Bennet and Moran 2013; Kobiałka et al. 2016). Moreover, Kobiałka et al. (2016) reported that sulcia cells in the bacteriocytes of *M. laevis* harbour γ-proteobacteria of the genus *Arsenophonus*. On the other hand, the co-primary symbionts of Deltocephalinae were not found in *Scaphoideus titanus* Ball, vector of "flavescence dorée" (FD) phytoplasma. The main bacterial symbionts in this leafhopper are in the genera *Cardinium* and *Asaia*. Both symbionts display high prevalence in *S. titanus* populations, and are vertically and horizontally transmitted (Marzorati et al. 2006; Sacchi et al. 2008; Crotti et al. 2009; Gonella et al. 2012, 2015). While *Cardinium* resides intracellularly in the fat body, salivary glands and gonads of the insect, being transovarially transmitted through

bacteriocytes in the ovaries (Sacchi et al. 2008), *Asaia* extracellularly colonizes guts, male and female reproductive systems and the salivary glands, and vertical transmission occurs through egg smearing (Crotti et al. 2009). Moreover, *Cardinium* can be horizontally transmitted through plants by *S. titanus* to different phloem sap-feeding leafhoppers, including the phytoplasma vector *Macrosteles quadripunctulatus* Kirschbaum (Gonella et al. 2015). Similarly, *Asaia* is horizontally transmitted among *S. titanus* individuals both by co-feeding and venereal transmission (Gonella et al. 2012). The role of *Cardinium* and *Asaia* in *S. titanus* has not been elucidated yet; however, their dominance in its microbiome, along with their capability to exploit multiple pathways for the colonization of the leafhopper's body, suggest that they could be obligate mutualist microorganisms. Besides primary symbionts, bacterial associates to leafhopper vectors include α-proteobacteria known to alter their hosts' reproduction, such as *Wolbachia, Arsenophonus* and *Rickettsia*; nonetheless at present no sexual manipulation have been recorded for these insects (Iasur-Kruh et al. 2011; Noda et al. 2012; Ishii et al. 2013). Additionally, some bacteria were reported, for which a role in transition from pathogenic to beneficial have been suggested. In *Euscelidius variegatus* Kirschbaum, natural vector of 16SrI (aster yellows) phytoplasmas, an endosymbiont named BEV displays symbiotic traits, but also features that are typical of pathogenic agents, such as the reduction of host longevity and fecundity (Cheung and Purcell 1999; Degnan et al. 2011). In *Orosius orientalis* (Matsumura), vector of phytoplasmas belonging to different ribosomal groups, a bacterium in the genus *Rickettsiella* was found with relatively high prevalence and was suggested to be vertically transmitted (Iasur-Kruh et al. 2011). Even though these traits support its non-pathogenic role, the genus *Rickettsiella* was described to include several pathogens of arthropods (Bouchon et al. 2011). Finally, some gut bacteria have been reported. *Spiroplasma*-like organisms were found in the gut lumen of several leafhoppers, including the phytoplasma vector *M. quadrilineatus*. These bacteria are non-pathogenic for the insect host, and can be horizontally transmitted by the oral way, causing no disease symptom in plants exposed to infected leafhoppers (Ammar et al. 2011). In *S. titanus*, many bacteria of endophytic origin colonize the alimentary canal, as they are transmitted from grapevine to grapevine through the insect. The fact that such transmission is selective suggests that only a portion of plant microbiome is able to stably colonize the leafhopper gut as well; however the specific interaction between grapevine endophytes and *S. titanus* has not been elucidated yet (Lòpez-Fernàndez et al. 2017).

Limited knowledge is available concerning the bacterial microbiomes of planthopper vectors. The most well studied model is *Hyalesthes obsoletus* Signoret, a major vector of phytoplasmas in the "stolbur" group (16SrXII-A). The co-primary symbionts sulcia and vidania were first identified by Gonella et al. (2011) in the gut, testicles, and oocytes of adult *H. obsoletus*, as well as in other species of the genera *Hyalesthes* and *Reptalus*. Then, Urban and Cryan (2012) demonstrated that the association with vidania is dated at the time of the diversification of the superfamily Fulgoroidea, and co-evolution with hosts and sulcia occurred. Other major symbionts detected in *H. obsoletus* include '*Candidatus* Purcelliella penstastirinorum', a

Fig. 10.1 A dividing *Dyella*-like bacterium (DLB) viewed under a scanning electron microscope. The length of one bacterium is about 1 μm (Courtesy of G. Mordukhovich and N. Mozes-Daube)

γ-proteobacterial primary symbiont, and bacteria known as reproductive manipulators such as *Wolbachia*, *Cardinium*, and *Rickettsia* (Gonella et al. 2011). Sexual manipulators seem to be common in planthoppers, as *Wolbachia*, *Cardinium*, *Rickettsia* and *Arsenophonus* were observed in other phytoplasma vectors as well, namely the cixiid *Haplaxius crudus* (Van Duzee), the flatid *Ormenaria rufifascia* (Walker), and the derbid *Omolicna joi* Wilson, Halbert and Bexine (Powell et al. 2015). Among other facultative bacteria is a cultured bacterium closely related to the genus *Dyella*, initially named a *Dyella*-like bacterium (DLB) and assigned to the Xanthomonadaceae (Iasur-Kruh et al. 2017) (Fig. 10.1). DLB is a gut bacterium, not vertically transmitted, but acquired, probably from plants, especially during the fall.

Bacteria in Sternorrhyncha The only group of phytoplasma vectors among the Sternorrhyncha is the family Psyllidae within the superfamily Psylloidea. All insect vectors known so far belong to the genus *Cacopsylla* (Alma et al. 2015). Like all psyllids, *Cacopsylla* species host the primary symbiont '*Candidatus* Carsonella ruddii' (hereafter *Carsonella*) (Thao et al. 2000; Raddadi et al. 2011; Cooper et al. 2015). Similarly to sulcia, carsonella has a long co-evolution history with hosts. The consequent strong genome reduction led this bacterium to loose many genes essential for its own survival. Hence, each psyllid species additionally harbours in the bacteriomes one or more secondary symbionts, generally belonging to γ-proteobacteria, including species in the genera *Arsenophonus* and *Sodalis* (Morrow et al. 2017). These symbionts show typical traits of obligate mutualism being as essential as carsonella for their hosts (Sloan and Moran 2012). As expected, carsonella was found in *Cacopsylla pyri* L. and *C. pyricola* (Förster), which are the

only vector species that have been screened for microbiome studies thus far (Raddadi et al. 2011; Cooper et al. 2015). In both *C. pyri* and *C pyricola*, carsonella was detected with high prevalence along with bacteria related to the genus *Arsenophonus* (Raddadi et al. 2011; Cooper et al. 2017), which could be the major secondary symbiont. A common trait observed in *C. pyri* and *C. pyricola* is the association with organisms related to disease agents, which are thought to act as facultative endosymbionts in these hosts. In *C. pyri*, the α-proteobacterium '*Candidatus* Liberibacter europaeus', closely allied to psyllid-borne plant pathogens associated with economically important diseases such as huanglongbing and zebra chip disease in citrus and in potatoes respectively (Wang et al. 2017), was detected with relative high prevalence and density (Raddadi et al. 2011). This bacterium was found in the ovaries and salivary glands of *C. pyri*, indicating vertical and horizontal transmission; moreover, it was successfully horizontally transmitted to pear, without causing disease symptom in inoculated plants (Raddadi et al. 2011). '*Ca*. L. europaeus' was detected in European populations of other psyllids, including *C. pyricola* and other insect phytoplasma vectors (Camerota et al. 2012); however it was not recorded in North American populations of *C. pyricola* (Cooper et al. 2017). On the other hand, in these populations high prevalence of '*Candidatus* Phytoplasma pyri', the agent of pear decline, were observed, suggesting that the plant pathogen could show endosymbiotic traits (Cooper et al. 2017). Finally, none of the studied vector species was shown to harbour reproductive manipulators like *Wolbachia* or *Rickettsia* (Raddadi et al. 2011; Cooper et al. 2017), even though these microbes have been described in other psyllids (Morrow et al. 2017). Further investigations of the occurrence of *Rickettsiales* sexual manipulators in psyllids vectors of phytoplasmas are needed to clarify if there is a negative correlation between pathogen infection and the presence of these bacteria.

10.5 Yeast-Like Symbionts

In contrast to bacterial symbionts, limited investigations have been carried out on the fungal partners of phytoplasma vectors. To our knowledge, the FD-transmitting leafhoppers *S. titanus* and *Orientus ishidae* (Matsumura) are the only phytoplasma vectors in which fungal symbionts have been identified (Sacchi et al. 2008; Kobiałka et al. 2018). Specifically, in *S. titanus* yeast-like symbionts (YLSs), members of the class *Sordariomycetes* (= *Pyrenomycetes*), are localized in the mycetocytes formed from the fat bodies and in the ovaries, both in follicular cells and eggs. The latter evidence has suggested the existence of a transovarial transmission of the symbionts to the offspring (Sacchi et al. 2008). However, there is no available information on the role of the YLSs in this host. Similarly to what observed in *S. titanus*, in the brown planthopper *N. lugens*, the most destructive phloem feeding pest of rice (*Oryza sativa*), YLSs have been detected in the fat bodies and ovaries and showed to use uric acid, a waste for the insect, as a nitrogen resource (Noda et al. 1995; Hongoh and Ishikawa 1997; Chen and Hou 2001). From the analysis of the

planthopper and YLS genome sequences it has been deduced that the ability to recycle the uric acid is complementary between the host and the fungal partner, which showed also the presence of an interdependent system for steroid biosynthesis. Moreover, researchers have inferred that the symbiont is able to provide the planthopper with essential amino acids absent in the phloem sap and not synthetized by the insect (Xue et al. 2014). A further phylogenomic analysis of the YLS genome supported its close relationship with entomopathogens and estimated its divergence from the ancestral species occurred approximately 99–203 million years ago (Fan et al. 2015). A symbiotic replacement of ancient bacteria with YLSs have been also hypothesized by Fan et al. (2015), similarly to what observed in other insects (Nishino et al. 2016; Vogel and Moran 2013). For instance, in the members of Cerataphidinae (Sternorrhyncha) clade, e.g. the aphid *Cerataphis brasiliensis* Hempel, *Buchnera* has been supplanted by a fungal symbiont (Fukatsu and Ishikawa 1992; Vogel and Moran 2013). The YLSs present in Delphacidae insects were proposed to be named '*Candidatus* Entomomyces delphacidicola' (Fan et al. 2015). Intracellular YLSs have been also found in the fat bodies of other Delphacidae planthoppers, i.e. *Sogatella furcifera* Horváth, *Laodelphax striatellus* Fallén, and in other Cicadellidae (subfamily Deltocephalinae) leafhoppers, i.e. *Fieberiella septentrionalis* Wagner, *Graphocraerus ventralis* Fallén, *O. ishidae* and *Cicadula quadrinotata* Fabricius (Noda et al. 1995; Kobiałka et al. 2018). Particularly, in *C. quadrinotata* numerous YLSs have been also localized in the cells of the midgut epithelium (Kobiałka et al. 2018). As mentioned above, the Asian species *O. ishidae*, supposed to be introduced first in US and then in Europe in the last century, has been recently described as a vector of FD phytoplasma in grapevines (Lessio et al. 2016). Microscopic observations suggested also the presence of a vertical (transovarial) transmission of YLSs in *F. septentrionalis*, *G. ventralis* and *O. ishidae*, while their inheritance in *C. quadrinotata* remains unknown (Kobiałka et al. 2018).

10.6 Interactions with Phytoplasmas

Although in recent years the work dedicated to the microbiome of phytoplasma insect vectors has consistently increased, a few studies directly investigated the interaction between microbial symbionts and these plant pathogens. At present, the most studied model is represented by *H. obsoletus* and its facultative symbiont. A previously un-described bacterium was isolated from that leafhopper and was temporarily named a *Dyella*-like bacterium (DLB) (Iasur-Kruh et al. 2017). Although it has been isolated from an insect, the newly described DLB possesses endophytic properties. In the wild bush *Vitex agnus-castus* it can be found virtually all year round, and the profile of the sugars it can utilize as food sources resembles that of the phloem. When applied exogenously, DLB can colonize the phloem of at least nine plant species, including annual and perennial crops such as grapevines, carrots and citrus. When sprayed on host plants it seems to penetrate the leaves via the

stomata, and can be detected up to 4 weeks post application in un-treated leaves both above and under the treated plant part (Lidor et al. 2018).

The unique ability of DLB to enter through leaves and colonize the vascular system of plants, provides it with the opportunity to interact with phytoplasmas. Indeed, when that bacterium was introduced into phytoplasma-infected grapevine plantlets, its presence significantly reduced the disease symptoms, including marked effects on the number of internodes and shoots, as well as plant and leaf length, to the level of healthy ones (Iasur-Kruh et al. 2018). These authors further report that bacterial introduction into healthy grapevine plantlets, did not affect any of the plant parameters tested. The mode of action used by DLB to reduce phytoplasma symptoms is yet unknown. Potential mechanisms used by beneficial bacteria for supressing phytopathogens have been listed in Eljounaidi et al. 2016. The influence of DLB on infected plants could be: (i) it competes with phytoplasma for nutrients and/or suitable colonization niches; (ii) it stimulates the plant's ISR; (iii) it secretes PGRs that enhance plant growth; (iv) it secretes substances that inhibit phytoplasma growth. So far, competition over essential metabolites was ruled out using genome-based systems biology tools (Iasur-Kruh et al. 2018). The production of phytohormones such as auxin does not seem likely based on the fully sequenced genome, and similarly, no evidence for genes involved in ISR or plant growth enhancement in general was detected (Lahav et al. 2016). The possible secretion of antimicrobial substances gained support by the finding that supernatant in which DLB was grown, inhibits the growth of *Spiroplasma melliferum*, a cultured mollicute that has been previously used as a model for phytoplasma inhibition studies (Naor and Zahavi 2011; Iasur-Kruh et al. 2017). The genome analysis revealed several secondary metabolites with potential antimicrobial activities including bacteriocin, terpenes and non-ribosomal peptide synthetases (NRPS) clusters (Lahav et al. 2016). The compounds responsible for the symptom reduction, as well as other mechanisms that might be involved in the phenomenon are yet to be determined.

Further study on the interaction between a microbial symbiont and a phytoplasma in an insect vector was recently carried out in the model leafhopper *E. variegatus*, which is an efficient vector of FD phytoplasma to broad bean in laboratory conditions. This insect was shown to be a suitable host for symbiotic *Asaia* strains isolated from mosquitoes and exhibiting different phenotypes (Gonella et al. 2018), similarly to *S. titanus*, which transmits FD phytoplasma to grapevine in the field (Crotti et al. 2009). FD phytoplasma transmission trials, using *E. variegatus* nymphs previously colonized by an exogenous *Asaia* strain producing an air-liquid interface biofilm (Fig. 10.2), showed that leafhoppers were significantly less infected by the pathogen with respect to insects not exposed to *Asaia*. However, the capability of transmitting the phytoplasma by *E. variegatus* specimens where the pathogen's infection succeeded was not divergent in insects colonized by the exogenous *Asaia* strain and control individuals. The alteration of the efficiency of phytoplasma to cross the gut barrier to spread in the whole leafhopper body was suggested, even though the mechanisms regulating this interference remain to be elucidated (Gonella et al. 2018).

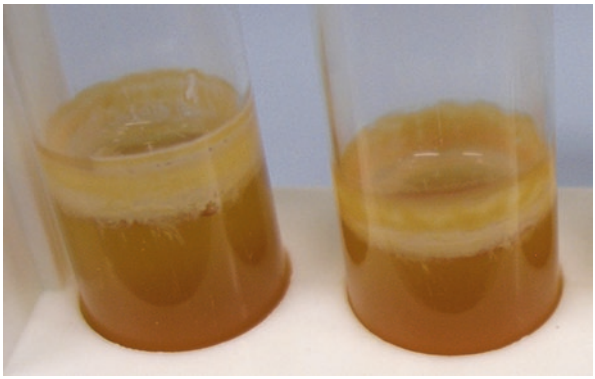

Fig. 10.2 Air-liquid interface biofilms produced by *Asaia* isolates when cultured in static condition. The isolates were inoculated in tubes containing the modified PDB medium (Gonella et al. 2018) and cultivated for 10–12 days

10.7 Endophytic Organisms in Phytoplasma Host Plants

In the plant, phytoplasmas share their habitat with other microorganisms, called endophytes. This term was used for the first time at the end of nineteenth century and its exact definition is still debated. The most common definition of endophytes is based on the description given by Hallmann et al. (1997), as "those (bacteria) that can be isolated from surface-disinfected plant tissue or extracted from within the plant, and that do not visibly harm the plant". However, this definition has some drawbacks. First, it does not account for endophytes that are uncultured. Indeed, the recent advances of new generation sequencers – that do not require the isolation and cultivation of the microorganisms – proved how plants are characterized by a "microbiota" composed by the bacteria and fungi that live inside it, as well as a "viroma", composed by the viruses present in the individual, that can be influenced by several factors, biotic, abiotic, or environmental (Bulgari et al. 2014; Campisano et al. 2014). Moreover, it is not always easy to assess phytopathogenicity and distinguish latent pathogens from endophytes, particularly for non-cultured fungi that are part of the microbiome community.The debate is supported by the fact that it has been known that in particular conditions there are pathogens that can lose their virulence (van Overbeek et al. 2004) or microorganisms considered beneficial that caused the development of severe symptoms (Kloepper et al. 2013). Therefore, a further endophyte definition has been proposed in a recent review, suggesting that the term "endophyte" should refer only to the habitat, not to the function, so the term should include "all microorganisms which for all or part of their lives, colonize the internal tissues of plants" (Hardoim et al. 2015), referring to the nature of the plant-endophyte interactions which can range from mutualism (positive effect) to pathogenicity (negative effect). An even more recent definition by Le Cocq et al. (2017) is similar to the definition of Hallmann et al. (1997), but considers all contributing microbes: "endophytes are microbes which occur within plant tissue for at

least part of their life cycle without causing disease under any known circumstances", meaning that some microbes may be currently considered endophytic, but this designation may be changed if it is subsequently proved that these microbes are harmful to the host plant.

Nowadays, it is well established that plants are inhabited by many types of microbial endophytes including bacteria, fungi, archaea, and unicellular eukaryotes (algae and amoebae) (Hardoim et al. 2015). Endophytes play many important beneficial roles in the metabolism and physiology of the host plant, including fixing atmospheric nitrogen, solubilizing phosphates, synthesizing plant-growth hormones, degrading toxic compounds, inhibiting or antagonizing fungal and bacterial pathogens (Yuan et al. 2017). On the other hand, the internal plant tissues provide a protective environment for endophytes, which colonize an ecological niche similar to plant pathogens, especially the vascular plant pathogens (Hallmann et al. 1997). The exploitation of the endophytic communities would allow to identify potential strains that could be applied as biocontrol agents useful to control also phytoplasma diseases.

Bacteria The first studies on the composition of bacterial community in relation to phytoplasma infections were carried out by Mocali and colleagues in 2003, analyzing elm plants that were either healthy or infected by elm yellows (EY) phytoplasmas. The results, achieved by identification and comparison of the bacterial isolates obtained starting from secondary roots and 2-years-old branches, highlight the high level of heterogeneity of the bacterial community present in the phloem tissues of elm trees. The differences in the composition of the bacterial community between healthy and infected trees are evident when the data are analyzed on the basis of the different season (April–June, with warm weather, or September–December, with cold weather) or on the basis of the starting organ (roots or branches). In both cases, the highest variability of bacterial species is registered in the healthy plants compared to the infected plants, and the most abundant genera among the identified bacteria are *Arthrobacter*, *Bacillus*, and *Streptomyces*. Data on the fluctuation of the bacterial communities in relation to the sanitary status of the plant are also reported in the works of Bulgari et al. (2011, 2014) in which a lower richness of bacterial species is registered in grapevine plants infected by phytoplasma associated with FD and in those that underwent spontaneous recovery from this disease, compared to the healthy grapevine plants. In particular, the endophytic bacteria identified in grapevine (Bulgari et al. 2009) are for the most part γ-Proteobacteria, in particular Enterobacteriaceae, and belong to the species *Pantoea agglomerans*. This bacterial species, already reported as a grapevine endophyte (Bell et al. 1995), is capable of activating the defence systems of the plant (Ortmann et al. 2006) and is therefore potentially useful for the control of pathogens, such as phytoplasmas. Other bacteria identified in the same study belong to the genera *Bacillus*, *Methylobacterium, Burkholderia*, *Paenibacillus* and *Agrobacterium*, which were already reported as part of the endophytic community of plants (Hardoim et al. 2015). An analysis using NGS of the same plants identifies bacteria of the genera *Burkholderia* and *Acinetobacter* to be mostly associated with healthy plants, while those of the

Methylocystaceae and Oxalobacteraceae families to be more present in the infected plants (Bulgari et al. 2015). The microbial communities of plants that underwent recovery were instead abundant in bacteria of the genus *Methylobacterium*. Analogous studies were carried out by the same authors to identify the endophytic community present in the roots of apple trees infected by '*Ca.* P. mali', showing symptoms of apple proliferation or asymptomatic (Bulgari et al. 2012). The use of cultivation-dependant methods allowed the identification of bacteria of the genera *Lysinibacillus* and *Paenibacillus* only in healthy plants, while *Bacillus* was detected in both infected and healthy plants. The cultivation-independent methods allowed to identify bacteria belonging to the classes β-proteobacteria, γ-proteobacteria both in infected and healthy plants, while bacteria belonging to α-Proteobacteria were more abundant in the healthy roots. The results obtained in apple confirm that in most cases, endophytes belong to the Proteobacteria phylum (Trivedi et al. 2010; Li et al. 2011; Hardoim et al. 2015).

Recent studies (Morales-Lizcano et al. 2017) describe the bacterial community associated with different organs (leaves, trunk) and in the rhizosphere of coconut palm trees affected by Côte d'Ivoire lethal yellowing (CILY). This study highlights the presence of different genera in different geographic areas (*Bacillus* in samples from Brafferdon, *Burkholderia* in samples from Yaokro), hypothesizing that the difference in the composition of the community is driven by the presence of phytoplasmas in all the samples from Yaokro, and the absence of the pathogen in the samples from Brafferdon, thus confirming the data reported (Bulgari et al. 2014). The most abundant genera found in the rhizosphere are *Bacillus*, *Burkholderia*, and *Pseudomonas*. In particular, *Burkholderia* and *Neodeightonia* were the most abundant bacteria identified from the rhizosphere in Yaokro, and *Burkholderia* was mostly isolated from symptomless palm trees.

Fungi The microbiome structure of endophytes in a single plant is usually composed by numerous and systematically diverse species of fungi, whose number and species composition is influenced by factors such as the environment, plant physiology, anthropogenic factors and pathogen infections (Varanda et al. 2016). Hardoim et al. (2015) determined that endophytes mainly belong to the Glomeromycota (40%), Ascomycota (31%), Basidiomycota (20%), unidentified phyla (8%), and, to a lesser extent, Zygomycota (0.1%) through the establishment of a data set of eukaryotic endophytic full-length internal transcribed spacer (ITS) regions with a total of 8439 sequences retrieved from the National Center for Biotechnology Information (NCBI) nucleotide database. The phylum Glomeromycota only comprises endophytes known as arbuscular mycorrhizal fungi (AMF), most of which (39%) can be assigned to the class Glomeromycetes, whose members form ubiquitous endosymbioses with most land plants and are of relevant ecological and economic importance. Among the Ascomycota, a large number of endophytes are identified in the class Dothideomycetes (15%), which contains many species of the genera *Alternaria* and *Epicoccum*. Many members of the class Sordariomycetes (9%) are endophytes, such as species of the genera *Balansia*, *Epichloë*, *Nemania*, *Xylaria*, and *Colletotrichum*. Among the Basidiomycota, the class Agaricomycetes

(18%) contains a large number of endophytes, mainly mushroom-forming (basidiome) fungi causing wood decay, white and brown rot saprotrophs, and the beneficial ectomycorrhiza (EMC) symbionts.

In grapevine the endophytic fungal community has been studied intensively with either culture-dependent or –independent methods and showed some very interesting and important results (Musetti et al. 2006; Martini et al. 2009; Grisan et al. 2011; González and Tello 2011; Pancher et al. 2012; Varanda et al. 2016; Pinto et al. 2014; Pinto and Gomes 2016). In these studies, the predominance of ascomycetes fungi over the basidiomycetes was found consistently with additional endophyte studies concerning other woody plants (Arnold 2007). Grisan et al. (2011) discovered 56 fungal endophytes grouped in OTUs on the bases of PCR/RFLP analyses of ITS region. The 27% of OTUs were obtained by culture-dependent method, the 48% by culture-independent method, and the 25% by both methods. Furthermore, the collected data revealed that fungal endophytes belonging to genera *Alternaria*, *Phoma*, *Epicoccum*, *Aureobasidium*, *Cladosporium*, *Pestalotiopsis*, and *Pestalotia* constituted respectively about the 89% of isolates obtained by the culture-dependent method, and the 79% of total clones obtained from the culture-independent method. Overall within Ascomycota the Dothideomycetes were the most representative and within those, the most representative order was Pleosporales; this is mainly due to *Alternaria* and *Epicoccum* species. Other important orders were Dothideales (mainly due to *Aureobasidium pullulans*), Capnodiales, Xylariales, Helotiales. *Alternaria*, *Epicoccum* and *Aureobasidium* species are the most frequent among fungal endophytes in grapevine, as well as in other plants (Pinto and Gomes 2016; Varanda et al. 2016) and have been studied as promising biocontrol agents. The effect of different cultivars and different viticulture managing practices on fungal community structure have been deeply studied by several authors. The composition of fungal endophytic communities did not show significant differences among cultivars, but differences were observed between fungal communities isolated from grapevines under biological or conventional management (Pinto and Gomes 2016; Varanda et al. 2016). These differences may be related to the use of chemical/organic products that directly affect microorganisms, or to alterations in plant physiology and consequently in plant- associated microorganisms. The diversity of culturable fungal community (together with the bacterial one as described above) was recently assessed also in coconut palms infected and non-infected by the CILY phytoplasma (Morales-Lizcano et al. 2017). Fungal microbes were isolated from leaves, trunk and rhizosphere samples and fungal endophytes identified in either Braffedon or Yaokro vary upon the microhabitat and location. The dominant fungal classes in Yaokro corresponded to Saccharomycetes for the symptomless palms and Dothiomycetes, Urediniomycetes and Sordariomycetes in symptomatic palms; whereas in Braffedon, the fungal dominant class was Saccharomycetes. Results show that there is more diversity for the fungal endophytic communities in CILY phytoplasma-infected coconut palms, which suggests that the presence of the CILY phytoplasma may influence also the composition of the fungal endophytic community.

10.8 Interactions with Phytoplasmas

The interaction between different bacterial and fungal strains of endophytic origin has been extensively investigated. Recent studies were carried out regarding the bacterial strain S1Pf1Rif of *Pseudomonas putida* (Gamalero et al. 2010; D'Amelio et al. 2011) isolated from soil near symptomless grapevines in vineyards where grapevine yellows disease was present. The effect of this bacterial strain was evaluated both when inoculated alone, or in association with a mycorrhizal fungus (*Glomus moasseae*), in plants of *Chrysanthemum carinatum* infected with chrysanthemum yellows (CY – '*Ca.* P. asteris'). The effects of the treatments show that, while the concentration of the phytoplasma in the host plant is not affected, the plants treated with the strain S1Pf1Rif and infected with the phytoplasma have total fresh weight, shoot fresh weight, number of leaves, and shoot length similar to that of the healthy control plants. Furthermore, comparison between CY-infected plants with or without S1Pf1Rif treatment, analyzed through electron microscopy, showed that in the treated plants some degenerated phytoplasma cells could be visualized. These results were also confirmed with the use of *P. putida* S1Pf1Rif together with *G. mosseae* (D'Amelio et al. 2011); with this treatment the number of vital cells of '*Ca*. P. asteris' was lower than in the control plants at 17 days after inoculation, while at 24 days there was no difference between treated and not treated plants. The authors did not investigate the mechanism behind the increased temporary tolerance to the pathogen after treatment with *P. putida* S1Pf1Rif, but do hypothesize that the production of IAA from the strain could balance the reduced endogenous auxin production due to the '*Ca*. P. asteris' virulence factor TENGU (D'Amelio et al. 2011). This hypothesis is in line with the results obtained from older studies that demonstrated how treatment with exogenous IAA could induce recovery in periwinkle plants infected by phytoplasma in *in vitro* conditions (Ćurković Perica 2008). *G. mosseae* was demonstrated to alter the level of defence-related hormones in tomato plants (López-Ráez et al. 2010), and therefore this same mechanism was hypothesized to be at the base of the effect in chrysanthemum. The tomato endophyte *Pseudomonas migulae* 8R6, has been used in the model system *Catharanthus roseus*/FD phytoplasma (Gamalero et al. 2017). The results of this study indicate that the bacterial strain can reduce by 50% the number of symptomatic plants, although it does not affect the concentration of the pathogen, while a mutant of this strain that can no longer encode the ACC deaminase gene does not show this activity. These results suggest that this enzyme, already known to reduce the level of the stress hormone ethylene by converting its precursor ACC into α-ketobutyrate and ammonia, plays a role in the reduction of FD symptoms. Production of this enzyme by plant-growth promoting bacteria, including those displaying endophytic lifestyles, can make host plants tolerant to a number of stresses (Hardoim et al. 2008; Nascimento et al. 2013).

The technical difficulties in obtaining plants infected by phytoplasmas in controlled conditions drew researchers in the field towards alternative model systems in which to test the efficacy of endophytic bacteria in controlling these pathogens. The

in vitro culture of apple shoots infected by '*Ca*. P. mali' allowed to evaluate the effect of bacterial strains isolated from apricot and peach trees that underwent natural recovery from European Stone Fruit phytoplasma (Jarausch et al. 2017). This study identified some endophytes that are able to colonize apple plants but have no effect on the phytoplasma, and others that are able to significantly reduce the concentration of the pathogen in the host plant. Likewise, some micropropagated grapevine plants FD-infected and treated with *Burkholderia* sp. strain R8, isolated from grapevine plants recovered from the disease (Bulgari et al. 2011), showed recovery from the disease (Passera et al. 2015), suggesting an involvement of this strain in the recovery. Furthermore, another bacterial strain isolated from grapevines that underwent recovery from FD, strain R16 of *Paenibacillus pasadenensis* (Bulgari et al. 2011), was shown to have traits typical of plant growth-promoting bacteria, being able to produce IAA and ACC-deaminase, and producing possible biocontrol enzymes such as chitinase (Passera et al. 2017, 2018). In particular, this strain was shown to produce farnesol, among other volatile organic compounds (VOCs), which is able to inhibit the growth of *Botryris cinerea* in *in vitro* conditions. VOCs in general, and terpenes in particular, are important molecules in plant resistance to stresses, as was demonstrated by Salomon et al. (2016). These authors showed that experimental inoculation of grapevine plants with *Kocuria erytromyxa*, *Microbacterium imperiale*, and *Terribacillus saccharophilus*, induced terpene synthesis in the leaves. Moreover, the photo-antioxidant properties of leaf extracts from bacterized plants were enhanced, while the diameter of *B. cinerea*-induced lesions was diminished in bacterized plants infected with the pathogen. These results are in line with the knowledge about VOCs, that can exert long- and short-distance effects on different plant-interacting organisms (Dudareva et al. 2006). Among their diverse roles, these compounds are essential components of the plant's defence repertoire and as airborne defence signals, they can prime defence responses against pathogens in plants distant from the emission source (Kishimoto et al. 2006; Yi et al. 2009).

To study the interactions occurring among fungal endophytes and phytoplasmas, an endophytic strain of *Epicoccum nigrum* (extensively reported as biocontrol agent or resistance inducer) was inoculated in *C. roseus*, chosen as a model phytoplasma host plant and experimentally infected with '*Ca.* P. mali'. The treatment reduced symptom severity and phytoplasma titre inside the plants (2.8 times lower compared to the untreated plants), inducing ultrastructural modifications both to the phytoplasma and to the host. The main cytological modifications were observed in the phloem, with abundant callose depositions, occasionally surrounding degenerated phytoplasma cells; P-protein aggregations were found in sieve tubes and occluding the sieve plates (Musetti et al. 2011). In further experiments, *E. nigrum* was inoculated in apple plants as a possible strategy for apple proliferation disease control (Farhan 2014). This inoculation did not induce disease symptoms (i.e. necrosis, rot) or other visible stress-related responses, confirming the endophytic life-style of this microorganism in the host plant. Ultrastructural analyses confirmed results described in *C. roseus* plants (Musetti et al. 2011). It has been reported that endophyte fungal inoculation could result in the priming expression of a set of stress-related genes or

eliciting stress hormone production compared with uncolonized plants (Sherameti et al. 2008). Molecular interaction between *E. nigrum* and the apple plant were studied by evaluating the expression levels of several selected genes, previously tested by studying recovery in apple trees (Musetti et al. 2010, 2013). Among the genes coding phloem proteins (Farhan 2014), two, *mdPP2-2* and *mdERG1*, were found significantly up-regulated in apple plants 3 day after *E. nigrum* inoculation and two callose synthases genes, *mdCaS2* and *mdCaS5*, resulted up-regulated upon endophyte inoculation. Interestingly *mdPP2-2*, *mdERG1* and *mdCaS5* were reported to be up-regulated in apple trees exhibiting recovery (Musetti et al. 2010). The aggregation of P-protein, which shifts from the unpolymerized to the polymerized form in sieve tubes, is one of the first responses of phloem cells to pH modification (Goleki et al. 1999), which can be correlated with different causes, among which is pathogen attack (Knoblauch et al. 2001). Callose synthesis is a key event among defence plant cell modifications (Skou et al. 1984), which may result in reinforcements of the cell wall at the attempted site of pathogen penetration, in providing a medium for the deposition of toxic compounds, and in blocking the transfer of nutrients from host cells to pathogen. Particularly, *mdCaS5* shows high homology with the *Arabidopsis* gene *atCalS7*, which is responsible for callose deposition in the phloem (Xie et al. 2011), the site where phytoplasmas accumulate and spread. Moreover, four PR genes (*pr1*, *pr2*, *pr5* and *pr8*) were over expressed in *E. nigrum*-inoculated apple trees, supporting the hypothesis of resistance mechanism activation, while jasmonate-pathway related genes resulted down-regulated (Musetti et al. 2013).

10.9 Conclusions and Perspectives

The awareness of microbial relationships in phytoplasma insect vectors and plant hosts has considerably increased in the last years. Recent findings concerning antagonistic interactions occurring in both insects and plants (Musetti et al. 2011; Gamalero et al. 2017; Jarausch et al. 2017; Iasur-Kruh et al. 2018; Gonella et al. 2018) open new insights that could support the current knowledge on the life cycle of phytoplasmas within their hosts, potentially contributing to the development of new tools for disease control. Moreover, even though the number of direct demonstrations of interference between insect symbionts/endophytes and phytoplasmas is still limited, the knowledge that has been gained in recent years concerning the microbiome of several plant species that are phytoplasma hosts and of insect vectors deserves attention. For example, many symbiotic organisms of insects were reported to co-localize with the pathogens in different organs, including gut and salivary glands. Possible interactions have been suggested between these symbionts and phytoplasmas in the insect's body, potentially affecting transmission (Gonella et al. 2011; Ishii et al. 2013). A survey on different vector and non-vector insects in the family Cicadellidae showed that nasuia, the co-primary symbionts of sulcia, was present in most of the vector species, whereas it could not be found in the species

not associated with phytoplasma transmission (Wangkeeree et al. 2012). Based on this evidence, the authors suggested that this or a related symbiont may be required for successful transmission. Conversely, symbionts of the genus *Asaia*, which are associated with the vectors *S. titanus* and *E. variegatus*, as well as with different mosquitoes, have been reported to modulate the immunity of *Anopheles stephensi* Liston (Capone et al. 2013). If this effect is exerted in the leafhoppers as well, the enhanced insect response could be one of the causes of reduced phytoplasma infection observed in *E. variegatus* (Gonella et al. 2018). Similarly, a role in promoting plant growth or induce defence mechanisms could be the cause of the recorded occurrence of different endophytic strains in healthy or recovered plants (Bulgari et al. 2011; Passera et al. 2015).

Much work is still needed in order to fully understand the mechanisms governing phytoplasma dynamics in their hosts, providing additional knowledge that could be exploited for control purposes. One particularly neglected research area is that of insect fungal symbionts. Indeed, YLSs, and, more generally, fungal symbionts, represent an intriguing example of eukaryotic symbiosis. Additionally, besides direct interactions between phytoplasmas and other microbes co-occurring in the same host, the multipartite relationships existing among all of the actors involved in the transmission process should be deeply investigated. For instance, when considering horizontally transmitted microorganisms that can be inoculated into the plant, the possible effects deriving from co-localization between symbionts and phytoplasmas could be exerted both in the insect's body and in the plant following co-transmission. Moreover, since the insect-mediated community shaping of the plant microbiome has been described (Lòpez-Fernàndez et al. 2017), the competition and mutual exclusion among microorganisms within the vector, including the phytoplasma, may alter plant growth and development in addition to directly affecting pathogen transmission. Furthermore, phytoplasma infection in plants was proposed to affect the whole microbiome composition (Morales-Lizcano et al. 2017), and the effects of such an alteration on the plant health status are still unexplored.

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Chapter 11
Integrated Management of Phytoplasma Diseases

Piero Attilio Bianco, Gianfranco Romanazzi, Nicola Mori, Wayne Myrie, and Assunta Bertaccini

Abstract Sustainable approaches to control phytoplasma-associated diseases are of utmost importance. The use of phytoplasma-resistant host plants and of phytoplasma-free material for new plantings could represent a starting point for phytoplasma disease management. The early identification of infected host plants and insect vectors represent necessary tools in preventing epidemics of diseases through the appropriate management of agro-ecosystems subjected to epidemic outbreaks. This approach can be integrated with the use of resistance inducers and of biocontrol microorganisms able to act as against phytoplasmas and/or their insect vectors. Furthermore comparative genomic approach facilitates the identification of candidate genes involved in the interactions with hosts, and together with the cultivation of phytoplasmas is a key point for improving the knowledge of these bacterial pathogens aimed to implement effective integrated management control strategies.

Keywords Phytoplasma associated diseases · Sustainable management · Prevention · Insect vectors · Propagation materials

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https://doi.org/10.1007/978-981-13-2832-9_11

11.1 Introduction

As the biological cycle of phytoplasmas includes plant hosts and insect vectors, the main conventional control strategies are not directed against the pathogen and include the compulsory eradication of the infected plants and the insecticide treatments against the insect vectors (Bianco et al. 2011). These treatments have a strong economic and environmental effect, representing a risk for operators, stakeholders and environment. Thus, sustainable approaches to control phytoplasmas are highly necessary. The use of phytoplasma-resistant host plants and of certified phytoplasma-free material for new plantings could represent a starting point for phytoplasma disease management. The early identification of infected hosts (plants and insect vectors) and molecular epidemiology, based on multiple gene typing and spatial analyses knowledge, allow to obtain accurate information concerning the biological cycle of these pathogens, and represent good tools in preventing epidemics of diseases through the appropriate management of agro-ecosystems subjected to epidemic outbreaks (Mori et al. 2015; Tedeschi et al. 2015; Kosovac et al. 2016). This approach can be integrated with the use of (i) resistance inducers that are molecules (elicitors) able to activate a non-specific form of disease resistance in plants (Romanazzi et al. 2009, 2013); (ii) microorganisms able to act as biocontrol agents against phytoplasmas (Bulgari et al. 2011) and/or their insect vectors (Gonella et al. 2011). Furthermore, the availability of genomic information supports new investigations dealing with phytoplasma biology and their effects in host plants and insect vectors. In detail, comparative genomic approaches facilitates the identification of candidate genes involved in the interactions with hosts (Minato et al. 2014; Quaglino et al. 2015), opening new avenues for the development of effective disease control strategies. Furthermore in the next years, the cultivation of phytoplasmas (Contaldo et al. 2016) should be a key point for improving the knowledge of these bacterial pathogens, to implement novel effective control strategies. In the following paragraphs some examples of integrated phytoplasma disease management are presented in order to illustrate the most effective practices implemented towards some of the most important phytoplasma diseases worldwide.

11.2 Almond Witches' Broom

Almond witches' broom (AlmWB) is a recent and currently geographically restricted disease that can prove to be an interesting case for the management of the emerging phytoplasma diseases. The first descriptions of the disease date back to 1990 in Southern Lebanon, with the detection of '*Candidatus* Phytoplasma phoenicium' in 1999, and the discovery of its association with the disease following in 2001 (Choueiri et al. 2001). As the name almond witches' broom implies, the most recognizable symptom of the disease is the development of witches' broom on the

main crop host of the pathogen, the almond tree. Still, the disease is also associated with other symptoms in almond including yellowing, early flowering, decline and dieback, in general it drastically reduces the productivity of infected plants and eventually kills the host plants in a few years (Abou-Jawdah et al. 2002; Molino Lova et al. 2011). '*Ca*. P. phoenicium' is likewise associated with similar, although slightly less severe, diseases in peach, nectarine, and apricot (Abou-Jawdah et al. 2010; Molino Lova et al. 2011). Taking into consideration the disease's severity on productivity of all the aforementioned crops, and the host range of the pathogen, it was suggested that this disease could become the worse threat to stone fruit cultivation if it become widespread (Abou-Jawdah et al. 2010).

The management of a possible emerging disease requires additional care, as preventing the establishment of the disease in new areas is definitely the first goal to pursue. Being a phytoplasma-associated disease, the two main risks concerning the diffusion of the disease are the distribution of infected propagation material, and the diffusion of the insect vector(s). The first is thought to be the main method of diffusion of the disease in the currently affected areas, Lebanon and Iran, but the export of propagation material from those countries is, at the moment, not intensive enough to be a great cause of concern. Still, propagation material could be critical in spreading the disease: '*Ca*. P. phoenicium' can be difficult to detect in some plant tissues even with molecular methods such as specific PCR. The visual assessment of the symptoms can be insufficient to clarify the sanitary status of a plant since the pathogen can have a latent period over one year long before the first symptoms appear. This facts can made the certification of healthy material complicated, and made also the unconscious diffusion of infected propagation material easier. Nevertheless, the use of certified plants from tested mother plants and healthy buds and avoid grafting (*i.e.* taking scions) from infected trees are considered. Also, weed control, constant monitoring of orchards and destruction of infected trees (including the roots to avoid the sprouting) and replacing of infected trees by non-host species are the measures currently applied in Lebanon (EPPO 2017).

While the epidemiology of '*Ca.* P. phoenicium' has not been fully elucidated, there are at least three proven insect vectors of the pathogen: the leafhopper *Assymetrasca decedens* and the planthoppers *Tachycixius viperinus* and *T. pilosus* cf. *cypricus*. For the latter two, further studies should be carried out to determine if both species are vector, as the proof of transmission came from an experiment using mixed populations of the two, closely related, species (Tedeschi et al. 2015). Furthermore, a number of other insect species were found positive to this phytoplasma, including some already reported as phytoplasma vectors (*Empoasca decipiens* and *Hyalesthes obsoletus*), however there is no demonstration of transmission from these insects. *A. decedens* is a very polyphagous leafhopper, that can be hosted by over 60 plant species belonging to 50 genera, enclosing herbaceous plants, as well as shrubs and trees, from both wild and cultivated species (Freitas and Aguin-Pombo 2006). This list of hosts includes some important crops or otherwise widespread plants: stone fruits (almond, apricot, cherry, peach, plum), citrus, grapevine, herbaceous crops (bean, beet, potato, raspberry), and spontaneous trees (*Populus*

spp., *Salix* spp.) among others. Furthermore, it is reported that *A. decedens* could adapt to new hosts when moving to new areas (Freitas and Aguin-Pombo 2006), making it an even more dangerous vector insect. To further aggravate the situation should the pathogen arrive in new areas, this vector insect was already reported in Middle, South, and Eastern Europe, the Middle East, and parts of Asia, including India and China (Alekseev et al. 1976; Chou and Ma 1981; Lodos and Kalkandelen 1983; Khan and Nighat 1990; Nickel 2010; Liu et al. 2014; Coutinho et al. 2015). While there is much information regarding *A. decedens*, *Tachycixius* is relatively unknown. The life cycle and behavior of these insect vectors are poorly investigated, although they were found in two weed species (*Smilax aspera* and *Antheris* spp.) in an almond orchard (Tedeschi et al. 2015). From the behavior of a related species, *T. pilosus*, one could assume that the larvae feed on weeds, while the adults move to deciduous woody plants, but this hypothesis should be confirmed.

In this complex scenario composed by a large number of possible hosts, possible vectors, and a high level of uncertainty regarding the whole epidemiology of the pathogen, it is difficult to come up with effective measures to control the disease. Still, the general guidelines followed to control other phytoplasma diseases are applied: since there is no curative method and the control in the field is difficult, the main tools that can be employed are the control of the insect vectors, the use of healthy propagation material, and some agricultural practices.

In Lebanon, where the first outbreak of the disease was registered, a combination of several measures was employed with the aid of extensive campaigns organized in order to improve awareness of farmers and nurserymen, mainly in areas of nursery production. In addition, control programs have extensively involved farmers, in particular where eradication measures have been compulsorily applied (i.e. pilot areas). From 2012 to 2014 more than 6,000 plants were eliminated: from 2014 onwards, the management has relied on growers to destroy infected trees. Now, the area where the certified nurseries are located is considered disease-free (EPPO 2017). Additional actions should be coupled, relying on the management of weeds, replacement with certified healthy material or with non-host plants, and control of the insect vectors. This last measure was initially carried out with traditional insecticide treatments, but it is now often shifting towards an integrated approach based on the habitat management (Molino Lova 2011). These measures gave some promising results, containing the spread of the disease from the North Lebanon to the South part. Since the disease started in the Northern part, it managed to establish there, but the knowledge obtained from the outbreak allowed slowing considerably its spread to the Southern part of the Country. One positive note is that many of the measures used against '*Ca.* P. phoenicium' are the same already employed against other phytoplasmas in stone fruits orchards (*e.g.* European stone fruit yellows), and this could contribute to an early management of the disease in orchards where stone fruits represent the major crop. This however could not be the case for almond orchards, which are not managed as intensively as the other stone fruits, not requiring irrigation in many cases and having fewer pests, or that are kept as a secondary crop in a farm. In the end, the best control solution for '*Ca.* P. phoenicium' would be the use of resistant cultivars (Ghayeb Zamharir 2011), but at the moment there is no record

of a resistant cultivar, and possible sources of resistance traits are still being researched. An alternative solution that might be viable in the short-term would be to graft almond plants on non-host rootstocks from other plant species (Tawidian et al. 2017).

11.3 "Bois Noir"

Grapevine yellows (GYs) are among the most damaging phytoplasma-associated diseases of grapevine throughout the world since they can cause serious yield losses (Fig. 11.1). They are associated with diverse phytoplasmas among which the most severe and widespread are the "bois noir" (BN) and the "flavescence dorée" (FD) (Belli et al. 2010; Maixner 2011; Zambon et al. 2018). BN is associated with the presence of '*Candidatus* Phytoplasma solani' (Quaglino et al. 2013), which belong to the "stolbur" group (16SrXII-A, -F, -G, -J, -K) (Quaglino et al. 2017). This is probably the most important, damaging and widespread grapevine phytoplasma disease in the Euro-Mediterranean area, since it has been recorded infecting the main viticultural areas. It causes important economic losses due to multiple physiological changes occurring in the host, including early drying of clusters, in all of the countries in which it has been identified as epidemic (Endeshaw et al. 2012; Ember et al. 2018). '*Ca*. P. solani' is usually transmitted to grapevine by the planthopper *Hyalesthes obsoletus* Signoret (Maixner et al. 1995), *Reptalus panzeri* Low and *Reptalus quinquecostatus* Dufour have also been recently reported as vectors of BN phytoplasma in Serbian and French vineyards, respectively (Cvrković et al. 2014; Chuche et al. 2016), while in some areas with poor presence of *H. obsoletus* other vectors still not determined may be present (Landi et al. 2015; Oliveri et al. 2015). BN has a complex epidemiology that involves different reservoir plants (e.g., *Urtica dioica*, *Convolvolus arvensis*, *Artemisia vulgaris*), insect vectors and grapevines. On the basis of selected molecular marker genes (e.g., *tuf*, *vmp1*, *stamp* genes from

Fig. 11.1 Severely GY infected vineyards of cultivar Chardonnay in the province of Verona, Italy (Courtesy of E. Marchesini)

phytoplasmas), previous studies have identified several '*Ca*. P. solani' genetically different strains in distinct ecological niches (Kosovac et al. 2016; Murolo and Romanazzi 2015; Pierro et al. 2018) and have contributed to a better understanding of the its ecology in the vineyard agroecosystem (Landi et al. 2015). Recent findings on GYs strongly readdressed their consideration in terms of control, pathways of introduction in countries and territories, pest risk assessment and categorisation, both in Europe and in all the viticultural areas around the globe (EFSA 2014).

The management of BN is complex, since it includes a list of preventive actions on the host plant(s), the insect vectors and the environment (Romanazzi et al. 2009). It is known since the mid of the last century that plants infected by phytoplasmas can undergo spontaneous symptom remission, or recovery (Caudwell 1961). In grapevines, this natural phenomenon has been observed in different varieties and viticultural regions (Osler et al. 1993; Garau et al. 2004; Maixner 2006; Romanazzi et al. 2013). The recovery phenomenon depends on factors such as phytoplasma identity, host-plant variety (*e.g.* cultivar Glera shows recovery, whereas cultivar Perera does not) (Garau et al. 2004; Bellomo et al. 2007), rootstock combination, it had higher incidence in plants grafted on Kober 5BB as compared to 420A (Romanazzi and Murolo 2008), environmental conditions (Braccini and Nasca 2008), and agronomic practices such as pruning (Fig. 11.2) or transplanting. Recovery can be complete or partial, temporary or permanent, and common or rare, and, consequently, it can be practically significant or not for an infected crop. Morone et al. (2007) recorded a productivity of recovered grapevines intermediate between healthy and symptomatic plants. Multiyear studies on cultivar Chardonnay reported that an average

Fig. 11.2 Grapevine pruning at the top (left) and at the bottom (right) of the plant (Courtesy of L. Milanesi)

recovery incidence of about 25%, and the 80% of these recovered plants maintained this status over time (Romanazzi et al. 2013). In recovered plants, molecular analysis of leaf veins has failed to reveal the presence of phytoplasmas in several Italian and German areas for "bois-noir"-infected and "flavescence dorée"-infected plants (Maixner 2006; Morone et al. 2007; Romanazzi and Murolo 2008).

The physiological basis of recovery is not known. It has been seen that in grapevine plants the recovery from phytoplasma-associated disease symptomatology was accompanied by an overproduction of hydrogen peroxide (H_2O_2) localized to the phloem tissues (Musetti et al. 2007). Such H_2O_2 accumulation was not detected in symptomatic diseased plants, or in healthy control plants. This led to hypothesize an active role of H_2O_2, and possibly of other reactive oxygen species, in counteracting the pathogen virulence and contributing to promote the recovery. Comparing the expression of a list of defense-related genes in cultivar Chardonnay (susceptible) and Sangiovese (moderately resistant), it was observed a higher reactivity of Sangiovese, with a number of enzymes overexpressed even in the absence of disease symptoms, or in symptomless portions of the plant (Landi and Romanazzi 2011). Together with the demonstration that recovered plants can be re-infected in nature to a lesser extent than plants that have never been previously infected, these observations indicate that a type of systemic acquired resistance (SAR) is involved in the recovery phenomenon.

The studies dealing with BN management were intensified in the last decade to address the losses of production of grapevine growers, which for susceptible cultivars as Chardonnay may have symptomatic plants percentages ranging from 20% to more than 50%. Hopefully not all clusters of symptomatic plants suffer from early drying; being the disease often localized to some of the canes, and in average a symptomatic plant has around a 30% reduction of production (Romanazzi et al. 2013). Severely infected plants can be lead to death, and if grower intends to replace them, considering a vineyard with 5,000 plants per ha, and 10% with severe BN symptoms, and 15 Euro per plant as replacement cost, the grower can be asked to cover a cost up to 7,500 Euro per year per ha. Those high costs open questions about the source of infection and the spread of the disease in the vineyard over time.

Management strategies tending to mitigate BN symptoms include measures to be applied to the grapevines and the insect vector(s). Regarding applications on the host, there are no protocols to be used in the field with a clear effectiveness. However, investigations were carried out to increase the naturally occurring recovery rate trying to increase the host resistance mechanisms. It is known that transplanting BN symptomatic plants induces a stress that may results in the recovery (Osler et al. 1993). Therefore, following those findings, application of a partial uprooting (practice poorly applicable by farmers) and pulling (that may have some applicability) to BN symptomatic plants increased the recovery rate, with a better performance when grapevines were grafted onto Kober 5BB compared to 420A (Romanazzi and Murolo 2008). The optimal BN management strategy should follow the general disease control strategies, mostly based on application of fungicides to the canopy. Following some preliminary trials in Sardinia (Italy) by using resistance inducers for BN management (Garau et al. 2008), several year investigations were run by

applying weekly from the beginning of May to the end of July to the canopy of BN symptomatic grapevines cultivar Chardonnay a number of commercially available resistance inducers (Bion, Kendal, Olivis, Chito Plant and Aliette) (Romanazzi et al. 2013). After a 4 year of investigations, the application of the first three formulations (Bion, Kendal, and Olivis) doubled the recovery rate, as compared to the untreated control (more than 50% versus 25%), and mitigated the disease symptoms in the plants that did not recover following the treatment. However, it is worth to note that such results obtained in experimental trials need to be confirmed with larger scale tests.

The strategies aiming to control the vector(s) resides mostly in the management of weeds within and around the vineyards. The main vector *H. obsoletus* spend its juvenile stages on the roots of bindweed and stinging nettle, harboring two molecularly distinguishable '*Ca*. P. solani', tuf-type a on *U. dioica* and tuf-type b on *C. arvensis* (Langer and Maixner 2004). An appropriate monitoring of the vineyard can therefore be helpful to find the main source of infection. The presence of *H. obsoletus* is not restricted to vineyards, but it depends on the occurrence and distribution of its natural plant hosts within and outside them, so that the infection pressure is not only determined by the specific conditions of a particular vineyard, but it depends also from the general biotic and abiotic conditions on a larger scale.

Insecticide sprays to the grapevine foliage, which are effective against the vector of "flavescence dorée", the other important grapevine yellows in Europe, are considered ineffective to control *H. obosletus* and the spread of BN (Pavan 1989; Mori et al. 2008). Consequently, strategies to control this planthopper focus on the nymphs on the roots of their host plants. Nettle and bindweed are common weeds of vineyards and surrounding structures and bindweed is also widespread inside the vineyards. A random or clumped distribution of BN infected grapevines corresponding to the presence of this plant host is often observed. While a well-managed green cover can suppress bindweed in the vineyard interrows (Maixner 2007), it remains a problematic weed in the undergrowth of the grapevine plants. Mechanical or chemical weeding of this area is advisable to avoid a high infection pressure close to the grapevine plants (Fig. 11.3). A non-chemical though laborious alternative to weeding of the undergrowth for organic viticulture, is the planting of ground covering rosette plants like *Hieracium pilosella* (Langer et al. 2003). Nettle is typically concentrated at the vineyard borders, along ditches or on fallow plots, although it is also found in small stands scattered over the vineyards. Disease gradients from the borders to the center of vineyards are characteristic for situations where the nettle is growing along the borders (Mori et al. 2008, 2012). Chemical weeding of nettles aims at depriving the root feeding *H. obsoletus* nymphs of their food source. Herbicide treatments proved to be equally effective when applied either in autumn or in early spring (Stark-Urnau and Kast 2008; Mori et al. 2014). However, the treatments in the spring need to be timed very early, so that the *H. obsoletus* nymphs are not able to complete their life cycle on the declining plants. It is advisable to treat the nettle plants not later than approximately 6 weeks before the anticipated start of adult emergence (Mori et al. 2014). A degree-day model is used in Germany to calculate the emergence date of *H. obsoletus* for both nettle and bindweed

Fig. 11.3 Example of vineyard grassing management: weeding (chemical/mechanical) on the row and frequent mowing of inter-rows and headlands. On the right in the presence of water bodies, as chemical weeding is not possible, the containment of the nettle must be carried out with mechanical means (Courtesy of Consorzio Fitosanitario Provinciale, Modena, Italy)

populations (Maixner 2007). Using this system, herbicide treatments should be carried out before 40% of the required temperature sums are accumulated. It is strongly advised to refrain from any kind of host plant control, either mechanical or chemical, short before and during the adult vector's period of activity. The unavailability of the original plant hosts would drive the vectors to disperse to the adjacent structures including vineyards, and would therefore increase the risk of BN infection to grapevine (Mori et al. 2012). To control the insect vector(s), given that '*Ca*. P. solani'-infected and associated weeds are dicotyledonous, it could be important to favor the vineyard ground cover with grasses instead of plants with broadleaves, by sowing selected grass species at transplanting, applying selective chemical treatments and frequent grass shearing (Mori et al. 2015). Moreover, in order to reduce the attractiveness of the grapevine towards the cixiid, the suckering is suggested as a suitable agricultural practice to reduce the disease spreading by decreasing the chance of contact between *H. obsoletus* and the grapevine (Picciau et al. 2010). Moreover, severe infections in young vineyards can occur when the grower completely eliminate the weeds, so immigrating insect vectors carrying the phytoplasma find just the grapevine as a feeding source. This can explain the high infection rate reported in the young vineyards where all the weeds were removed.

An alternative source of infection could be the propagation materials. It was observed that when the multilocus sequence typing of selected '*Ca*. P. solani' genes reveal a high variability, there are high chances of infections in the field by complex inoculum sources already in the area, while in case of high uniformity, then an high chance of infection through contaminated propagating materials can be speculated (Murolo and Romanazzi 2015). The management of BN is complex and requires an integrated approach, based mostly on preventive actions aiming to reduce the inoculum sources, starting from the use of clean propagation materials, which needs to be carefully controlled especially if growers select a susceptible cultivar as Chardonnay for the vineyard.

11.4 "Flavescence Dorée"

"Flavescence dorée" (FD) is a phytoplasma-associated disease of grapevine, transmitted by the leafhopper *Scaphoideus titanus* Ball. Two distinct FD strains, 16SrV-C and -D have been found associated with infected grapevines, both transmitted by *S. titanus* (Mori et al. 2002). Even if others leafhopper species, *Dictyophara europea* (L.) and *Orientus ishidae* (Matsumura) proved to be able to transmit FD (Filippin et al. 2009; Lessio et al. 2016), *S. titanus* is the only FD vector that feeds and breeds on grape and therefore it can be considered the specific vector of the disease. Due to the biology of *S. titanus*, that is strictly associated with grapevine and has only one generation per year, the control of FD spread mainly relies on the control of this leafhopper, *S. titanus* population level and disease spread are infact clearly correlated.

An area-wide and prompt monitoring of the insect vector is the pre-requisite to design a rational control strategy. *S. titanus* has a biological cycle characterized by two extended periods of nymph and of adult presence that largely overlap. To monitor nymphs, visual inspection and direct counts on grapevine leaves (on the underside of the leaves), are carried out. Nymph monitoring generally takes place on June, with the purpose of recording the developmental stage to time the first insecticide application of the year. Another purpose of the nymph monitoring is to estimate the population level and, for this, a sequential sampling, based on fixed precision level (75%) threshold lines, has been proposed (Lessio and Alma 2006). To monitor adults, different methods can be applied: direct counting on the leaves, "frappage" (beating tray), sweep-net and yellow sticky traps. This latter method is by far the most effective, comparable and used (Bosco and Mori 2013).

Among the different insect vector control techniques, only insecticides and agronomic methods are generally applied. Biological control with predators and parasitoids, though naturally acting in the field, is not effective enough. To reduce the vector population, canes from winter pruning should be destroyed (minced and/or buried under the soil), since they host leafhopper eggs (Fig. 11.4). Suckers should

Fig. 11.4 Mechanical collection of pruning residues infested with *S. titanus* eggs (Courtesy of M. Jermini)

Fig. 11.5 Correct shredding of the strains (left) as agronomic practice of reducing the populations of *S. titanus* in comparison with uncontained vineyard (Courtesy of G. Posenato)

be also removed (Fig. 11.5) because they are likely to host several nymphs (Trivellone et al. 2015). Also, a very important prophylactic measure is the cleaning of uncultivated areas surrounding the vineyards (hosting often wild American grapevines), as well as the prompt removal of abandoned vineyards, that host important *S. titanus* populations (Lessio et al. 2007).

Insecticides are generally applied twice a year in the areas characterised by a high incidence of the disease. In the areas where the disease is under control only one application is suggested. The first treatment, targeted to immature forms only, is applied in the second or in the last decade of June, depending on the mode of action of the active substance, in any case after the end of the grapevine flowering in order to avoid the poisoning of the honeybees. For the same reasons, the growers are advised to cut grasses in the interrows before spraying the insecticide to avoid insecticide drift on flowers. For the best timing of the first insecticide treatment, a phenology model has been developed (Rigamonti et al. 2011). The second treatment is generally applied one month after the first one, and is targeted against adults and late nymph populations, using neurotoxic active ingredients. When the vector population level does not exceed the threshold of 0.02 nymphs per 5 leaves per plant and of 2 adults captured on 3 traps per season (*e.g.* if little FD symptoms occurs in the vineyard), the two treatments can be reduced to one. When only one treatment per year is applied the first treatment at the end of the spring is skipped, matching the application with grapevine berry moth (*Lobesia botrana* Den & Schiff) strategy control (Pavan et al. 2005). Organic grapevine growers can apply spinosad or pyrethrum, provided that they apply the insecticide at the sunset in a slightly acidic suspension (pH 6–6.5); the addition of piperonyl butoxide is also recommended for pyrethrum. Due to the risk of incoming infected leafhoppers, a third insecticide treatment to the vineyard borders surrounded by wild vegetations or by abandoned vineyards is suggested in August. Actually, untreated areas represent refuges for the vector that can re-colonize the cultivated vineyards (Pavan et al. 2012). It is worthy to note that the reduction in vector population is higher following a wide area treatment rather than multiple treatments.

A rising concern for the winemakers and phytosanitary service offices is related to the presence and management of the new insect vectors, and in particular of *Orientus ishidae* Matsumura since its presence is clearly associated with a significant spread of the disease as reported already in Switzerland (Casati et al. 2017).

As a long term perspective, control by using symbionts and vibrational mating disruption strategies are under investigation. The control of phytoplasma vectors by using symbionts has been envisaged (Alma et al. 2010) and for this purpose the microbiome of *S. titanus* has been studied (Pajoro et al. 2008; Gonella et al. 2012). The vibrational communication behaviour of *S. titanus* has been studied (Mazzoni et al. 2009) and the possibility of disrupting this behaviour in order to stop mating has been proposed (Eriksson et al. 2012).

The role of the propagating material in the disease spread for the long distance dispersal is well known; less clear is the role attributed to new planting material (canes and buds) collected from grapevines infected by FD. Probably due their erratic distribution and low concentration *in planta*, the rate of phytoplasma transmission to new grapevines is very low (Constable et al. 2003). Nevertheless, the quarantine rules in Europe require the production of FD phytoplasma-free cuttings, and a rigorous management of the propagation material production is applied in all the Countries where the nursery activity is conducted.

Since no curative treatments are available for phytoplasma elimination *in planta* prevention practices are then applied including, in some way, thermotherapy, the hot water treatment of dormant woody and cuttings. The efficacy if this process is a controversial issue, even if its use is mandatory in several quarantine regulations worldwide as safeguard measure.

11.5 Lethal Yellowing

Diseases and injuries have affected the production of coconut palms for many decades, reducing fruitful lands to brushes and forcing good coconut producing lands into housing development. The rural poor people have moved away from coconut production as diseases have devastated the coconut industry. Coconut palms and approximately 35 other palms species are affected by lethal yellowing disease, making it a truly devastating malady (Fig. 11.6).

Symptoms matching the profile of the lethal yellowing (LY) disease were first reported in the Cayman Islands in 1834 (Martyn 1945) and then in Jamaica beginning in 1884 (Coconut Industry Board 1971). There were descriptions of the disease in the early 1900s in Cuba (Johnston 1909, 1912; Eden-Green 1997). Symptoms of the disease were first reported in the Belize and Bahamas in 1946; from Dominican Republic in 1915 later confirmed in 1962. Similar symptoms were reported from Haiti in the 1920s. Descriptions of LY were reported from Yucatan Peninsula, Mexico in 1974 and were observed in the Honduran Bay Islands, Roatan, in 1995. Later on the symptoms of the disease were observed on the mainland of Honduras (Roca de Doyle 2001). Lethal yellowing was first confirmed in St Kitts and Nevis in

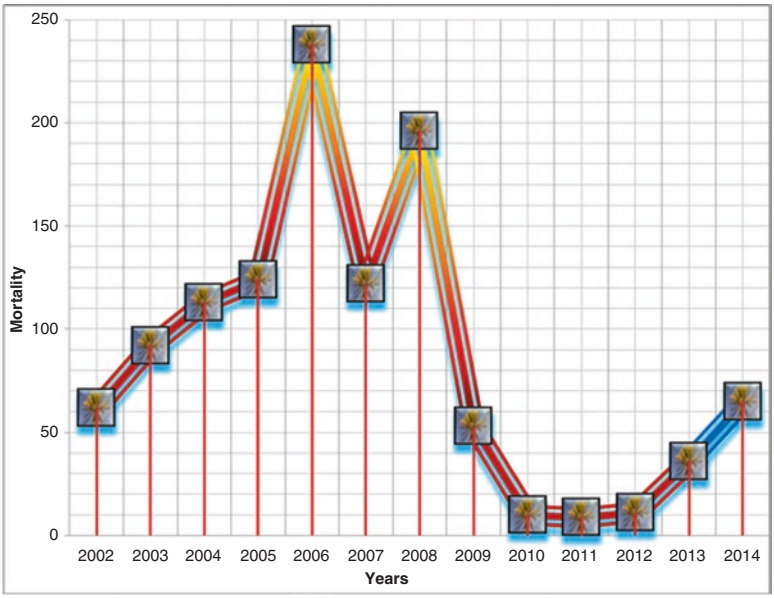

Fig. 11.6 Lethal yellowing diseased mortality at Nutts River Farm, St. Thomas, Jamaica

2005 (Myrie et al. 2006). Similar lethal diseases have affected coconut palms in Kenya, Mozambique, Tanzania, Nigeria, Ghana, Togo and Cameroon (Eden-Green 1997). "Awka" wilt or lethal yellowing disease of coconut palm was first reported in Nigeria in 1917 at the Awka district in the former Eastern region (Ekpo and Ojomo 1990).

Many years of research efforts to find a cure for lethal yellowing were undertaken, but it is still an elusive achievement. To date, the best approach for its control is an integrated program, which includes the use of resistant varieties (when available), quarantining new plants, antibiotic therapy, vector control and sanitation. Breeding of a resistant variety can provide a more direct and efficient approach to combat phytoplasma diseases. This was achieved two times in Jamaica with the production of the Maypan hybrid and the special Malayan Yellow Dwarf.

There is no artificial inoculation technique for lethal yellowing disease, hence the survival of coconut palms after many years of field exposure where other varieties succumb is presumed to be due to resistance. The level of resistance for a given coconut population is expressed as its percentage survival after years of field exposure to the disease. In the 1960's and 70's the Malayan Yellow Dwarf genotypes showed a high level, 85% or greater, of resistance to lethal yellowing. In comparison, the West African Tall, Panama Tall, Atlantic Tall and Malayan Tall have shown the lowest level of resistance, 15% or less (Been 1982; Dery et al. 1997; Kullaya et al. 1997; Schuiling et al. 1992; Zizumbo et al. 1999). Research in finding

the genes for lethal yellowing disease resistance or other desirable traits is difficult due to the reproductive biology of the coconut palm (Cardeña et al. 1999). Depending on the variety, coconuts reproduce 3–7 years after planting and have not been successfully propagated vegetatively.

Antibiotic therapy using tetracyclines has proven successful in suppressing symptom development in palms infected by phytoplasmas. Typically, these antibiotics are injected prior the expression of systemic foliar yellowing (McCoy 1972; Been 1995). In Florida, palms are mainly produced for aesthetic purposes rather than utilitarian, and antibiotic therapy has been used as a partly successful, temporary control measure. In Jamaica, where the coconut palm is produced commercially for its fruit, antibiotic therapy was tried, but due to the high cost and perceived health risks, this option of controlling this phytoplasma disease was rejected (Been 1995; Myrie 2005).

The lethal yellowing disease remains a significant threat to the global coconut industry and has been causing the death of coconut trees in some coconut producing areas. It is assumed that lethal yellowing is only insect vector-borne; therefore, its spread depends on the population density of the insect vectors. Although the use of insecticide sprays was able to reduce the levels of the known vector *Haplaxius crudus* van Duzee populations in Florida (McCoy et al. 1983), Been (1995) stated that the reduction of the vector population was not significant enough for this method to be recommended and attempts were made in the past to control the probable vector in Jamaica without success.

Research and experience over the years have taught how the disease can be reduced in the affected areas. According to Been (1995), the movement of living plant material from lethal yellowing infected areas to lethal yellowing free areas should be avoided. Quarantine measures depend on the ability and willingness of countries and persons to enforce them and abide by them.

Considerable work has been carried out to breed and identify coconut varieties resistant to the devastating lethal yellowing disease. However, the Malayan Yellow Dwarf and the Maypan varieties which demonstrated some resistance in the past have now shown high susceptibility to the disease. While intensive studies are being carried out by the Coconut Industry Board's (CIB) research department in Jamaica to better understand the disease and develop new resistant varieties, cultural methods are also being sought to diminish the devastating effects of the disease on the industry. Reduction of the disease increases the income to the small coconut growers and provides incentive for expanding the coconut populations.

The following management strategies are being used to significantly reduce the spread of the disease in Jamaica: planting several varieties and hybrids in the same field: (i) immediate identification and removal of symptomatic coconut trees, (ii) replacement of infected trees, (iii) removal of alternate/alternative hosts by proper weed control, and (iv) planting other susceptible palms as indicator plants in plantations and around the boundary of the plantations.

The "Black" approach developed in Jamaica fell trees at the earliest signs of the disease and replace each felled tree (at times with an over compensation for lost trees) to ensure sustainability of the crop. Close monitoring and prompt removal of diseased trees, cultural practices and prompt replanting are measures used to revive

the local industry. Other agronomical practices such as a sound fertilizer program and weed management are key factors in this approach. This method requires the immediate removal and replacement of infected trees. It is also advised that weeds must be removed. In addition, farmers must plant other susceptible palms as indicator plants in plantations and around their boundaries.

Michael Black Farms Ltd. in Nuts River, St. Thomas started its tree felling program in 1998, to reduce the spread thereby minimizing the effects of the disease. The farm is 70,000 coconut trees and has 0.015% of the trees affected by lethal yellowing. The integrated approach to the management of LY disease can lead to sustainable coconut production. When the approach is practiced efficiently and accurately, it has reduced the spread of the disease.

In the integrated approach, use of herbicide plays a critical role and must be carefully managed. Glyphosate, a systemic, broad-spectrum herbicide, is widely used for weed control in coconut orchards. It is considered dangerous for the environment and toxic for aquatic organisms and should be used with care.

The roles of cultural practices and environmental factors in the spread of lethal yellowing disease are not yet properly understood.

Farmers should intercrop their coconuts with other crops that can yield additional income so, if the field is affected by lethal yellowing the farmer will continue to get on income. The wide spacing in coconuts has made intercropping easy and many crops can be suited for this purpose. In the Caribbean, the farmers mostly intercrop with banana (Fig. 11.7) or cacao plants. Spacing between a well-estab-

Fig. 11.7 Intercropping of coconut with banana trees

Fig. 11.8 Coconut plantation being used as pasture for cow

lished coconut orchard can be used for grazing livestock, the coconuts must be tall and should not be reachable by the animals (Fig. 11.8). Intercropping is known to reduce weed control, morevoer common fertilizers utilized by both crops can be beneficial and increase the overall production. The coconut pantations, when well established and designed, also provides shading for the cacao trees.

11.6 Conclusions

To conclude some issues are common and should be considered for all the phytoplasma associated epidemic outbreaks in the different crops/orchards after having identified the involved pathogens: (i) identification and breeding of crop plant varieties (cultivars and rootstocks) that are resistant (or less susceptible) to the phytoplasma or phytoplasma strain involved in the epidemic, (ii) development of new resistant cultivars/rootstocks by conventional breeding or transgenic approaches, (iii) examination of the effects of biotic and abiotic environmental factors on disease and symptom development and analysis of the effectiveness of plant resistance inducers and similar bioactive compounds, (iv) analysis of the influence of climatic conditions on symptom development (predictions of the effect of climatic change); (v) insect vector control with special attention to low-impact insecticides and

treatment schedules, as well as the use of environmentally sustainable insect vector control strategies; (vi) application of effective strategies to control alternative host plants for the phytoplasmas and/or the insect vectors. The increased knowledge about the pathogen biology and genetic are playing a key role in the devising and applying the above practices together with the use of phytoplasma-free propagation materials and the early detection of phytoplasma presence in crops/orchards.

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