New perspectives in phytoplasma disease management

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Integrated Management of Phytoplasma Epidemics
in different Crop Systems

New Perspectives in Phytoplasma Disease Management

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New Perspectives in Phytoplasma Disease Management

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Preface

The goal of COST action FA0807 “Integrated Management of Phytoplasma Epidemics in Different Crop Systems” is to promote information exchange in order to design integrated management strategies to protect crops from phytoplasmas-associated disease toward the sustainable production of high-quality plant products by reducing pesticide use, resulting in less residues in fresh market products.

Phytoplasmas are insect-transmitted plant pathogenic prokaryotes associated with serious diseases in important crops such as grapevine, vegetables, corn, sugar beet, oil-seed crops and fruit trees. New approaches for disease management, improved diagnostic methods; reduction of disease spread; improvement of insect-vector monitoring and a reduction in the pesticides used for control were achieved and harmonized.

The COST action FA0807 is composed of four working groups:

WG 1: Early detection and diagnostics

WG 2: Epidemiology and vector ecology

WG 3: Crop systems and control

WG 4: Phytoplasma/host interactions

This workshop aims to disseminate the information collected in the COST network to plant protection services, plant health inspectors, advisors and growers. The most recent information obtained with special attention to the management of phytoplasma diseases were collected and presented (WG2-WG3).

Working group 3 leaders

Wolfgang Jarausch and Ester Torres

INDEX

Invited presentations of grower's associations from different European countries

PROGRAM

9:00 – 10:30

- Welcome (**Assunta Bertaccini**, Wolfgang Jarausch)
    - Outlook on relevant phytoplasma diseases (**Assunta Bertaccini,** Bojan Duduk)
- Spread of phytoplasma diseases by insect vectors: consequences for risk assessment
    - Introduction (**Barbara Jarausch,** Phyllis Weintraub)
    - Actual distribution of fruit tree and grapevine phytoplasma diseases and their vectors (**Rosemarie Tedeschi, Barbara Jarausch,** Duska Delic, Phyllis Weintraub)
    - Spread of fruit tree phytoplasma diseases (**Barbara Jarausch,** Nicolas Sauvion, Wolfgang Jarausch)
    - Spread of grapevine phytoplasma diseases (**Xavier Foissac,** Michael Maixner)

10:30 – 11:00 Coffee break

11:00 – 13:00

- Management of phytoplasma diseases through vector control
    - Fruit tree phytoplasma diseases
        - Integrated control of psyllids (**Tim Belien**, Eva Bangels, Gertie Peusens)
        - Innovative vector control (**Astrid Eben,** Jürgen Gross)
    - grapevine phytoplasma diseases
        - "Flavescence dorée" vector control in Italy (**Nicola Mori, Domenico Bosco**)
        - Management of "bois noir" through vector control (**Michael Maixner,** Nicola Mori)
- Role of propagation material in phytoplasma dissemination (**Bojan Duduk,** Nicola Mori)
- Rules and regulations related to phytoplasma-free materials: European regulation on plant quarantine (**Jordi Giné**, Ricard Sorribas)

13:00 – 14:30 Lunch

14:30 – 16:00

- Invited presentations of representatives of grower's associations from different European countries
    - Phytoplasma effect on grapevine nursery and Italian laws (Claudio Colla, **Gian Luca Mordenti**)
    - Controlling Flavescence Dorée with less insecticides: local scale strategy developed in Bordeaux vineyard (**Antoine Verpy**, Frédéric Gil, Séverine Mary, Carine Garcia, Dominique Vergnes, Maarten Van Helden)
    - Phytoplasma control in the production of fruit trees in Agromillora (**Mariàngela Mestre**)
- General discussion

16:00 – 17:30

- Perspectives for the management of phytoplasma diseases through genetic or induced resistance
    - Management of fruit tree and grapevine phytoplasma diseases through genetic resistance (**Wolfgang Jarausch,** Elisa Angelini, Sandrine Eveillard, Sylvie Malembic-Maher)
    - What can we learn from the phenomenon of “recovery”? (**Rita Musetti,** Paolo Ermacora, Marta Martini, Nazia Loi, Ruggero Osler)
    - Perspectives for the management of phytoplasma diseases through genetic or induced resistance: what can we expect from resistance inducers? (**Gianfranco Romanazzi**)
    - Perspectives of endophytes as biocontrol agents in the management of phytoplasma diseases (**Piero Bianco,** Cristina Marzachì, Rita Musetti, Vered Naor)

17:30 Cloncluding remarks

Outlook on relevant phytoplasma diseases in Europe

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Introduction

Phytoplasmas are microorganisms associated with a number of severe plant diseases affecting many areas of diverse agricultural cultivations worldwide. The discovery of a new group of plant pathogens related to bacteria led to the finding of polymorphic, wall-less prokaryotes, located in the phloem of many plant species affected by yellows-type diseases believed to be caused by viruses, considering their infectious nature, and transmission by insects. Molecular data have provided considerable insights into their molecular diversity and genetic interrelationships and significant taxonomic progress has been achieved by the study of the 16S ribosomal gene with full sequencing of four strains. However there is a gap between taxonomy and diseases since it is not uncommon that the same disease is associated with molecularly differentiable phytoplasmas and this is quite common with diseases described in different areas of the world (Lee *et al*., 1998). During the last ten years, the use of molecular methods has enabled the detection and characterization of phytoplasmas associated with major fruit tree and grapevine diseases in Europe (table 1).

Ribosomal group	'Candidatus' species	disease	Literature
16SrV-C; -D	'*Ca*. P. vitis'*	'Flavescence dorée'	Martini *et al*., 1999
16SrX-A	'*Ca*. P. mali'	Apple proliferation	Seemüller *et al*., 2004
16SrX-B	'Ca. P prunorum'	European stone fruit yellows	Seemüller *et al*., 2004
16SrX-C	'*Ca*. P. pyri'	Pear decline	Seemüller *et al*., 2004
16SrXII-A	'*Ca*. P. solani'**	'Bois noir'	Quaglino *et al*., 2013

*, not officially published; **, published according to unique phytoplasma transmitted by *Hyalesthes obsoletus* as differential biological property.

Table 1. Nomenclature of phytoplasma associated with relevant diseases in European countries.

On the other hand the inability to fulfil Koch's postulates severely restricts the understanding of the real roles of phytoplasmas in plant disease and in plant–insect-phytoplasma interaction, however very recently it was demonstrated that phytoplasmas, similarly to mycoplasmas, can grow independently from the host(s) (Contaldo *et al*., 2012). This success will eventually allow deeper biological studies on basic phytoplasma properties, increasing knowledge about their life cycle and epidemiology. This knowledge will improve detection, aetiology and consequently allow a better management of these diseases.

Phytoplasma diseases of grapevine

Grapevine yellows (GY) are widespread infectious diseases of grapevine associated with molecularly distinguishable phytoplasmas in the most important grape growing areas worldwide (Bertaccini *et al*., 1995; Boudon-Padieu, 2003). The most important diseases in main viticultural areas of Europe are 'flavescence dorée' and 'bois noir'.

'Flavescence dorée' (FD) is a quarantine disease still dangerous in spite all the mandatory measures that over the last 30 years allow to reduce its impact in affected grape growing areas. Symptoms of disease are similar to those associated with other phytoplasma diseases in grapevine, and mainly involve plant decline, leaf rolling, shrivelled grapes, unripened shoots and reddening or yellowing of leaves on red or white cultivars respectively (Fig. 1). The severity and increasing presence of this disease has prompted extensive efforts for its detection and differentiation from the phytoplasmas associated to 'bois noir' (BN) disease and for the fine identification and characterization of strains of both phytoplasmas. The major problem viticulturists are facing is the great ability of FD phytoplasmas to differentiate new strains in short periods of time. The molecular

differentiation of FD strains present in infected grape growing areas is therefore of major relevance towards a correct disease management. Phytoplasma strains FD associated belong to ribosomal subgroups 16SrV-C and 16SrV-D and they are further differentiated using polymorphisms detected in rpS3, SecY genes as well as other genes (Bertaccini *et al*., 1997; Angelini *et al*., 2001; Martini *et al*., 2002; Botti & Bertaccini, 2007; Arnauld *et al*., 2007). Both FD types resulted to be experimentally transmissible by the same vector *Schapoideus titanus* (Mori *et al*., 2002). Strains of FD 16SrV-D were detected in Northern Italy (Martini *et al*., 1999), France and Spain (Angelini *et al*., 2001; Torres *et al*., 2005) where the disease showed the highest epidemic outbreaks. In other grape producing areas such as North-central Italy and Serbia the 'flavescence dorée' strains associated with disease outbreaks belong to ribosomal subgroup 16SrV-C (Marzachì *et al*., 2001; Duduk *et al*., 2004; Botti & Bertaccini, 2006).

In the same areas as well as in almost all other grapevine growing regions worldwide the presence of **'bois noir'** disease associated with phytoplasmas belonging to ribosomal subgroup 16SrXII-A and having symptoms undistinguishable from FD, is widespread. BN phytoplasmas are transmitted to grapevine by *Hyalesthes obsoletus* Signoret (Homoptera, Cixiidae) using as source of inoculum *Convolvulus arvensis* L. (Maixner, 1994; Sforza *et al*., 1998) and *Urtica dioica* L. (Alma *et al*., 2002). However, these phytoplasmas were also detected in other plants and auchenorrhyncha species that are supposed to be involved in BN epidemiology. Over the last fifteen years a severe spreading of BN disease was described in several European grapevine-growing areas and the usefulness of tuf gene for epidemiological studies was shown since three strains, named tuf-types, were differentiated (Langer & Maixner, 2004; Mori *et al*., 2008). Recent findings indicate that there is molecular variability also inside the 16S gene of BN and of other stolbur-related phytoplasmas indicative for the presence of diverse strains, possibly relevant to study BN epidemic outbreaks.

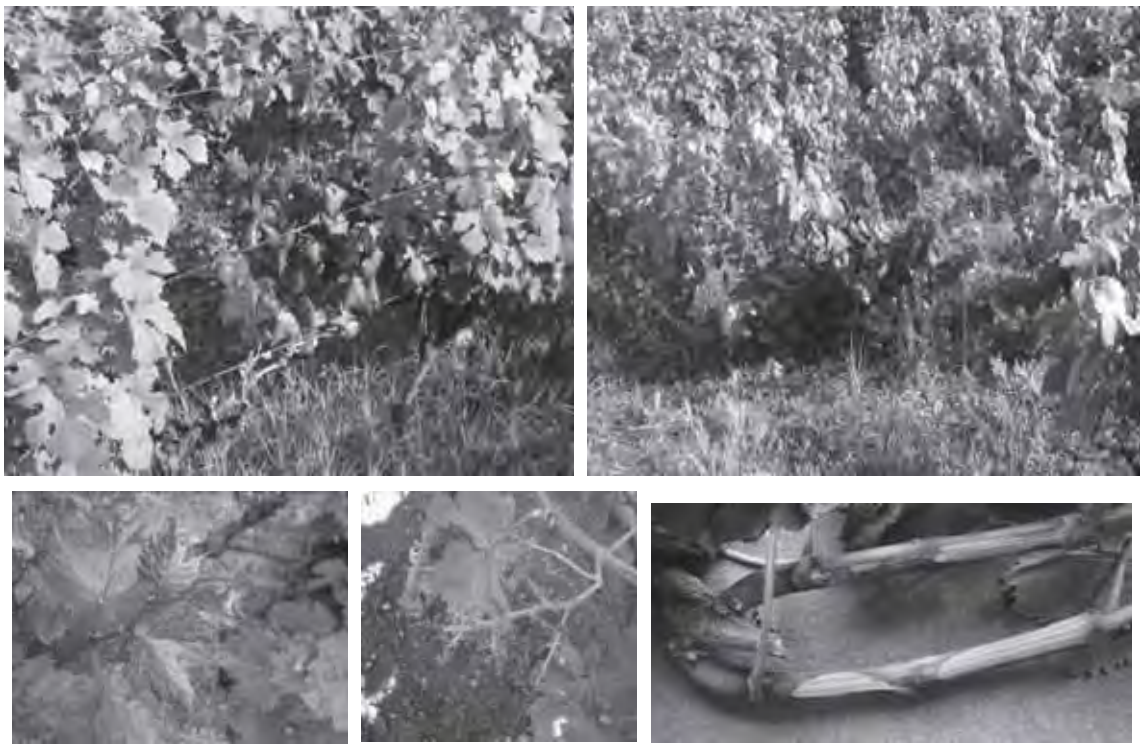

Figure 1 Symptoms of FD and BN in grapevine. In the infected plants the production is dramatically reduced, and the frequent lack of lignification also reduces the possibility to produce cuttings.

Phytoplasma diseases of pome and stone fruit

Phytoplasma diseases of fruit trees in Europe include three economically important disorders: apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY). Phylogenetic analyses revealed that the 16S rDNA sequences of strains of each of these pathogens are identical or nearly identical. Seemüller and Schneider (2004) showed that the differences between the three phytoplasmas range from 1.0 to 1.5% of nucleotide positions and are thus below the recommended threshold for assigning species rank to phytoplasmas under the provisional status '*Candidatus*'. However, supporting data for distinguishing the AP,

PD and ESFY agents were obtained by examining other molecular markers, including protein-encoding genes and randomly cloned DNA fragments. However the most important feature is that the three phytoplasmas show clear differences in psyllid vector transmission and host-range specificity. Therefore the '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', respectively, were proposed as temporary nomenclature for the phytoplasma associated with the three diseases all belonging to ribosomal group 16SrX (table 1). In some cases other phytoplasma in single or mixed infection were detected in European fruit trees showing the same symptomatology (Lee *et al*., 1995) but they never reached epidemic level of importance.

Apple proliferation is only reported in Europe, it was for the first time described in Trentino, north Italy in 1950, and its major impact on the agriculture is that the infected plants produce small and unmarketable fruits. Affected apple cultivars are almost all those present in the main apple growing areas of Europe regardless whether they are grafted on different rootstocks. AP is one of the most important disease of apple, affecting almost all cultivars, reducing size (by about 50%), weight (by 63-74%) and quality of fruit, as well as reducing tree vigour and increasing susceptibility to powdery mildew. The most typical symptom associated with '*Ca*. P. mali' presence is witches' broom at the end of shoots; on diseased trees, leaves are generally smaller and more dented, with unusually enlarged stipules. Fruits are smaller and flattened, and peduncles longer. Early leaf reddening is a good indication of the presence of the disease (Fig. 2) but can be induced also by other factors. The distribution pattern of '*Ca*. P. mali' in the tree is depending by the season as well as by temperature. Very recently a link was demonstrated between the diverse '*Ca*. P. mali' strains colonizing apple trees and their pathogenicity (Seemüller *et al*., 2011). In France, phytoplasmas could be found throughout the trees at temperatures of 21-25°C, causing symptoms; at 29-32°C symptoms were inhibited and phytoplasmas were found only in the roots (Ducrocquet *et al*., 1986). Although in Europe AP disease affects most or all varieties of apple, it is associated with relatively genetically homogeneous phytoplasmas. However from various isolates of '*Ca*. P. mali' three subtypes named AP, AT-1 and AT-2 were distinguished which in some cases were associated with specific epidemics. Apple is the main host of '*Ca*. P. mali'. Cultivars vary in reaction but most, including seedlings, appear to be susceptible. The disease can be observed on cultivars or on rootstocks, as well as on wild and ornamental *Malus* spp. Also, '*Ca*. P. mali' was found in hazelnut (*Corylus* spp.), cherry (*Prunus avium*), apricot (*P. armeniaca*) and plum (*P. domestica*). The psyllid *Cacopsylla picta* (Forster) (synonym: *C. costalis*) is a vector of apple proliferation in north-eastern Italy and in Germany, respectively. However in north-western Italy *C. melanoneura* is the most abundant psyllid and the overwintering adults of *C. melanoneura* are the responsible of the diffusion of AP in apple orchards in this area. In addition, the leafhopper *Fieberiella florii* Stal (Homoptera: Cicadellidae) has been demonstrated to be able to vector '*Ca*. P. mali'.

Figure 2 Apple proliferation symptoms in an old tree (right) and in productive trees showing witches' broom and reddening of the leaves (left).

Pear decline was firstly reported after the Second World War in western USA and Canada, but since fifty years it is of relevant importance also in European pear orchards. In certain regions of the USA, pear production has been reduced by half. In Italy, during 1945-47, over 50,000 trees were destroyed. Pear decline causes some economic loss in all the countries in which it is present. Main symptoms enclose poor shoot and spur growth, dieback of shoots, premature reddening and upper rolling of leaves, reduced leaf and fruit size and number in a tree, and premature leaf drop. Sudden tree collapse can result from hypersensitive tissue damage at the graft union on highly susceptible Asian rootstocks such as *Pyrus serotina* or *P. ussuriensis*.

The typical symptoms of pear decline on trees grafted to tolerant rootstocks are a very slow decline when trees are not receiving adequate water and nutrition. There is a progressive weakening of the tree, which may fluctuate in severity. Terminal growth is reduced or may cease completely. Leaves are few, small, leathery and light-green, with slightly up-rolled margins; they become abnormally red in early autumn and drop prematurely. Although blossoming is heavy in the early stages of attack, later on, fewer flowers are produced, fruit set is reduced and fruit does not attain the normal size. The reduced growth in successive season's results in shoots appearing as tufts of leaves. Most of the feeder roots are killed, while larger roots may appear normal (Fig. 3). On removing the bark at the graft union, a brown line may be visible on the cambial face in the bark surface at or directly below the union, and vertically fluted ridges may also be seen. The coloration is not consistently recurrent and can fade during the growing season. It should be noted that symptoms similar to those of pear decline described above can be produced by other factors, such as rootstock-scion incompatibility, girdling, bad drainage, malnutrition, winter injury and drought. Care should be taken to eliminate these possible causes when diagnosing the disease. In North America and UK the known vector is *Cacopsylla pyricola* (Foerster) but in other parts of Europe *Cacopsylla pyri* (L.) has been found as the main vector. Some studies indicate that '*Ca*. P. pyri' is able to overwinter in the body of *C. pyri* (Carraro *et al*., 2001) and spread the disease all over the vegetative period. The psyllid does not migrate from pear, but overwinters in the adult stage in bark crevices; the phytoplasma can be acquired in a few hours and persists in the vector for at least 3 weeks. The disease has been transmitted by grafting with rates up to 33%. In experiments with the pear psyllid, symptoms appear approximately 2 months after the infective insects have fed. Age of the tree and scion variety do not seem to influence the occurrence of the disease, while differences in susceptibility to the disease among varieties and rootstocks were reported (Pastore *et al*., 1998; Seemüller *et al*., 1998).

Figure 3 Pear decline symptoms in an old tree (left) and in productive trees showing sparse foliage, lack of production and reddening of the leaves (from second picture to right).

European stone fruit yellows. Several stone fruit species are affected by severe diseases associated with phytoplasmas. These include apricot chlorotic leaf roll, plum leptonecrosis, peach yellows and peach decline. Considering the common aetiology of these diseases the name of European stone fruit yellows was adopted. '*Ca*. P. prunorum' induces economically important diseases in apricot, Japanese plum and peach and can infect several other *Prunus* spp. Among these, apricot and Japanese plum are the most susceptible and sensitive. Although symptom severity is fairly variable, infected *P. armeniaca* and *P. salicina* trees in general show typical yellows accompanied by leaf roll followed by leaf reddening, reduction or suppression of dormancy with the consequent risk of frost damage, severe and progressive necroses, decline and eventual death of the tree (Fig. 4). Within the most sensitive cultivars, 100% of the infected plants can die. European plum is susceptible but generally tolerant to ESFY, some cultivars however, can show weak symptoms (Carraro *et al*., 1988a). The susceptibility of the rootstocks to ESFY varies according to genotypes: some are highly sensitive others are tolerant. Wild or cultivated *Prunus* species, such as *P. cerasifera*, *P. mahaleb*, *P. padus*, *P. spinosa*, *P. tomentosa*, are highly tolerant to the disease and the presence of specific symptoms is rare (Carraro *et al*., 2002); however *P. cerasifera* and *P. spinosa* are fairly susceptible. *P. avium* demonstrated a high level of resistance. ESFY presence was reported also in *P. serrulata*, *P. amygdalus*, *P. insititia* and *P. cerasus*, *Celtis australis*, *Fraxinus excelsior* and *Rosa canina* growing inside or in the surroundings of infected apricot orchards. The exact role played by these non-*Prunus* species in the epidemiology of the disease is not yet clear. Presence of '*Ca*. P. prunorum' strains differentiable for virulence intensity was also described (Ermacora *et al*., 2010)

ESFY is characterised by rapid and widespread diffusion especially when the conditions are favourable for

host-plants and vectors. *Cacopsylla pruni* is the known vector (Carraro *et al*., 1988b): the insect completes one generation per year and overwinters as adult on shelter plants (conifers). In areas with high infection pressure, the natural infectivity of *C. pruni* reaches levels greater than 10% and the annual rate of newly infected plants the 20%. The important role played by wild *Prunus* species - such as *P. spinosa* and *P. cerasifera*, both hosts for the vector and the agent of ESFY was demonstrated in the epidemic cycle of the disease; the phytoplasma can survive and persist independently of the presence of cultivated susceptible plants. It should also be noted that tolerant *Prunus* spp can act as sources of inoculum for the pathogen spread. ESFY control is mainly based on prevention but precise knowledge of the disease epidemiology in the different environments is necessary. In areas with low infection pressure, where the disease is absent and the presence of the vector is very low (or absent), the use of healthy plants can be sufficient. In areas with medium or high infection pressure, where ESFY is endemic (present on wild plants) and the populations of *C. pruni* are abundant, the control of the vector is necessary. In areas with high natural pressure of ESFY the cultivation of tolerant species (ex. *P. domestica*) instead of sensitive ones (ex. *P. salicina*) is often advisable. In addition plants with induced-resistance can be used especially in already infected areas.

Figure 4 ESFY symptoms in peach (left) Japanese plum (center) and apricot (right).

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Spread of phytoplasmas by insect vectors: an introduction

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Introduction

Phytoplasmas are phloem-limited plant pathogenic bacteria, therefore only phloem-feeding insects can potentially acquire and transmit them. All known phytoplasmas are transmitted by insects in the order Hemiptera. However, vector species are restricted to only a few families of the suborder Auchenorrhyncha: namely, Cercopidae, Cixiidae, Derbidae, Delphacidae, Cicadellidae and in the Sternorrhyncha, Psyllidae (Weintraub & Beanland, 2006). Within a family, some species are known to be phytoplasma vectors, while others are not. Transmission specificity defines the fact that only selected species can act as vectors of a pathogen (Bosco & D'Amelio, 2010). But even within the same species different populations, mostly geographically separated, may occur which are competent pathogen vectors in one area but not in another.

Vector identification

Commonly, search for insect vectors of phytoplasmas starts with the determination of insect species found in the vicinity of diseased plants (Weintraub & Beanland, 2006). In general, all phloem-feeding insect species are putative candidates for phytoplasma dissemination, therefore all collected insect species, considered as phytoplasma vectors, are analysed for phytoplasma infection by molecular means (usually by PCR). Hence, when an uninfected insect feeds in the phloem of an infected plant it may acquire the phytoplasma passively during this process and the pathogen can be detected in the insects' body. However, ingesting phytoplasmas, or any other pathogen, does not mean that the insect is a competent vector. In phytoplasma disease systems that have been characterized, a specific sequence of events is necessary for insects to transmit the pathogen to new hosts (Weintraub & Wilson, 2010).

Pathogen acquisition

The first step in the vector transmission process is the acquisition of the pathogen. The least common type of acquisition, generating the fewest confirmed vectors, is the **cell rupture feeding strategy** (formerly known as lacerate-and-flush) (Backus *et al*., 2005). In this method, the insect is feeding primarily in the mesophyll cells. Multiple cells are punctured by the stylets and watery saliva is released. The insect then ingests the liquefied cells. During this process phloem cells are also sometimes hit during the stylet puncturing of the leaf cells and their contents are ingested.

The more usual type of acquisition, and used by the vast majority of competent vectors, is the **salivary sheath strategy**. In this method gel saliva is egested while the insect is searching for phloem cells. The gel saliva hardens to a sheath to protect the delicate stylets. Once the phloem cells are found, watery saliva containing digestive enzymes is released and the cell contents imbibed. Hence, with the salivary sheath feeding strategy the contents of phloem cells are directly ingested. Insects feeding in this fashion are the most effective vectors. However, it is important to emphasize that ingesting phytoplasmas, or any other pathogen, does not mean that the insect is a competent vector because in the insects' body there are many barriers to overcome. Thus, the phytoplasmas must:

1. Survive the digestive enzymes and arrive in the midgut
2. Pass through the midgut to the hemocoel
3. Move to the critical area of the salivary gland
4. Replicate in the salivary glands to a minimum titer for transmission.

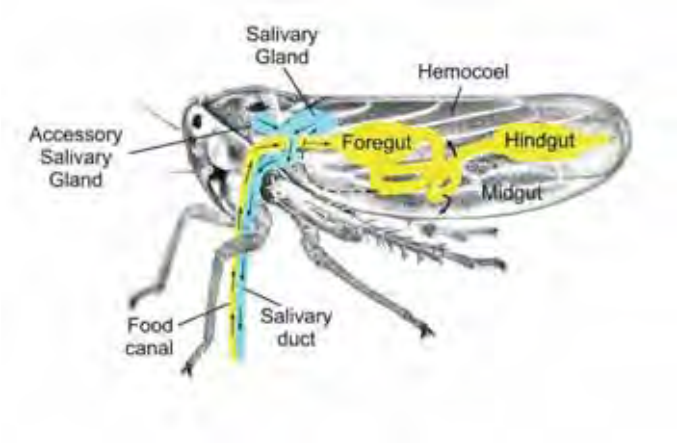

Figure 1. Schematic diagram of the ingestion of mollicutes by a competent vector. Mollicutes pass into the midgut where they penetrate the cells gaining access to the hemocoel. Once in the hemocoel they may invade other tissues but must eventually arrive and replicate in the salivary glands to be transmitted.

To move and replicate in the competent vectors' body it is supposed that phytoplasmas adhere to the midgut epithelial cell membrane and enter the midgut intra- or intercellularly (by endo- or diacytosis) (Bosco & D'Amelio, 2010). After entering the hemocoel, phytoplasmas circulate in the hemolymph, where they may infect other tissues such as the Malpighian tubules, fat bodies and brain, or reproductive organs. Further details are described in Weintraub and Beanland (2006) and in Bosco and D'Amelio (2010). Each of the steps in route to the critical area of the salivary gland constitutes a barrier to transmission. If the phytoplasma replicates in the fat bodies, or muscles, or reproductive system it may test positive to phytoplasmas presence by PCR. However a positive PCR analysis **does not** mean that the insect is a potential or competent vector. Only if the pathogen is able to arrive at the critical part of the salivary gland and to replicate in the salivary glands to a minimum titer for transmission an insect can become a competent vector. At each point of this process should the phytoplasma fail to enter or exit a tissue, the insect would become a dead-end host and would be unable to transmit the pathogen (Weintraub & Beanland, 2006).

Experimentally acquisition can be conducted by two approaches: i) healthy imagines or nymph instars (from a colony) are transferred on infected plants to acquire the pathogen during a certain acquisition access period (AAP) and then put again on healthy plants which will be observed for symptom development; ii) rearings are directly installed on infected plants (tested by PCR) where the insect performs its whole development from egg stage, through larval instars until emergence of new imagines. Individuals of these offspring are then transferred to healthy plants and symptom expression will be controlled. Although larval instars as well as new adults usually feed intensively the acquisition is not always successful and influenced by multiple factors, e.g. the fitness of the individual or the resistance of the salivary glands to phytoplasma infection which may explain the fact that some species acquire phytoplasmas but are not vectors (Bosco & D'Amelio, 2010). Hence, acquisition of a phytoplasma by an insect does not imply that the insect is a vector, since phytoplasmas may be acquired but not re-injected by feeding. Therefore, the natural infection rate of an insect species is not necessarily correlated with its transmission capacity, which has to be proven by transmission trials (Jarausch & Jarausch, 2010).

Transmission features and specificity

Phytoplasmas are reported to be transmitted in a persistent, propagative manner (Marzachì *et al*., 2004). Once a pathogen has overcome all vector barriers described above and has achieved a critical level in the salivary glands of the insect, it can be transmitted to further hosts by excreting of saliva during successive nutrition. The period of time that elapses from initial acquisition to the ability to transmit the phytoplasma is known as latent period (LP) or incubation period. The excreted phytoplasma takes residence in the plant host and begins to multiply. After a latent period, the plant begins to develop symptoms of disease. Once the titre of phytoplasma is sufficiently high, this plant can serve as an acquisition host for any vector species feeding upon it (Weintraub & Wilson, 2010).

Phytoplasma transmission can be demonstrated experimentally when field collected insects are allowed to feed on healthy plants and symptoms of phytoplasma infection are observed after a latent period. Detection of the pathogen can be performed by PCR on the plant and in the insect vectors.

Transmission of phytoplasmas by insects involves, at several levels, elements of host-pathogen specificity. Often phytoplasma infection is due to a single phytoplasma strain/species and vector insects can acquire this single phytoplasma and transmit it to other plants of the same species (Bosco & D'Amelio, 2010). In this case the epidemiological cycle is simple, since a single phytoplasma type is vectored among susceptible plants of one or more botanical species. For example, the psyllid *Cacopsylla pruni* Scopoli transmits the phytoplasma '*Ca*. P. prunorum' to different *Prunus* species and the leafhopper *Scaphoideus titanus* transmits the FD ("flavescence dorée") phytoplasma exclusively to grapevine. Otherwise, insect vectors can acquire more than one phytoplasma species/strains, either by feeding on multiple-infected source plants or by feeding sequentially on different plants infected by different phytoplasmas (Bosco & D'Amelio, 2010). In this case a competitive situation arises in the insect which may provoke epidemiological consequences for the vector. Thus, the transmission pattern over time is determined by the different strains and by the possibly diverse latent periods of the different phytoplasmas (Bosco & D'Amelio, 2010).

Vector-phytoplasma relationship

Vector-host plant interactions play an important role in limiting or expanding the spread of phytoplasmas. Polyphagous vectors have the potential to inoculate a wider range of plant species, depending on the resistance to infection of each host plant. Hence, the host range of a vector limits the spread of phytoplasmas by that species (Weintraub & Beanland, 2006). Several studies have shown that insects that normally do not feed on certain plant species can acquire and transmit phytoplasmas to those plants under laboratory conditions. This can also occur under field conditions: the vector of "bois noir" (BN) disease of grapevine, the cixiid *Hyalesthes obsoletus* Signoret, feeds accidentally on different crops and thus transmits the phytoplasma from weeds to grapevine although it cannot live on grapevines (Maixner, 1994; Weintraub & Wilson, 2010). *Euscelidius variegatus* is a natural vector of Chrysanthemum yellows phytoplasma (CY) and is an experimental vector of "flavescence dorée" (FD). In nature, FD infects grapevine and *E. variegatus* does not feed on grape. Therefore, this leafhopper and FD have no history of evolutionary interaction and this may explain the less efficient colonization of the host insect and the very high level of phytoplasma multiplication in the vector, which also results in pathogenic effects (Bressan *et al*., 2005). Conflicting reports show that the phytoplasma-vector relationship can be beneficial, deleterious or neutral in terms of its impact on the fitness of the insect host. While early reports suggested that infection by phytoplasmas was harmful to the insect hosts, more recent reports describe the tendency that phytoplasmas may confer increased fitness to their insect hosts. Thus, insect hosts infected with phytoplasmas may even show improved overwintering and increased fertility and longevity (Beanland *et al*., 2000; D'Amelio *et al*., 2008).

Another important factor in the vector-host plant relationship is the vector's biology and its life history. Some vector species generate several generations per year (polyvoltine) and appear in high population densities on its host plant species while others only produce one generation (univoltine) and may appear in more or less important numbers of individuals. Vector abundance is recognized as a determinant of disease risk in general (Girod *et al*., 2011). However in agricultural systems, there are seasonal factors that play a major role in disease risk: the economic host plant may not be available year-round. Fruit trees and grapevines are deciduous and from the autumn to spring are inaccessible to pathogen transmitting insects. Therefore, even if the vector was present, the risk of pathogen transmission is minimal. There may be abundant populations of leafhoppers or psyllids present, but they cannot transmit the phytoplasma until they have acquired it and it has migrated to the salivary glands and developed to a sufficient degree for the insect to become a competent vector. Some univoltine psyllid species occur in very low population densities and there are vast fluctuations among the years depending on the environmental conditions mainly during their larval development and their hibernation period. But they have been proven as only and highly efficient phytoplasma vectors in certain regions while other known vector and also non-vector species are much more abundant in this area. For instance, the monitoring of *Cacopsylla picta*, the main vector of '*Ca*. P. mali' on apple in Germany, revealed very low population densities over several years in all investigated zones in Germany as well as in different neighboring European countries. Amazingly, the natural infection rate of overwintered individuals collected in orchards with high incidence of apple proliferation was maintained over the years at a constant level of 10% (Jarausch *et al*., 2011). During transmission trials in laboratory the high vector capacity of *C. picta* was confirmed (Jarausch *et al*., 2004; 2011) indicating that vector abundance and competence for phytoplasma

transmission are not positively correlated. More long-term studies on the abundance of phytoplasma vectors and the development of diseased plants are necessary to confirm this relationship.

In conclusion, the spread of phytoplasmas by their insect vectors is on one hand strictly determined by an strict vector definition based on specific acquisition and transmission characteristics which *a priori* exclude certain insect species as competent vectors. But once a species fulfils the basic vector criteria multiple biotic and abiotic factors may influence the vector-host plant interactions and hence the epidemiology of a disease.

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Actual distribution of fruit tree and grapevine phytoplasma diseases and their vectors

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Introduction

Phytoplasmas of fruit trees and grapevine are quarantine organisms causing several economically important losses in all Europe. Fruit tree phytoplasmas belong to the apple proliferation ribosomal group (16SrX), which include '*Candidatus* Phytoplasma mali', the agent associated with apple proliferation (AP), '*Candidatus* Phytoplasma prunorum', the agent of European stone fruit yellows (ESFY), and '*Candidatus* Phytoplasma pyri, associated with pear decline (PD). The most widespread grapevine related phytoplasma diseases in Europe are "flavescence dorée" and "bois noir" associated respectively with phytoplasmas belonging to the 16SrV and 16SrXII groups. Fruit tree phytoplasmas are transmitted mainly by psyllids, while "flavescence dorée" phytoplasma is transmitted by the leafhopper *Scaphoideus titanus* and the "bois noir" phytoplasma by the cixiid *Hyalesthes obsoletus*. Transmission by insects and long-distance spread by infected planting material lead to a rapid spread of these phytoplasmas. For this reason, updated knowledge on their distribution as well as on the presence of their vectors is very important for pest risk assessment and phytosanitary decisions. Therefore, within WG2 (Epidemiology and Vector Ecology) of the COST Action FA0807, a questionnaire about distribution of phytoplasma diseases and their putative vectors throughout European regions have been drafted and given out to all members of the COST action. As a result, a database, consisting of 8 maps and detailed tables from 28 European and Middle East Countries, was produced.

Phytoplasma distribution data presented in the maps are based on the molecular detection of the agent in plant samples in the different countries. Vector data are mainly based on the presence of species proven as competent vectors during transmission trials in single countries and found infected with the agent in a couple of other countries. As transmission trials are difficult and time consuming, the presence of an important number of infected individuals may be considered as indication that this species is also a vector for a particular phytoplasma in a neighbouring country without having been proven by transmission trials. These species were considered as confirmed vectors, while species that tested positive by PCR analysis were considered putative vectors.

The maps report the spread of the five phytoplasmas described above and of their confirmed and putative insect vectors. The database is available at the following web-site: http://www.costphytoplasma.eu/InsectVectors.htm and the detailed information it provides on phytoplasma presence (strain/s, incidence, host/s symptoms, detection methods) as well as on the confirmed and putative vectors (population density, infection rate, phytoplasma detection tools, host plant/s, collection method/s, identification method/s) are here summarized.

It is important to note that the objective of the survey was to achieve update information about the distribution of the fruit tree and grapevine diseases and that the information given here is dependent on the records given by the COST action members which might be sometimes unpublished and unofficial. For this reason, we can also not pretend that the dataset is complete but it probably gives the best available overview of the situation in the COST areas.

Fruit tree phytoplasma distribution maps

Apple proliferation has been recorded in Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, Finland, France, Germany, Hungary, Italy, Norway, Poland, Romania, Serbia, Slovenia, Spain, Switzerland, The Netherlands and Turkey.

The confirmed vectors of '*Ca.* P. mali' are the psyllids *Cacopsylla picta* and *Cacopsylla melanoneura*: *C. picta* has been reported in Austria, Bosnia and Herzegovina, Germany, Italy, Poland, Serbia and Turkey and *C. melanoneura* has been reported in Austria, Bosnia and Herzegovina, Italy, Poland and Serbia, while this species is considered non-vector in Germany.

Pear decline has been recorded in Austria, Azerbaijan, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, France, Germany, Hungary, Italy, Lebanon, Poland, Romania, Serbia, Slovenia, Spain, Switzerland, The Netherlands and Turkey.

The confirmed vectors of '*Ca.* P. pyri' in Europe are the psyllids *Cacopsylla pyri* and *Cacopsylla pyricola*: *C. pyri* has been reported in Croatia, France, Hungary, Italy, Bosnia and Herzegovina Poland, Romania, Slovenia, Spain, The Netherlands, while *C. pyricola* has been reported in Croatia, Hungary, Italy and Slovenia. Although not part of our questionnaire action, *C. pyricola* has formerly been reported as vector also in North America and UK.

European stone fruit yellows has been recorded in Austria, Azerbaijan, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, France, Germany, Hungary, Italy, Poland, Romania, Serbia, Slovenia, Spain, Switzerland and Turkey.

The confirmed vector of '*Ca.* P. prunorum', the psyllid *Cacopsylla pruni*, has been reported in Austria, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, France, Germany, Italy, Poland, Slovenia, Spain and Turkey.

Many other psyllid species are putative vectors of fruit tree phytoplasmas all over Europe.

Grapevine phytoplasma distribution maps

"Flavescence dorée" has been recorded in Austria, Croatia, France, Italy, Portugal, Romania, Serbia, Slovenia, Spain and Switzerland.

The acknowledged vector *Scaphoideus titanus* has been reported in Croatia, France, Italy, Portugal, Romania, Serbia, Slovenia, Spain and Switzerland.

"Bois noir" has been recorded in Austria, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, France, Germany, Hungary, Iran, Israel, Italy, FYR of Macedonia, Lebanon, Portugal, Romania, Serbia, Slovenia, Spain, Switzerland and Turkey.

The acknowledged vector *Hyalesthes obsoletus* has been reported in Austria, Azerbaijan, Bulgaria, Croatia, Czech Republic, France, Germany, Hungary, Italy, FYR of Macedonia, Portugal, Romania, Serbia, Slovenia, Spain and Turkey.

Spread of fruit tree phytoplasma diseases

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Introduction

The genetically closely related '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma prunorum' and '*Candidatus* Phytoplasma pyri' cause economically important diseases such as apple proliferation (AP), European stone fruit yellows (ESFY) and pear decline (PD) in European fruit tree areas. While most phytoplasmas are transmitted by insect vectors of the hemipteran suborder Auchenorrhnycha (leafhoppers, planthoppers, treehoppers), all these fruit tree agents are spread by psyllid vectors of the superfamily Psylloidea (Hemiptera, Sternorrhyncha). Interestingly, the psyllid vector species all belong to the genus *Cacopsylla* and both, phytoplasmas and psyllid vectors, were geographically limited to Europe and the Palearctic region. Although the relationship between these phytoplasmas, their hosts and the vectoring psyllids is almost highly specific, there are some interconnections and biological factors which influence disease epidemiology and vector ecology and which should be taken into consideration for risk assessment and vector control. For instance, a psyllid species can be a proven vector in one region or country but not in a neighbouring one because insect populations are genetically different; a *Cacopsylla* species can even split into two different subspecies with potentially opposed characters regarding transmission capacities. Furthermore, a vector may transmit only a particular phytoplasma strain or subtype but not another.

Therefore, for a reliable risk assessment of a putative vector is important to know its population dynamics, its transmission efficiency, its host plant preferences and its epidemiological cycle. For example, a highly competent vector may occur only in small numbers, but is able to spread the disease much faster than high populations of an inefficient vector. Although psyllid vectors usually show a high host plant specificity, being either monophagous or oligophagous on closely related plant species, shelter plants may serve as sources of inoculum and hence interfere with the disease spread. Dispersal activity of summer generations, as well as the migration to and from specific hibernation sites at elevated areas, favour disease dissemination (Sauvion *et al.*, 2007) and have important consequences for the spread of the disease on a local and/or a regional scale (Thébaud *et al.*, 2009). Therefore, the knowledge of the epidemiological cycle of both, the phytoplasma and its main vector is crucial for the development of efficient control measures.

Table 1 gives an overview of the most important European phytoplasma diseases of fruit crops, their agents, their psyllid vectors and the vectors' host plant.

Psyllid species	Phytoplasma	Disease	Reproduction host plant
Cacopsylla picta	'*Ca.* P. mali'	Apple proliferation	*Malus*
Cacopsylla melanoneura	'*Ca.* P. mali'	Apple proliferation	*Crataegus, Malus*
Cacopsylla pruni	'*Ca.* P. prunorum'	European stone fruit yellows	*Prunus* species
Cacopsylla pyri	'*Ca.* P. pyri'	Pear decline	*Pyrus*
Cacopsylla pyricola	'*Ca.* P. pyri'	Pear decline	*Pyrus*
Cacopsylla pyrisuga *	'*Ca.* P. pyri'	Pear decline	*Pyrus*

* putative but not yet confirmed vector

Table 1. Overview of the most important European phytoplasma diseases of fruit crops, their agents, their psyllid vectors and the vectors' host plant.

Apple proliferation

Two psyllids, *Cacopsylla picta* (Foerster) (syn. *C. costalis*) and *Cacopsylla melanoneura* (Foerster) are recognized vectors of '*Ca*. P. mali' (Frisinghelli *et al*., 2000; Jarausch *et al*., 2003; Tedeschi *et al*., 2002). *C.*

picta is distributed only in Europe and is monophagous on *Malus* spp. The insect completes one generation per year and hibernates as adult on conifers (Mayer and Gross, 2007; Mattedi *et al.*, 2008). At the end of winter (March/April), *C. picta* remigrates from the overwintering sites to apple trees for oviposition.

C. melanoneura has a similar life cycle as *C. picta* but the overwintered adults reappear earlier in the year on *Crataegus* or apple trees and the new generation leaves its host plant earlier than *C. picta* to its aestivation and overwintering habitats (Mattedi *et al*., 2008; Tedeschi *et al.*, 2002, 2009). *C. melanoneura* has a Palaearctic distribution and is oligophagous on *Rosaceae*. A significant difference is that the principal host plant of *C. melanoneura* is not apple but *Crataegus monogyna* (hawthorn), a common shrub. In most of the studied areas both species are present (Carraro *et al*., 2001; Jarausch *et al*., 2003; Delic *et al*., 2005; Mattedi *et al*., 2008), in others so far only *C. melanoneura* has been found on apple (Tedeschi *et al*., 2002).

Comprehensive studies on the vector capacity of *C. picta* and *C. melanoneura* and on the role of hawthorn as source of '*Ca*. P. mali' in different European regions led to contradictory results. Thus, *C. picta* has been proven main vector of '*Ca.* P. mali' in Germany (Jarausch *et al.*, 2003, 2011) and northern Italy (Frisinghelli *et al*., 2000; Carraro *et al*., 2008). In contrast, *C. melanoneura* was identified as main vector in north-western Italy (Tedeschi *et al*., 2002); whereas the German population of *C. melanoneura* hardly acquired '*Ca*. P. mali' from infected apple and was not able to transmit the phytoplasma (Mayer *et al.*, 2009). Furthermore, the German population preferred hawthorn as host plant which, however, was not found infected with the phytoplasma whereas the north-western Italian population seems to be able to move between apple and hawthorn. Accordingly, hawthorn has been found infected with '*Ca*. P. mali' and, thus, may play a role in the epidemiology of AP in this region (Tedeschi *et al*., 2009).

'*Ca.* P. mali' is the only phytoplasma which has been reported to be transmitted by psyllids as well as by a leafhopper. A previous report that *Fieberiella florii* (Stål) is able to transmit the phytoplasma has recently been confirmed by Tedeschi and Alma (2006). Although the significance of this species as a vector of AP is considered to be inferior, the leafhopper could disseminate the agent to other host plants due to its polyphagous feeding behaviour (Tedeschi and Alma, 2006).

'*Ca.* P. mali' was also detected by molecular means in different aphid species captured on infected apple trees (Cainelli *et al.*, 2007). But failure of transmission trials and a low phytoplasma titer in the aphids as measured by quantitative PCR clearly indicate that aphids are not able to transmit AP.

European stone fruit yellows

The only described vector of '*Ca.* P. prunorum' is *C. pruni* Scopoli, a strictly oligophagous species on *Prunus* (Carraro *et al.*, 1998a) reported in 15 of the 27 EU countries (Steffek *et al.*, 2012). Like the vectors of AP, *C. pruni* hibernates on conifers and returns to its feeding hosts in early spring (Jarausch and Jarausch, 2010; Sauvion *et al.*, 2012). The hibernated specimens are already infective (Carraro et al., 2001; Thébaud *et al.*, 2009). Interestingly, the natural infection rate and the transmission capacity of *C. pruni* can vary stunningly. For instance, low natural infection rates of 1-3% were reported in Germany (Jarausch *et al.*, 2007, 2008) and in France (Thébaud *et al.*, 2008), while ten times higher natural infection and important transmission rates are described by Carraro *et al.* (2004) in north-eastern Italy. Sauvion *et al.* (2007) found indications for molecular divergences among different populations and suggested the existence of two sister species of *C. pruni*, shown to be able to transmit the phytoplasma (N. Sauvion, unpublished data.)

Wild *Prunus* species play an important role in the epidemiology of '*Ca*. P. prunorum' (Marcone *et al.*, 2010; Sauvion *et al.*, 2012). Although the highest vector densities were mainly recorded for wild *Prunus* such as *P. spinosa*, *P. cerasifera* or *P. salicina* these natural host plants rarely show symptoms (Carraro *et al.*, 2002; Jarausch *et al.*, 2008). Nevertheless, wild *P. spinosa* are infected by ESFY and an epidemiological cycle of ESFY independent from cultivated *Prunus* exists. Some cultivated as well as uncultivated *P. domestica* are tolerant carriers of '*Ca*. P. prunorum' and, as additional sources of inoculum, could be an unrecognized disease reservoir and a threat to orchards with susceptible fruit trees (Carraro *et al.*, 2002).

Pear decline

Three psyllid species live on pear as reproduction host plant: *C. pyri* (L.), *C. pyricola* (Förster) and *C. pyrisuga* (Förster). *C. pyri* is reported from Europe, the Caucasus, Central Asia, the Russian Far East and China. *C. pyricola* naturally occurs in the western Palaearctics and has been introduced into the USA and Canada in the early 19th century (Jarausch & Jarausch, 2010). The two species are oligophagous on *Pyrus* species such as *P. communis*, *P. eleagrifolia*, *P. pyraster*, *P. amygdaliformis* and *P. salicifolia* where they produce several generations per year while *C. pyrisuga* is univoltine; the adults overwinter on conifers and

remigrate to *Pyrus* by middle March to April. For Great Britain (Davies *et al.*, 1992) and North America (Jensen *et al.*,1964) only *C. pyricola* has been described as vector of '*Ca*. P. pyri' while *C. pyri* was identified as main vector in France (Lemoine, 1984), Italy (Carraro *et al*., 1998b) and Spain (Garcia-Chapa *et al*., 2005). Although naturally infected individuals of *C. pyrisuga* have been found (Kucerova *et al*., 2007) its vector capability has not yet been proven (Jarausch & Jarausch, 2010).

Epidemiological cycles and its consequences for control strategies

The basic epidemiological system of phytoplasma diseases consists of at least three components: the phytoplasma itself, a susceptible host plant and a competent vector feeding on the host plant. In the case of fruit tree phytoplasmas the complexity of this system is increased by multiple vector species, differing host plant preferences and long-distance migration to hibernation sites. Furthermore, the impact of the genetic variability of both the phytoplasma (Danet *et al.*, 2011) and the vector species on the system is only starting to be discovered.

Multiple vectors are known for '*Ca*. P. mali' but their respective risk for the spread of AP seems to be very different. Extensive studies in Germany showed that *C. picta* is the most important vector with a high rate of infective individuals whereas *C. melanoneura* plays no role for the disease spread despite its higher abundance (Jarausch *et al.*, 2011; Mayer *et al.*, 2009). This situation seems to be similar in most other apple growing regions except for north-western Italy where *C. picta* is not found but *C. melanoneura* is the most important vector (Tedeschi *et al.*, 2004). An explanation for this situation can be the existence of different populations of *C. melanoneura*. Indeed, genetic differences among populations of *C. melanoneura* have been detected (Malagnini *et al.*, 2007; Tedeschi & Nardi, 2010). Further studies are needed to link a molecular marker to the phytoplasma transmission capacity of a population of *C. melanoneura*.

Multiple vectors are also known for '*Ca*. P. pyri' and their respective risk for the spread of PD seems to be more related to the geographic region: *C. pyricola* has been described as vector in North America and Great Britain while *C. pyri* seems to be the dominant vector in continental Europe. Further studies are needed to assess the transmission capacities of the different pear psyllids in the different geographic regions of Europe.

Information about the different risks of multiple vectors for the disease spread should lead to consequences in the strategies for vector control: the control measures should be focused on the species with the highest risk, even if it is not very abundant and difficult to treat.

Differing host plant preferences may influence the spread of AP by *C. melanoneura* as this species seems to migrate from hawthorn to apple and backwards (Tedeschi *et al.*, 2009). Host plant preferences play also an important role in the spread of ESFY by *C. pruni*: the different *Prunus* species vary in their attractiveness for *C. pruni* and are consequently colonised by this vector in different densities (Carraro *et al.*, 2002; B. Jarausch, unpublished data). In this regard, rootstock suckers are often much more attractive than the cultivar (e.g. apricot or peach) and have to be eliminated to avoid important colonisation of the trees by the vector.

Univoltine vector species like *C. picta*, *C. melanoneura* or *C. pruni* hibernate on conifers in higher altitudes and migrate to the hibernation sites sometimes for long distances. The available data indicate so far that conifers are not a source of fruit tree phytoplasmas but that emerging individuals acquire the phytoplasma from infected host plants before migration (Fig. 1). The phytoplasma is retained in the insects during hibernation and is even multiplied to higher concentrations (Carraro *et al.*, 2001; Thébaud *et al.*, 2009). This means that remigrating individuals arrive in the orchards already highly infective and may remain their as infective individuals for up to 8 weeks (Jarausch *et al.*, 2011). Remigrant *C. picta* were highly efficient in transmitting '*Ca*. P. mali' to test plants (Jarausch *et al.*, 2011). Thus, treatments against these remigrants have to be effective over the entire period of orchard colonisation. An important consequence of the long-distance migration is the dispersal of infective remigrants over an entire fruit growing region. The available data for the disease spread of AP and ESFY indicate that there is a more or less random distribution of infected trees in a given region and a less evident spread within a single orchard. In this regard, a monocyclic spread (from one season to the next) has been proposed for ESFY (Thébaud *et al.*, 2009) whereas the spread of AP may be polycyclic in untreated areas (Jarausch *et al.*, 2011). The latter is based on the observation that '*Ca*. P. mali' multiplies very rapidly in its vector *C. picta* so that emigrant individuals become infective within two weeks and are able to transmit the disease inside the orchard where they developed before migrating to their hibernation sites. The consequences for the vector control differ according to the model: whereas treatments against the remigrants are most important according to the monocyclic model, treatments against both remigrants and emigrants are recommended with respect to the polycyclic model. Uprooting of infected trees to prevent phytoplasma acquisition by the vector is of benefit in both cases.

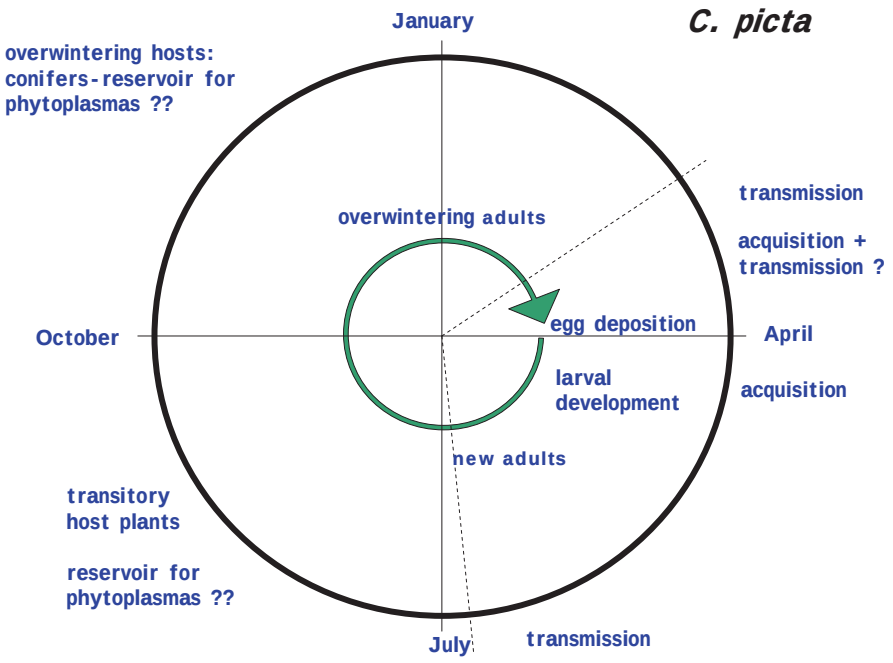

Figure 1. Epidemiological system of the spread of apple proliferation disease based on the ontogeny of its agent '*Ca*. P. mali' and the biological cycle of its main vector in Germany *C. picta*.

Polyvoltine vector species such as the pear psyllids *C. pyricola* and *C. pyri* spend their whole life cycle on their host plant generating several generations per year. Hence, theoretically there is a high risk for transmission all year long. But variations in the transmission efficiency could be studied with regard to plant phenology and populations dynamics. In California, Blomquist and Kirkpatrick (2002) concluded from their observations on *C. pyricola* that psyllid-mediated spring infections could happen well before '*Ca*. P. pyri' would normally recolonize the upper part of the tree from the roots. For Italy, the data by Carraro *et al*. (1998, 2001a) suggested that *C. pyri* retained infectivity during winter but could not transmit PD to dormant plants. Garcia-Chapa *et al*. (2005) found that the percentage of infected individuals is similar from June to August but reaching a rate of almost 100% in September coinciding with the maximum phytoplasma titer in the aerial plant parts. These data indicate that the infectivity and transmission capacity is not homogeneous along the phenological season and among the different generations these species generate. With regard to control strategies these data suggest an high risk for transmission in spring and in autumn while there is a period of reduced risk in summer and during winter. But as long as it is not clear which generations are the most efficient for disease spread it is not possible to find a satisfactory timing for treatments. Therefore further studies are necessary to analyse the seasonal transmission capacity for polyvoltine vector species.

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Spread of grapevine phytoplasma diseases

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Introduction

Two grapevine yellows due to phytoplasma infection are mainly affecting the European vineyards. Both diseases are spreading by sap feeding hemipteran insect vectors and at some extend by exchanges of infected planting material from grapevine nurseries. "Flavescence dorée" (FD) is a quarantine disease in Europe as it is epidemically transmitted by the grapevine leafhopper *Scaphoideus titanus*, an insect of North American origin now widely distributed in the vineyards of Southern Europe. "Bois noir" disease (BN) is endemic in the Euro-Mediterranean area and is mainly transmitted by *Hyalesthes obsoletus,* a planthopper residing in weeds such as bindweeds *(Convolvulus arvensis)* and stinging nettles *(Urtica dioica)* which also act as plant reservoirs for the BN phytoplasma. Present knowledge on the etiology and epidemiology of these grapevine yellows has been recently reviewed (Constable, 2010; Belli *et al.*, 2010).

Three strains of FD phytoplasma mainly spread from grapevine to grapevine but surrounding wild *Vitis* regrowth, infected alders and *Clematis* may constitute potential epidemic reservoir

S. titanus is an efficient vector of FD phytoplasma (Schvester *et al.*, 1961; Mori *et al.*, 2002). Since its introduction in Europe certainly in South-Western France (Papura *et al.*, 2012), *S. titanus* has expanded its geographical distribution and represents an important risk factor for FD spreading. Its current distribution ranges from South Italy to Hungary and from Portugal to Romania.

The genetic diversity of FD phytoplasmas has extensively been studied. It appeared that three main genetic clusters of FD phytoplasmas are present in Europe. The sequencing or the restriction map of the gene *map* allows differentiating three genetic clusters causing FD outbreaks (Arnaud *et al.*, 2007). In France, the genetic cluster mapFD2 is clonal and represents 85% of the disease cases, whereas the cluster mapFD1 only represents 15% of the FD cases and is mainly detected in South-Western France (Salar *et al.,* 2009). In Italy, mapFD3 strains (also called FD-C) are detected in addition to some cases of mapFD2 (also called 16SrV-D or FD-D strains) and mapFD1 strains (mostly present in North-Western Italy). In North-Eastern regions of Italy and in Slovenia mapFD3 strains are usually more abundant than mapFD2 strains. In Serbia, only mapFD3 strains have up to now been detected. In Northern Spain, Portugal and Switzerland only the mapFD2 strains have been detected so far (Figure 1).

Despite the control measures such as the pulling out of the infected grapes and the spread of insecticide, the disease is difficult to control due to the large viticulture areas involved. In order to use less insecticide, some growers organizations, as well as extension services in some counties as Italy, decided to improve the disease management by monitoring the insect vector populations and extensively surveying the vineyards for disease symptoms.

In South-western France, along rivers, uncontrolled rootstock regrowths (Figure 1) have shown to constitute in many places a reservoir for FD phytoplasmas and *S. titanus* populations escaping the insecticide treatments.

If the leafhopper vector *S. titanus* is of North American origin, the FD phytoplasma is widespread in alders (*Alnus glutinosa*) in South-Western France (Malembic-Maher *et al.,* 2007) but also all over Europe. Sixty to eighty percent of the alders are healthy carriers of the phytoplasma. More than 140 *map* genotypes have been detected in *A. glutinosa* in France, Italy, Germany, Serbia and Hungary, including the three mapFD1, mapFD2 and mapFD3 clusters. Transmission from alder to alder is achieved by *Oncopsis alni* (Maixner and Reinert, 1999), which occasionally transmits the phytoplasma to grapevine (Maixner *et al.*, 2000). In Italy and Serbia, mapFD3 strains are present in wild clematis (*Clematis vitalba*) from which they can be transmitted to

grapevine by *Dictyophara europea* (Filippin *et al.*, 2009). The importance of phytoplasma transmission from alders and clematis to grapevine remains to be determined but it cannot provoke a FD outbreak in the absence of the leafhopper *S. titanus*.

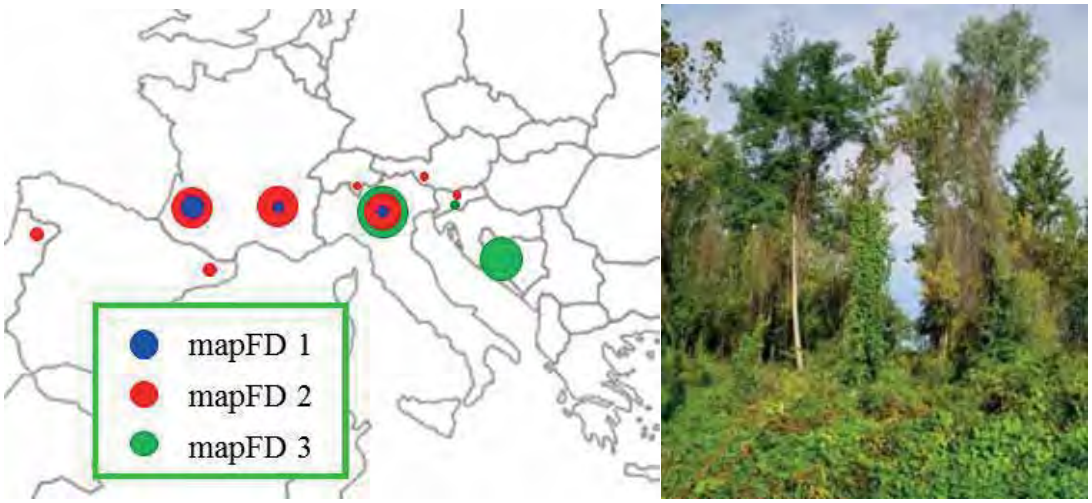

Figure 1. Geographical distribution of FD phytoplasma genetic clusters in Southern European vineyard (left) and wild vitis reservoir of FD phytoplasmas and *S. titanus* populations along Garonne river in South-Western France (right).

"Bois noir" is caused by the accidental transmission of different "stolbur" phytoplasma strains that are maintained by epidemiological systems based on different endemic weed species as reservoir plants

"Bois noir" (BN) is associated with '*Candidatus* Phytoplasma solani' presence (Quaglino *et al.*, 2013). These phytoplasmas and their vectors are endemic to Europe. The phytoplasmas are present in the natural vegetation and transmitted from and to herbaceous plants mainly by planthoppers of the family Cixiidae (summarized in Cvrkovic *et al.*, 2011). One planthopper, *H. obsoletus,* is the only species known so far to transmit BN by occasional feeding on grapevine. However, it does not take up phytoplasmas from infected grapevines. Instead, the root feeding nymphs acquire the pathogen from their herbaceous plant hosts nettle and bindweed. BN is considered the result of an occasional branching of the natural transmission cycles to grapevine as a dead end host for the pathogen. Spread of BN is less epidemic than FD and not affected by infected grapevine presence in the vineyard. Typical for BN are long term fluctuations of disease incidence with short epidemic outbreaks and decreasing disease levels during endemic stretches (Fig. 2). The reasons for the periodic outbreaks of BN are not yet understood and require further investigation.

The genetic variability of BN strains is high and exhibits geographical patterns (Fabre *et al.*, 2009, 2011; Johannesen *et al.*, 2012; Quaglino *et al.*, 2009). Most important for the epidemiology of BN is the fact, that genetic diversity is linked to plant host specificity. The variability of the *tuf* gene is diagnostic for the host plant association of BN strains (Langer & Maixner, 2004), since tuf-type a strains are specific to nettle while tuf-type b strains are typical for bindweed, though associated with other weeds, too. In addition, populations of *H. obsoletus* from nettle and bindweed exhibit signs of adaptation to their respective host plants, e.g. differences in phenology or survival (Cargnus *et al.*, 2012; Johannesen *et al.*, 2011; Maixner, 2007). Genetically distinct host races of *H. obsoletus* have been identified in Central Europe (Imo *et al*., 2013). The host affiliation of stolbur strains and vector populations results in distinct epidemiological cycles based on the different plant host species. This implies the risk that new plant/vector or plant/stolbur-strain combinations could result in altered disease cycles and changing infection pressure to grapevine. Possible reasons for such changes include altering environmental conditions or cultural practice, host plant shift of phytoplasmas or vectors, and their range extension or dissemination. The phenomenon of the recent severe outbreaks of the nettle type (tuf-type a) of BN in Central Europe was likely the result of the host shift of local populations of *H. obsoletus* from bindweed to nettle in combination with the range extension of Italian populations and associated tuf-type a strains to the north (Johannesen *et al.*, 2012).

The nature of BN epidemiology with grapevine being just an accidental host prevents epidemic outbreaks on the one hand, but impedes effective disease control on the other hand, since reservoir plants and vectors are common in the natural vegetation and not restricted to vineyards. Detailed information about the elements involved in local disease spread (predominant host plants, vector species, stolbur strains) is necessary to setup selective control measures. The biology of the vector is the most important factor for BN epidemiology. Where other vector species are probably involved in BN transmission, e.g. in south-eastern Europe (Cvrkovic *et al.*, 2011), their vector status and their life history need to be investigated carefully as a prerequisite for appropriate risk analysis as well as effective control of BN.

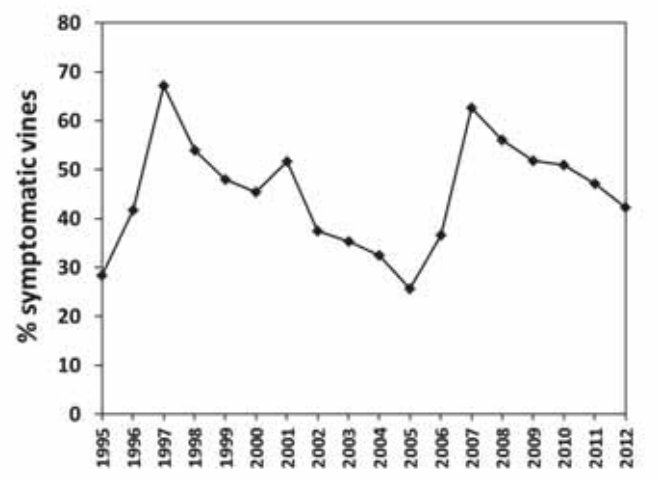

Figure 2. Temporal variation of BN incidence in a Riesling vineyard planted in 1993 in an area with high infection pressure. Typical for BN is the alternation of outbreaks with endemic periods.

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Integrated control of psyllids

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Introduction

As there is no applicable means to cure a phytoplasma-infected fruit tree, insecticide treatments were the first measures to control the spread of fruit tree phytoplasma diseases whenever a vector species was identified. Although it is impossible to eliminate all vectors from the environment, well-managed vector control strategies significantly reduce the chance of an epidemic outbreak of phytoplasma diseases. However, important differences exist between the control of univoltine and polyvoltine psyllid vectors. Highly efficient univoltine vectors, e.g. *Cacopsylla picta*, the vector of apple proliferation disease, might be present in the orchards in very low abundance, appropriate and efficient insecticides might miss or might not be homologated in all countries (Jarausch & Jarausch, 2010). Therefore, control of univoltine vectors is not well studied as it is the case for *Cacopsylla pruni*, the vector of European stone fruit yellows (Jarausch & Jarausch, 2010). On the contrary, polyvoltine pear psyllids, vectors of pear decline, are pests on their own and, therefore, control strategies and efficacies of different insecticides are well studied. The work presented here, will therefore focus on recent progress made in integrated control of pear psyllids.

Control of pear psyllids vectoring pear decline: an example from Belgium

Pear decline is associated with the presence of '*Candidatus* Phytoplasma pyri' which is transmitted by pear suckers (psyllids). In England *Cacopsylla pyricola* (Foerster) is known as vector species of pear decline, while in other parts of Europe *Cacopsylla pyri* (L.) has been described as the main vector (Garcia-Chapa *et al.*, 2005). This pear sucker species is the most common pest in pear orchards in Belgium, and one of the most important pests of commercial pears across Europe (Bangels *et al.*, 2008). Although it is impossible to eliminate pear psylla from the environment, well-managed control strategies significantly reduce the chance of an epidemic outbreak.

C. pyri overwinters as adults and mating takes place as soon as the adults regain activity (Kryson & Higbee, 1990), when the average temperature reaches at least 10°C for two successive days. According to pcfruit vzw monitoring data of the past decades, the mean date for the start of activity is February 3rd, soon followed by egg laying. At about the 1st of April, the first larval stages emerge in the field. Larvae develop through 5 instars into a winged imago and over time become less mobile and secrete more honeydew. This cycle is repeated several times per year, resulting in 3 to 5 or even more succeeding generations. The development stages of the first two generations can be distinguished easily while from third generation on in summer, they largely cover each other.

Currently, ten active compounds are registered for psylla control in pear production in Belgium: diflubenzuron, abamectin, thiacloprid, spinosad, deltamethrin, spirotetramat, spirodiclofen, aluminium silicate, thiamethoxam and potassium bicarbonate (Table 1). Despite the availability of several products with active ingredients belonging to various chemical classes, many farmers face problems to reach a satisfactory control. Therefore, we continually evaluate the efficacy of pear sucker control products in field trials.

We here present a general overview of the mean efficacies of crop protection agents reached in field trials on pear psyllids conducted between 2006 and 2012. Efficacy field trials were executed according to EPPO standards, following the guidelines as described in PP1/44 (2). Assessments were done on 10 marked shoots per replicate. For each marked shoot the number of eggs, the number of young larvae (L1-L3, yellow, L1 and L2 do not show any wing formation) and old larvae (L4-L5, dark brown, with wing formation) are assessed before application and followed up 1 to 21 days after application (DAT). Efficacies are calculated according to the Abbott formula (Abbott, 1925). As trials were conducted on several fruit grower's farms under practical circumstances, with local differences in pest occurrence and population dynamics, a certain degree of variability was obtained in the overall analyses of the product efficacies. However, general effects and observed trends were quite consistent, enabling us to generate comprehensive insights into the usage of the

different products with respect to their optimal implementation in pear sucker control schedules. A key factor for reaching a good control is the timing of application.

Active ingredient	Commercial name	dose (g or ml/ha leaf wall)	max # applications	remark
ABAMECTIN	Vertimec 18 EC, Agrimec, Acaramik, Inter Abamectine, Safran, Vargas, Zamir	500	2	
ALUMINIUM SILICATE	Surround 95 WP	13,300-20,000	5	
DELTAMETHRIN	Decis EC 2.5, Patriot, Splendid	300 - 400	1	exceptional use in IPM
DIFLUBENZURON	Dimilin SC-48,	600	1	
POTASSIUM BICARBONATE	Atilla 85 SP	4,700	9	
SPINOSAD	Tracer 480 SC, Conserve Pro	300	2	after flowering
SPIRODICLOFEN	Envidor 240 SC	400	1	after flowering
SPIROTETRAMAT	Movento 100 SC	1,500	1	after flowering BBCH 69-73
THIACLOPRID	Calypso 480 SC	250	2	before flowering or after harvest
THIAMETHOXAM	Actara 25 WG	220	1	After Harvest

Table 1. Registered insecticides against *Cacopsylla pyri* in Belgium (February 2013).

a. Natural control compounds

Aluminium silicate -better known as kaolin clay- and potassium bicarbonate are two natural compounds having an impact on pear psylla populations. Spraying of the former results in a white coating of the pear trees that discourages pear psyllids from laying eggs in the pear trees. Potassium bicarbonate is unfavourable for population development of pear suckers. It is assumed that it has a repellent as well as an irreversible physico-chemical effect, although the exact mechanism of how this salt operates is not yet known. In Fig. 1 efficacy results for both compounds are represented. Aluminium silicate (kaolin) drastically reduces the number of eggs corresponding to ~80% Abbott efficacy after 1-2 applications and ~95% Abbott efficacy after 3-4 applications (Fig. 1.A). The effect is most clear during the first 10 days after treatments (1-10 DAT). A prolonged effect necessitates multiple applications (>95% efficacy after 4 applications vs ~70% efficacy after 2 applications when assessed 11-20 DAT). The impact of potassium bicarbonate also largely depends on the number of treatments, as efficacy clearly increases when repeatedly is treated in a 5-7 days interval schedule (Fig. 1.B). Results for reduction in eggs, young (L1-3) larvae and old (L4-5) larvae are indicated and remaining effects can be interpreted as the combination of activity on earlier development stages or long-lasting effects on present stages or the combination of both.

b. Chemical control compounds

In Fig. 2 the results for chemical compounds are displayed. In each graph efficacies are presented on the assessed life stage, i.e. young larvae (L1-L3) or old larvae (L4-L5) indicated by dark grey and light grey bars, respectively. The results clearly show that products behave different according to efficacy, speed of action and residual activity.

Thiacloprid is a neonicotinoid insecticide with systemic and translaminar characteristics. It acts as an agonist of the nicotinic acetylcholine receptor in the central nervous system, thus paralyzing insects by disturbing synaptic signal transmissions. Thiacloprid reaches a rather fast and high efficacy in the second generation. However, due to possible side effects on beneficials, this compound is only allowed in IPM before flowering in pear, thus on the first generation, and only when pressure is high. The effect on young larvae is better than on older larvae as seen in the knock down effect and the number of old larvae that appeared in the later assessments.

Another recently registered neonicotinoid insecticide for control of pear suckers is thiamethoxam. This compound is only allowed after harvest, but as it shows a high control activity, with a strong knock-down effect, it is able to erase the psyllid population to a large extent, preventing potentially phytoplasma-infected psyllids to pass winter and cause new infections in next year early spring.

Spirodiclofen and spirotetramat both start slowly, which is typical for their mode of action. Both compounds are tetramic acid derivatives, acting as lipid biosynthesis inhibitors (Brück *et al.*, 2009). For spirodiclofen as well as spirotetramat a clear augmentation of control efficacy was assessed several days after application. In contrast to spirodiclofen which remains on the leaf surface, spirotetramat penetrates into the leaves and is distributed throughout the plant via the spirotetramatenol. As a weak acid this compound is mobile within the phloem of the plant, enabling it to move acropetally as well as basipetally. Due to this two-way systemicity, spirotetramat reaches a higher and longer lasting effect on psyllids than spirodiclofen, acting mainly by contact. As a consequence, optimal timing of applications differs between both products. While spirodiclofen reaches the best results when applied at a maximal presence of orange eggs, spirotetramat spraying is highly effective when applied on orange eggs but also at hatching of the eggs and massive presence of L1 larvae. Due to the ambimobilic systemicity of the latter, newly-grown leaves that developed after the spirotetramat application are also protected. One could have reservations about the usefulness of tetramic acid derivatives in phytoplasma vector control in view of the slow-acting nature of these compounds. On the other hand, considering the fact that spirotetramat is present in the phloem favours its use as phytoplasma vector control agent, as highly active phloem sucking (and, hence, likely highly phytoplasma infected) psyllids are preferentially killed.

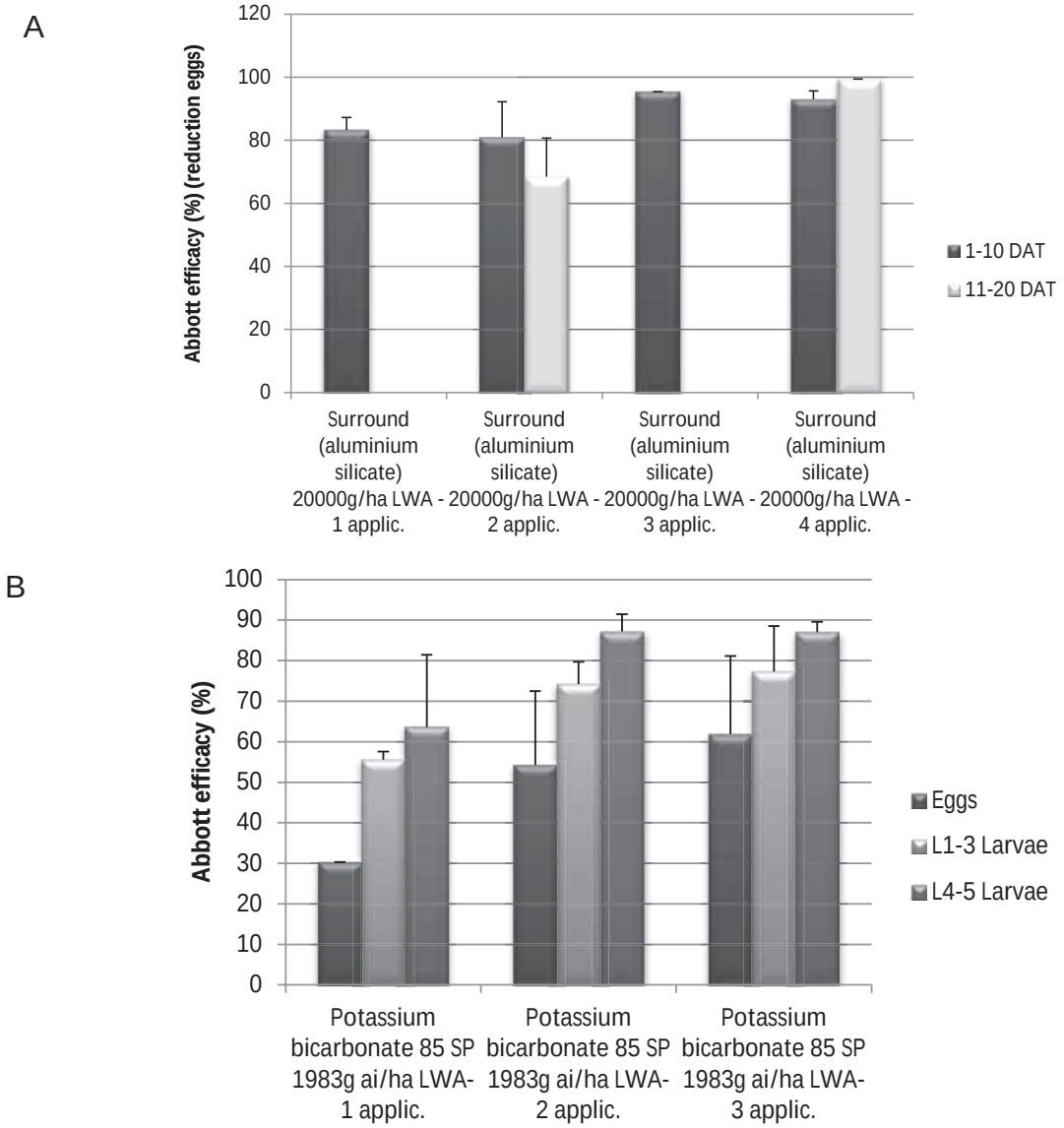

Figure 1. A. Efficacy results of Surround (aluminium silicate) on pear psyllid eggs (in case of multiple treatments, DAT indicates assessments on days after the last treatment). **B.** Efficacy results of potassium bicarbonate on pear psyllid eggs, young and old larvae after 1-3 treatments (assessed in a range of 5-13 days after the last treatment).

Compared to lipid biosynthesis inhibitors, spinosad and abamectin show a quite different working profile. Consistent with their mode of action on the nervous system, treated larvae find a quicker death. Spinosad displays an important knock down efficacy as it has rapid contact and ingestion activity in insects, causing paralysis by excitation of the nervous system. Abamectin is the common name of a mixture of avermectin Bla and Blb, affecting the chloride channels in the central nervous system. Spinosad as well as abamectin are linked to micro-organisms (in nature they are produced by the soil bacteria *Saccharopolyspora spinosa* and *Streptomyces avermitilis*, respectively) and rather susceptible to degradation. In the *Cacopsylla pyri* field trials analysed in this study later assessments show a decrease in efficacy for spinosad at 5 to 8 days after treatment while the effect of abamectin increased. One week after application, high and quite consistent mean efficacies of about 80% were generated over the different trials with abamectin, but a couple of days later its effect on young larvae also started to decrease. Spinosad is registered as a double application and therefore, in practical use, higher efficacies can be reached with this product than in a single application.

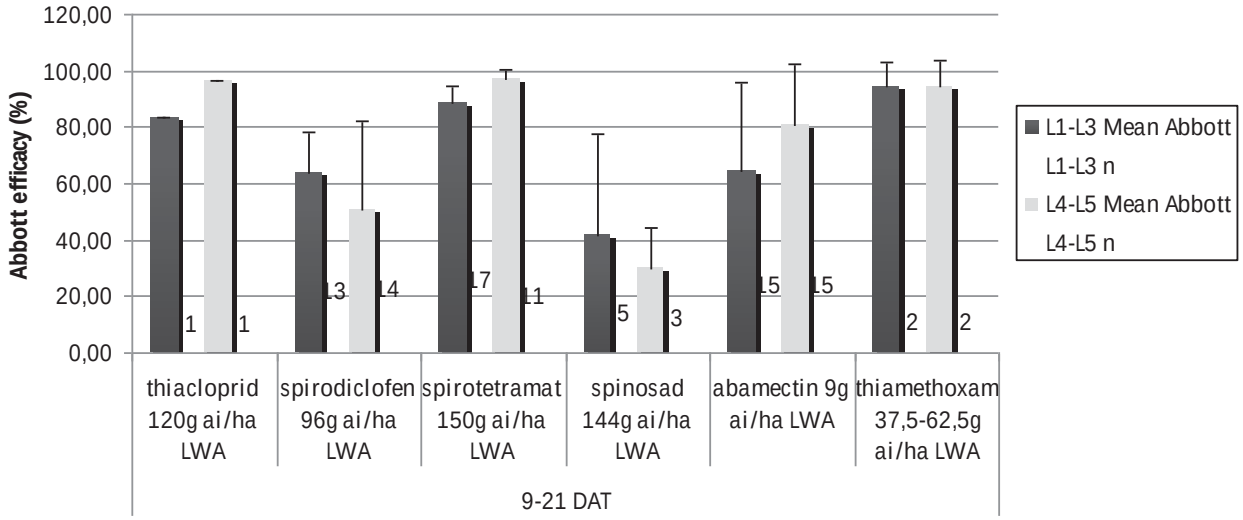

Figure 2: Efficacy results of thiacloprid, spirodiclofen, spirotetramat, spinosad and abamectin on the second generation of *C. pyri*. The number of trials is indicated at the right side of each block.

c. Optimal *C. pyri* control schedules

In order to limit potential phytoplasma spread as much as possible multiple control actions throughout the season against psyllids are often required. When a high pressure of pear sucker is present within the orchard IPM pear growers can get a special permission to use pyrethroids (active ingredients bifenthrin and deltamethrin) early in the season or after the harvest of the previous season. A very effective way to prevent population build-up early in the season is by multiple sprayings of kaolin (aluminium silicate), considerably preventing egg-laying in pear trees through its repellent effect. Another important suppression of the pear sucker population in early spring can be achieved by multiple mineral oil applications. Horticultural mineral oil prevents hatching of eggs, acts by suffocation and also causes repulsive effects, but less clear. Both alternative non-chemical control methods can offer a very important contribution to pre-bloom pear sucker control in pear orchards. When just before flowering still high numbers of psyllids are present an application of thiacloprid is allowed in IPM, with the restriction that 30% of the shoots should have infestation at least evolving to 30% of L1-larvae. However, from a phytoplasma management viewpoint it would be better if these constraints were less restrictive. After all, the lower the number of mobile adults entering the blossoming and past-bloom period the less the likelihood of phytoplasma dispersion. After blossoming the second generation of *C. pyri* provides the best conditions for optimal timing of insecticides, since there is the least overlap between the different life stages in this generation. Hence, this provides an excellent opportunity for a perfectly tuned treatment of spirodiclofen at massive presence of orange eggs. Also for spirotetramat there is an optimal application timing at massive egg hatching and presence of first larvas, but treatments at eggs or massive presence of larvae result in high efficacies as well, reflecting the fact that for this compound the growth condition of the tree is more critical than the precise pest stage at the timing of application. Some days later at peak presence of young larvae an abamectin or spinosad application reaches its highest efficacies, as shown by the results presented above. During summer, predation by predatory bugs (*Anthocoris* sp.) is of major importance to reduce the following pear sucker generations till the end of September (Bangels *et al.*,

2009). In this period new build-ups of the psyllid population can be prevented by sprayings of potassium bicarbonate on a regular basis (5-7 days interval) until harvest. These potassium bicarbonate sprayings can also be continued after the harvest, but in orchards with moderate to high pressures an intervention with thiacloprid or thiamethoxam (or pyrethroids) might be necessary to substantially decrease the population of overwintering psyllids.

Efficient control of pear suckers relies on a perfect tuning of treatment schedules, taking into account efficacies of (at preferably) low-impact insecticides and side-(repellent)-effects of alternative products (e.g. kaolin, potassium bicarbonate and mineral oils), the optimal positioning of these crop protection agents, and the best possible presence of beneficial predators. From a phytoplasma management viewpoint the economic threshold of *C. pyri* should be lowered if there are any indications of pear decline in the close environment. Indeed, a control treatment that eliminates low numbered but phytoplasma infected psyllids will provide an economic return, possibly not on short term by lowering honeydew-linked damage, but certainly on long term by preventing new infections of healthy pear trees.

Control of vectors of apple proliferation

Two univoltine psyllids, *Cacopsylla picta* and *Cacopsylla melanoneura*, are acknowledged vectors of '*Candidatus* Phytoplasma mali' (reviewed by Jarausch & Jarausch, 2010). As both species hibernate on conifers in larger distances from the orchards, their control is possible only when the insects are present on cultivated plants. In Trentino (north Italy), since 1999 field trials were carried out in order to find efficient insecticides to control *C. picta* and *C. melanoneura* and to determine the timing of the treatments. Ethofenprox was found to be the most efficient product to control the overwintering adults of both species before blossom (Mattedi *et al*., 2007). *C. melanoneura* was also efficiently depleted with organophosphates. The control strategy aimed to prevent the reproduction of both species on apple. A particular problem arose for the control of overwintered adults of *C. picta* in years when oviposition coincided with the period of blossom when insecticides cannot be applied. In this case the strategy can be focused on the control of the development of the new generation. Organophosphates as well as neonicotinoids (thiametoxan, thiacloprid) were found to be appropriate products to control the larval development of *C. picta* (Mattedi *et al*., 2007). The results of the transmission trials showed that both generations of *C. picta* can transmit the phytoplasma. Consequently, in areas where the disease is present, both the remigrants and the new generation must be controlled. Therefore the precise prediction of the migration phase and the larval development is indispensable for an efficient control of the vectors of '*Ca*. P. mali'.

Control of *Cacopsylla pruni*, the vector of European stone fruit yellows

Only very few attempts have been undertaken to control *Cacopsylla pruni*, the vector of '*Candidatus* Phytoplasma prunorum', by classical means of spraying. *C. pruni* is an univoltine species strictly oligophaguous on *Prunus* species which hibernates on conifers. Also for this psyllid, possible control is restricted to the period when it reproduces on its host plant. However, the disease might be endemic (present on wild plants) and the populations of *C. pruni* might be abundant (Jarausch & Jarausch, 2010).

Poggi Pollini *et al*. (2007) conducted a trial for vector control after a severe outbreak of ESFY disease in the Trentino (north Italy). They treated 4 different experimental orchards with diverse pesticides to control the vector *C. pruni*. The monitoring of ESFY-like symptoms during the following seasons demonstrated, however, that most of the applications had no efficacy in controlling the disease.

Jarausch *et al*. (2010) tested directed control strategies against adults and the various larval instars of *C. pruni* in semi-field trials. By this means the most promising results could be obtained with the substance abamectin by affecting the larval development and thereby reducing the emergence of new imagines of *C. pruni*. The most efficient application period for abamectin was between mid April and mid May when the first nymphs (L1+L2) started to hatch. Actually, abamectin can be used with exemption certificate for the control of the larval development of *C. pruni* in Germany.

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Innovative vector control

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Introduction

Phytoplasmas are worldwide responsible for more than 700 different plant diseases and have an important economic impact (Weintraub & Beanland 2006). For example, the apple proliferation disease causes an annual loss of about 25 Mio Euro in Germany and 100 Mio Euro in Italy due to the production of witches brooms and tasteless undersized fruits (Strauss, 2009). Phytoplasma species belonging to the apple proliferation group are the economically most important fruit tree phytoplasmas and are widespread in the temperate regions of Europe. The univoltine psyllid species *Cacopsylla picta* was identified as vector for '*Candidatus* Phytoplasma mali', the microbial agent of the apple proliferation (AP) disease, in most parts of Europe, while the psyllid *Cacopsylla melanoneura* was identified as vector of AP exclusively in northwestern Italy (Mayer *et al.*, 2009). *Cacospylla pruni* is the only known vector of '*Candidatus* Phytoplasma pruni' (European Stone Fruit Yellowing, ESFY). These three psyllid species move during their life cycles between two groups of host plants, the reproduction hosts (fruit trees) used for mating and oviposition, that also provides food for the offspring, while several species of conifers are used as overwintering hosts. All three psyllid species use chemical cues for the identification of their host plants during migration (Gross, 2011).

For these multitrophic systems only limited information is available for ecology and evolution of interactions between plants, phytoplasmas, and vector insects. Basic research on several phytoplasma – vector – plant systems are currently carried out with the aim to develop species specific traps for monitoring and mass trapping of different vector species of fruit tree phytoplasmas. Here we present results of our research on the chemically mediated interactions of the different phytoplasmas affecting apples and stone fruits, their host plants, and the vectors *C. picta, C. melanoneura* and *C. pruni* (Hemiptera: Psyllidae). Furthermore, we present preliminary data on the application of these findings for the development of innovative biotechnical control methods for phytoplasma vectors using sticky traps equipped with newly detected infochemicals.

The approach

Volatile compounds from host plants under field and laboratory conditions were collected using different headspace sampling methods. Odour compounds sampled were analyzed after thermodesorption by gas chromatography coupled with mass spectrometry. The olfactory preferences of psyllids for certain plants and volatile chemical compounds were investigated in Y-shaped olfactometer bioassays. Several compounds were tested in field traps in apples, stone fruit and pears in various locations (Fig. 1). Host plants were selected based on their attractiveness to psyllids under field conditions.

Figure 1. JKI field trap prototype for monitoring of psyllids.

Chemically mediated multitrophic interactions in a plant-insect vector-phytoplasma system

We could show that '*Ca.* P. mali' directly manipulates plant physiology and indirectly vector behaviour by attracting *C. picta* to infected plants and thus increasing its spread within the host plant population (Mayer *et al.*, 2008a; b). In contrast, the vector developed mechanisms to minimize harmful effects by a phytoplasma infection: the infection is tolerated by adult *C. picta* and detrimental effects to the offspring are avoided by a preference to oviposit on healthy trees (Mayer *et al.*, 2011). When developing on apple plants infected by '*Ca.* P. mali', the nymphs suffered higher mortality and reached lower body weights compared to conspecifics reared on uninfected plants. We conclude that *C. picta* evolved mechanisms to minimize harmful effects for its offspring through avoiding an infection by '*Ca*. P. mali'. In contrast, infection by the phytoplasma is tolerated by adults and seems to have no detrimental effects. The chemical compound responsible for the attraction of the vector to infected trees could be collected from the headspace of infected apple trees. It was identified as β-caryophyllene by gas chromatography coupled with mass spectrometry. We found that this sesquiterpene is attractive for both sexes of *C. picta*. The compound will be used for the development of traps for monitoring and/or mass trapping of *C. picta* (Weintraub & Gross, 2013).

Analyzing the complex chemically mediated interactions between '*Ca.* P. mali', its two vectors *Cacopsylla picta* and *C. melanoneura*, and their host plants (reproduction host and overwintering host) for the first time, we were able to show that this phytoplasma lures the highly adapted vector *C. picta* to infected apple plants by changing its odour (Mayer *et al.*, 2009). The phytoplasma induces apple trees to produce more ß-caryophyllene which preferentially attracts new generation adults of *C. picta* (emigrants) just before their emigration to the overwintering host (Mayer *et al.*, 2008). By feeding on infected plants, the probability of an acquisition of the phytoplasma increases. In contrast, the hawthorn psyllid *C. melanoneura* did not react to this sesquiterpene. After overwintering, the psyllids return to apple plants (remigrants), but now prefer to lay their eggs on uninfected plants. By doing so they transmit the phytoplasma to previously healthy hosts. Which infochemical(s) may regulate the observed oviposition behaviour still remains unknown.

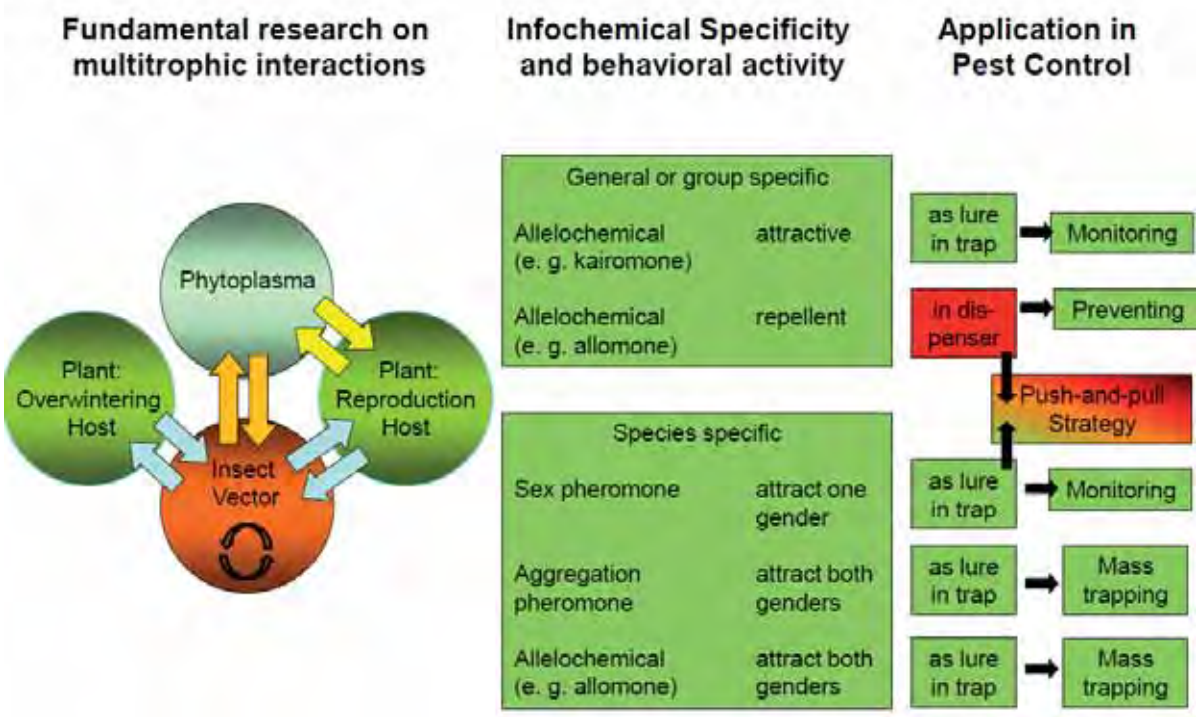

Figure 2. Scheme of chemically mediated multitrophic interactions between host plants, phytoplasmas, and vector insects. Behavior modifying compounds can be used in biology based pest control programs (Weintraub & Gross, 2013).

Development of innovative control strategies based on plant infochemicals

In recent years we started to exploit our findings by constructing traps with attractive components like ß-caryophyllene for the capture of psyllids (Gross *et al.*, 2011). Psyllids are small insects and the different species are morphologically very similar. Thus, the development of species specific traps could greatly reduce the efforts necessary for identification. Furthermore, through this monitoring the amount of chemical insecticides can be reduced by detecting the adequate date for spraying. Because in the AP-system the infochemical produced by infected plants is attractive to both sexes of psyllids, it could also be possible to develop mass trapping systems for a sustainable control of these insects in the future.

For *C. melanoneura* and *C. pruni* we have identified potentially behavior modifying compounds. To date, however, we have not found a species specific attractive volatile compound from their respective host plants. Interestingly, we could identify potential repellent chemicals for emigrants of *C. pruni*. We are currently working with the olfactory behavior of remigrants of this species. Furthermore, we are collecting the headspace of various host plants in the field to be able to compare field and laboratory data on volatile compounds with insect behavior. When identified, attractive compounds could be used in traps as lures for monitoring and mass trapping purposes and combined with repellent compounds these chemicals can be used in complex push-and-pull strategies (Fig. 2).

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"Flavescence dorée" vector control in Italy

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Introduction

"Flavescence dorée" (FD) is a phytoplasma-associated disease of grapevine, specifically transmitted by the leafhopper *Scaphoideus titanus* Ball. Two distinct FD strains, 16SrV-C and –D have been found associated with infected grapevines but both are transmitted by *S. titanus* (Mori *et al.*, 2002). Even if another hopper species, *Dictyophara europea* (L.), proved to be able to transmit FD to grape (Filippin *et al.*, 2009), *S. titanus* is the only FD vector that feeds and breeds on grape and therefore can be considered the specific vector of FD in the field. Due to the biology of *S. titanus*, that is strictly associated with grape and has only one generation per year, the control of FD spread mainly relies on the control of this leafhopper and *S. titanus* population level and disease spread are clearly correlated. To prevent FD spread in Italy, a ministerial decree has been published in 2000 (D.M. n. 32442, 31/05/2000) and detailed guidelines have been enacted by the different Italian Regions concerned by FD every year since then. According these guidelines, insecticide applications against the vector, as well as removal of symptomatic plants in the vineyards, are compulsory in Northern Italy since the early 2000's. However, in compliance with the national legislation, each Regional Phytosanitary Service designed specific guidelines considering the specific viticultural conditions of the regional areas, so that slightly different FD control approaches are acting in the different Central and Northern Italian Regions.

In this contribution we aim to review current monitoring and control techniques as well as the new perspectives in the control of the vector *S. titanus* in the Italian vineyards.

Vector monitoring

An area-wide and prompt monitoring of the vector is the pre-requisite to design a rational control strategy. Monitoring has several different purposes: to detect the presence of the vector in a given area, to establish the developmental stage of the population in order to well-time the insecticide application, to detect the population level to eventually reduce the number of insecticide applications, to check the application of compulsory insecticide treatments, and to evaluate the efficacy of the insecticide applications.

S. titanus is monovoltine and its biological cycle is characterized by two extended periods of nymph and of adult presence. The two periods 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 population level and, for this, a sequential sampling, based on fixed precision level (75%) stop lines, has been proposed (Lessio & Alma, 2006). Basal leaves (close to the oviposition sites) should be preferably inspected in the early morning when leafhoppers are less mobile and counting should involve grapevine plants scattered over the whole vineyard (e.g. visual inspection of one plant out of ten along the row, one row out of three...). To monitor adults, different methods can be applied: direct counting on the leaves, "frappage" (beating tray), sweep-net and yellow sticky traps (YST). This latter method is by far the most effective, comparable and used. YST are placed on the canopy, preferably in the shadow, vertically or horizontally, generally hanged at 1-1.5 m above the ground. Different types of YST can be used, but yellow 24x40 cm are the most widely used, even if smaller Rebel traps (8x16 cm) may be more efficient, per surface unit, in capturing *S. titanus* (adults per cm^2). A minimum of three traps per vineyard with 1 trap/2,000 m^2 density is suggested. Traps are changed every one or two weeks and are placed in the vineyards starting from the end of June. Adult monitoring allows the estimation of the vector presence and of population peak occurrence, so the second insecticide application can be timely targeted against an important proportion of adults as well as against the late nymphs that co-occurred together with the adults until the beginning of August. In many Northern Italian Regions, in order to monitor the actual application of the compulsory insecticide treatments, removal of the infected grapevines and of the

abandoned vineyards, thousands of vineyards have been surveyed starting from 2001. Among these, in the Piemonte Region, in the early 2000's about 400 vineyards were surveyed every year with YST, while only 150-200 vineyards have been surveyed in the last three years. The decrease in monitoring activity reflects the decrease in public funding due to budget cuts to the Regional governments. These surveys allowed identifying hundreds of non-fulfilments every year, with 150 injunctions and 60 charges to defaulting vine growers.

Vector control

Among the different 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, branches from winter pruning should be destroyed (minced and/or buried under the soil), since they host leafhopper eggs. Suckers should be also removed because they are likely to host several nymphs. Also, a very important prophylactic measure is the cleaning of uncultivated areas surrounding the vineyards (hosting wild American grapes), as well as the prompt removal of abandoned vineyards, that host important *S. titanus* populations. In the Piemonte Region growers are advised: i) to destroy the creeper wild vegetation surrounding the vineyard in wintertime (at least the 10 m surrounding the vineyard) using boom mulchers to eliminate wild grapes climbing on the trees, ii) to spray with glyphosate (high concentration: 4-8% a.i.) the wild grape sprouts in spring and eventually repeat the herbicide application in autumn, iii) to repeat the mechanical and chemical removal of the wild vegetation once a year.

Insecticides are generally applied twice a year in the areas characterised by a high incidence/prevalence of the disease. In the areas where the disease is under control only one application is suggested. The first treatment, targeted to immatures 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 grapevine flowering in order to avoid poisoning of honey bees. For the same reasons, growers are advised to cut grasses in the inter rows before spraying the insecticide to avoid insecticide drift on flowers. Insecticides that can be used in the first treatment are the insect growth regulator buprofezin, the organophosfate chlorpyrifos-ethyl, chlorpyrifos-methyl and the systemic neonicotinoid thiamethoxam. This last one is by far the most widely used in the Piemonte Region. 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 adult and late nymph populations, using chlorpyrifos-ethyl, chlorpyrifos-methyl, thiamethoxam, etofenprox. Thiamethoxam, as well as the other a.i. should only be applied once a year. In the early 2000's thiamethoxam was always chosen for the first treatment in June. Now, in some farms and for late ripening varieties, this a.i. is applied as the second treatment, to ensure a prolonged protection of grapevines from *S. titanus* adults. 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 occurs in the vineyard), the two treatments can be reduced to one only. When only one treatment per year is applied the first treatment in June is skipped, avoiding the use of insect growth regulator and matching the application with Grape Berry Moth strategy control (e.g. using a mixture of thiamethoxam and chlorantraniliprole) (Pavan *et al.*, 2005).

Organic vine 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 wide area treatment rather than multiple treatments (Pavan *et al.*, 2004). In the Piemonte Region, in the last years several "pilot projects" have been started on a voluntary basis thanks to the vine growers of some viticultural areas. So far the pilot projects are 10 and involve more than 100 municipalities in the provinces of Alessandria, Asti and Cuneo. At first, local representatives together with vine growers, supported by the extension services, establish a working group. Both professional and part-time vine growers are involved. *S. titanus* is monitored by direct sampling of nymphs in early June for the best timing of the first insecticide application. The vector is then monitored by YST in July, August and September in a variable number of vineyards. YST are provided by the Servizio Fitosanitario of the Piemonte Region and the traps are placed and changed in the vineyards by the growers, then checked for the presence-counting of *S. titanus* by technicians of the extension service, when needed. Within the frame of these pilot projects, 400 vector monitoring sites have been established and more than 5,500 YST are checked for *S. titanus* every year. Besides these activities, volunteers of the pilot projects also survey and register all the abandoned vineyards, and woodlands hosting wild grapevines. As a further step, the owners of abandoned vineyards or uncultivated areas hosting wild grapevines are identified and

involved in the cleaning and removal of wild vegetation, with mechanical and/or chemical methods. Finally, training stages are organised to teach the correct identification of the infected plants, of the vector and to explain the FD control techniques to the vine growers.

New perspectives in vector control

In the short term, the main goal is to optimize *S. titanus* control by extending insecticide applications to all the vineyards, since some vine growers, especially part-time growers, are not yet concerned by the problem and do not respect compulsory treatments.

In the very last years, the presence of an important number of *S. titanus* adults late in the season (late August and September) has been recorded. Often these leafhoppers harbour FD phytoplasmas (author's unpublished data) and therefore can be infectious. On the other hand, insecticides are applied at the end of July at latest so that the plants are not protected anymore after that time. Since many of these "late flying" leafhoppers colonize the vineyards from wild areas or abandoned vineyards (Lessio & Alma, unpublished), a further insecticide treatment limited to the border rows/grapevines, can be implemented in the control strategy.

As neonicotinoid insecticides appear the most active in protecting plants from phytoplasma transmission (Saracco *et al.*, 2008) but their use may affect honey bees and other pollinators, a new thiamethoxam application targeted to the trunk of the grapevine is under investigation. Actually thiamethoxam (and other neonicotinoids) can be absorbed via the trunk and their activity following this delivery is very persistent. Trunk applications of neonicotinoids are commonly used in citrus plantation in Brazil for the control of *Diaphorina citri* Kuwayama, the vector of huanglongbing (citrus greening) and have been suggested also for the control of *Homalodisca vitripennis* (Germar) the vector of *Xylella fastidiosa* (Castle *et al.*, 2005). The main improvements of this technique are a very targeted application to the plant trunk without insecticide drifting to the herbaceous plants, avoiding poisoning of pollinators and of non-target insects, and a longer persistence of activity that may eventually allow to reduce the two treatments to one only.

As a long term perspective, symbiotic control and vibrational mating disruption strategies are under investigation. The symbiotic control of phytoplasma vectors 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).

Obviously, these latter perspectives are far from the field application and therefore the optimization of chemical and agronomical control techniques is imperative to limit the spread of FD in the next years.

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Management of "bois noir" through vector control

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Introduction

"Bois noir" (BN) is a grapevine yellows disease of great economic importance in European viticulture. It is associated with '*Candidatus* Phytoplasma solani' (16SrXII-A) presence (Quaglino *et al.*, 2013). This phytoplasma is endemic in the natural vegetation and transmitted naturally from and to herbaceous plants by the Cixiid planthopper *Hyalesthes obsoletus* Signoret. Grapevine is infected through the occasional feeding of this vector. Since *H. obsoletus* does not acquire BN phytoplasmas from grapevine, it is considered a dead end host for the phytoplasma (Maixner, 2010). Consequently, BN incidence does not influence the further disease spread and there is no need to remove infected vines from vineyards (Pavan *et al.*, 2012). Infection pressure is rather determined by the presence and infestation of natural host plants and the associated vector and their density and distribution patterns within and around the vineyards. This implies, that attempts to reduce infection pressure need to overstep the vineyard borders and have to focus on the natural phytoplasma-vector-host-systems.

Control strategies for *Hyalesthes obsoletus*

H. obsoletus has one generation per year in Europe. Eggs are deposited in the soil where the five larval instars feed on the roots of their host plant, of which field bindweed (*Convolvulus arvensis* L.) and stinging nettle (*Urtica dioica* L.) are the most important species (Langer & Maixner, 2004). The second and third instar nymphs move to deeper soil layers for hibernation and resume their feeding activity in spring (Cargnus *et al.*, 2012; Langer *et al.*, 2003). BN is acquired by the nymphs from the roots of infected hosts (Kaul *et al.*, 2009). The life cycle is completed with the emergence of the adult planthoppers, which live on the aerial parts of a wide range of host plants and occasionally feed on grapevine, too. The presence of *H. obsoletus* is not restricted to vineyards but depends on the occurrence and distribution of its natural plant hosts within and outside the vineyards, so that infection pressure is not only determined by the specific conditions of particular vineyards, but it depends also on the general biotic and abiotic conditions on a larger scale. Light and permeable soils, sparse vegetation and high insolation resulting in a favorable microclimate are key factors for the presence of *H. obsoletus.* Young vineyards, fallow fields and other uncultivated areas around vineyards are typical habitats of this vector. Insecticide sprays to the grapevine foliage, which are effective against the vector of 'flavescence dorée', another important grapevine yellows in Europe, are therefore ineffective to control *H. obsoletus* 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. Since bindweed and nettle harbor different strains of BN (Langer & Maixner, 2004), typing of BN isolates from infected vines allows the identification of the locally predominant reservoir plants.

Control of host plants

Nettle and bindweed are common weeds of vineyards and surrounding structures. Bindweed is widespread inside the vineyards. A random or clumped distribution of BN infected grapevines corresponding to the presence of this plant host is often the result. While a well-managed greencover can suppress bindweed in the vineyard interrows (Maixner, 2007), it remains a problematic weed in the undergrowth of the grapevines. Mechanical or chemical weeding of this area is advisable to avoid a high infection pressure emerging close to the grapevine plants. 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 situation where nettle is growing along the borders (Mori *et al.*, 2008; 2012), since adult *H. obsoletus* disperse from these sites to adjacent vineyards (Mori *et al.*, 2011). 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 & Kast, 2008; Mori *et al.*, submitted). Thus, the decision on autumn or spring treatment can be made with respect to practical needs and the requirements of environmental protection. However, treatments in spring need to be timed so early, 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 six weeks before the anticipated start of adult emergence (Mori *et al.*, submitted). A degree-day model is used in Germany to calculate the emergence date of *H. obsoletus* for both nettle and bindweed 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 for grapevine (Mori *et al*., 2012).

Where nettles grow in little stands scattered around in the vineyards or on the embankments of terraced vineyards, point applications, either by brushing or spraying herbicides, are the appropriate measure. The selective elimination of nettles can save the surrounding green cover but reduces the number of emerging vectors (N. Mori *et al.*, unpublished).

Alternative control approaches

Alternative approaches beside host plant control have been evaluated with more or less success. Although they might be not practicable in general, they may provide solutions to specific problems.

Insecticides that are traslocated to the roots after foliar application on host plants proved to kill the nymphs and to reduce the numbers of emerging adult *H. obsoletus* (N. Mori *et al.*, unpublished) with an efficiency that was comparable or slightly lower than the early chemical weeding. The use of insecticides could be considered as an "emergency treatment" shortly before the start of the emergence of adult vectors when herbicide treatments are not efficient anymore. However, the use of insecticides on uncultivated areas is restricted in many countries.

Alternatives to the chemical control of host plants or vectors are required in organic viticulture in particular and for areas where herbicide treatments are not applicable. Repeated mowing reduces nettle density gradually over time (Mori *et al.*, 2011; 2012). It is therefore suitable as a long term management tool for areas where herbicide treatments are not possible. However, numbers of *H. obsoletus* turn down only slowly corresponding to the decrease of nettle density (Mori *et al.*, 2012). Mechanical weeding could be an alternative, but it is not applicable on ditches and embankments because of soil landslide. Plowing of host plant stands in winter significantly increases the mortality of hibernating nymphs because they are moved to the soil surface and the numbers of emerging adults are significantly reduced (Maixner, 2007). Fences of plastic insect nets have been used as flight barriers to prevent immigration of adult vectors into vineyards (M. Maixner, unpublished). They are costly but could provide a solution were other measures are not applicable, e.g. where conservation areas are bordering the vineyards.

Extended uncultivated areas such as fallow fields can be important sources of BN inoculum. Since herbicide or insecticide treatments on such non-cultivated areas are impossible, both from the economic and from the environmental point of view, habitat management techniques are an alternative. Cultivation of a competitive green cover by sawing out seed mixtures of biannual and perennial herbs after clearing of the plots reduces the density of host plants and deteriorates the microclimatic conditions for *H. obsoletus* with an acceptable effort. If endemic plant species are chosen that require no further cultivation this method can provide a long term solution.

Conclusions

Direct and indirect strategies to control *H. obsoletus* interfere with the life cycle of this vector and aim at the best possible reduction of infection pressure. Given the complex epidemic system of BN effective control attempts can reduce but not eradicate the problem. Coordinated actions of the growers to reduce sources of inoculum at a viticultural site on a larger scale are more promising on the long run than plot-related activities. Further investigations are necessary to identify the factors that drive the long term temporal variation of BN infection pressure in order to setup and improve appropriate preventive measures.

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Role of propagation material in phytoplasma dissemination

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Introduction

Grapevine yellows (GY) are widespread diseases of grapevine associated with molecularly distinguishable phytoplasmas. In Europe, the most important grapevine yellows are “flavescence dorée” (FD) and “bois noir” (BN). While both diseases are economically important on grapevine, FD is also a quarantine disease. The BN-associated phytoplasma has very wide host range, while host range of FD is more restricted (Boudon-Padieau, 2003). For FD both phytoplasma and eggs of the vector can be spread with symptomless planting material.

The three economically important disorders of temperate fruit trees associated with phytoplasmas, present in Europe are apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY). The psyllid vectored phytoplasmas associated with AP, PD and ESFY are ‘*Candidatus* Phytoplasma mali’, ‘*Candidatus* Phytoplasma pyri’ and ‘*Candidatus* Phytoplasma prunorum’, respectively. They are considered quarantine organisms and are almost restricted to the genera *Malus* (apple), *Pyrus* (pear) and *Prunus* (stone fruits), respectively (Seemüller & Schneider, 2004).

Together with insect vectors, propagation material plays a main role in phytoplasma disease dissemination in woody host plants, especially for long-distance transmission and introduction of diseases in new geographic areas. Although the importance of planting material for long-distance transmission is clear, its relevance for some diseases, when planting in areas with high infection pressure is still controversially discussed. For example, in areas with BN epidemic outbreaks low amount of infected planting material should be a minor problem, while only a single grapevine, infected by FD, imply a high risk for areas where it’s vector is present (Maixner, 2006).

Infected planting material

The source of infected planting material can be both, infected scions and infected rootstock, and despite the high mortality of GY infected grafting (up to 85% for BN, Zorloni *et al*., 2011) the surviving plants can be infected. Grapevine rootstocks (*Vitis rupestris*, *V. riparia*, etc.) are mainly tolerant to both FD and BN, so they can carry latent infection. There is also a threat for using scions from infected plants, since the scions are usually taken during the dormant period, when symptoms are very difficult to be observed.

Prunus cerasifera (myrabolan) and genotypes of *P. domestica* (European plum) are little affected by ESFY and, when infected, they are very often asymptomatic therefore can be a source of *inoculum* when used as rootstock for susceptible stone fruits (Kison & Seemüller, 2001; Paltrinieri *et al*., 2004). On the other hand AP seems not to be transmissible by scions, at least under the central European climate, due to low phytoplasma titer in the aerial part of the plant during winter (Seemüller *et al*., 1984). ‘*Ca.* P. pyri’ is shown to be present in the aerial parts of trees for a longer period in Mediterranean climates than in central European climates and, although shown to be not transmissible during winter, there are reports of its transmissibility by scions in Mediterranean climates (Schaper & Seemüller, 1982; Garcia-Chapa *et al*., 2003). This must be considered when collecting plant material during winter for vegetative propagation.

When planting material is infected from rootstock or scion, delay in symptom expression can be from few months up to years (depending on species and variety used), which makes it difficult to determine the source of infection when symptoms appear (Giunchedi, 2003). Because of this latent period even apparently healthy plants may carry phytoplasmas.

Although the molecular techniques for phytoplasma detection are extremely sensitive, due to strong seasonal variation, irregular distribution and low concentrations of phytoplasmas in woody hosts (during some periods) and especially in young, grafted material there are difficulties in their detection (Seemüller *et al*., 1984; Garcia-Chapa *et al*., 2003).

In case of grapevine, planting material can also carry under the bark the eggs of *Scaphoideus titanus*, vector of FD (Fig. 1). This is very likely the way how *S. titanus* arrived to Europe from North America, where it is a native species (Caudwell, 1983; Bertin *et al*., 2007). A recent study by Papura *et al*. (2012) used a combination of nuclear and mitochondrial markers to trace back the invasion history of *S. titanus* to Europe and confirmed the American origin of this leafhopper, and suggested that the introduction of this pest happened once or several times from the same area.

Fig. 1: *Scaphoideus titanus* eggs on grape under bark.

Resistant rootstocks

Some of the most promising approaches to control phytoplasma diseases of woody hosts is the use of resistant plants.

Natural resistance to AP, characterized by the absence of symptoms and by a strongly reduced concentration of the pathogen, is demonstrated in experimental rootstocks, with *M. sieboldii* in their parentage. The fact that the pathogen overwinters in the roots and that non-infected scions can be taken during winter opened the possibility that growing scion cultivars on AP resistant rootstocks can prevent or reduce the impact of disease. Promising genotypes are selected from *M. sieboldii*–derived rootstocks as resistant to AP and were then employed for further agronomic evaluation of their productivity and vigor, what is currently in progress (Seemüller *et al*., 2008; Jarausch *et al*., 2011).

For PD promising genotypes - in terms of disease resistance, vigor and yield efficiency - were observed in progeny derived from a Muscovite *P. communis* seed parent (Seemüller *et al*., 2009), while for ESFY no significant advantages in this field were yet achieved although some differences in sensitivity of rootstocks and varieties were observed (Kison & Seemüller, 2001; Laimer & Bertaccini, 2006).

Specific studies about grapevine rootstock resistance to FD and BN phytoplasmas were not carried out, but differences in phytoplasma susceptibility were identified; however, since very often rootstocks are asymptomatic, the visual assessment is not the best tool for this screening (Borgo *et al*., 2009).

Phytoplasma and vector free propagation materials

In order to control the pathogen presence and to prevent its spreading in the propagation material it is mandatory to exclude the presence of quarantine phytoplasmas such as FD, AP and ESFY and it is also advisable to exclude pathogens relevant for quality, such as BN, by using the most sensitive detection techniques available applied in the appropriate time of the year and to the correct plant tissue. It is advisable however to acquire every possible information concerning the phytosanitary status of the material used for propagation in order to prevent possible unforeseen outbreaks of disease such as those occurred for FD disease when a grapevine insect such as *S. titanus* was introduced in Europe. It is known in fact that a number of different phytoplasmas are able to infect grapevine worldwide in the presence of appropriate insect vectors or by grafting or micropropagation technique application to infected starting materials. To maintain the orchards and vineyards clean from phytoplasmas, elimination of symptomatic plants, identification and elimination of insect vectors and elimination of alternative host plants should be routinely carried out.

To avoid possible vector (*S. titanus*) infestation in the grapevine propagation material it is necessary to:

(i) apply the appropriate insecticide during the whole vector flight period
(ii) locate the nursery vineyards away from potential sources of *S. titanus* that could be vineyards not treated with insecticides or headlands and woodlands hosting wild grapes
(iii) use rootstock mother plants, scion mother plants and rootling plantlets training systems that allow effective insecticide treatments in nursery industry.

In the case that there is no germplasm with clean plants available, it is possible to clean the material using **thermotherapy** and/or **meristem-tip culture** in order to eliminate the pathogens. However these techniques are not eliminating the pathogens from all the produced material, therefore molecular tests are again necessary to assess the plant health status before the material can be employed for field dissemination. The clean germplasm should also be protected under insect proof environment in order to avoid its re-infection. It is very important to keep also the propagated material clean from insects that are phytoplasma vectors.

Graft transmissibility and the vegetative propagation of grapevine imply the risk of dissemination of GY by propagation material. The propagating material may carry, beside the phytoplasma, also the overwintering eggs of *Scaphoideus titanus*; Caudwell *et al*. (1997) conducted through the years several experiments on the elimination of those eggs with the use of hot water (50°C for 45 minutes).

Several experiments and a lot of experiences are available regarding grapevine thermotherapy to eliminate phytoplasmas: not only in France but also in Italy researches were carried out to verify the effectiveness of this method in phytoplasma elimination by testing the treated material with PCR/RFLP analyses (Borgo *et al*., 1999; Bianco *et al*., 2000).

One example is that GY infected and healthy self-rooted cuttings of varieties Chardonnay, Prosecco, Garganega and other were treated by thermotherapy in hot water at different temperatures for different time, and kept in an insect proof screenhouse for two to four years. In the same conditions were also maintained treated grafted plants and plants in which only the scion was subjected to thermotherapy. The survival over the years of treated material was only about 40% (Fig. 2) and none of the plants showed symptoms during this period. The molecular tests carried out to verify GY phytoplasma elimination showed only 5% of phytoplasma elimination in treated plants (Fig. 3). While the FD phytoplasma was never detected, BN and aster yellows (16SrXII-A and 16SrI-B) phytoplasmas resulted to be present in the treated materials (Bertaccini *et al*., 2001).

The hot water treatment is particularly useful as a quarantine measure where highest phytosanitary standards are required to prevent dissemination of GY (Maixner, 2006). BN phytoplasmas were more difficult to completely eradicate than FD phytoplasma (Borgo *et al*., 1999; Bianco *et al*., 2000; Bertaccini *et al*., 2001; Mannini & Marzachì, 2007). Hot water treatment against FD phytoplasma is considered a reliable technique and is compulsory for the basic propagation material in France (Ministère de l'Agriculture France, 2003). It is recognized as a phytosanitary treatment in EU Directive 2000/29/EC (EU, 2000) and EPPO Standard PM 4/8, as well as by other organizations (Frison and Ikin, 1991; ICA, 2007). This hot water treatment is also effective in eliminating eggs of the leafhopper *S. titanus* (Caudwell *et al*., 1997). Hot water treatment is most effective on 1-year-old grapevine propagation material (Linder *et al*., 2010), while the 2-year wood materials may have higher levels of *S. titanus* eggs, and are not fully controlled by this thermotherapy.

Some reluctance concerning the use of hot water treatment exists in some countries because of its negative effect on the vitality of woody propagation material. It was therefore hypothesized that some varieties may be more susceptible than others.

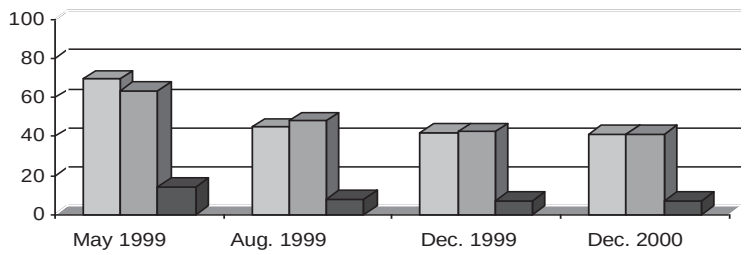

Fig. 2. Effects of hot water treatments on survival of grapevine grafted (light grey: untreated, grey: rootstock untreated and scion 50°C for 40', black: rootstock and scion 50°C for 40 min).

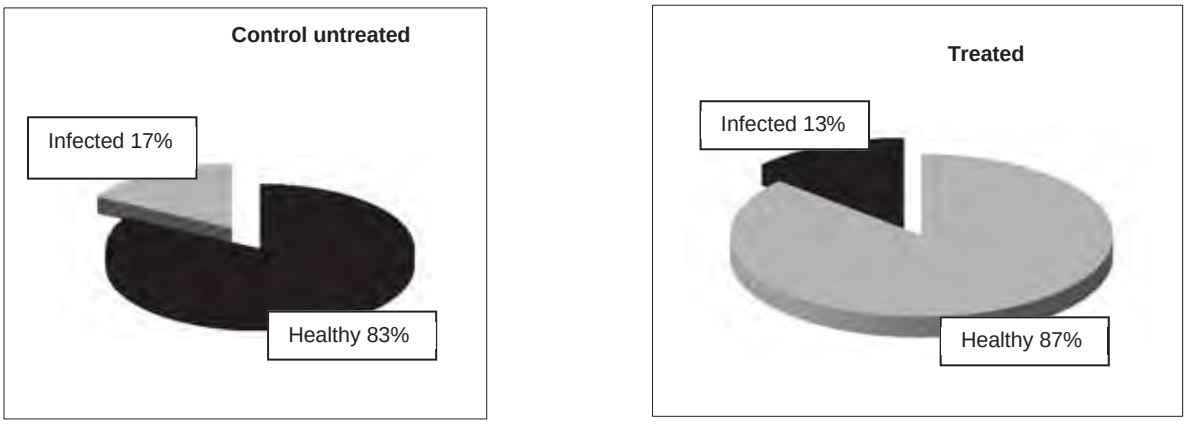

Fig. 3. GY phytoplasma elimination from grapevine by thermotherapy (50°C for 40 min) as determined with molecular testing of the surviving plants maintained under insect proof screenhouse.

Conclusions

Fruit tree and grapevine phytoplasma-associated diseases are difficult to control. As direct measures against the pathogens are not available or not registered, while controlling the vector often resulted quite ineffective under field conditions, the most promising means is to prevent the introduction of the pathogen(s) and vector(s), where not present, and employ resistant plants/varieties. The best way to prevent phytoplasma outbreaks is the production and introduction of planting material clean of pathogen and vector. For this, the use of thermotherapy and meristem-tip culture in order to eliminate the pathogens together with the application of the most sensitive detection techniques for selection of phytoplasma-free material is of outstanding importance. Great differences in susceptibility and tolerance to phytoplasma infection were observed among different varieties and plant species grown as rootstocks and scions. These characteristics can be used as source for selection and production of plant material which is less susceptible or more tolerant, although tolerant material may be nevertheless a source of *inoculum* for the sensitive grapevine or fruit trees species or varieties in some environmental conditions.

Acknowledgements

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Rules and regulations related to phytoplasma-free materials: European regulation on plant quarantine

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Introduction

The main European regulation on plant quarantine is the Council Directive 2000/29/EC of 8 May on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community.

This Directive lays down measures designed to protect Member States against the introduction of organisms harmful to plants and plant products from other Member States or third countries. Also lays down measures designed to protect Member States against the spread of harmful organisms within the European Union (EU).
Annexes I and II list the harmful organisms banned in the EU, either altogether or when they are on certain plants or plant products.
Annex IV list the special requirements for the introduction and movement of plants, plant products and other objects in all member states.

The Directive regulates several phytoplasmas in the annexes listed below. N.B. the nomenclature is the original in the Directive it is obvious to which phytoplasma is referred to.

Annex I

Part A: Harmful organisms whose introduction and spread should be banned in all member states

- Section I: Harmful organisms not known to occur in the Community and relevant for the entire Community
    - Elm phloem necrosis mycoplasma
    - Peach rosette mycoplasma
    - Peach X-disease mycoplasma
    - Peach yellows mycoplasma
    - Strawberry witches' broom mycoplasma
- Section II: Harmful organisms known to occur in the Community and relevant for the entire Community
    - Apple proliferation mycoplasma
    - Apricot chlorotic leafroll mycoplasma
    - Pear decline mycoplasma

Annex II

Part A: Harmful organisms whose introduction into, and spread within, all member states shall be banned if they are present on certain plants or plant products.

- Section I: Harmful organisms not known to occur in the Community and relevant for the entire Community
    - Palm lethal yellowing mycoplasma: Plants of *Palmae* intended for planting, other than seeds, originating in non-European countries

- Witches' broom (MLO): Plants of *Citrus* L., *Fortunella* Swingle, *Poncirus* Raf., and their hybrids, other than fruit and seeds

- Section II: Harmful organisms known to occur in the Community and relevant for the entire Community

  - Grapevine "flavescence dorée" MLO: Plants of *Vitis* L., other than fruit and seeds Potato stolbur mycoplasma Plants of *Solanaceae*, intended for planting, other than seeds.

  - *Spiroplasma citri* Saglio *et al*.: Plants of *Citrus* L. *Fortunella* Swingle, *Poncirus* Raf., and their hybrids, other than fruit and seeds.

PART B: Harmful organisms whose introduction into, and whose spread within, certain protected zones shall be banned if they are present on certain plants or plant products.

- Flavescence golden vine (MLO): Plants of *Vitis* L., other than fruit and seeds Czech Republic, France (Alsace, Champagne-Ardenne and Lorraine) and Italy (Basilicata).

Annex IV

Part A: special requirements to establish the member states for the introduction and movement of plants, plant products and other objects in all member states.

- Section II: Plants, plant products and other objects originating in the Community

  - Apricot chlorotic leafroll mycoplasma

    Plants of *Prunus* L intended for planting other than seeds.
    Official statement that:
    a) The plants originate in areas known to be free from the relevant harmful organisms or
    b) No symptoms of diseases caused by the relevant harmful organisms on plants at the place of production since the beginning of the last complete cycle of vegetation

  - Apple proliferation mycoplasma

    Plants of *Malus* L intended for planting other than seeds.
    Official statement that:
    a) the plants originate in areas known to be free from apple proliferation mycoplasma or
    b)
       a. the plants, other than those raised from seed, have been:
          - Either officially certified under a certification scheme requiring derived in direct line from material which is maintained in good condition and nematological tested using appropriate indicators or methods equivalent to detect at least apple proliferation mycoplasma found, in these tests, from those agencies harmful or
          - Derived in direct line from material which is maintained under appropriate conditions and subjected at least once during the last six complete cycles of vegetation, to official testing to detect at least apple proliferation mycoplasma using appropriate indicators or equivalent methods, revealing in these tests, from those harmful organisms
       b. no symptoms of diseases caused by apple proliferation mycoplasma have been observed on the plants at the place of production, or on the susceptible plants in its immediate vicinity, since the beginning of the last three complete cycles of vegetation.

- Pear decline mycoplasma

  Plants of *Cydonia* Mill., and *Pyrus* L., intended for planting, other than seeds
  Official statement that:
  a) the plants originate in areas known to be free from pear decline mycoplasma; or
  b) the plants at the place of production and in its immediate vicinity, which have shown symptoms giving rise to the suspicion of contamination by pear decline mycoplasma, have been rogued out at that place within the last three complete cycles of vegetation.

- "Flavescence dorée" MLO

  Plants of *Vitis* L., other than fruit and seeds
  Official statement that no symptoms of grapevine "Flavescence dorée" MLO have been observed on the mother-stock plants at the place of production since the beginning of the last two complete cycles of vegetation.

  Special requirements protected zones: Czech Republic, France (Champagne-Ardenne, Lorraine and Alsace), Italy (Basilicata).
  Official statement that:
  a) the plants originate and have been grown in a place of production in a country where grapevine "flavescence dorée" MLO is not known to occur; or
  b) the plants originate and have been grown in a place of production in an area free from grapevine "flavescence dorée" MLO established by the national plant protection organisation in accordance with the relevant international standards; or
  c) the plants originate and have been grown in either the Czech Republic, France (Champagne-Ardenne, Lorraine and Alsace), or Italy (Basilicata); or
  d) the plants originate and have been grown in a place of production where:
     a. no symptoms of grapevine "flavescence dorée" MLO have been observed on the mother-stock plants since the beginning of the last two complete cycles of vegetation; and
     b. either
        i. no symptoms of grapevine "flavescence dorée" MLO have been found on the plants in the place of production; or,
        ii. the plants have undergone hot water treatment of at least 50°C for 45 minutes in order to eliminate the presence of grapevine "flavescence dorée" MLO.

- Potato stolbur mycoplasma

  Plants of Solanaceae, intended for planting, other than seeds:

  Official statement that:

  a) the plants originate in areas known to be free from potato stolbur mycoplasma or

  b) no symptoms of potato stolbur mycoplasma on plants at the place of production since the beginning of the last complete cycle of vegetation

Management of fruit tree and grapevine phytoplasma diseases through genetic resistance

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Introduction

Phytoplasma diseases are difficult to control due to their hidden growth inside the sieve tubes of the plants. Up to now, preventive phytosanitary measures and control of the insect vectors are the only means to restrict the spread of fruit tree and grapevine phytoplasma diseases. As these strategies are difficult to implement and costly, resistant plant material would be of great benefit in fruit and grapevine production. Despite the numerous diseases associated with phytoplasmas on cultivated and wild plants worldwide, few resistant species or varieties have been obtained. Some studies performed on woody plants, like fruit or coconut trees are the exception (for review see Seemüller & Harries, 2009). Indeed, exploitation of genetic resistance for phytoplasma disease management is most advanced in apple.

Identification of natural genetic resistance is often hampered by the lack of an efficient resistance screening system. A defined phytoplasma inoculum should be efficiently inoculated to the plant genotype to test and the infected plant should be evaluated with a defined system. In this regard, important differences between fruit trees and grapevine have to be considered: whereas phytoplasmas can easily be maintained in fruit trees and can efficiently be transmitted by graft-inoculation, this is not the case in grapevine. Here, inoculation by the insect vector is most efficient. This makes the resistance screening of a large number of genotypes more difficult and a distinction between a resistance to the phytoplasma or to the insect vector cannot easily be made. The evaluation of the resistance of the inoculated plant has become much more precise since the measurement of phytoplasma concentration by quantitative real-time PCR has been applied. In this regard it is important to note that **resistance** to phytoplasmas in fruit trees and grapevine is defined as absence of symptoms and growth alteration and low titer of the pathogen, whereas **tolerance** is absence or mild symptoms but high titer of the pathogen.

Further studies focus on the development of model systems to study the resistance and its molecular basis under controlled conditions in the laboratory. Here, plant tissue culture of fruit trees has been used.

A practical application of genetic phytoplasma resistance in fruit trees and grapevine is also largely dependent on the physiology of the plant, in particular the phloem renewal in the aerial parts (=cultivar). The complete replacement of the previous year's phloem by the new year's phloem takes place every year in late winter in pome and stone fruits. This leads to a natural elimination of the phytoplasmas from aerial parts of the trees which is complete in apple and pear but less complete in stone fruits. Phloem renewal in the root system is a constant process permitting the survival of the phytoplasmas in the roots of infected trees. The actual resistance strategy for the management of fruit tree phytoplasma diseases is therefore based on the development of resistant rootstocks which prevent a recolonisation of the aerial part of the trees by the phytoplasma in springtime. This strategy has the great advantage that the pomological traits of the cultivar remain unchanged. This resistance strategy can probably not be applied in grapevine as the phloem in the aerial parts of grapevine is not completely replaced every year. In this case, resistant cultivars are needed. However, not only a resistance to the phytoplasma has to be considered but also a resistance to the insect vector.

Genetic resistance in pome and stone fruits

a. Identification of natural resistance

Search for natural genetic **resistance to '*Candidatus* Phytoplasma mali'** within the taxa *Malus* has been extensively carried out in the eigthies and nineties of the last century, in particular by the working group of Erich Seemüller (Kartte & Seemüller, 1991; Seemüller *et al.*, 1992). Hundreds of genotypes consisting of established, more recent and experimental rootstocks of *Malus* x *domestica* as well as wild and ornamental *Malus* genotypes were tested by graft-inoculation but satisfactory resistance was only observed in some experimental apomictic rootstocks derived from *Malus sieboldii*. All other rootstocks favored the growth of the pathogen and the development of the disease, many wild and ornamental genotypes proved to be even highly susceptible, responding by dwarfing and decline. As '*Ca.* P. mali' is eliminated in the aerial parts of *Malus* trees due to the phloem degeneration during winter and recolonises the tree in spring from the root system (Schaper & Seemüller, 1982; Seemüller *et al.*, 1984), a strategy to prevent the establishment of apple proliferation (AP) in the tree by using resistant rootstocks was adopted. To proof this strategy, several *M. sieboldii*-derived rootstocks were grafted with AP-infected susceptible cultivars and the disease development was examined in several long-term field trials. Symptom expression of the susceptible cultivar was monitored and the phytoplasma concentration was determined in stem and roots. Satisfactory resistance was shown by trees on *M. sieboldii* and *M. sieboldii* hybrids 3432, 4551, 4556, 4608, 4637, C1907, D1131, D2118, D2212, Gi477/4, H0801, and H0909 (Bisognin *et al.*, 2008; Seemüller *et al.*, 2008; Seemüller & Harries, 2009). These trials confirmed that a low starting concentration of the phytoplasma in the roots and poor host suitability of resistant genotypes have a negative effect on the spread of the pathogen from the roots into the scion in spring and can, thus, prevent the establishment of the disease.

These AP-resistant *M. sieboldii*-derived rootstocks, which develop via apomixis seeds that are genetically identical to the mother, have been developed in the 1950s and 1960s for easy propagation by seeds, virus-free plants, better anchorage and higher resistance to some fungal and bacterial diseases such as crown rot, apple scab, powdery mildew and fire blight (Schmidt, 1988). However, these apomictic rootstocks did not succeed in European apple growing because the trees grown on them were mostly more vigorous and less productive than trees on standard rootstock M 9. Therefore, a breeding program was started in 2001 to improve the agronomic value of these AP-resistant genotypes (see below).

As pear decline (PD) is a devastating disease not only in Europe but also in North America, screening for natural **resistance to '*Candidatus* Phytoplasma pyri'** has been first done in America by studying different *Pyrus* taxa and clonal rootstocks under natural infection conditions (Westwood & Lombard, 1982). Some of these results could be confirmed in later studies carried out in Europe but also contradictory data was obtained, e.g. for OHxF clonal rootstocks derived from the cross of the *P. communis* cvs Old Home x Farmingdale. Contradictory results were also obtained in Germany and Italy for different quince rootstocks (reviewed in Seemüller & Harries, 2009). In a more recent trial carried out in Germany progenies of 39 open pollinated genotypes belonging to 26 *Pyrus* taxa were graft-inoculated and observed for at least 18 years (Seemüller *et al.*, 2009). Also this study revealed considerable differences in PD resistance between and within the progenies. The authors concluded a segregation of the resistance trait within the seedling progenies and recommended a careful selection of suitable genotypes. Although identification of genetic resistance to '*Ca.* P. pyri' is not as clear as for '*Ca.* P. mali', its potential for a durable management of PD disease is similar. As for AP the use of resistant rootstocks would be sufficient to reduce the impact of PD, because the winter elimination of the phytoplasmas in the aerial parts of *Pyrus* is similar to the conditions observed in *Malus* (Schaper & Seemüller, 1982; Seemüller *et al.*, 1984).

Identification of natural **resistance to '*Candidatus* Phytoplasma prunorum'** is less advanced. '*Ca.* P. prunorum' infects several cultivated stone fruit species and wild *Prunus* whose response to the infection ranges from susceptible (apricot, peach, Japanese plum) to tolerant (European plum) and highly resistant (cherry) (Marcone *et al.*, 2010; Jarausch *et al.*, 1999a). Since '*Ca*. P. prunorum' is able to overwinter in the above-ground parts of *Prunus* (Jarausch *et al.*, 1999b), resistance of both rootstock and scion cultivars is required to prevent damage by European stone fruit yellows (ESFY) disease. However, the concentration of the phytoplasma in the upper parts of the tree is highly reduced by the phloem degeneration which also takes place in *Prunus* and the recolonisation of the phytoplasmas from the roots to the aerial parts of the tree is important in spring (Jarausch *et al.*, 1999b). Therefore, the rootstock significantly affects the response of grafted cultivar to ESFY infection and the choice of a suitable rootstock may prolong the productivity of infected trees. Kison and Seemüller (2001) examined by graft inoculation established and experimental rootstocks and scion cultivars and observed considerable differences in their response to ESFY infections. However, satisfactory resistance to '*Ca*. P. prunorum' could not be detected. Only trees on *P. domestica*

stocks Ackermann's, Brompton and P 2175 and *P. cerasifera* stock Myrabi were little affected. Slightly more symptoms occurred in trees on rootstocks GF 677, GF 8/1, and *P. insititia* stocks St Julien A and St Julien GF 655/2. By contrast, an interesting level of resistance has been observed in several hybrids and γ-ray mutants of Reine Claude cultivars (Jarausch *et al.*, 2000).

b. Breeding of apple proliferation-resistant rootstocks

Important efforts have been invested in the exploitation of the genetic resistance to '*Ca.* P. mali' identified in *Malus sieboldii* and its hybrids. A classical breeding program has been started in 2001 to integrate the agronomic values of established apple rootstocks into the AP-resistant genotypes (Seemüller *et al.*, 2010; Jarausch *et al.*, 2011). The major objectives were the reduction of the vigour of the resistant genotypes and the improvement of their productivity to a level which is competitive to established apple rootstocks. However, this breeding program faced several obstacles which drastically reduced the output of selected genotypes: a high level of apomixis of *M. sieboldii* and its hybrids made it difficult to obtain a sufficient number of recombinant genotypes for further selection, the different degrees of polyploidy of the resistance genitors made it impossible to develop molecular markers for assisted selection and finally a high sensitivity of some genotypes to latent apple viruses (Seemüller *et al.*, 2008) was observed during the resistance evaluation of the breeding progeny. So far, 36 different crosses were evaluated. Approximately 3,000 offspring were genotyped by locus-specific simple sequence repeats (SSR) markers to distinguish sexually derived seedlings from apomictically derived seedlings (Bisognin *et al.*, 2009). 535 recombinant seedlings were graft-inoculated and observed at nursery scale for 2-3 years and further on an orchard scale for additional 3-5 years for AP resistance and pomological traits (Jarausch *et al.*, 2011). Screening during the nursery phase revealed considerable differences in the inheritance of AP resistance of the various apomictic parents used. The best donors of this trait were selections 4608 and D2212. Crossings of these genotypes with M9 yielded 60 to 70% recombinant offspring classified as resistant. The resistant genotypes showed no symptoms and had a 10-1,000 times lower phytoplasma titer than the susceptible controls (Jarausch *et al.*, 2011). At the end of the observation period in the orchard scale the selected trees on recombinant rootstocks differed considerably in size and productivity. Nevertheless, several resistant genotypes with promising agronomic values could be selected.

Recent research activity evaluated the impact of latent apple viruses on AP-resistant rootstocks as sudden decline of *M. sieboldii*-hybrids has been observed in the field when co-infections of '*Ca.* P. mali' with apple stem grooving virus (ASGV) or apple stem pitting virus (ASPV) occurred (Seemüller *et al.*, 2008; Liebenberg *et al.*, 2010). Unpublished data indicate that *M. sieboldii* and some specific hybrids react hypersensitive to a phytoplasma/virus co-infection whereas an other specific group of *M. sieboldii*-hybrids does not. This further reduces the number of exploitable AP-resistant genotypes. A further obstacle for the use of *M. sieboldii*-derived genotypes for AP disease management is their recalcitrance to standard propagation methods. This problem, however, could be overcome by the use of micropropagation methods (Ciccotti *et al.*, 2008).

In conclusion, several selected genotypes are now available from the breeding program which are resistant to '*Ca.* P. mali' but tolerant to latent apple viruses and which have promising pomological values. These genotypes can easily be micropropagated and will now be assessed in further agronomic trials.

Genetic resistance in grapevine

a. Identification of natural resistance to FD phytoplasma in *Vitis* species and *V. vinifera* cultivars

Intraspecific (*V. vinifera* cultivars) and interspecific (hybrids and rootstocks) variability in plant susceptibility to the grapevine yellows (GY) diseases is well known from field experience and observations, but literature references are scarce and information are often reported in local publications (Borgo *et al.*, 2002). Differences between cultivars in terms of symptom frequency, intensity but also in the ability to recover were recorded for "bois noir" (BN) (Maixner *et al.*, 2006; Murolo *et al*., 2010) and "flavescence dorée" (FD) (Schvester *et al.*, 1967; Boudon Padieu *et al.*, 1996; Pavan *et al.*, 2012). Globally, the grapevine varieties seem to show the same relative susceptibility to the various GY diseases occurring all over the world, although FD phytoplasma is indeed the most aggressive. Different susceptibilities were also observed in diverse clones of the same *V. vinifera* variety (Borgo *et al.*, 2006). Rootstocks appeared as a promising source of resistance because only a few rootstock genotypes were found to be naturally infected (Borgo *et al*., 2009) and some inoculated rootstocks showed few or even no symptoms of the disease (Boudon-Padieu *et al.*, 1996; Moutous *et al.*, 1977). Moreover, no rootstock infected with BN phytoplasma has ever been reported. However, the variability in GY susceptibility has not been studied in controlled conditions of inoculation and has not been

characterized in terms of phytoplasma titer in the plant, which is an important factor for the propagation of the disease.

A study was recently initiated in order to evaluate the susceptibility to the FD disease of major *V. vinifera* cultivars, *Vitis* hybrids used as rootstocks, but also wild *Vitis* species originating from North American and Asian continents. FD phytoplasma (FDp) was inoculated to young plants obtained from *in vitro* multiplication by experimental transmission with the vector *Scaphoideus titanus* in insect proof greenhouse. The susceptible cultivar Cabernet Sauvignon (CS) was included in each experiment as a positive control. Five and 10 weeks post-inoculation (wpi), disease development was evaluated by recording the symptoms, the percentage of infected plants and by measuring the mean phytoplasma titer in the whole plants by quantitative real-time PCR. For each experiment, the ratio between the mean phytoplasma titer in the tested genotype and in the reference CS was calculated.

First results are presented in figure 1. For the susceptible control CS, 87% of the plants were infected with a mean titer of $3.7x10^7$ phytoplasma cells per g of fresh weight at 10 wpi. Symptoms appeared at 6 wpi. The wild species *V. labrusca* and *V. longii* showed high FDp multiplication rates, similar to CS, but without symptoms at 10 wpi. *V. vinifera* cultivars Grenache and Chardonnay presented FDp multiplication rates and symptoms timing close to those measured in CS. Phytoplasmas multiplied 9 times less in Pinot Noir than in CS and only 47% of the plants were infected. No symptoms were recorded for Merlot and Syrah at 10 wpi, only 50% and 26% of plants were infected respectively and FDp hardly multiplied in infected plants (165 and 300 times less than in CS respectively). None of the rootstock hybrids exhibited symptoms but differed in terms of FDp multiplication and percentage of infection which were higher for 3309 Couderc (3309C) and Riparia Gloire de Montpellier (RGM) but lower for 41 Berlandieri (41B) and Kober 5BB. Interestingly, none of the Nemadex rootstocks could be infected by FDp.

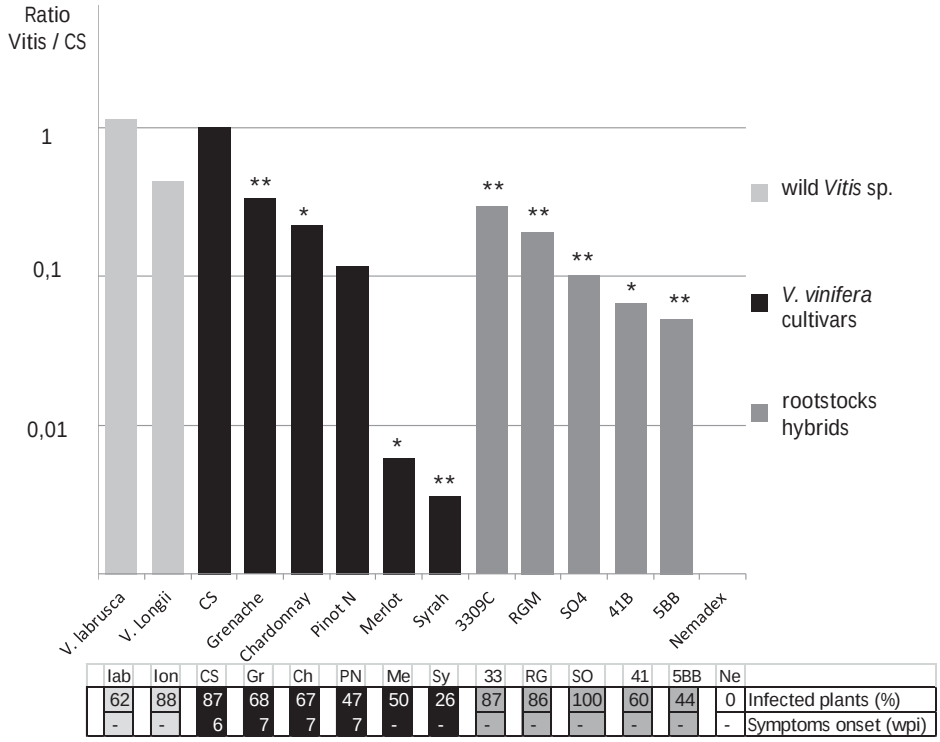

Figure 1: Evaluation of the susceptibility to FD of different *Vitis* genotypes in terms of expression of symptoms, percentage of infected plants and phytoplasma multiplication in the plant after inoculation by *S. titanus* under controlled conditions. Y-axis represents the mean phytoplasma titer in the *Vitis* tested expressed as ratio to the titer in the susceptible CS. Statistical significance between *Vitis* and reference CS were determined using the Wilcoxon rank sum test. * $p<0.05$; $p<0.01$. wpi: weeks post-inoculation.

As a conclusion, the phenotyping of major grapevine cultivars and rootstocks allowed to identify highly and poorly susceptible genotypes. CS, Chardonnay and Grenache can be considered as highly susceptible to FD

as they show symptoms and high phytoplasma titer. Pinot Noir can be considered as intermediate. Merlot and Syrah are the less susceptible with no symptoms observed in controlled conditions and very low phytoplasma titer. This ranking is in accordance with former field observations based on symptoms (Boudon-Padieu, 1996). Furthermore, observations and analysis carried out in infected grapevine plots confirmed that the level of symptoms and the phytoplasma titer were significantly lower in Merlot than in CS. Even if they did not present symptoms, *V. labrusca*, *V. longii*, 3309 C and RGM rootstocks permitted high multiplication of the phytoplasma and can be considered as tolerant to FDp. The recent finding of high FDp titres in non-symptomatic "wild" rootstocks surrounding FD outcasts support this. On the contrary, the lower FDp multiplication in 5BB and 41B makes these rootstocks a source of partial resistance to FD disease and need to be confirmed. Insect survival rate on *Muscadinia rotundifolia*-derived intergenic hybrid Nemadex was quite low and it could not be infected by FDp. It was shown that muscadine hybrids are a source of resistance to the nematode *Xiphinema index* (Esmenjaud *et al.*, 2010). Nemadex could eventually constitute a source of resistance to the insect vector *S. titanus*.

In perspective, other *Vitis* species and *M. rotundifolia* will be evaluated for their susceptibility to FD in order to complete the ranking. Assemblies between the less sensitive rootstocks and cultivars will then be tested before and after a wintering period. But the influence of resistant rootstocks on the disease development in susceptible cultivars will also be analysed. Indeed, differences in susceptibility between vine varieties grafted on different rootstocks have not been investigated in the past; however it is known that phytoplasmas can hardly be detected in roots of symptomatic grapevine even using very sensitive detection methods.

The resistance observed can be a combination between plant responses to the phytoplasma and to the insect vector. This will be discriminated by inoculating FDp by grafting and separately, by studying the behavior of *S. titanus* on healthy genotypes in terms of attractiveness, feeding, development and survival.

Molecular basis of genetic phytoplasma resistance

a. Grapevine genetic traits as candidate for GY resistance

Several studies aimed to find out genes and proteins up and down regulated during GY infection, and thus potentially involved in grapevine resistance to phytoplasmas, were carried out in the last few years.

A few researches were focused on Chardonnay, which is a very sensitive cultivar; indeed, investigation on the defence signalling pathway in susceptible varieties can help in the identification of candidate genes for resistance. Qualitative and quantitative changes in the global gene expression profiles of BN-infected and healthy plants of cv. Chardonnay were performed using microarray technology. Results showed 2,456 genes differentially expressed in the infected plants: 54% genes up-regulated and 46% genes down-regulated. Also some genes involved in defence-signalling were differentially expressed (Hren *et al.*, 2009). Modulation of genes expressed in the phloem tissue of BN-infected and healthy Chardonnay was examined by laser microdissection pressure catapulting and real-time PCR. Site-specific expression analysis showed a differential regulation and specificity of two pathogenesis-related thaumatin-like genes of the PR-5 family (Santi *et al*., 2012). Total transcriptome in aster yellows-infected Chardonnay plants was explored by deep sequencing; preliminary results showed that 119 genes were induced and 56 were repressed (Snyman *et al*., 2012).

Other researches compared the molecular response to GY infection of two grapevine varieties with different susceptibility to the GY disease. Transcriptional responses of BN-infected Chardonnay (very susceptible) and Manzoni Bianco (moderately susceptible) were analyzed using microarray technology. Expression levels of a few hundred genes were altered in infected plants and some genes related to defence pathways were induced or repressed specifically in only one cultivar (Albertazzi *et al.*, 2009). The variation of some defence-related gene expression in Sangiovese (moderately susceptible) and Chardonnay (highly susceptible) cultivars infected with BN were investigated by real-time PCR. The defence genes in Sangiovese were generally up-regulated in both symptomatic and symptomless leaves, while this behaviour was not observed in Chardonnay, in which changes in gene expression were linked to symptom display (Landi and Romanazzi, 2011). Other authors investigated the proteome responses of Nebbiolo (moderately susceptible) and Barbera (susceptible) varieties to FD phytoplasma. Beside common spots, some proteins were exclusively expressed in one cultivar (Margaria & Palmano, 2011a, 2011b; Margaria *et al.*, 2013). Thus, all genes and proteins differentially expressed in varieties showing different susceptibility are potentially associated to the differential susceptibility to the phytoplasma and could be candidate genetic traits for GY resistance.

Another study aimed to identify candidate resistance genes for GY in grapevine is in progress. The goal is to find out quantitative and qualitative differences in total mRNA expression from grapevine plants of varieties

displaying different susceptibility to FD phytoplasma infection using deep sequencing. Micropropagated plantlets of cv Chardonnay (very susceptible) and cv Tocai (very poorly susceptible) were infected with FD in controlled conditions using the vector *S. titanus.* Differently from the previous works, which took in account field plants infected and symptomatic since a long time, the current experimental plan is focused on the analyses of the first moments after the inoculation of the phytoplasma.

A different approach to discover genetic traits associated to resistance or tolerance to GY is the quantitative trait loci (QTL) analysis, which includes accurate phenotyping and the use of genetic markers. This analysis can be performed on a wild population showing different degrees of susceptibility or on a population segregant for the character of interest. A reciprocal cross between very susceptible (Chardonnay) and very poorly susceptible (Tocai and Moscato) varieties was carried out, in order to obtain a population segregating for the characters related to GY resistance. This will be the basis for future field and molecular studies, aimed to localize the QTLs for resistance in the grapevine genome.

In conclusion, no resistance genetic locus or gene has been found so far. Anyhow, all the collected data provide an interesting picture of the grapevine response to GY diseases and allow the selection of several candidate genes for future functional analysis.

b. Analysis of the molecular basis of genetic resistance to AP

So far, the resistance mechanism against '*Ca*. P. mali' is still poorly understood. As all of the screening work has been done under field conditions with entire trees, a model system to study the resistance under controlled conditions was needed. For this, plant tissue culture was used. '*Ca*. P. mali' can be easily maintained in micropropagated *Malus* and can be transmitted to test plants by *in vitro* grafting (Jarausch *et al.*, 1999c). Using this approach, it was shown that the resistant phenotype could be reproduced in micropropagated plants (Bisognin *et al.*, 2008). *In vitro* graft-inoculation allowed working with defined infections. Furthermore, *ex vitro* plants could be produced from the inoculated micropropagated plants to study also the root system. Differences in the gene expression between '*Ca*. P. mali'-resistant and -susceptible genotypes during infection were investigated through cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) technique in this model system (Moser *et al.*, 2010). This study showed that a broad range of metabolisms is affected by the presence of the phytoplasma. A general response against stress and pathogen is activated in the resistant genotype. This resembles a type of reaction that was also observed in the "recovery" phenomenon. In order to gain more information on ultrastructural features and modifications at cellular level a cytochemical analysis was performed (Moser *et al.*, 2011). Preliminary results indicated a dramatic change in the cellular organisation in phytoplasma-infected susceptible genotypes while in resistant genotypes this reaction was localised to very few cells. Thus, structural features of the phloem cells could be correlated to the ability of the resistant genotype to contain the phytoplasma spread.

Conclusions and outlook

In view of the great economic and social importance of fruit tree and grapevine phytoplasma diseases and the difficulties to confine these diseases the use of resistant plant material would be of great benefit. Natural genetic resistance could be identified in the germplasm of fruit trees and grapevine, but only recently molecular work started to elucidate the mechanism of this resistance. As phytoplasmas alter dramatically the metabolism of the plant its reaction to the infection is also multiple – also in the resistant genotypes. This makes it difficult to identify the resistance mechanism and the available preliminary data indicate that it might be complex. Knowledge about the mode of action of the resistance is, however, needed to evaluate the stability of an identified resistance and the possibility that the genetically highly variable phytoplasmas might break it. In this direction further studies are needed.

The practical application of genetic resistance is most advanced in apple where promising AP-resistant rootstock genotypes have been selected and can now pass to the final step of agronomic evaluation. This example demonstrates that it is possible to manage a phytoplasma disease by genetic resistance.

In grapevine the research is actually focused on different topics: on one hand on the definition of a standard protocol for assessing the level of susceptibility, the phytoplasma titer in the infected plants, and thus the identification of truly tolerant and resistant varieties, and on the other hand the identification of the molecular traits associated with susceptibility, resistance and tolerance to GY; this will lead to find molecular markers to be used in new breeding programs based on molecular assisted selection, as it is already in course for grapevine downy and powdery mildew.

Furthermore, it is important to highlight that any knowledge and subsequent use of moderately resistant scion or rootstock cultivars can contribute to limit the impact and the spread of phytoplasma diseases. Their

use has to be flanked in practice by other means of control, e.g. vector control. This applies equally well for the management of fruit tree and grapevine phytoplasma diseases.

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What can we learn from the phenomenon of "recovery"?

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Introduction

Phytoplasma diseases of fruit trees and grapevine are very dangerous because economically significant, epidemic and not curable without antibiotics that are currently forbidden to be used in our Countries. None of the cultivated varieties is resistant and the disease control is exclusively based on preventive insecticide treatments against the insect vectors and on the eradication of the infected plants. The recommended use of healthy plants as propagation material is not enough to limit the phytoplasmas associated disease spread: healthy resistant (difficult to obtain) or tolerant plants are needed in such as situation.

"Recovery" has been defined as the spontaneous remission of disease symptoms in plants that previously showed them. It has been reported in grapevines, apple and apricot trees infected by phytoplasmas (Musetti *et al.*, 2007; 2010). In apple trees and grapevines "recovery" is associated with the disappearance of phytoplasmas from the crown (Carraro *et al.*, 2004). Interestingly, Osler *et al.*, (2000) demonstrated that recovered plants can be re-infected in nature in a lesser extent than the never infected ones, indicating that a resistance could be involved in the phenomenon.

In this context, the interest about "recovery" becomes not only of scientific interest but also important from a practical point of view. In fact this phenomenon could play a role in the management of phytoplasma diseases of perennial woody crops.

Recovery: physiological and molecular bases

The physiological basis of "recovery" is not completely known, however, during the last years, studies have been carried out with the aim to add new insight about the phenomenon. Cytochemical analyses revealed that it is accompanied by biochemical changes in the phloem. In fact, it has been demonstrated that recovered plants are able to accumulate, in the sieve elements, H_2O_2, a stable reactive oxygen species whose antimicrobial as well as signaling roles are well known, because in these plants the activities of two main enzymatic H_2O_2 scavengers (catalase and ascorbate peroxidases) are selectively and stably down-regulated (Musetti *et al.*, 2004; 2005; 2007). The variation of sieve-element oxidative status leads to modifications of phloem protein (P-protein) conformation and in phloem occlusion expression patterns. An anomalous accumulation of callose and protein, associated with the up-regulation of callose synthase- and P-protein-coding genes, has been observed in the sieve elements of recovered apple trees (Musetti *et al.*, 2010), supporting the hypothesis that recovered plants are able to develop resistance mechanisms depending on Ca^{2+} signal activity (Musetti *et al.*, 2010; 2013). Recently, the activation of jasmonate (JA)-related defense mechanism, via JA gene up-regulation, has also been demonstrated in apple trees recovered from apple proliferation disease (Patui *et al.*, 2012; Musetti *et al.*, 2013).

Epidemiological and practical aspects of recovery

"Recovery" is a complex phenomenon still not fully understood even from the epidemiological point of view. The difficulty of studying these aspects of "recovery" is probably derived from the complexity of the trinomial: host species/phytoplasmas/vectors. As a current opinion, the permanent or stable "recovery" is considered a minimum asymptomatic period of two-three years after the expression of the phytoplasma disease symptoms (Maixner *et al.*, 2011). In the case of grapevine yellows, phytoplasmas are not detectable by nested-PCR in the canopy of recovered grapevines cvs. Prosecco, Chardonnay, Pinot noir, Barbera, (Osler *et al.*, 2003;

Morone *et al.*, 2007); this suggests that recovered plants are not source of inoculum for the potential spreading of the disease.

A management of phytoplasma disease based on vector control and on "recovery" expectance of the symptomatic plants, avoiding rouging, can be conveniently applied when the expected rate of the phenomenon is high (Pavan *et al.*, 2012).

"Recovery" also occurs in apple trees, in which the phytoplasmas remain confined in the roots (Carraro *et al.*, 2004); also in this case, the epidemiological risk related to the presence of recovered plants is not relevant because vectors are not able to acquire the pathogen from the roots (Seemüller *et al.*, 2008).

Researches on the stability of "recovery" over the time are still in progress; preliminary results on grapevines recovered from "bois noir" disease showed that the probability of these plants to become infected and to show again the disease symptoms is 4.9% in 5 years; the percentage of the new symptomatic plants in the same period is 8.4% (Ermacora *et al.*, 2012).

Conclusions

Given that "recovery" is a natural, spontaneous event, still not experimentally reproducible, the explanation of the phenomenon is not simple. Thanks to the recent researches about physiological and biochemical aspects of "recovery", the hypothesis that the phenomenon is due to the expression of an acquired resistance by the plant was confirmed.

About the epidemiological and practical aspects, recovered plants exhibit similar behavior to healthy never infected plants, but with the important advantage of the resistance acquisition towards the disease. The main difficulty to include "recovery" as an integrated measure for phytoplasma disease management consists in the evaluation of the expected rate of spontaneous remission of symptoms under specific environmental conditions. However, in some areas, natural "recovery" occurrence, combined to vector control, was decisive for phytoplasma disease control.

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Perspectives for the management of phytoplasma diseases through genetic or induced resistance: what can we expect from resistance inducers?

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Introduction

Phytoplasma disease can cause severe losses to a long list of crops, and qualitative and quantitative production parameters of those can be affected. Nowadays we do not have protocols to control phytoplasma diseases, and preventive measures rely on the use of healthy propagating materials and in the control of vectors. One of the few possibilities to contain the occurring of disease symptoms is the use of resistance inducers (Romanazzi *et al.*, 2009a). Resistance inducers, called also elicitors, can be of abiotic or biotic nature and challenge the plant, leading to a reaction, often linked to the production of antimicrobial compounds and/or the elicitation of plant defense mechanisms. The treatment with resistance inducers can be successful in i) decreasing the number of infected plants, ii) reducing the severity of disease symptoms, and iii) delaying the appearance of disease symptoms.

Abiotic resistance inducers

Abiotic resistance inducers are usually chemical compounds able to start a reaction of the plant. The most common elicitors used to control phytoplasma diseases are: benzothiadiazole (BTH), Phosetyl-Al, prohexadione calcium, indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), chitosan, salicylic acid (SA), mixture of glutathione and oligosaccharines (GOs). Most studies were carried out on experimental hosts infected with a phytoplasma, e.g. *Arabidopsis thaliana* challenged with X-disease phytoplasma (Bressan & Purcell, 2005), *Catharantus roseus* inoculated with chrysanthemum yellows (CY) or elm yellows (EY) phytoplasma (Prati *et al.*, 2004; Chiesa *et al.*, 2007), or with aster yellows (AY), EY or "stolbur" (Curkovic Perica, 2008; Leljak-Levanic *et al.*, 2010). SA was applied in crops as tomatoes infected potato purple top phytoplasma (Wu *et al.*, 2012). Trials on woody crops infected by phytoplasmas can be more difficult because it is not easy to find a high number of plants that after application allow getting significant differences in recovery induction. First evidence of resistance inducer application was obtained in Sardinia on cvs Chardonnay and Vermentino infected with "bois noir" (BN), that were sprayed with two commercial formulations based on active ingredients Phosetyl-Al and GOs (Garau *et al.*, 2008). Application of humic and fulvic acids, and algae extracts gave promising results but they were not able to contain BN infections on two different cultivars (Mazio *et al.*, 2008). When five commercial resistance inducers, based on chitosan, Phosetyl-Al, BTH and two GOs formulations were applied weekly in a vineyard cv Chardonnay naturally infected by BN, BTH and the two GOs provided a significant reduction of number of symptomatic plants (Romanazzi *et al.*, 2009b).

Biotic resistance inducers

The role of arbuscular mycorrhizal (AM) fungi in phytoplasma infection has been investigated in several pathogenic systems. In "stolbur" infection of tomato, agglutinations and degeneration of phytoplasma cells, coupled to reduced symptom expression, was seen in plants treated with AM fungi (Lingua *et al.*, 2002). In a different pathogen–host system, inoculation with *Glomus intraradices* increased tolerance to pear decline in infected pear trees (Garcia-Chapa *et al.*, 2004). Significant protection from '*Candidatus* Phytoplasma asteris' (the chrysanthemum yellows, CY strain) infection of chrysanthemum plants, coupled to lower symptom severity, has also been reported (D'Amelio *et al.*, 2007). Recently, the evidence that *G. mosseae* BEG 12 inoculation does not decrease periwinkle tolerance to mild and severe '*Ca*. P. asteris' strains (Kaminska *et al.*,

2010) has indicated that the effects of AM fungi on phytoplasma infection are complex and probably dependent on a combination of host plant, AM fungus and phytoplasma isolate (Romanazzi *et al.*, 2009a).

Plant-growth-promoting rhizobacteria represent another group of microorganisms that can activate plant-defence responses. Indirect stimulation relies mainly on plant health improvements through biocontrol of plant pathogens or enhancement of plant tolerance to environmental stress. The application of a pseudomonad reduced the number of CY-infected chrisanthemum and extended the life span of infected plants (D'Amelio *et al.*, 2007; 2010).

Concluding remarks

The possibility to contain phytoplasma diseases within an integrated approach that include, together with the applied control measures based on clean propagating materials, vector control and eventually weed management, a stimulation of plant defences is of great interest for researchers and can become practically important for growers. This can be particularly true where phytoplasma diseases can be a limit for the growing of some species or cultivars in areas with high disease pressure, as it occurs for grapevine cv Chardonnay and BN combination. Although research in this field made important progress in the last ten years, we are still far from protocols ready to be applied by growers. Further research is still needed to find the best treatments, schedules of applications and mechanisms of action involved to optimise phytoplasma disease control through the use of induced resistance.

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Perspectives of endophytes as biocontrol agents in the management of phytoplasma diseases

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Introduction

The control of the plant diseases is a fundamental task of the agriculture as economical and productive activity. The need to use low impact protocols for the environment is becoming the main goal for the next generation farming.

Among the plant diseases, those associated with phytoplasmas presence are assuming an emerging importance for their detrimental effects. Phytoplasmas are wall-less bacterial plant pathogens that are causing economically relevant yield losses in annual and perennial crops worldwide. Phytoplasmas are transmitted in nature by phloem feeders, mostly leafhoppers, planthoppers and psyllids. Thus, conventional strategies for phytoplasma containment are based on pesticide application against insect vectors and uprooting of infected plants; for quarantine pathogens these rules are mandatory and their application is compulsory and regulated by EU and national legislation. Then, their absence from propagation materials is essential for sustainable plant production and particularly important for the vegetative propagated crops in which infected planting materials transmit the pathogen to the new crop. Treatments against phytoplasmas, including antibiotic applications (i.e. injection of antibiotics to the trunk), revealed none positive effects for their eradication from the plant or the containment of the disease and are not allowed in agriculture in Europe. Most promising results are arising from the treatments with inducers of the plant resistance (Romanazzi *et al.*, 2009) and the use of resistant varieties (Jarausch *et al.*, 2011).

In the last decade, a curious and interesting phenomenon, called "recovery*",* has been reported in several plant species belonging to the *Rosaceae* family (apple and stone fruits) and in grapevine. Spontaneous remission of symptoms accompanied by lack of detectable phytoplasmas in recovered plants prompted several researches to study more about this phenomenon opening new perspectives on phytoplasma containment. Different research groups focus their work on the study of endophytes associated with different plants and on the possibility to use them as biocontrol agents.

Endophytes are microorganisms residing inside the plant without inducing disease symptoms. In nature, endophytes can promote plant growth by reducing the deleterious effects of plant pathogens through direct or indirect mechanisms. Bacteria and fungi can directly antagonize pathogens by competition for nutrients and production of allelochemicals and indirectly through the induction of systemic resistance (ISR) (Lugtenberg and Kamilova, 2009; Shoresh *et al.*, 2010).

Endophytic bacteria

Endophytic bacteria promote plant growth and protect plants against pathogen infections. Endophytes-plant and endophytes-pathogens interactions are poorly explored. In particular, the ability of endophytes to control pathogens that are not managed directly is still new. Recently, *Pseudomonas putida* S1Pf1Rif was tested, alone or in combination with the mycorrhyzal fungus *Glomus mosseae* BEG12, against chrysanthemum yellows (CY) phytoplasma infection of chrysanthemum. Plant biomass, root architecture, symptom severity, phytoplasma titer, and viability were evaluated in inoculated and control plants. Moreover, *P. putida* S1Pf1Rif was able to reduce and delay symptom expression in non-resistant plants; when *P. putida* S1Pf1Rif was

applied in combination with *G. mosseae* BEG12 some resistance to phytoplasma infection was observed. Single or combined inoculation with the two microorganisms did not alter CY multiplication rate and viability although, in both cases, in inoculated and infected plants, phytoplasma morphology was typical of senescent cells. A more active and efficient root system in double-inoculated plants probably mediated the effects of the two microorganisms in the infected plants (Gamalero *et al*., 2010; Sampo *et al*., 2012).

The practical application of rhizospheric microorganisms for mitigating phytoplasma symptom expression, following evaluation under field conditions, may represent an additional tool for the integrated management of phytoplasmas-associated diseases.

A basic point for the success of sustainable management of plant diseases based on biocontrol agents is the study of endophytic bacterial community associated with plants. Recently endophytic bacteria associated with healthy, phytoplasma-infected plants have been described suggesting some putative biocontrol agents (Bulgari *et al.*, 2011; 2012). Interestingly, the study of recovery phenomenon shed new light on the possibility to use endophytic bacteria as biocontrol agent of phytoplasmas. These promising studies showed the presence of three main groups of bacteria (*Proteobacteria*, *Actinobacteria* and *Firmicutes*) and allowed to isolate from recovered plants some endophytes (*Burkholderia* sp., *Bacillus pumilus*, *Curtobacterium*) reported as biocontrol agents of several diseases (Raupach & Kloepper, 2000; Compant *et al*., 2008). Endophytic bacteria isolated from healthy, diseased and recovered grapevine plants were characterized for five beneficial traits related to mineral nutrition (phosphate solubilization, siderophores), development (indolacetic acid-IAA synthesis), stress relief (1-amino-cyclopropane-1-carboxylate deaminase and catalase activity), disease control (chitinase activity, siderophores). In detail, five bacterial isolates showed the ability to solubilize phosphate, reacted to stress and were able to synthesize IAA. In addition, some of these strains were able to produce siderophores. To verify the ability of endophytic bacteria in phytoplasma control, a model system should be developed. To date, the experiments with biotic and abiotic inducers were performed on *Catharanthus roseus* infected by '*Candidatus* Phytoplasma mali' or by '*Ca.* P. asteris' (Leljak-Levanic *et al.*, 2010; Musetti *et al.*, 2011) or *Chrysanthemum carinatum* infected by Chysanthemum yellows phytoplasma (D'Amelio *et al.*, 2011). Nevertheless, to be suitable for biocontrol a bacterium should not only produce secondary metabolites but it should compete with indigenous microorganisms and maintain the interactions with the host. For that reason, it is worth to develop a model system with grapevine plants. Unfortunately, the transmission and the stability of phytoplasma infection in grapevine plants is difficult to obtain *in vitro* or in greenhouse (Gribaudo *et al.*, 2007). Recently, Naor *et al.* (2011) published a model system for maintaining phytoplasmas in Chardonnay and Cabernet sauvignon *in vitro*. Using this system, one isolate improved growth parameters of grapevines and *C. roseus* potted plants phytoplasma-infected (Naor, 2012).

Endophytic fungi

Among beneficial microorganisms interacting with plants there are also fungal endophytes that can result extremely diverse in the different plants, colonizing all or part of the host. In spite of the knowledge about their biology is still incomplete, it is recognized that fungal endophytes are important source of secondary metabolites and compound of biotechnological value as antibiotics or antitumor drugs (Gimenez *et al.*, 2007).

Fungal endophytes establish mutualistic relationships with plants also inducing physiological modifications in their hosts making them more resistant against biotic or environmental stresses. Fungal endophyte strains have been identified from grapevines and apple plants grown in areas where recovery phenomenon was recurrent. For fungal community description, a combination of culture-dependent and culture-independent methods has been set up (Grisan *et al.*, 2011). The combined use of the two methods allowed to discover 56 different fungal endophytes grouped in OTUs on the bases of PCR/RFLP analyses of ITS region. 27% of OTUs were obtained by culture-dependent method, 48% by culture-independent method, and 25% by both methods. Furthermore the collected data revealed that fungal endophytes belonging to genera *Alternaria* sp., *Phoma* sp., *Epicoccum* sp., *Aureobasidium* sp., *Cladosporium* sp., *Pestalotiopsis* sp. and *Pestalotia* sp. constituted respectively, about 89% of total isolates obtained by the culture-dependent method, and 79% of total clones obtained from the culture-independent method.

Strains of *Epicoccum nigrum* and *Aureobasidium pullulans*, were chosen for further research activities because extensively reported as biocontrol agents or resistance inducers. Using the model plant *C. roseus* infected with '*Ca*. P. mali', it was observed that reduction in symptom severity and lower phytoplasma titre in host tissues occurred when the plants were previously inoculated with an endophytic strain of *E. nigrum* (Musetti *et al.*, 2011.). Aiming to assess the possibility of using *E. nigrum* in phytoplasma disease control, new investigations are in progress in *Malus domestica,* the natural host of '*Ca.* P. mali'.

Conclusion

As phytoplasma diseases are not curable and vector control is the main containment strategy applied so far, the use of endophytic microorganisms could represent an alternative strategy against phytoplasmas-associated diseases. The concept of "management" and control of plant diseases implies the application of modern measures compatible with the environment.

Molecular analyses and phytoplasma genome sequencing allowed to define the basis of phytoplasma-host plant interaction. Furthermore, recent studies on the recovery phenomenon open new possibilities to develop control strategies using biocontrol agents. Recently, the endophytic communities associated with healthy and phytoplasma diseased plants were described through cultivation-dependent and –independent methods. Intriguingly, these analyses allowed identifying putative biocontrol agents that will be tested *in vivo* against phytoplasmas. Although the mechanisms of phytoplasma-endophyte interaction are not clear, preliminary experiments in controlled condition reported a mitigation of phytoplasmas-asssociated symptomatology mediated by endophytes. On the basis of these data field experiments should be carried out in order to evaluate the possibility to use endophytes for phytoplasma containment.

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Phytoplasma effect on grapevine nursery and Italian laws

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Grapevine nurseries have a key role in the modern agricultural production chain because they transfer innovations from the "research sector" to the "production sector" and spread highly qualified plant propagation materials. In special wine vocated areas, usually, winery and strong professional nursery coexist because they grow together: as shown in fig. 1, in Italy more than 99.5% of nursery are located in the most advanced wine-production districts.

Figure 1. Wine (green) and table (yellow) grape district in Italy, related to professional grapevine nursery diffusion (red).

The presence of "flavescence dorée" (FD) and "bois noir" (BN) were reported in Italy since 1970 (Belli *et al*., 2010). These two phytoplasmas have different behaviour and different dangerousness for vineyard therefore are subject to different laws: FD is a quarantine pathogen and is subject to a specific Ministerial decree law "DM 32442 – 31 may 2000: Misure per la lotta obbligatoria contro la Flavescenza Dorata della vite". This law includes several measures against FD and its insect vector *Scaphoideus titanus*.

On the other side, BN is considered a "quality pathogen": and every year the Phytosanitary Services controls nursery's mother plant fields to ensure that they are free from BN, but nothing is specifically done or recommended to control its known insect vector *Hyalesthes obsoletus*.

Regards the possibility of spreading these diseases with plant propagating material, several studies have demonstrated the low percentage rate of grafting transmissibility of these pathogens (Credi *et al*., 2012) so that a professional nursery, supported by phytosanitary services, should be sure to reduce almost to zero the possibility of graft transmission of these pathogens.

Despite this, MIVA and Italian nursery are still interested in every new technique that can improve the grapevine young plants quality. Once that the pathogen and the insect vector are widespread in an area, phytoplasmas can cause big economic damages to all the actors of the production chain (nursery – vineyard - winery), that's why MIVA and Italian grapevine nursery association hope that European researchers can continue to work on grapevine phytoplasmas, especially on BN, until they will find sure and effective strategies and solutions to reduce the disease incidence. The best solutions could be new treatments against pathogen or insect vector, or even new resistant plants, preventing phytoplasmas further spread and reducing the economic damages.

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Controlling 'flavescence dorée' with less insecticide: local scale strategy developed in Bordeaux vineyard

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The "flavescence dorée" (FD) disease is present in the South of France. As this is a quarantine disease, plant protection regional services are in charge of controlling this threat. The discovery of a new infected plant leads to the creation of an Imposed Sprayed Area (ISA). Each ISA is a large territory limited by town frontiers. Inside an ISA, the leafhopper control requires from one to three mandatory insecticides sprayings, depending on the importance of the initial contamination. The neurotoxic insecticides are applied systematically each year on ISA unless a large scouting proves the total absence of FD disease. This strategy was initially developed to be very effective and to reduce the spreading of the disease. However, this program was a failure due to non-respect of obligatory sprayings by winegrowers and absence of scouting.

Another approach has been developed in Gironde since 2007. This strategy is based on local organization named GDON (Groupement de Défense contre les Organismes Nuisibles). The GDON are association financed by winegrowers and covering a small territory with the agreement of plant protection services. Each GDON is in charge of managing FD disease on its own territory. In 2012, 99% of winegrowers from Gironde are part of a GDON. Each GDON develops in own strategy and particularities. The data presented are based on results obtained by the GDON du Libournais, which is the oldest organization in Gironde (created in 2007). The GDON du Libournais works on a territory covering 16 different towns around the Saint Emilion region, equivalent to a 12,000 acres vineyard and 1,200 different winegrowers.

Local strategy developed

General method

The FD management strategy applied in GDON du Libournais is based on a different definition of the ISA. Each ISA is a buffer area centered on the initial FD contamination. Buffer sizes and spraying frequencies are defined by different technical parameters: importance of FD primary contamination, trap catches during past year, "bois noir" (BN) disease presence during last scouting and grape varieties repartition. Buffer sizes are never smaller than a 500 meters radius and can range up to a 2,000 m radius buffer. There is no mandatory insecticide sprayings outside buffers.

Vector control

Leafhopper control is mainly based on an important yellow delta traps network, with a density of one trap per 50 acres of vineyard covering all the territory (250 traps). Inside buffers, the trap network is heightened up to a density of one trap per 5 acres of vineyard. Outside buffers, the catches permit to estimate the historical population of leafhopper. Inside buffers, traps are a useful tool to check the sprayings' efficiency. Mandatory insecticide sprayings are stopped outside buffers but they can also be reduced inside buffers thanks to the use of threshold. If trap catches are almost nonexistent (<3 catches per week for each trap), this result is considerated as a proof of sufficient control of the vector and the sprayings targeted on adults is cancelled inside buffer. Larvae observations are done to monitor spraying dates and to control traps values.

Scouting method

The aim is to localize plants infected by FD and BN. Scouting is done by crossing vineyard by walk and by localizing suspect plants with GPS. Samples analyzed in laboratory permit mapping disease repartition. Then winegrowers are informed of laboratory results and have to uproot infected plants. Each ISA have to be scouted in totality 2 times, without finding FD infected plant before being suppress.

Respect of obligatory insecticide sprayings

The different controls done to estimate leafhopper population permits to count (and localize) the number of winegrowers that do not respect the insecticide spraying program. Depending on year studied, we record from 2 to 5% controls that clearly indicate failure in insecticide sprayings, which is a low rate compared to other territories without local action supported by GDON.

Insecticide sprayings reduction

The reduction of obligatory insecticide sprayings range from 53 up to 82% compared to sprayings strategy developed in the classical ISA. During the period 2007-2012, this insecticide reduction is equal to 70,000 acres of vineyard untreated.

Action cost and winegrowers satisfaction

The program costs 18 € / acre and is essentially financed by winegrowers. The final cost depends on the importance of scouting, which is a big expense because it requires human labor. This action is a success with winegrowers because they prefer paying a monitoring than spraying insecticides.

FD disease eradication

This experiment shows great efficiency to decontaminate ISA when the initial contamination is quickly detected, and infected plants are not too numerous (< 30 infected plants). However, we note an important increase of isolated infected plants (outside existent buffers area). We have to face the problem of creating more new ISA that we are able to clean old ones.

Prospects and conclusion

Considering this latest result, we modified our method on two different points. We introduced a cyclical scouting to check at minimum all fields every 4 year. We also study the multi-annual effect of neurotoxic insecticide sprayings on leafhopper population, trying to determine an estimation of vector speed colonization after the end of sprayings. This data is used to choose a multi-annual insecticide spraying frequency. It should permits to maintain leafhopper population to low level, and reduce the FD dispersion risk. This local scale strategy is a short term success and was duplicated in other French vineyards. The possibility of stopping FD spreading with that method remains to be evaluated on a long period (10-15 years).

Phytoplasma Control in the production of fruit trees in AGROMILLORA

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AGROMILLORA initiated its activities in 1986 in Subirats (Barcelona, Spain). It has grown to an international nursery specialised in the production and marketing of high quality plantlets and young trees. Since the very beginning, we have kept faithful to the values of quality, continuous improvement and innovation. Our long experience, along with the most advanced technology and spirit of expanding internationally, has positioned us as a reference in the nursery industry worldwide.

Leading the propagation of stone-fruit trees and olive trees in the world, we have been established in several countries located in Europe – Spain-, Asia – Turkey-, North and South America – USA, Chile, Brazil, Argentina, Uruguay-, Africa – Morocco, Tunisia -, and Australia.

The aim of the company focuses mainly on the quality of plant material in terms of sanitary conditions and genetical guarantee. For these purposes in 1996 the Quality Control Department was created and its function began with the development of an own Quality Control Lab.

Different methods of diagnosis were established to determine the presence or absence of different pathogenic organisms that affect fruit trees, mainly viruses and phytoplasmas.

In 2001 we start working with PCR techniques for the universal detection of phytoplasmas in stone-fruit and olive plants, based in DNA extraction, amplification using nested-PCR and electrophoresis in agarose.

Nowadays we have a Real-Time PCR equipment to carry out test to verify phytoplasma presence. The probe and primers we are using were designed to amplify DNA from as many different plant species as possible (Christensen *et al*., 2004). This analysis is included in our Control Plan for Fruit Trees and allows us to exclude any material which does not comply with our standards of health quality.

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