Conventional and novel approaches for managing “flavescence dorée” in grapevine: knowledge gaps and future prospects

Conventional and novel approaches for managing Flavescence dorée in grapevine: knowledge gaps and future prospects

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Abstract

Flavescence dorée (FD) is a grapevine quarantine disease associated with phytoplasmas and transmitted to healthy plants by insect vectors, mainly *Scaphoideus titanus*. Development of efficient methods for its control has been hampered by the lack of knowledge about phytoplasma biological properties, linked also to difficulties in its *in vitro* cultivation. Conventional management strategies rely mainly on the application of insecticide treatments, rouging of infected plants and production of phytoplasma-free propagation material. However, these strategies are costly and could have undesirable environmental impacts. Novel approaches are being investigated using transcriptomic and proteomic tools that can assist in identifying key regulators expressed by diseased, recovered and healthy plants. These studies allowed the identification of molecular profiles linked to the grapevine cultivar diverse susceptibility, that are of great interest for the development of FD tolerant plants by breeding programs. Other promising FD management strategies include the use of grapevine endophytic microorganisms with known biocontrol properties and endophytes living inside specialized insect cells, which can be potential candidates for FD vector control. Finally, the application of plant defense elicitors might be interesting tools for FD containment but more research is needed before they can be potentially implemented. In this review, the methodologies used for detecting and confining FD dispersion are discussed, focusing mainly on conventional tools, current research perspectives and knowledge gaps.

Introduction

Phytoplasmas are phytopathogenic prokaryotes responsible for more than 700 different plant diseases worldwide (Bertaccini *et al.*, 2014); they belong to the Mollicutes class and are phylogenetically related to the Gram-positive bacteria (Weisburg *et al.*, 1989; EFSA Journal, 2014).

The most important grapevine yellows diseases in the main viticultural areas of Europe are FD and Bois noir (BN) (Bertaccini and Duduk, 2013) associated with the presence of 16SrV-C/-

phytoplasmas (Martini *et al.*, 1999) and '*Candidatus* Phytoplasma solani' (Quaglino *et al.*, 2013), respectively. The FD phytoplasmas are the most devastating, leading to drastic grapevine yield losses and even to the death of the infected plants (Chuche and Thiéry, 2014). They are quarantine organisms included in the EU2000/29 Council Directive on Harmful Organisms and the EPPO A2 list of pests (Prezelj *et al.*, 2012).

Although symptoms of FD had been observed in France since the beginning of the 20th century, it was only around 1950 that FD was named, after spreading in the vineyards of south-west France (especially in Gascogne and Armagnac) (Caudwell, 1957; 1983). Currently, FD is reported also in the other wine-producing European regions, including Austria, Croatia, Hungary, Portugal and Switzerland (CABI, 2015).

The molecular classification of FD occurred for the first time in Italy, being assigned to group 16SrV (Bertaccini *et al.*, 1995). Flavescence dorée strains are divided into two ribosomal subgroups, 16SrV-C and 16SrV-D, and are experimentally differentiated using polymorphisms in specific genes, such as *rpS3, SecY,* amongst others (Angelini *et al.*, 2001; Martini *et al.*, 2002; Botti and Bertaccini, 2006; Arnauld *et al.*, 2007). Generally, FD types have different geographic distributions, although they may co-exist in the same geographic areas. For example, Angelini et al. (2001), showed that FD70 (from 16 SrV-C group), and FD88 and FD92 (from 16SrV-D group) are present in France. Still, strains of FD 16SrV-D are prevalent in Northern Italy (Martini *et al.*, 1999), France, Spain (Angelini *et al.*, 2001; Torres *et al.*, 2005) and Portugal (Sousa *et al.*, 2007), whereas FD 16SrV-C was detected in Northern Italy (Piedmont and Veneto regions), France (Caudwell, 1957). Martini *et al.*, 1999; Martini *et al.*, 2002), Serbia (Duduk *et al.*, 2004), Macedonia (Filippin *et al.*, 2009) and Slovenia (Foissac and Maixner, 2013).

Both FD strains are vectored by the leafhopper *Scaphoideus titanus* Ball (Schwester *et al.*, 1962; 1969; Mori *et al.*, 2002; EFSA Journal, 2014), which acquires the phytoplasma during its first larval stage while feeding on infected plants (Boudon-Padieu *et al.*, 1989). The sap-feeding process of *S. titanus* was recently studied by electropenetography technique (Chuche *et al*., 2017a). Previously

characterized as a phloem feeding insect, this technique showed that *S. titanus* can ingest a mix of both xylem and phloem sap, thereby potentially also being able to spread *Xylella fastidiosa* in vineyards (Chuche *et al.*, 2017b).

In Europe, *S. titanus* was monitored for the first time in the late 1950s in France (Bonfils and Schvester, 1960). Flavescence dorée dispersal is closely linked to this vector spread (Bertin *et al.*, 2007; Papura *et al.*, 2009), which was favoured by European climatic conditions (Bertin *et al.*, 2007; Chuche and Thiéry, 2014) and then potentially spread to Northern Asia (Ge and Wen, 2006; Steffek *et al.*, 2007).

Although *S. titanus* is considered the main FD vector, recent studies showed that the polyphagous *Orientus ishidae* leafhopper (Mehle *et al.*, 2011) can also transmit the disease (Lessio *et al.*, 2016; Trivellone *et al.*, 2016). Moreover, other leafhoppers like *Dictyophara europaea* (L.) (Filippin *et al.*, 2009) can harbour FD phytoplasma (EFSA Journal, 2014).

The main host plant species for FD are *Vitis vinifera* and *V. riparia*, but the two FD strains differ in their host specificity. While for the FD-D strains no alternative plant host or insect vectors are reported, for FD-C alternative host plants such as *Clematis vitalba*, *Alnus glutinosa* (EFSA Journal, 2014; Malembic-Maher *et al.*, 2009), and *Ailanthus altissima* (Filippin *et al.*, 2011) have been identified (Angelini *et al.*, 2004).

Infected plants usually develop symptoms characterized by leaf rolling, yellowing or reddening (in agreement with berry colour), stunted growth, unripe cane wood and shrivelled berries (Figure 1). The shoots of susceptible grapevine varieties also fail to lignify, are thin, rubbery, and hang pendulously (Caudwell, 1957).

Flavescence dorée has been subjected to mandatory control measures so that over the last 15 years its impact in affected grapevine growing areas has reduced. However, FD phytoplasmas have the ability to differentiate into new strains in short periods of time, challenging the current disease management strategies (Bertaccini, 2015). The FD management strategies, which are currently based on insecticides application against *S. titanus*, uprooting of symptomatic plants and utilization of

disease-free propagation material (Marzorati *et al.*, 2006; Margaria *et al.*, 2014; Roggia *et al.*, 2014), in several circumstances, are not sufficient to contain this disease. Since *S. titanus* and FD continue to expand to new areas it is of utmost importance to explore more sustainable, innovative and effective approaches to manage their dispersal.

The aim of this paper is to critically review the current knowledge on the methodologies for FD detection and for potentially managing the disease and vector dispersal. To that end, this review will focus on describing the conventional approaches and current research perspectives for management FD. The knowledge gaps and future challenges related to the implementation of such methodologies are also discussed.

Detection and quantification of Flavescence dorée phytoplasmas

Phytoplasma identification is based on its molecular detection in plant tissue samples or in insect vector specimens, and it can be done using low to high-throughput screening methodologies. In general, although the molecular techniques for FD detection are sensitive, there can be some problems due to the seasonal variation in the phytoplasma titre, their irregular distribution within the plant and their low concentration in woody hosts, especially in young grafted plant material (Duduk and Mori, 2013). Therefore, visual inspection remains the basis for the routine practice.

Serology techniques, such as double-antibody sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA), have also been successfully applied to FD detection using polyclonal or monoclonal antibodies and molecular probes (Boudon-Padieu *et al.*, 1989; Daire *et al.*, 1992; Bertaccini *et al.*, 1993). Since the early 1990s, several PCR-based methodologies (Deng and Hiruki, 1991; Lee *et al.*, 1995; Lee *et al.*, 1998), have been optimized, allowing new insights into the diversity and genetic interrelationships among phytoplasmas (Bertaccini and Duduk, 2009). Several quantitative PCR (q-PCR) protocols have been developed as fast routine detection methods for FD (Angelini *et al.*, 2007),

with q-RT-PCR being five orders of magnitude more sensitive than q-PCR (Margaria *et al.*, 2009; Salar *et al.*, 2013). Also, a Triplex RT-PCR for FDp detection have been tested with increased sensitivity (Pelletier *et al.*, 2009). Besides these approaches, LAMP based assays have been developed which are able to detect 16SrV group phytoplasma presence in grapevine in only about one hour (Kogovsek *et al.*, 2015).

Approaches to study FD pathogenicity and FD-host interactions

Having accurate genomic information regarding FD phytoplasma strains may be a very powerful tool. It not only gives the possibility to design more specific primers and better molecular probes, allowing strain discrimination and classification (Bertacini, 2007), but it may also provide valuable information regarding FD pathogenicity mechanisms (Mehle *et al.*, 2014, Roggia *et al.*, 2014). A physical map of the 671 kbp chromosome of FD strain FD92 including the two rRNA operons, tuf, uvrB-degV and secY-map genes was produced (Malembic-Maher *et al.*, 2008). Sequence of the 671 kb chromosome of the same strain was later partially obtained by pyrosequencing (Carle *et al.*, 2011). Out of the 464 chromosomal coding sequences, 38% were involved in information transfer, 19% encoded metabolic enzymes, 9% corresponded to transporters, 1% were related to cellular processes, and 31% remained cryptic. It was shown that this FD phytoplasma strain possesses a complete glycolytic pathway and the authors suggested the presence of a prominent system for proteolysis that may have resulted from the adaptation to its woody hosts (Carle *et al.*, 2011). Once the full genome of FD is sequenced, even more information will be available regarding other genes and molecular pathways which may contribute to the pathogenicity of FD. More recently, RNA-Seq technology was successfully applied for the first time to analyse the global transcriptome profile of an FD strain during grapevine infection (Abbà *et al.*, 2014). This work provided new insights into the transcriptional organization and gene structure of FD, generating about 8300 FD-mapped reads assembled in 347 sequences, corresponding to 215 annotated genes (Abbà *et al.*, 2014). Functional classification revealed that most of the expressed genes were either related to translation and protein biosynthesis or hypothetical proteins

with unknown function. Some of the latter were predicted to be secreted, acting as effectors with a potential role in modulating the interaction with the host plant. Interestingly, qRT-PCR validation of the RNA-Seq expression values confirmed that a group II intron represented the FD genomic region with the highest expression during grapevine infection. This mobile element may contribute to the genomic plasticity increasing its fitness towards host-adaptive strategies (Abbà *et al.*, 2014).

Proteomics studies may also be very informative indicating different molecular level of response to pathogen infection. But despite their potential, so far only a few studies have looked at the defense protein production triggered by FD infection. Margaria and Palmano (2011) monitored the effects of infection on the plant protein expression profile. Among the 576 analyzed spots, 33 proteins were differentially regulated in infected grapevines. In a posterior study, Margaria *et al.* (2013) uncovered novel aspects of grapevine response to phytoplasma infection using a proteomic and phospho-proteomic approach. They identified 48 proteins that differentially changed in abundance, phosphorylation, or both in response to FD infection. Amongst others, fifteen differentially phosphorylated proteins were identified in infected compared to healthy plants, including proteins involved in photosynthesis, response to stress and the antioxidant system. Further work will be necessary to assess whether the differences in proteome profiles are conserved among different grapevine cultivars showing similar levels of susceptibility to the disease, and under different environmental conditions.

Finally, metabolomics studies may also contribute to a better understanding of the molecules involved in plant susceptibility to FD. Recently, FD-grapevine interaction mechanisms were studied in infected grapevines of cv. Modra Frankinja under field conditions, and a non-targeted metabolomics analysis was conducted (Prezelj *et al.*, 2016). The analysis identified 22 significantly changed compounds with increased levels during infection. Flavescence dorée infection was shown to inhibit phloem transport, resulting in accumulation of carbohydrates and secondary metabolites that provoke a source-sink transition and a defense response status.

In order to better understand the role of the identified proteins in the FD infection pathway, a new system has been employed using spiroplasmas as the model organism. The construction of recombinant spiroplasmas exhibiting FD variable membrane proteins (Vmp), present in the midgut and salivary glands of *S. titanus*, provided a new biological approach for studying interactions of phytoplasma surface proteins with host cells (Renaudin *et al.*, 2015). A recent study showed that a *Spiroplasma citri* mutant G/6 for VmpA expression and used VmpA-coated fluorescent beads interacted with *Euscelidius variegatus* insect cells in culture and promoted the retention of VmpA-coated beads to the midgut of insect. Thus, VmpA act as an adhesin that could be essential in the colonization of the insect by the FD phytoplasmas (Arricau-Bouvery *et al.*, 2018).

The abovementioned molecular studies are of great value in the comprehension of FD phytoplasma and how it interacts with its grapevine host. The discovery of the differential genomic, transcriptomic or metabolic profiles from different grapevine cultivars showing contrasting susceptibilities to the disease may help in understanding the underlying causes for susceptibility, and can assist in the development of breeding programs for FD tolerant grapevine genotypes. The knowledge obtained by looking at the pathogen, and at which genes, transcripts, proteins or metabolites are being expressed and secreted during its infection, will enhance the development of novel, more effective and specific control methodologies for FD management (as discussed in a section below) (Figure 2).

Conventional approaches for managing Flavescence dorée

The treatment of infected vineyards with insecticides is mandatory in several countries, and remains the most important tool for *S. titanus* elimination, and for significantly reducing disease pressure (Posenato *et al.*, 1996; Girolami *et al.*, 2002). Insecticide treatments are essentially directed at the insect mobile instars (nymphs and adults) and are applied to the vineyards up to two or three times a year in epidemically infected areas (Chuche and Thiéry, 2014). Nonetheless, because the effect of insecticides is not immediate, and phytoplasmas can be rapidly acquired by the insect vector, this strategy has limited efficacy (Weintraub and Wilson, 2010). Moreover, other important aspects,

such as the grapevine production system (e. g. organic production) and the leafhopper density limit the possibility and the efficacy of insecticide treatments (Seljak, 2008; Zezlina *et al.*, 2013). Additionally, the intensive use of insecticides is costly and has a negative impact on the environment (due to their soil persistence and water contamination); it may also have deleterious effects on human health (Compant *et al.*, 2012). Although there is a high number of diversified active substances used as insecticide against *S. titanus*, their intensive application can favour the persistence in soils, contamination of the environment, as well as appearance of resistant pathogenic soil strains (Compant *et al.*, 2012). In a two-year experiment, Zezlina *et al.* (2013) found that thiamethoxam, a substance that remains for a long time in the plants due to its low metabolization rate, produced the best results against *S. titanus* compared with other four neurotoxic substances. Also, chlorpyrifos-methyl (active compound of Reldan 22 EC) significantly reduced the leafhopper nymph numbers and had lower persistence in the environment compared with thiamethoxam (Zezlina *et al.*, 2013). In organic vineyards, the control of *S. titanus* is restricted to natural products, mostly relying on the use of pyrethrins. In a recent study, the efficacy of several natural products (e.g. pyrethrins, kaolin, orange oil, insecticidal soap and spinosad) has been analysed (Tacoli *et al.*, 2017). These authors have concluded that kaolin and pyrethrins are effective products in the FD management. Nevertheless, their efficacy is lower compared to the synthetic insecticides used in conventional agriculture.

The application of hot water treatment (HWT) to the propagation material (rootstocks, scions or grafted cuttings) is an important measure for preventing FD dissemination. The process consists of soaking dormant grafted cuttings in water at 45-50ºC for 40-45 minutes and is effective at eliminating FD phytoplasmas and *S. titanus* eggs (Caudwell *et al.*, 1997; Borgo *et al.*, 1999; Bertaccini *et al.*, 2001; Tassart-Subirats *et al.*, 2003). However, HWT may impose a severe stress to the treated plants if a homogenous temperature inside the treatment tanks is not achieved (Mannini, 2007; Waite and Morton, 2007).

The removal of infected plants (rouging) is also a regular practice for FD management. This procedure is mandatory in the cases of severe outbreaks of the disease, affecting more than 20% of the plants in the same vineyard. However, when the symptomatic plants have a patchy distribution, it is

advisable to inspect and analyse asymptomatic grapevines also, since they may act as FD reservoirs contributing to the disease spread. In this context, abandoned vineyards should also be regularly inspected by the National Agricultural Departments and possibly uprooted or sprayed, because they are relevant FD sources of inoculum. Moreover, after rouging, chemical treatment against the vector must be applied to maintain a low incidence rate of FD (Pavan *et al.*, 2012).

The removal of wood from vineyards after winter pruning and the removal of suckers growing along the vertical trunk, which are abundantly colonized by nymphs that hatch from the eggs laid into the bark of the trunk, can be used to reduce *S. titanus* populations (Trivellone *et al.*, 2015).

Alternative approaches for managing Flavescence dorée

The conventional strategies for FD management described above are costly, difficult to implement, and have a high environmental impact, therefore alternative management strategies must be developed. Below we describe current research perspectives with potential for managing FD spread. It is expected that emerging tools may have the potential for managing FD if some of the knowledge gaps and limitations described below are properly addressed.

Use of tolerant cultivars

Empirical evidence has shown that it is possible to identify grapevine cultivars with differential susceptibility to FD (Jarausch *et al.*, 2013). Susceptibility may be linked to morphological and physiological differences in the plant, which are ultimately determined by the genetic background of each cultivar, and on how they respond to the environment in which they are grown. It may also be linked to their response to specific FD effectors, and how these interact with the host plant (Eveillard *et al.*, 2016). Thus, in order to understand the differences in susceptibility to FD, it is important to look at the molecular aspects of the host as well as of the pathogen at a genomic, transcriptomic, proteomic and metabolomic level, as described above.

Jarausch *et al.* (2013) have defined grapevine´s field resistance to phytoplasmas as the absence of symptoms and of growth alterations combined with a low titre of the pathogen, whereas tolerance reflects the absence or mild symptoms but with a high titre of the pathogen. The use of tolerant plant material is often referred to as a promising approach for phytoplasma-associated field disease control (Seemüller and Harries, 2010; Jarausch *et al.*, 2013). However, care must be taken, since asymptomatic infected grapevines and rootstocks may contribute to disease dispersion (Eveillard *et al*. 2016). Therefore, although tolerant plants might be an appealing complement to other FD management methodologies, it is important to guarantee that these plants would not represent phytoplasma reservoirs constituting a masked hotspot. Thus, ideally, tolerance should be associated with phytoplasma absence in plant tissues. In recent experiments different susceptibilities were found in plants infected with FD. From Table 1 it is possible to observe that globally, most cultivars have been reported as highly susceptible or intermediately susceptible and only a very limited number were referred to as low susceptible cultivars. Nonetheless, many economically relevant cultivars have not yet been studied for tolerance/susceptibility to FD. The variability in FD susceptibility has hardly been addressed because of the difficulties in cultivating and transmitting phytoplasmas under controlled conditions of inoculation. Efforts at their *in vitro* culture and mutagenesis have been scarce since phytoplasmas are phloem limited (Maejima *et al*., 2014) and an axenic cultivation method has only recently been characterized (Contaldo *et al*. 2016). Additional studies need to be conducted before the physiological and molecular mechanisms of susceptibility to FD can be fully understood. Moreover, grapevines are produced in a vast range of environmental conditions, thus the interaction between genotype and the environment needs to be considered when studying such mechanisms. Also, the replacement of traditional European grapevine cultivars with tolerant ones might not be compatible with the high-quality wines (that are usually obtained from old vineyards) neither with appellations (that traditionally use specific cultivars). To this end, a better option could be to use rootstocks as sources of field resistance, since natural infection and disease symptomatology has only been verified in a few genotypes (Jarausch and Torres, 2014). This source of resistance would have

the great advantage that the commercial traits of the cultivar (scion) remain unchanged. However, a recent study has shown that rootstocks may carry high phytoplasma titres, potentially constituting a silent reservoir of contamination (Eveillard *et al*., 2016).

Use of naturally recovered plants

The spontaneous remission of the disease symptoms, known as recovery, has been reported following the first year of symptom expression in grapevines infected by phytoplasmas (Caudwell, 1990; Morone *et al.*, 2007; Gambino *et al.*, 2013; Vitali *et al.*, 2013), but the mechanisms and the dynamics behind it are largely unknown (Gambino *et al.*, 2013; Vitali *et al.*, 2013). Recently, the seasonal time course of gas exchange rates in healthy, FD-infected and recovered grapevines from cultivars Barbera and Nebbiolo subjected to water stress and a control with no drought were studied (Vitali *et al.*, 2013). This study showed that in FD-infected plants, net photosynthesis and transpiration gradually decreased during the growing season and this effect was stronger when water availability was not a limiting factor. During recovery, plants that had been infected two years before (but not the ones infected the year before) regained the gas exchange performances, reaching values comparable to the ones measured before FD infection. This can explain why the recovered grapevines usually show higher yield than the symptomatic ones (Morone *et al.*, 2007). Vitali *et al.* (2013) concluded that metabolic, not stomata, leaf gas exchange limitation in FD-infected and recovered grapevines is the basis of the plant response to FD disease. Moreover, they also suggested that such response is dependent upon water availability since water stress superimposes on FD infection in terms of stomata and metabolic non-stomata limitations to carbon assimilation. A study conducted by Margaria *et al.* (2014) provided a molecular and biochemical description of the flavonoid pathway in response to FD phytoplasma in infected and recovered plants. This study suggested that proanthocyanidins accumulation, mainly in healthy and recovered grapevines, could help minimize further infection by the insect vector (Margaria *et al.*, 2014). Some researchers have studied the role of hydrogen peroxide (H_2O_2) in the recovery phenomenon of FD infected plants using qRT-PCR to

investigate the H_2O_2 expression in production related genes in 2-year-old-recovered plants (i.e., plants found positive for FD in the past but FD-negative and symptomless for the last 2 years) of *V. vinifera* cv. Barbera and cv. Prosecco (Musetti *et al.*, 2007; Gambino *et al.*, 2013). In these studies, recovered plants produced larger amounts of H_2O_2, compared with healthy and infected plants for the transcriptional activation of *gox* and *glp* genes involved in the H_2O_2 production and a downregulation of a *apx2* gene coding H_2O_2 scavenging was also observed.

Endophytes as elicitors of plant defense against FD

Exploiting the use of beneficial microorganisms with potential biocontrol properties is recently attracting the interest of several researchers (Pinto *et al.*, 2014). According to Petrini (1992) the term endophytes refers to all microorganisms that can colonize internal plant tissues without causing visible harm to their host. Several studies have shown that endophytes act as plant protectors against different pathogens and as elicitors of plant defenses (Compant *et al.*, 2005). The effects of these organisms as promoters of plant growth due to a reduction of disease impact is well known (Bianco *et al.*, 2013). Their use for plant disease control offers numerous advantages: i) they do not interfere with the environment and human health; ii) they are targeted and efficient in low amounts; iii) they have a slow degradation rate as they can multiply and are less subjected to selection for resistance and, iv) their application can be extended to conventional and integrated pest management production systems (Berg, 2009). However, the success of these microorganisms as biocontrol agents depends on the study of endophyte-host interactions and on the effective production of chemical formulations with these organisms (Hallmann *et al.*, 1997). Endophytes may be of bacterial or fungal nature, and they may be associated not only with the host but also with the insect vector of FD. Below some of the most well-known endophytes having the potential to be used against FD are reported.

Rhizospheric and endophytic bacteria – Plant growth-promoting rhizobacteria (PGPR) are present in root surfaces and in the rhizosphere (Kloepper *et al.*, 1978; 1999), and when these bacteria enter the root they are called endophytic bacteria. These are important for plant defense, being shielded from the competitive soil environment. Endophytic bacteria can also originate from the phyllosphere, anthosphere or spermosphere (Hallmann *et al.*, 1997), and can be found in the vascular system, intercellular space or cell cytoplasm (Bulgari *et al.*, 2009; Romanazzi *et al.*, 2009) of several plant parts (roots, tubers, stem and leaves) (Hallmann *et al.*, 1997).

Several studies have suggested the involvement of endophytic bacteria in the recovery phenomenon against phytoplasma diseases in grapevine (Bulgari *et al.*, 2009; Romanazzi *et al.*, 2009; Grisan *et al.*, 2011; Musetti *et al.*, 2011). Bulgari *et al.* (2011a; 2011b) showed that the diversity of the grapevine's endophytic bacterial community is greater in recovered grapevines previously infected with FD as compared to the one observed in diseased and healthy plants.

There is still only a limited number of studies addressing the biocontrol properties of endophytic bacteria against phytoplasmas. Gamalero *et al.* (2017) concluded that 1-aminocyclopropane-1-carboxylate (ACC) deaminase synthetized by *Pseudomonas migulae* can limit the phytoplasma damages in periwinckle. Endophytic bacteria have been studied also in BN infected grapevines (Bulgari *et al.*, 2011b), in apple proliferation disease associated with the presence of '*Ca.* P. mali' (Bulgari *et al.*, 2012), and in the chrysanthemum yellows phytoplasma (Gamalero *et al.*, 2010). Some of the mechanisms used to promote plant growth and to protect against plant pathogens are the same for free living PGPR and endophytic bacteria (Kloepper *et al.*, 1991; Höflich *et al.*, 1994). Competition for niches, carbon sources, production of inhibitory allelochemicals and triggering of induced systemic resistance are the main biocontrol mechanisms activated by the presence of these bacteria (Lugtenberg and Kamilova, 2009; Shoresh *et al.*, 2010; Bulgari *et al.*, 2011). The direct effects of these microorganisms on plants are related to the production of indole acetic acid, which

stimulates root development (Patten and Glick, 2002; Romanazzi *et al.*, 2009), induces changes in the root architecture (Gamalero *et al.*, 2002; 2004; Romanazzi *et al.*, 2009), improves nutrient uptake by solubilization of phosphate (Gyaneshwar *et al.*, 2002; Romanazzi *et al.*, 2009) and increase nitrogen fixation (Romanazzi *et al.*, 2009; Vessey *et al.*, 2005).

The use of endophytic bacteria might be a promising tool for programs aiming at enhancing grapevine resistance against FD, however further studies are needed before these bacteria can be applied in a field scale.

***Endophytic fungi* –** Endophytic fungi have also been isolated from FD recovered grapevines (Martini *et al.*, 2009; Bianco *et al.*, 2013); they are sources of secondary metabolites and antibiotics (Gimenez *et al.*, 2007) and are symbiotically associated with plants, improving their mineral nutrition (Smith and Read, 1997), protecting against pathogens (Azcon-Aguilar *et al.*, 2002) and improving plant resistance against environmental stresses (Turnau *et al.*, 2002; Bianco *et al.*, 2013). *Epicoccum nigrum* strains, isolated from infected *Catharanthus roseus*, have been reported as biocontrol agents against '*Ca.* P. mali'. Inoculation of *C. roseus*, colonized by *E. nigrum* resulted in significant reduction of disease symptoms and '*Ca.* P. mali' phytoplasma titre (Musetti *et al.*, 2011). In the study of Pinto *et al.* (2014) two other important protectors agents were identified in *V. vinifera*: *Bulleromyces albus* and *Dioszegia* spp.. These fungi induce plant cell modifications indicative of plant cell defense activation, such as deposition of phloem protein plugs, deposition of callose in sieve tubes and synthesis of phenolic compounds in companion cells and sieve tubes (Musetti *et al.*, 2007). Plant inoculations with combinations of bacteria and fungi can enhance their beneficial effect (Russo *et al.*, 2012), as it has already been shown that rhizobacteria interact with arbuscular mycorrhizal fungi (Romanazzi *et al.*, 2009). The inoculation of *Pseudomonas putida* S1Pf1Rif alone or in combination with the mycorrhyzal fungus *Glomus mosseae* BEG12 effectively reduced chrysanthemum yellows phytoplasma infection in chrysanthemum (Gamalero *et al.*, 2010).

Fungi can thus be considered an interesting tool for FD biocontrol, but extensive research on this

topic is still warranted. The study of the relationship between phytoplasmas, fungal endophytes and host plants is essential for the success of this application.

Endosymbiont microorganisms living inside the insect vector – The endosymbiontic microflora of insects is of extreme importance for their ecological and evolutionary success as they influence host growth, nutrition and fertility (Sachi *et al.*, 2008; Ishikawa, 2003). These microorganisms are present in specialized insect cells, like mycetocytes and bacteriocytes, and can colonize different insect organs or tissues or form aggregates (Sacchi *et al.*, 2008). Thus, the knowledge about the microbiological community that influences the insect vector ecological success is crucial for the search of innovative biocontrol methodologies (Beard *et al.*, 1998; Chuche *et al.*, 2017). The use of microorganisms that are not pathogenic to the plant host but can interfere with the pathogen life cycle could be a good possibility (Baldridge *et al.*, 2004). The genetic manipulation of the insect microflora in order to select the microorganisms which have, for example, the capacity to spread to the insect progeny, is a promising approach since in this case, these microorganisms can block a specific genetic trait (Zabalou *et al.*, 2004). However, the chosen endophyte must: have a stable relationship with the insect vector, prevail within its population, cohabitate with the pathogen, be able to be manipulated in laboratory and be efficiently distributed within the insect community (Alma *et al.*, 2010). Also, it is important to keep in mind that a single symbiont strain can affect many vector species (Chuche *et al.*, 2017). Marzorati *et al.* (2006) identified an endosymbiont (ST1-C) belonging to the genus *Cardinium* in *S. titanus*. These bacteria are known to influence reproduction and behaviour of insect hosts (Zchori-Fein *et al*., 2001; Kenyon and Junter, 2007), suggesting they could be a possible FD control agent. Moreover, species of the acetic acid bacteria genus *Asaia* identified in *S. titanus* (Gonella *et al*., 2012) can be involved in its feeding metabolism (Crotti *et al.*, 2010) having the potential to control the insect vector.

Use of plant defense elicitors

Elicitors are signalling molecules that stimulate the plant's natural defense mechanisms against biotic and abiotic stresses (Belhadj *et al.*, 2008), without environmental impact and risk of selecting resistant pathogen strains, that is usually obtained with the use of conventional pesticides and antimicrobial compounds for pest and disease control (Ruiz-García and Gómez-Plaza, 2013). Current concerns about human health, food safety, and respect for the environment are stimulating the search for these alternative protection strategies. A variety of molecules can act as elicitors, including hormones, oligo- and polysaccharides, peptides, proteins and lipids (Côté *et al.*, 1998).

Jasmonates are endogenous plant hormones derived from fatty acids (Koda, 1992; Creelman and Mullet, 1997; Repka *et al.*, 2004). After their production, jasmonates, such as jasmonic acid (JA), induce phytoalexin biosynthesis, which acts in the general plant response to pathogens (Gundlach *et al.*, 1992). The involvement of JA and its more active derivative methyl jasmonate (MeJA) in the signal transduction cascade has stimulated the use of this molecule as an inducer of plant defense mechanisms in several pathosystems (Belhadj *et al.*, 2008). The application of this compound has been studied due to its capacity to activate gene expression (e.g. *PAL*, *STS*, *PIN*, *PGIP*) and biosynthesis of secondary metabolites (e.g. phenolic compounds, alkaloids, pathogen-related proteins) in many plant species, including grapevine, and triggering significant protection against diverse pests and diseases such as *Erysiphe necator and Plasmopara viticola* (Pérez *et al.*, 1997; Belhadj *et al.*, 2006; Wen *et al.*, 2012; Thiruvengadam *et al.*, 2016). In grapevine, pre-harvest treatments with MeJA revealed that it activated chalcone synthase (CHS), stilbene synthase (STS), UDP-glucose: flavonoid-*O*-glucosyltransferase (UFGT), proteinase inhibitors and chitinase gene expression. Such activations triggered the cellular accumulation of both stilbenes and anthocyanins (Belhadj *et al.*, 2008). Treatment with MeJA has also been applied in leaves and suspension-cultured cells of grapevine *V. vinifera* L. cv. Limberger (Repka *et al.*, 2004). MeJA triggered a cascade of events which induced necrotic lesions, similar to those normally associated with resistance to avirulent pathogens, causing hypersensitive cell death, stimulating medium alkalinisation accompanied by massive callose and phenolic compounds deposition (Repka *et al.*, 2004) and expression of PR genes (PR-1, PR-2, and

PR-3) (Yang *et al.*, 1997; Repka, 2001). After exposure to a biotic or abiotic stress, the transcription of defense genes is activated in plants. Belhadj *et al.* (2008) used qRT-PCR to study the expression of eight genes related with defense mechanisms triggered by MeJA. Application of MeJA also triggered the activation of transcription pathways whose products act in grapevines against pathogens.

Besides MeJA, salicylic acid (SA) also attracted the attention of the researchers as a potential plant elicitor against several grapevine diseases including: *Botrytis cinerea* (Reanult *et al.* 1996), *Sphaceloma ampelinum* (Prakongkha *et al.*, 2013), *Plasmopara viticola* (Thiruvengadam *et al.*, 2016) and Bois noir (Paolacci *et al.*, 2017). These studies have reported the acquisition of a systemic resistance related with SA application, which acts as a transcription factor of pathogenesis-defense genes. Moreover, some studies have been conducted using other elicitors, such as chitosan (Romanazzi *et al.*, 2002) and benzothiadiazole (Iriti *et al.*, 2004) in grapevines against *B. cinerea*.

Although these findings indicate that elicitors might be good candidates for enhancing grapevine tolerance to FD, several studies are needed for better understanding the plants' genetic and physiological responses and to find the best timing, frequency and dosage of each elicitor, adjusted to different cultivars and environmental conditions.

Models to predict disease and vector spread

Prediction models that integrate information on climate change and its relation to disease and vector spread are scarce. Rigamonti *et al.* (2011) developed a phenology model to improve the understanding of the insect-vineyard dynamics and the better timing of application of an insect growth regulator, considering the development of insect eggs, nymphal instars and their transition to the adult stage. This task was improved since the discovery of the importance of the temperature during oviposition (Rigamonti *et al*., 2013). An FD epidemiological model was developed, incorporating different parameters of the transmission process (acquisition of the disease, latency and expression of

symptoms, recovery rate, removal and replacement of infected plants, insecticidal treatments, and the effect of hotbeds) (Lessio *et al.*, 2015). This model showed the risks of establishing new vineyards in locations where strong hotbeds of FD are present.

Recently, Maggi *et al.* (2016) suggested that the FD epidemic is multivariate, depending on infection incidence, vector population and flight behaviour as well as plant position in the vineyard. The researchers related these parameters with newly infected and recovered plants and presented a space-time epidemic model, where it was concluded that FD infection could come from outside (primary) or within (inside) the vineyard and the spread of FD is more effective in healthy plants nearby infected plants, due to typically short-distance mobility of vector.

Other possible FD management methodologies

Physical control methodologies including insect-exclusion screening for field coverage of mother plant vineyards (Mannini, 2007), and the use of reflective synthetic mulches covering the soil surrounding potential host plants, have been used to repel insect vectors (Weintraub and Wilson, 2010) and avoid phytoplasma infections. However, the high costs associated with these techniques makes them unpractical for wide scale application (Setiawen and Ragsdale, 1987). Moreover, there is the risk that screening application in the vineyards where *S. titanus* eggs have potentially been laid, could work counterproductively, restricting the insect habitat to the areas close to the crop.

Insect vector mating disruption relies on breaking the substrate-borne vibrational signals in the mate recognition and localization process through emission of recorded calling songs (Mazzoni *et al.*, 2009; Eriksson *et al.*, 2012; Polajnar *et al.*, 2014). Other possible limitations with field implementation of this technique include the energy costs, the attenuation of vibrations with the distance from the source of vibration, and the need for adjusting the timings and frequencies of the vibrational signal emissions according to the insect's natural activity pattern. Still, Polajnar *et al.* (2016) after conducting a small-scale field trial concluded that the approach appears robust enough to merit a scale-up testing in the future.

It has been suggested that the risk of exposure to pests and diseases in viticulture is a function of climate (Bois *et al*., 2017) and that the ecological control methods (e.g. pheromones, mating disruption) will possibly be affected by thermopluviometric, wind and hygrometry changes (Reineke and Thiéry, 2016). For example, Chuche *et al*. (2015) showed that *S. titanus* insect hatchings were synchronized with grapevine bud break, which varies in cold and mild winter vineyards. This illustrates that putative changes in the number of yearly insect generations are complex and difficult to predict for future global warming scenarios (Reineke and Thiéry, 2016). In a study looking at the impact of temperature increase on the establishment of *S. titanus*, Quiroga *et al.* (2017) concluded that a 3ºC increase of average daily temperature increases the probability of insect establishment, due to a shorter diapause period following warmer winters (Chuche and Thiéry, 2009; Reineke and Thiéry, 2016). Also, climate change might increase population densities of alternative insect vectors of grapevine phytoplasmas (Reineke and Thiéry, 2016). Hence, research on the interactions between vector life cycle and climate fluctuations is needed before any strategy of disruptive mating can be applied.

Recently several developments on imaging technologies (multispectral, hyperspectral or thermal imaging) are taking place in order to support vineyard management, namely to enhance water use efficiency (e.g. Gutiérrez *et al.*, 2018; Poblete *et al.*, 2018), to access optimal harvest time (Piazzolla *et al.*, 2013; Chen *et al.*, 2015) and to improve disease detection (Bock *et al.*, 2008; Oerke *et al.*, 2016; Al-Saddik *et al.*, 2017). Although the results obtained so far seem to be promising in terms of disease detection, identification and monitoring in precision agriculture (Al-Saddik *et al.*, 2017) more studies are needed before these non-invasive technologies can be fully applied.

Applied perspectives for growers

Some of the aforementioned research perspectives are closer than others in terms of their practical implementation (e.g. vibrational signalling and imaging technologies as compared to elicitor application). Until those research perspectives become actual alternative methods for managing FD,

and in order to prevent disease dispersal, growers should continue performing the conventional approaches discussed above. These include hotwater treatment of propagating material and applying the mandatory treatments against *S. titanus*. The utilization of certified plant material will continue being an important measure, ensuring their phytosanitary quality. Prophylactic measures include the destruction of infected vines, infected pruning wood and abandoned vineyards. In case there is a suspicion of infection, growers should perform a close visual monitoring of phenotypic symptoms of the disease, such as leaf rolling and leaf colouring changes, stunted growth of the putative infected plants, and at a later stage, the presence of shrivelled berries.

Conclusions

The search for effective FD management measures has been hampered by several constraints including: the difficulties in cultivating phytoplasmas and in transmitting FD from plant to plant under controlled laboratory conditions. The recent findings on host-phytoplasma relationships and the preliminary evidence that phytoplasmas can be maintained in a defined medium open new gates for the development of powerful tools for FD management. Since FD has been spreading to new wine growing regions, even with application of mandatory treatments against *S. titanus*, urgent measures for its effective containment are crucial. Nowadays, the best tool for FD management still relies on the mandatory application of insecticides to reduce *S. titanus* populations. Although these insecticides can be effective, they are clearly not enough as this epidemy is still expanding. Moreover, the increasing costs and the social pressure against health and environmentally hazardous treatments, has led to the search for new ways to contain FD. The evaluation of the effect of FD infection at transcriptional and proteomic levels and the identification of FD defense-related genes will help understand differential susceptibilities of grapevine cultivars. Identification of molecular traits associated with the susceptibility to FD would allow the development of markers to be used in breeding assisted selection programs. Another promising alternative for FD containment is biocontrol using endophytic microorganisms, which have been shown to reduce disease pressure by directly or

indirectly affecting pathogen or insect activity. Although the potential of endophytic microorganisms in disease protection is recognized, the plant-pathogen-endophytic microorganism interactions are not fully explored or understood. Furthermore, there is a growing evidence that exogenous application of elicitors (e.g. MeJA and SA) can trigger the defense mechanisms of several plant species, enhancing the level of defensive compounds. Thus, the application of these substances in diseased plants might also become a good alternative for FD management, but more research is needed to explore the physiological and plant responses and to optimise elicitors' application. These options are sustainable approaches to avoid the use of insecticides and rouging for FD containment and should be further evaluated at the experimental level. Table 2 summarizes the main advantages and drawbacks of FD managing methodologies.

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Table 1. Summary of susceptibilities of different *Vitis vinifera* subsp. *vinifera* cultivars' to Flavescence dorée according to different bibliographic sources.

Degree of susceptibility	Grapevine cultivar	Source
Highly susceptible	Barbera	Belli *et al.*, 2000; Vercesi & Scattini, 2000; Vitali *et al.*, 2013
	Cabernet Sauvignon	Jarausch *et al.*, 2013; Eveillard *et al.*, 2016
	Chardonnay	Belli *et al.*, 2000; Vercesi & Scattini, 2000; Jarausch *et al.*, 2013
	Grenache	Jarausch *et al.*, 2013
	Pinot Blanc	Bressan *et al.*, 2005
	Pinot Gris	Belli *et el.*, 2000
	Plovdina	Kuzmanovic *et al.*, 2011
	Prosecco	Pavan *et al.*, 1997; Steffek *et al.*, 2007
	Sangiovese	Belli *et al.*, 2000
	Ugni Blanc	CABI, 2015
	Sauvignon B	Eveillard *et al.*, 2016
Intermediately susceptible	Pinot noir	Vercesi and Scattini, 2000
	Riparia Gloire de Montpellier	Eveillard *et al.*, 2016
	Cabernet Franc N	
	3309 Couderc	
	Grenache N	
	Chardonnay B	
	Sélection Oppenheim 4	
	41 B Millardet et de Grasset	
	110 Richter	
Less susceptible	Cortese	Belli *et al.*, 2000
	Erbamat	Belli *et al.*, 2000
	Moscato	Belli *et al.*, 2000
	Merlot	Belli *et al.*, 2000

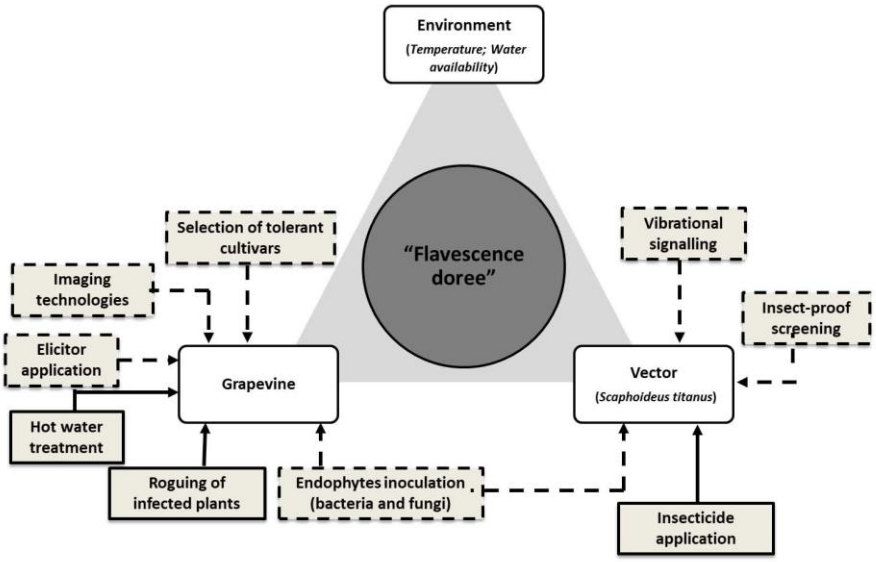
		Jarausch *et al.*, 2013; Eveillard *et al.*, 2016
	Syrah	Jarausch *et al.*, 2013; Eveillard *et al*. 2016
	Magdeleine Noire des Charentes	Eveillard *et al*. 2016
	Pinot Noir N	
	Merlot N	
	Kober 5 BB	
	Nemadex Alain Bouquet	

Table 2. Summary of the main advantages and drawbacks of different Flavescence dorée management methodologies.

Method	**Advantages**	**Drawbacks**
Insecticide application	• Significant reduction of disease pressure	• Limited application in organic grapevine production • Efficacy depends on leafhopper density • Costly (needs regular application) • Environmental contamination (soil and water) • Appearance of resistant soil pathogenic strains • Possible deleterious effects on human health
Hot water treatment	• Effective elimination of phytoplasma and vector eggs	• Risk of reducing plant viability and productivity when treatment is not properly applied
Tolerant cultivars	• Harmless to the environment and human health	• Most cultivars have not yet been screened for susceptibility/resistance traits • Grapevine and wine production chain value is rather closed to the introduction of new cultivars • Interaction with new environment may influence susceptibility
Endophytes (bacteria and fungi)	• Harmless to the environment and human health • Efficient in low amounts • Slow degradation	• More research is need to: – Understand endophyte-host interaction; – Develop chemical formulations • Legislation hurdles

	rate • Less susceptible to induce resistances • Can self-multiply • Can be applied in integrated pest management production systems	
Elicitors application	• Harmless • Efficient induction of natural plant defenses	• Reported antagonistic effects between jasmonates, salicylate and abscisic acid • Optimization of frequency and dosage application must be verified
Prediction models	• Harmless • Higher precision in FD management	• Do not represent a direct control strategy, but only a support for decision making • It takes a lot of time to develop a new model
Vibrational signalling	• Harmless • Significant reduction of *S. titanus* populations • Affect the behaviour of other pests	• Does not eliminate vectors or FD • Proximity between males and females may still allow short-range chemical or visual cues which enable partner recognition • May have negative effects on beneficial fauna

Figure 1. Grapevine with FD symptoms: severely affected plants showing down word curling of the leaves that are red in a red cultivar (a and b). In (c) the cane has yellow leaves (white cultivar) and lack lignification.


Environment
(Temperature; Water availability)
"Flavescence doree"
Selection of tolerant cultivars
Imaging technologies
Elicitor application
Hot water treatment
Grapevine
Roguing of infected plants
Endophytes inoculation (bacteria and fungi)
Vibrational signalling
Insect-proof screening
Vector
(Scaphoideus titanus)
Insecticide application