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Insights on insect vector-plant-phytoplasma interactions: the Flavescence dorée of grapevine

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I have a friend who's an artist and has sometimes taken a view which I don't agree with very well. He'll hold up a flower and say "look how beautiful it is," and I'll agree. Then he says "I as an artist can see how beautiful this is but you as a scientist take this all apart and it becomes a dull thing," and I think that he's kind of nutty. First of all, the beauty that he sees is available to other people and to me too, I believe, although I might not be quite as refined aesthetically as he is, I can appreciate the beauty of a flower.

At the same time, I see much more about the flower than he sees. I could imagine the cells in there, the complicated actions inside, which also have a beauty. I mean it's not just beauty at this dimension, at one centimeter; there's also beauty at smaller dimensions, the inner structure, also the processes. The fact that the colors in the flower evolved in order to attract insects to pollinate it is interesting; it means that insects can see the color. It adds a question: does this aesthetic sense also exist in the lower forms? Why is it aesthetic? All kinds of interesting questions which the science knowledge only adds to the excitement, the mystery and the awe of a flower. It only adds. I don't understand how it subtracts.

Richard Feynman, *Ode to a flower* (1981)

Summary

Introduction

Phytoplasmas

Phytoplasmas are phytopathogenic organisms, belonging to the Bacteria Domain, class Mollicutes (IRPCM, 2004). The large, monophyletic phytoplasma clade represents a unique and highly specialized group, defined by its adaptation to obligate intracellular parasitism (Cao *et al.*, 2020). Indeed, all known phytoplasmas live as parasite of insect and plant phloem cells, and have evolved mechanisms for evading host defenses (Tomkins *et al.*, 2018). Infected plants show a wide range of symptoms including stunting, yellowing, witches' broom (development of numerous tiny shoot branches with small leaves), phyllody (formation of leaf-like tissues instead of flowers), virescence (greening of floral organs), proliferation (growth of shoots from floral organs), purple top (reddening of leaves and stems), and phloem necrosis (Maejima *et al.*, 2014).

So far, more than 100 phytoplasma distinct subgroups have been described, belonging to more than 44 '*Candidatus* Phytoplasma' species (Namba, 2019). The '*Candidatus*' genus designation is maintained since the 'IRPCM Phytoplasma taxonomy group' phytoplasma description (IRPCM, 2004). The motivation behind this nomenclature lays in the impossibility of *in vitro* cultivation of phytoplasmas, thus failing in fulfilling Koch's postulates. Despite few attempts of phytoplasma *in vitro* cultivation (Contaldo *et al.*, 2012, 2016), more convincing results need to be achieved (van Bel and Musetti, 2019). The characterization and description of

phytoplasmas largely relies on the percentage of identity of the 16S rRNA gene sequence (16Sr). Phytoplasmas sharing more than 97.5% identity for this gene belong, for convention, to the same '*Candidatus* Phytoplasma spp.' (IRPCM, 2004). Moreover, at least 36 groups resulted from Restriction Fragment Length Polymorphism (RFLP) on 16Sr gene (Naderali *et al.*, 2017), and are indicated by a roman number (from I to XII) based on their RFLP profile (Lee *et al.*, 1998). Subgroups are indicated consequently with a letter following the group number, e.g. 16Sr-V –C or –D.

Phytoplasmas share an extremely reduced genome size, with a range of 530–1350 kb (Marcone *et al.*, 1999; Christensen *et al.*, 2005; Kube *et al.*, 2012; Oshima *et al.*, 2013; van Bel and Musetti, 2019). Their genome lacks different metabolic related genes, so these bacteria must rely on their hosts to compensate for these losses. An exemplar case is the absence of ATP synthase complex F1-F0 genes, suggesting a complete dependence on glycolysis for the production of cellular energy (Oshima *et al.*, 2007). Interestingly, phytoplasmas harbor multiple copies of transporter-related genes. These genomic features suggest that phytoplasmas are highly dependent on metabolic compounds from their hosts (Oshima *et al.*, 2013). Consistently, their main ecological niches are represented by plant host phloem and insect haemolymph, that are rich in sucrose and trehalose, respectively.

Phytoplasmas need to maintain high level of plasticity, since they are transmitted from plant to plant by insect vectors. After the ingestion of phloem sap of an infected plant, the insect vector gut is

colonized, followed by the haemolymph. During a latency period, tipically from two- to four-weeks after the first infectious feeding, phytoplasmas invade the vector salivary glands, making it infectious for life in a persistent propagative manner (Bosco and D'Amelio, 2010; Alma *et al.*, 2018).

Interestingly, extant phytoplasmas apparently evolved from a single common ancestor that acquired a specific association with phloem-feeding insects and their host plants, presumably a single ancestral host plant and vector combination (Cao *et al.*, 2020).

In nature, phytoplasmas are transmitted from plant to plant mainly through insect vectors, belonging to the Hemiptera order, namely leafhoppers (Auchenorryncha: Cicadellidae, Deltocephalinae), planthoppers (Auchenorryncha: Cixiidae and Derbidae), and psyllids (Sternorryncha: Psyllidae) (Weintraub and Beanland, 2006). The highly specialized nature of phytoplasma-plant-insect associations, the widespread geographic occurrence of phytoplasmas, and the extensive phylogenetic diversity of the phytoplasma lineage suggest that this group of bacteria has been co-evolving with its plant and insect hosts for a very long time, feasibly for millions of years (Cao *et al.*, 2020).

Phytoplasmas depend on their insect vector for dispersion, and their potential distribution is directly dependent on the feeding preferences and ecological niches of the vectors. Transmission of phytoplasmas by insects involves, at several levels, elements of host–pathogen specificity. The host range of both phytoplasmas and insects greatly influences the chances that a phytoplasma and

a potential vector will come into contact (Bosco and D'Amelio, 2010). Vector insects can be polyphagous, oligophagous or monophagous, according to their ability to feed and reproduce on many, few or one host plant, respectively. Similarly, phytoplasmas may be generalists, infecting several different plant species, or specialists, infecting one or a few related plant species. Insect feeding behavior (the tissues explored by the stylets for nutrition) plays a major role in transmission competence. The transmission of phloem-restricted pathogens like phytoplasmas correlates with the phloem-feeding behavior. Yet, it is known that aphids, whiteflies and mealybugs, in spite of their phloem-feeding behavior, are not vector of phytoplasmas. Therefore, a phloem-feeding habit is necessary but insufficient for phytoplasma transmission. Finally, phytoplasmas must adhere and invade insect cells. The specificity of the interaction between phytoplasmas and vector cells/membranes is mediated by the molecular interaction between phytoplasma membrane proteins and insect proteins (Rossi, Samarzija, *et al.*, 2019). Since phytoplasmas are endo-cellular parasites that lack a cell wall, their membrane proteins and secreted proteins function directly in the host cell (Maejima *et al.*, 2014). In many phytoplasmas, a subset of membrane proteins (usually referred to as immunodominant membrane proteins, IDP) accounts for a major portion of the total cellular membrane proteins (Kakizawa et al., 2006). IDP were classified into three types: immunodominant membrane protein (Imp), antigenic membrane protein (Amp), and immunodominant membrane protein A (IdpA) (Kakizawa *et al.*, 2006). The three IDP types share a central hydrophilic region, with

a predicted external orientation, and one or two transmembrane domains (Rossi, Samarzija, *et al.*, 2019). In addition, the Amp protein forms a complex with insect microfilaments (Suzuki *et al.*, 2006), and the formation of Amp–microfilament complexes correlates with the phytoplasma-transmitting capability of leafhoppers (Suzuki *et al.*, 2006). The specific interaction between phytoplasma Amp and actin (a component of microfilament), as well as the ATP synthase β subunit of insect vectors, has also been observed for the CYP strain of '*Ca*. P. asteris' (Galetto *et al.*, 2011), and it is probably involved in determining the transmissibility of phytoplasma. In Flavescence Dorée (FD)-related phytoplasmas, a variant of the Variable membrane protein A predicts the inability of the phytoplasma to be transmitted by *Scaphoideus titanus*, the natural vector of FD (Arricau-Bouvery *et al.*, 2018; Malembic-Maher *et al.*, 2020). Moreover, specific binding of the phytoplasma membrane protein IMP by insect proteins seems to be related to vector status of leafhopper species (Trivellone *et al.*, 2019). All this information again suggest that the specific interaction may be crucial for transmission competence and efficient phytoplasma spread by vectors.

Flavescence dorée

Flavescence dorée (FD) is a grapevine disease caused by phytoplasmas belonging to the 16Sr-V group, -C and –D subgroups; for these phytoplasmas the '*Candidatus* Phytoplasma vitis' (https://gd.eppo.int/reporting/article-4783) taxon has been

proposed but not accepted yet. The disease was first described in the middle of the XX century in French vineyards (Caudwell, 1957), and since then it spread to viticultural areas of several other European countries (EFSA, 2014). Flavescence dorée may have different severities depending on the grapevine variety (Morone *et al.*, 2007; Eveillard *et al.*, 2016; Ripamonti *et al.*, 2021). On the most susceptible varieties, the first symptoms appear in May–June, with stunting and lack of bud break. Later, they evolve into leaf yellowing or reddening (depending on the variety), downward leaf curling, drying of inflorescence and bunches, lack of cane lignification, presence of black spots on new canes and premature leaf fall, on the entire plant or just on individual branches (Caudwell, 1990; EFSA, 2020; Galetto et al., 2016). Both cultivated and wild species of the genus *Vitis* can host FD phytoplasmas (FDp), along with some broadleaved trees or shrubs, such as *Alnus* spp., *Ailanthus altissima*, *Corylus avellana*, *Salix* spp. and *Clematis vitalba* (Filippin, 2011; Casati *et al.*, 2017; Malembic-Maher *et al.*, 2020). Indeed, the almost asymptomatic presence of FD phytoplasmas in feral plants other than grapevine, and the genetic variability of the phytoplasmas in the wild compartment (Rossi, Pegoraro, *et al.*, 2019; Malembic-Maher *et al.*, 2020) support the paleoarctic origin of this phytoplasma. On the other hand, the severe losses caused by FDp to European viticulture suggest a more recent association between these two species.

After the RLFP taxonomic description based on 16SrRNA of the two FD subgroups, 16SV-C and 16SV-D (Martini *et al.*, 1999), several

attempts were tried to describe FDp more in detail. Sequencing of two non-ribosomal loci, *sec*Y and *rps*C, allowed for the identification of three genetic clusters within FDp populations belonging to France and Italy (Martini *et al.*, 2002). Sequencing of *map* and *deg* loci confirmed the existence of three genetic clusters of FDp, characterized by different geographical distribution and genetic variability (Arnaud *et al.*, 2007). More recently, *dna*K, *mal*G, and *vmp*A genes were used for an in-depth description of FDp genetic variability at a small geographic scale (Rossi, Pegoraro, *et al.*, 2019).

In the EFSA risk assessment of FD for the EU territory (2016), different scenarios were drawn, differing in the strength of application of FD control measures. Regardless to the yield losses, disease management has high costs in term of prevention (monitoring, insecticide applications, production of phytoplasma-free propagation material) and agronomic measures (removal of infected plants and their replacement with new rooted cuttings). In some cases, depending on the attitude of the grapevine variety to recover from the disease and on the age of the vineyard, replacing symptomatic plants may decrease vineyard productivity (Pavan, Mori, S, *et al.*, 2012). Prevention and control measures must be adopted wherever the pathogen and the vector are present, because the disease has the potential to destroy whole vineyards in few years, if left uncontrolled (EFSA, 2016). So far, the control of FD relies on prophylactic measures, such as the use of healthy propagation material, as well as on compulsory measures in

infected vineyards, namely roguing of infected plants, and insecticide treatments against the vector(s) (Bosco and Mori, 2013).

Insect vectors

Scaphoideus titanus (Ball) is a grapevine feeding leafhopper belonging to the Deltocephalinae subfamily, and the natural vector of FD to grapevine. Native of the Nearctic region, it was probably introduced in Europe at the beginning of the 20th century (when a lot of American rootstocks were imported to confer resistance to the *Phylloxera*) (Bertin *et al.*, 2007). However, since the species was first identified in Europe in 1958 (Bonfils and Schvester, 1960) the actual time of introduction is unclear. Due to its ecological niche, strictly associated with *Vitis* plants and the fortuitous match with FDp, it soon became the main vector of FD to grapevines (Chuche and Thiéry, 2014; Schvester et al., 1963). According to the recent findings on the origin of FDp, before the introduction of *S. titanus*, the phytoplasmas was associated with non-cultivated plants (mainly *Alnus* spp.) and only seldom transmitted to cultivated grapevines by polyphagous leafhoppers. In this epidemiological situation, infected grapevine plants were dead-end hosts for the phytoplasmas. The introduction of *S. titanus* in Europe prompted the vine-to-vine spread of FDp within the vineyard, thus producing epidemics. *Scaphoideus titanus* is an efficient vector both inside the vineyard (vine-to-vine, secondary infections), and from the wild vegetation surrounding the vineyard (abandoned vines, wild *Vitis* spp., feral American rootstocks) to the cultivated vines (primary infections) (Pavan, Mori, G, *et al.*, 2012; Maggi *et al.*, 2017; Malembic-Maher

et al., 2020; Ripamonti *et al.*, 2020). The wild compartment represents a reservoir for both *S. titanus* and FDp and, since insecticide treatments on the wild vegetation are prohibited, the only option is host plants removal, although this is challenging in many situations, due to the large extent of land covered by feral *Vitis* and the difficulty to reach them.

As previously described for phytoplasma transmission, following feeding on an infected plant, FDp is ingested, crosses the midgut epithelium and, via the haemolymph, invades the haemocoel and finally the salivary glands, making the leafhopper infectious for life in a persistent-propagative manner (Figure 1; Bosco and D'Amelio, 2010; Kube et al., 2012). FD is then transmitted by the infective leafhopper, after a latency period of about 4 weeks, to healthy plants.

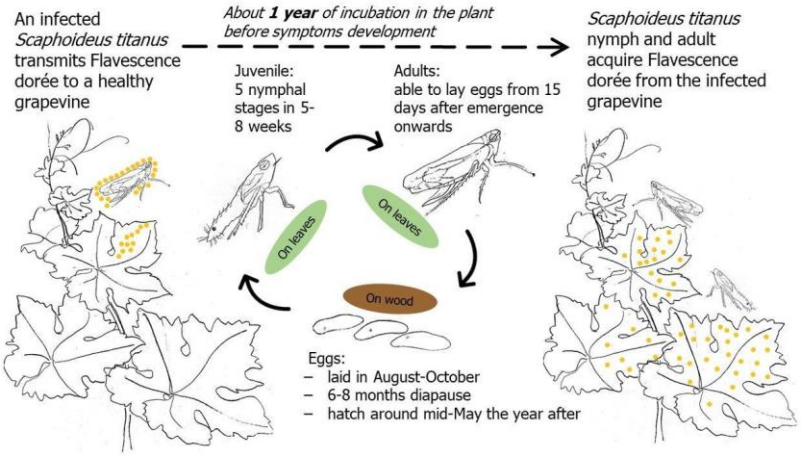

Figure 1. Schematic representation of the life cycle of *Scaphoideus titanus* and its role in transmitting grapevine Flavescence dorée (EFSA, 2020).

The disease spread is influenced by the movement of infective *S. titanus.* Different studies highlighted the limited flight capability of *S. titanus*, with an active dispersal of most adults of 30-40 meters (EFSA, 2016). However, few individuals can move up to few hundred meters (Lessio *et al.*, 2014). Moreover, as most insects, *S. titanus* can be passively transported by wind or by human activities, reaching longer distances and allowing the disease to “jump” from one place to another (Steffek *et al.*, 2007; Lessio *et al.*, 2014).

The alternative vector species are the Deltocephalinae leafhoppers *Allygus modestus, A. mixtus* and *Phlogotettix cyclops* (indigenous and polyphagous), *Orientus ishidae* (exotic and polyphagous species recently introduced in Europe) and the planthopper *Dictyophara europea* (indigenous and polyphagous) (Filippin *et al.*, 2009; Strauss and Reisenzein, 2018; Malembic-Maher *et al.*, 2020). All these alternative vectors sustain primary infections from alternative hosts (i.e. *Alnus* spp. and *Clematis* spp.) to grapevine (Malembic-Maher *et al.*, 2020). A possible involvement in the epidemiological cycle of FDp of hazelnut and willow, common host plants of *O. ishidae*, has also been suggested (Casati *et al.*, 2017).

The specificity of interaction between FD and its vectors is granted by membrane proteins, involved in the molecular interaction with host partner proteins. FD Imp was found interacting with insect protein extracts in two FD vector species, the laboratory vector *Euscelidius variegatus* and the natural vector *S. titanus* (Trivellone *et al.*, 2019). In *E. variegatus*, insect actin and ATP synthase β were confirmed as target protein for protein-protein interaction with

phytoplasma membrane proteins, allowing adhesion and subsequent internalization and multiplication of FDp in the insect host (Galetto *et al.*, 2020). Moreover, the phytoplasma membrane proteins VmpA and B act as adhesins for vector cells (Arricau-Bouvery *et al.*, 2018), and together with the FD *map* sequence, their sequence can predict the transmissibility of a given FD phytoplasma strain by the vector (Malembic-Maher *et al.*, 2020).

To test whether insect proteins could be necessary for efficient phytoplasma acquisition and transmission, double stranded RNAs (dsRNAs) triggering RNA interference response were designed and applied against both *E. variegatus* and *S. titanus*. Indeed, the RNAi machinery works in both *E. variegatus* (Abbà *et al.*, 2019; Galetto *et al.*, 2020) and *S. titanus* (Ripamonti *et al.*, in preparation). Two insect proteins interacting with phytoplasma membrane proteins were selected for silencing, ATP-synthase β and muscle actin. The results of the RNAi experiments showed effective silencing, increased mortality of the insect, reduced phytoplasma multiplication (Galetto *et al.*, 2020) and sterility of silenced females (Galetto *et al.*, 2021). Along with other innovative control measures against insect, RNAi could be implemented in an integrated pest management strategy for a more sustainable viticulture.

Aim and thesis structure

So far, the prophylactic control measures against FD (Bosco and Mori, 2013) are not effective enough, given the active spread of the disease. Although the activity of some insecticides with low-persistency can mitigate the impact of insecticide applications (Tacoli *et al.*, 2017), their efficacy is only partial and, to achieve a more sustainable viticulture, new environmental friendly control measures are needed. A valuable contribution in this direction can be represented by plant tolerance or resistance against FDp or the vector. Resistance to pathogens is the host plant ability to limit pathogen multiplication, while tolerance is the host ability to reduce the effect of infection (=symptoms) regardless of the level of pathogen multiplication (Pagán and García-Arenal, 2018). Resistance to insects occurs when plant structural or chemical traits deter herbivore feeding and thus minimize the amount of herbivore damage experienced by the plant, while tolerance occurs when plant traits reduce the negative effects of herbivore damage on crop yield (Mitchell *et al.*, 2016). Summarizing, resistance deters (for insects) or limits (for pathogens) the presence of the unwanted species, while tolerance is the ability to live with it. A pioneering work on the different susceptibilities to FDp in *Vitis* spp. (Eveillard *et al.*, 2016), suggests that some tolerance/resistance traits involved in insect-mediated phytoplasma transmission, multiplication, circulation and symptom development exist within the genus *Vitis.* The molecular interplay among the different partners of this plant/phytoplasma/vector interaction are difficult to study, and transcriptome analyses has shown that different grapevines

genotypes activate complex active and passive defences against *S. titanus* and FDp, while the phytoplasma itself manipulates the plant reaction to the insect (Bertazzon *et al.*, 2019).

The present thesis aims at exploring different aspects of the FD pathosystem, as depicted in Figure 2. In particular, special focus has been placed on i) describing the genetic variability of FDp in the wild compartment nearby cultivated vineyards and highlighting the importance of primary infection in the disease epidemiology under compulsory insecticide treatment regime, ii) proving the existence of tolerance or resistance mechanisms against FD and/or *S. titanus* in grapevine varieties cultivated in the Piemonte Region of Italy, iii) exploring the feeding behavior of *S. titanus* on grapevine varieties with different susceptibilities to FD, iv) comparing the fitness of the insect upon feeding on grapevine varieties with different susceptibilities to FD.

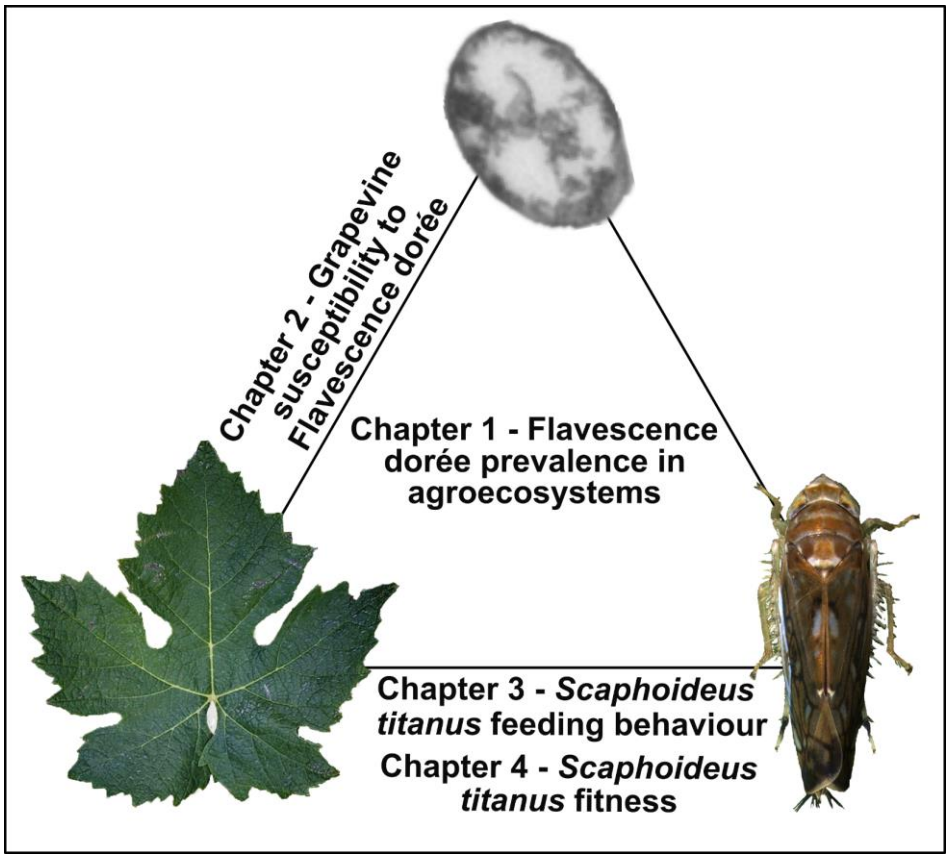

Figure 2. Graphical representation of the main actors of the pathosystem and the focus of each chapter.

Chapter 1 (Ripamonti *et al.*, 2020) provides a picture of FDp epidemiology in the Piemonte Region, with emphasis on the role of the wild compartment on the incidence of the disease in the vineyards. The conclusions of this paper highlight the lack of knowledge on the different varietal susceptibilities, that may, at least partly, explain the prevalence of the disease in different vineyard agroecosystems. Chapter 2 (Ripamonti *et al.*, 2021), describes the controlled FDp transmission experiments with infectious *S. titanus* to a set of 14 typical Piedmontese varieties. Substantial differences in susceptibility were found among three groups of varieties, with

two of them at the extremes of the susceptibility range: one in the susceptible and one in the tolerant group. No FD resistant cultivars were found, consistently with the field observation. In order to discriminate if the FD tolerance acts directly against the phytoplasma or it is mediated by a tolerance/resistance towards the vector, *S. titanus* feeding behavior (Chapter 3) and its fitness parameters (Chapter 4) on the most susceptible and tolerant varieties were investigated. In Chapter 3 (Ripamonti *et al.*, submitted to Journal of Insect Physiology) a differential *S. titanus* feeding behavior on FDp susceptible and tolerant varieties is demonstrated, suggesting that a vector-mediated tolerance against FDp occurs. In Chapter 4 (Ripamonti *et al.*, in preparation) *S. titanus* fitness parameters (developmental time, longevity and prolificacy), are investigated to identify the suitability of different grapevine varieties, susceptible or tolerant to FDp, for the vector. As a whole, the research provides a comprehensive scenario on the susceptibility of Piedmontese grapevine varieties to FD, and provides some insights on the mechanisms that underline susceptibility/tolerance to this insect transmitted phytoplasma disease.

Addendum. During my PhD, I have contributed to other research activities, mainly on Flavescence dorée and its vectors, but since these activities were not fully consistent with the main topic of the thesis, the related publications were excluded from the PhD dissertation. The complete list of publications can be found in the Appendix B.

Chapter 1 – Flavescence dorée prevalence in agroecosystems

Article, Insects 11(5): 301

Prevalence of Flavescence dorée phytoplasma infected *Scaphoideus titanus* in different vineyard agroecosystems of Northwestern Italy

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Abstract

Quantitative estimates of vector populations and of their infectivity in the wild and cultivated compartments of agroecosystems have been carried out to elucidate the role of the wild compartment in the epidemiology of Flavescence dorée. Seven sites were selected for the investigations in the Piedmont Region of Italy. They were characterized by a high variety of agricultural and ecological landscape features, and included a vineyard surrounded by wild vegetation. In order to describe abundance and prevalence of FD-infected vectors in the cultivated and wild compartments of the vineyard agroecosystem, adults of *S. titanus* were collected by yellow sticky traps inside and outside the vineyard over the period July 10th-September 9th 2015. They were counted and singly analyzed for the presence of FD phytoplasmas by PCR. Multifactorial correlations among vector population level, prevalence of infected insects inside and outside the vineyards, disease prevalence in cultivated and wild *Vitis* plants, location of wild *Vitis* plants with respect to the vineyard were analyzed. Abundance of *S. titanus* adults significantly decreased from the end of July onwards, particularly inside the vineyard (average range 22.7 ± 2.5 insects/trap). Percentage of FD-positive *S. titanus* was significantly higher outside the vineyard (up to 48% on average) compared to inside the vineyard (up to 34% on average), and increased during the season in both compartments.

1. Introduction

Flavescence dorée of grapevine (FD) is a phytoplasma-associated disease present in several European countries. The disease has a major impact on viticulture because inflorescences and berries dry up, shrivel and cannot be further processed. Other symptoms include downward leaf rolling with yellowing (in white varieties) or reddening (in red varieties), leaf vein necrosis, lack of lignification and presence of black spots on the new canes and premature leaf fall (EFSA, 2014); on the most susceptible varieties stunting or lack of bud break is also observed early in the season (Morone *et al.*, 2001). Plants can either be persistently infected over several years and eventually die or recover (Caudwell, 1961; Maggi *et al.*, 2017). Phytoplasmas are phloem obligate, non-culturable pathogens described under the provisional genus "*Candidatus* Phytoplasma" mainly based on 16S rRNA gene phylogeny. By definition, FD phytoplasmas (FDp) are those belonging to the 16SrV-C and –D ribosomal subgroups transmitted by the American grapevine leafhopper, *Scaphoideus titanus* Ball (Caudwell, 1990; EFSA, 2014). The vector transmits FDp according to a persistent propagative modality; a long latent period, approximately one month, is required for the insect to become infectious (Chuche and Thiéry, 2014). The vector remains infectious for life. *S. titanus* is the main vector of FDp, as it feeds and breeds on *Vitis* species and can transmit FDp following acquisition on either cultivated grapevine inside the vineyard or from infected, naturalized *Vitis* rootstock plants outside the vineyard, thus sustaining both secondary (within vineyard) and primary (from outside the vineyard) infections.

Abandoned (or untreated) vineyards and wild *Vitis* rootstock plants in the areas surrounding vineyards are frequently infected and represent a reservoir of both FDp and *S. titanus* (Pavan *et al.*, 2012; Rossi *et al.*, 2019). American *Vitis* spp. do not show symptoms but are susceptible to FDp (Eveillard *et al.*, 2016) and are attractive host plants for *S. titanus*, which co-evolved with them in the Nearctic Region. Besides *S. titanus*, other leafhopper and planthopper species have been identified as FDp vectors, among these *Orientus ishidae* (Lessio *et al.*, 2016), *Dictyophara europaea, Allygus* spp. (Malembic-Maher *et al.*, 2020) and *Phlogotettix cyclops* (Strauss and Reisenzein, 2018). However, these latter species are polyphagous rather than grapevine feeders and are likely to spread phytoplasmas in the wild compartment and, only occasionally, transmit FDp to cultivated grapevines (primary infections). *S. titanus* is regarded as the main vector associated with all the major epidemics of the disease (EFSA, 2014). FD was first identified in the Piedmont Region of Italy in 1998 (Morone *et al.*, 2001); soon after its discovery, dramatic epidemics occurred because vector populations were not controlled, and the disease progressed rapidly because of vine-to-vine transmission within the vineyard. Following the enforcement of compulsory control of FD, mandatory uprooting of infected plants and insecticide applications against the vector, secondary infections were substantially suppressed. However, over the years a number of new infections took place, namely in the vines close to vineyard borders. These observations suggested that the wild compartment, represented by naturalized *Vitis* and associated *S. titanus*, was the major source of infection. To clarify the

epidemiology of FD in the area, genetic tracking of phytoplasmas in the vineyard agroecosystems was carried out (Rossi *et al.*, 2019), and results showed that FD haplotypes identified in cultivated and wild *Vitis*, as well as in *S. titanus* collected inside and outside the vineyards, largely overlap, a further proof of the wild compartment role in the FD epidemiology. The comparative analysis of population level and of proportion of infected *S. titanus* in the cultivated and wild compartments of the vineyard agroecosystem is almost unexplored and represents an essential information for management of FD. The aim of the work is to fill this knowledge gap by conducting systematic investigations in representative sites of the Piedmont Region, where FD is a major problem for viticulture. Our results substantially improve the understanding of the epidemiology and contribute to design rational and effective control programs of FD and its vector.

2. Materials and Methods

2.1. Sites, vineyards and FD prevalence

The same seven sampling sites as described by Rossi and co-workers (Rossi *et al.*, 2019) were selected in an important winegrowing area of the Piedmont Region, north-western Italy. They were characterized by a high variety of agricultural and ecological landscape features, but all included cultivated *Vitis vinifera* (several cvs, see Table 1) with different prevalence of FD disease, presence of the FD vector *S. titanus*, and potential alternative host plants for the FDp (eg. abandoned *V. vinifera*,

naturalized rootstocks of *V. riparia* and hybrids of different *Vitis* species, and *Clematis vitalba*). The sites were named after the villages closest to them using the following abbreviations: AT, CI, CR, LM, MO, PA, and PC, as previously detailed (Figure 1) (Rossi *et al.*, 2019).

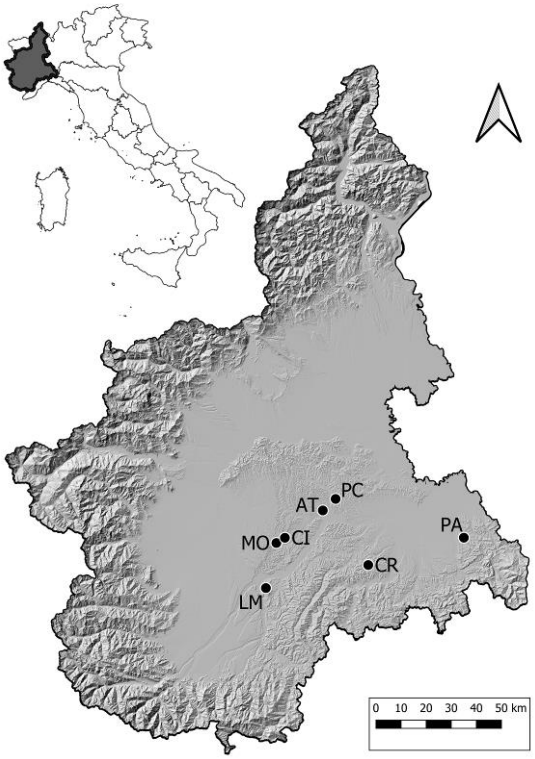

Figure 1. Location of the seven experimental sites within the Piedmont Region of Italy. AT, Asti, CI, Cisterna d'Asti; CR, Castel Rocchero; LM, La Morra; MO, Montà d'Alba; PA, Paderna; PC, Portacomaro.

FD prevalence was calculated by visual inspection for FD-specific symptoms, as described in Morone *et al.* (2007). Prevalence of FD in the vineyards was ranked in four categories, spanning from about 1% to more than 30% (Table 1).

Table 1. Main characteristics of the seven vineyards.

Site	Surface (ha)	Grapevine cultivars	Inside traps (n)	Outside traps (n)	Naturalized *Vitis* (FDpos/tested) [1]	FD-infected grapevines (%)
AT	2.6	Albarossa, Barbera, Chardonnay, Cortese, Incrocio Manzoni, Syrah	4	4	6/19	5-10
CI	0.9	Croatina	9	5	7/43	10-15
CR	1.9	Barbera, Dolcetto	3		0/19	>30
LM	1.1	Nebbiolo	5	5	1/20	≤1
MO	0.1	Nebbiolo	3	5	7/21	10-15
PA	1.5	Freisa, Merlot, Dolcetto	4	4	8/28	≤1
PC	1.2	Barbera, Grignolino, Ruché	3	3	9/39	>30

[1] Data extracted from (Rossi *et al.*, 2019).

The vineyard in Asti (Figure 2, AT) was a multi varietal experimental plot of 2.6 ha with several red (Albarossa, Barbera and Syrah) and white (Chardonnay, Cortese and Incrocio Manzoni) cvs. A forested area bordered the vineyard to the north, and to the south a tree line separated it from a meadow. On the western side, a large abandoned vineyard was present, and to the east a grassy area separated the vineyard from a dense edge of naturalized rootstocks. FD prevalence was in between 5 and 10%. At Cisterna d'Asti (Figure 2, CI), the 0.9 ha vineyard of cv Croatina was characterized by a forested area on the steep south facing slope to the north of the vineyard, with an abandoned vineyard where wild rootstocks were present. At this site, FD prevalence in the vineyard ranged between 10 and 15%. At Castel Rocchero (Figure 2, CR),

the 1.9 ha vineyard consisted of Barbera and Dolcetto cvs and the FD prevalence was about 30%. There were very few trees or wild vegetation around the vineyard as the surrounding area was characterized by intensive viticultural practices. A few naturalized grapevine plants were found and sampled along the roadside to the west and on the top of a mild west-facing slope on the eastern side of the vineyard. The vineyard of cv Nebbiolo at La Morra (Figure 2, LM) was 1.1 ha in size and surrounded on three sides by dense forestation and on the south-western edge was separated from another vineyard by a narrow windbreak of trees. Around the vineyard edges of La Morra were a few sparse populations of *C. vitalba* plants and numerous wild rootstocks, from an old abandoned vineyard. At La Morra, less than 1% of the plants showed FD symptoms. At Montà d'Alba (Figure 2, MO), the small vineyard (0.1 ha) of cv Nebbiolo was on the middle of a mild slope bordered by hazelnut orchards to the north and dense forestation bordering the roadway that wrapped around it. Along the western side of this forested edge, wild rootstocks from abandoned vineyards were found. Prevalence of FD at this site was between 10 and 15%. Paderna's vineyard (Figure 2, PA; 1.5 ha) was planted with cvs Freisa, Merlot, Dolcetto. Forested edges bordered the vineyard to the east and south, grassy plains and herbaceous crops surrounded the vineyard to the north and west. Within these forested edges, there were several abandoned *V. vinifera*, and *C. vitalba* plants. No more than 1% of the vines showed FD symptoms. The Portacomaro vineyard, of cvs Barbera, Grignolino and Ruché, (Figure 2, PC; 1.2 ha) was situated at the top of a steep sloped hill

and was bordered by land for livestock production to the north, a narrow forest and civic housing to the west, a dense forest to the east, and the town of Portacomaro to the south. The western and southern edges of the vineyard were surrounded by hazelnut orchards. Within the southwestern forest, substantial populations of wild rootstocks were found. Both *C. vitalba* and wild rootstock plants were also found along the northern tree line that separated the viticultural and livestock production areas. Visual estimates of FD prevalence at this site was about 30%. Out of the seven sampled sites, only Castel Rocchero and Paderna were not subject to conventional chemical control of insect pests of viticulture (that includes two insecticide applications against *S. titanus*, the first against nymphs and the second against adults), and were managed according to guidelines for organic viticulture (based on three applications of pyrethrins in June-July).

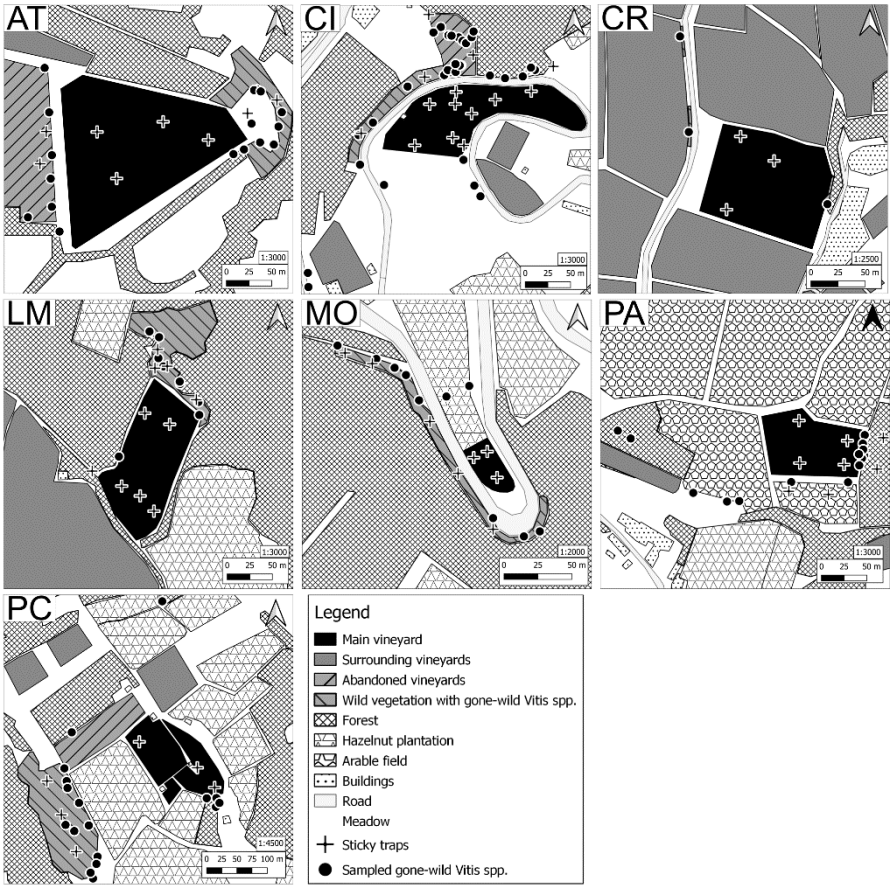

Figure 2. Stylized vegetal composition of the seven vineyards and surroundings. Acronyms: AT, Asti; CI, Cisterna d'Asti; CR, Castel Rocchero; LM, La Morra; MO, Montà d'Alba; PA, Paderna; PC, Portacomaro.

2.2. Insect monitoring and collection

S. titanus populations were monitored at each site both inside and outside the cultivated vineyards by means of yellow sticky traps (YST), 25 × 40cm (0.1 m^2) (Figure 2) during summer 2015. Traps were hung at 1.5 m high during July-beginning of September, the best period to collect adult of this species according to its life cycle (Bosio and Rossi, 2001). They were replaced for three trapping

periods: July 10th to 31th, July 31st to August 21th, and August 21st to September 9th, from now on defined as period A, B and C, respectively. Climatic conditions of these periods are summarized in Table S1, where minimum, maximum and average monthly temperatures, as well as rainfall, are reported for three sites close to the investigated ones. Following counting, the *S. titanus* adults were removed from sticky traps with a paintbrush and a drop of vegetal solvent. At Castel Rocchero, due to the absence of wild vegetation around the vineyard, traps were hung inside the vineyard only. At the Portacomaro and Montà sites, due to the very high number of *S. titanus* found outside the vineyard, some adults were also collected by sweep net with the purpose of molecular detection for FDp presence. All insect samples were stored under ethanol in glass vials at -20°C until nucleic acid extraction.

2.3. Nucleic acid extraction and FDp detection

Total nucleic acids were extracted from single leafhoppers according to the method of Pelletier (Pelletier *et al.*, 2009), then suspended in 75 µL of Tris-HCl 10mM pH 8. DNA concentration was measured with NanoDrop 2000TM Spectrophotometer (Thermo Scientific, Waltham, MA), and all samples were then diluted to 20 ng/µL. The presence of FDp was detected by Real-Time PCR (CFX Connect Real-Time PCR Detection System, Bio-Rad) with primers mapFD-F/mapFD-R and the TaqMan probe mapFD-FAM (Pelletier *et al.*, 2009). The PCR mix (10 µl) contained 1x iTaq Universal Probe Supermix (Bio-Rad), together with 300 nM primers, and 200 nM probe, and 20 ng of total nucleic acids.

Samples were run in triplicate, together with a negative control, with double distilled water instead of template nucleic acid. All insects collected at AT, CI, CR, LM, and PA were from sticky traps. About 40 samples from MO and all those from PC were collected by sweep net (Table S2).

2.4. Data analyses

2.4.1. Vineyard mapping

Schematic maps were produced with the software QGIS v 3.2.3 'Bonn' (QGIS, 2019) (Figure 1-2).

2.4.2. Statistical analyses and graphical representation

The dataset consists of multiple captures of *S. titanus* through yellow sticky traps hung at fixed places inside or outside each vineyard (Figure 2). *S. titanus* were then pooled for vineyard, time period, and trap position for analyses of FDp status.

To model the number of *S. titanus* individuals trapped as a function of the covariates, a negative binomial GLMM with a log link function was used. Fixed covariates were Trap position (categorical with two levels – "inside" and "outside" the vineyard), and Time period (categorical with three levels). The interaction terms were Trap position × Time period. To incorporate the dependency among observations of the same vineyard, we used Vineyard as a random intercept.

Model assumptions were verified by plotting residuals versus fitted values, for each covariate in the model and for each covariate not in the model. We assessed the residuals for temporal dependency.

Model validation did not raise any significant concern with normality of residuals and linear relationship among variables (Table 2, Figure 4).

To model the proportion of FDp-positive *S. titanus* as a function of the covariates, a binomial GLMM with a logit link function was used. The logit link function ensures fitted values among 0 and 1, and the binomial distribution is typically used for proportion data. Fixed covariates are Trap position (categorical with two levels – “inside” and “outside” the vineyard), Time period (categorical with three levels). The interaction terms were Trap position × Time period. To incorporate the dependency among observations of the same vineyard, we used Vineyard as a random intercept. Overdispersion was accounted by using a quasi-GLM model and correcting the standard errors accordingly (Table 3, Figure 6).

The package lme4 (Bates *et al.*, 2015) and glmmPQL (Venables and Ripley, 2002) in the software R (R Core Team., 2020) were used to fit the models.

Correlation between proportion of infected *S. titanus* and proportion of infected grapevines measured inside the vineyards was estimated using nonparametric Spearman’s rank correlation (*cor.test* in stats R package) (R Core Team., 2020).

Wilcoxon rank-sum test was applied to the comparison of *S. titanus* numbers trapped inside vs. outside the vineyard at each time period (Figure 3). Z- test was used to compare the proportion of infected *S. titanus* collected in the same compartments of the vineyard agroecosystems (Figure 5). Plots were constructed using package

ggplot2 (Wickham., 2016) and lemon (McKinnon Edwards, 2019) in the software R.

3. Results

S. titanus adults were collected in all sites during July and August both inside and outside the vineyards (Table S2). At Asti, sticky traps collected many more samples outside the vineyard, in the canopy of naturalized rootstocks climbing on broad leaved trees. At Cisterna, a similar population level of *S. titanus* was estimated inside and outside the vineyard, although late in the season more adults were collected outside the vineyard. At Castel Rocchero all samples were collected inside the vineyard, as no uncultivated areas were present around the investigated vineyard. At La Morra, sticky traps collected more *S. titanus* outside compared to the inside of the vineyard; at this site the highest vector population was recorded. At Montà a substantial amount of *S. titanus* adults were collected both inside and outside the vineyard. At Paderna, similar numbers of leafhoppers were trapped inside and outside the vineyard, although they were more abundant outside the vineyard in August and beginning of September. At Portacomaro, similar numbers of *S. titanus* were collected by YST inside and outside the vineyard. To this purpose, it should be mentioned that, in the previous year, the population of *S. titanus* in the wild compartment surrounding this vineyard, was much higher, and leafhoppers could be collected directly from the leaves with a mouth aspirator. The population then declined in 2015 as most of the wild vines were

uprooted during the winter. In all the vineyards, the highest levels of population were recorded in July, and then decreased rapidly in the following months (Figure 3).

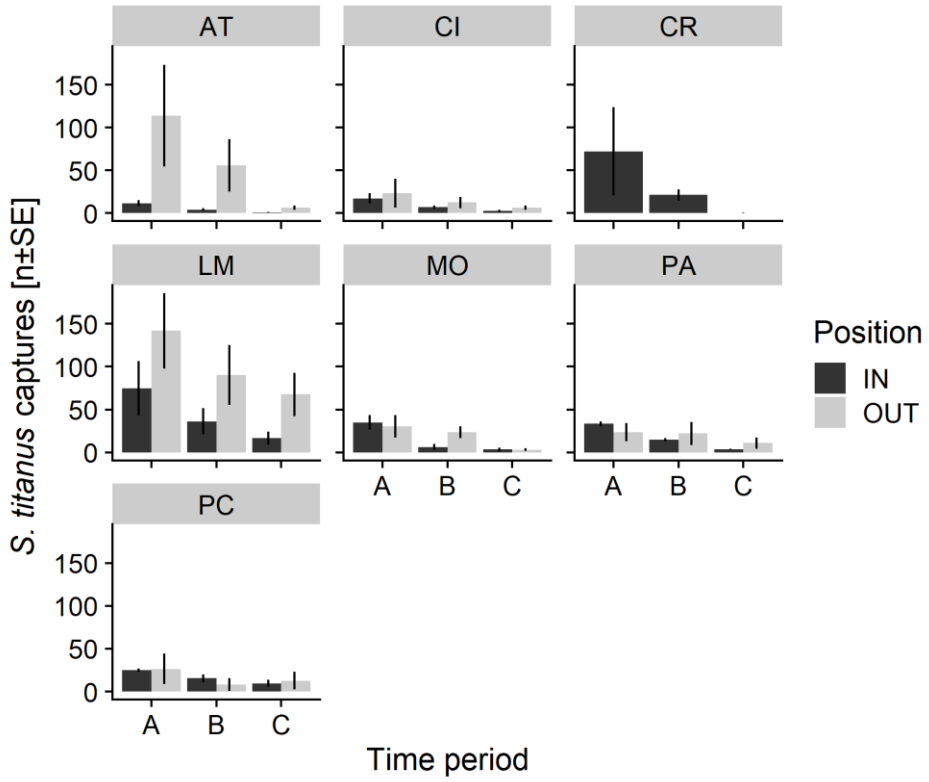

Figure 3. Number of *S. titanus* adults trapped in the three summer periods ± Standard Error (A = July 10th-July 30th; B = July 31st–August 20th; C = August 21st-September 10th) over the different sampling sites. Inside (IN) and outside (OUT) captures in the same vineyard are represented (Table S2). Asterisk indicates significant difference between the number of *S. titanus* collected inside and outside the vineyard ($p < 0,05$).

The vector population level decreased from period A to periods B and C with a significantly different rate inside and outside the vineyard. There was a significant interaction between position and sampling time on the number of trapped *S. titanus*. That is, the *S. titanus* captures significantly decreased as the season progressed,

especially inside the vineyards. Indeed, the *S. titanus* counts were mostly similar among traps located inside and outside the vineyards on the first period (July), but differed for later sampling periods (August-early September), with traps located outside collecting more insects compared to the ones located inside the vineyards (Table 2 and Figure 4).

Table 2. Estimated regression parameters (log), standard errors, z-values and P- values for the negative binomial GLMM of *S. titanus* counts with covariates *Trap position* and *Time period* and their interaction. The estimated value for $\sigma_{Vineyard}$ is 0.334 and σ_{Trap} is 1.007.

	estimate		**std.error**	**statistic**	**p.value**	**conf.low**	**conf.high**
Intercept	3.143	***	0.262	11.981	> 0.001	2.629	3.657
PositionOutside	0.32		0.338	0.948	0.343	-0.342	0.982
Time_periodB	-0.923	***	0.196	-4.719	> 0.001	-1.307	-0.54
Time_periodC	-2.118	***	0.22	-9.638	> 0.001	-2.549	-1.688
PositionOutside:Time_periodB	0.422	*	0.289	1.461	0.144	-0.144	0.988
PositionOutside:Time_periodC	0.801	**	0.312	2.57	0.01	0.19	1.412

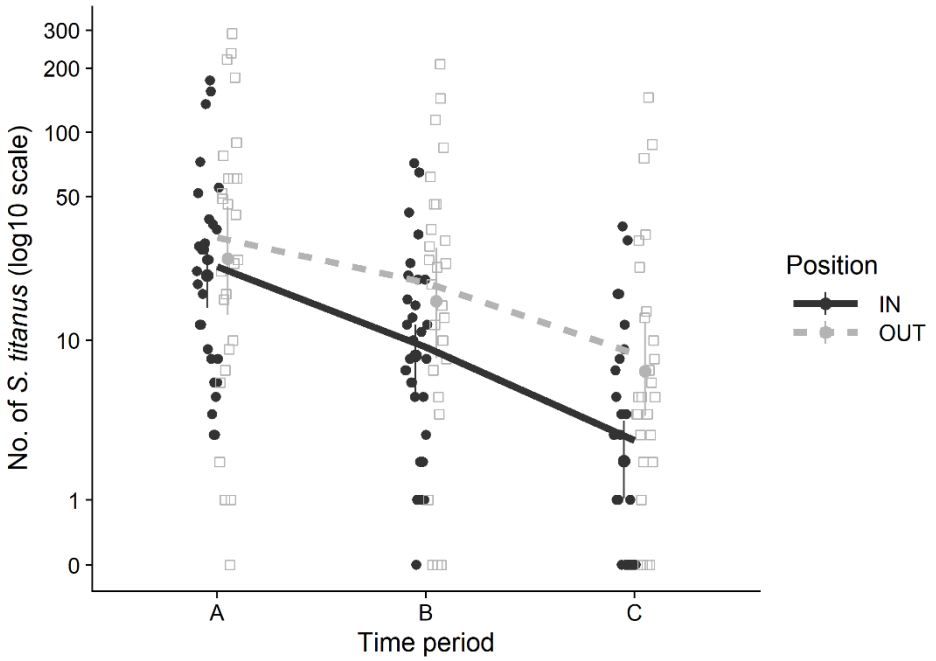

Figure 4. Number of *S. titanus* collected along the season in Piedmont vineyards. Black points and continuous line represent data and GLMM model of traps located inside the vineyard, whereas grey squares and grey dashed line represent data and model of traps located outside the vineyard.

The proportion of FDp-infected leafhoppers varied according to the vineyard, the trap position (inside/outside) and the sampling time. More than 40% of infected leafhoppers was recorded at Cisterna, La Morra (outside the vineyard only), Montà and Portacomaro, while at Paderna, about 10% of leafhoppers (both from inside and outside vineyard traps) were FDp carriers. Similarly, inside the vineyard of La Morra, only 7% of tested leafhoppers were infected (Table S2). Overall, more leafhoppers collected in the wild compartment were FDp-infected compared to those collected inside the vineyard (Figure 5). Although leafhoppers collected later in the season were more frequently infected, a remarkable proportion of adults

collected in July tested positive for FDp (Figure 5-6), thus suggesting that many, if not most, leafhoppers acquired phytoplasmas at the nymphal stages.

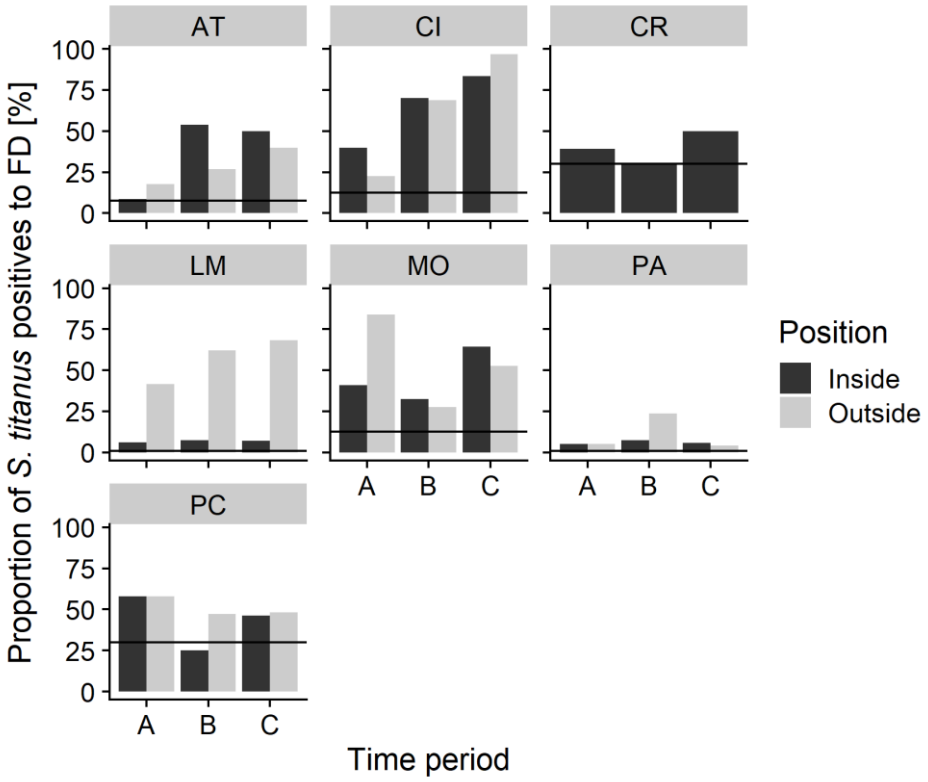

Figure 5. Flavescence dorée phytoplasma infection of *S. titanus* populations sampled with yellow sticky traps exposed for two weeks inside and outside the different vineyards. Inside and outside levels of infection in the same vineyard are represented. The horizontal black line represents the prevalence of FD-symptomatic grapevines in the vineyard (Table 1). The total number of FDp tested *S. titanus* at each vineyard, time point, and trap position is reported in Table S2. Asterisk indicate significant difference between the proportion of FD-infected *S. titanus* collected inside and outside the vineyard (*, p<0,05; ***, p<0,001).

The proportion of FDp-positive *S. titanus* was significantly higher for individuals trapped outside than inside the vineyards, irrespective of the period of trapping (Table 3 and Figure 6). The proportion of

FDp-positive *S. titanus* per vineyard was higher for the 2nd and 3rd time periods, although not significantly (Table 3).

Table 3. Estimated regression parameters (odds ratio), standard errors, t-values and P- values for the binomial GLMM of proportion of FDp-positive *S. titanus* with covariates *Trap position* and *Time period* and their interaction. The estimated value for $\sigma_{Vineyard}$ is 0.412.

	Estimate	Std. Error	t value	P-value
Intercept	-1.341	0.229	-5.85	<0.001
PositionOutside	0.370	0.173	2.14	0.041
Time_periodB	0.191	0.188	1.02	0.317
Time_periodC	0.203	0.227	0.896	0.378

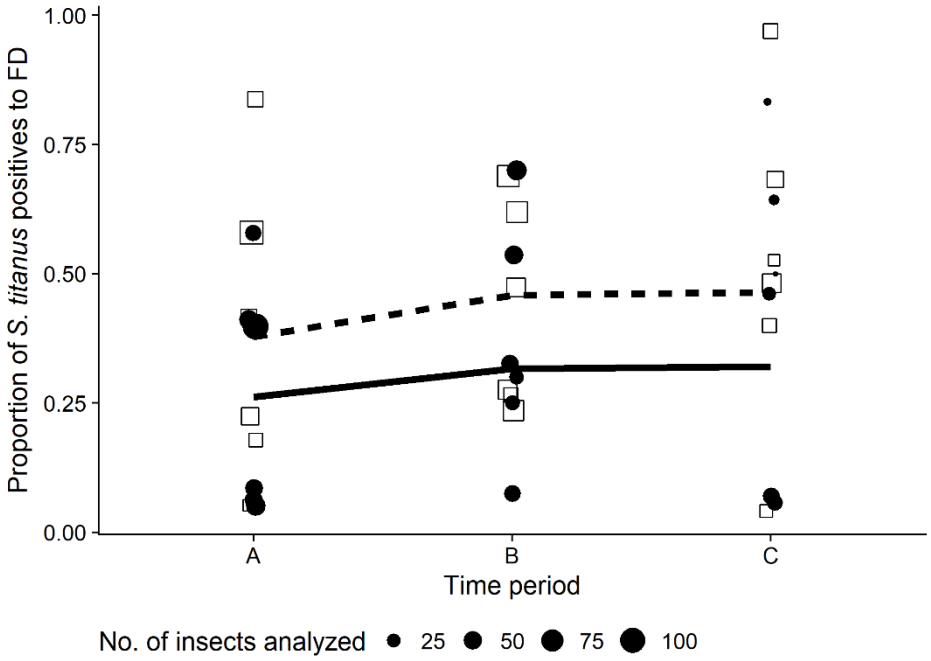

Figure 6. Proportion of FDp-positive *S. titanus* along the season in Piedmont vineyards. Black points and continuous line represent data and GLMM model of traps located inside the vineyard, whereas white squares and dashed line represent data and model of traps located outside the vineyard. Size of points and squares is proportional to number of insects analyzed for each trap.

A positive correlation was found between the proportion of FDp-infected leafhoppers collected inside the vineyard and the

proportion of infected grapevines in the same vineyards (ρ = 0.75, p = 0.051, R^2 = 0.57; Figure 7).

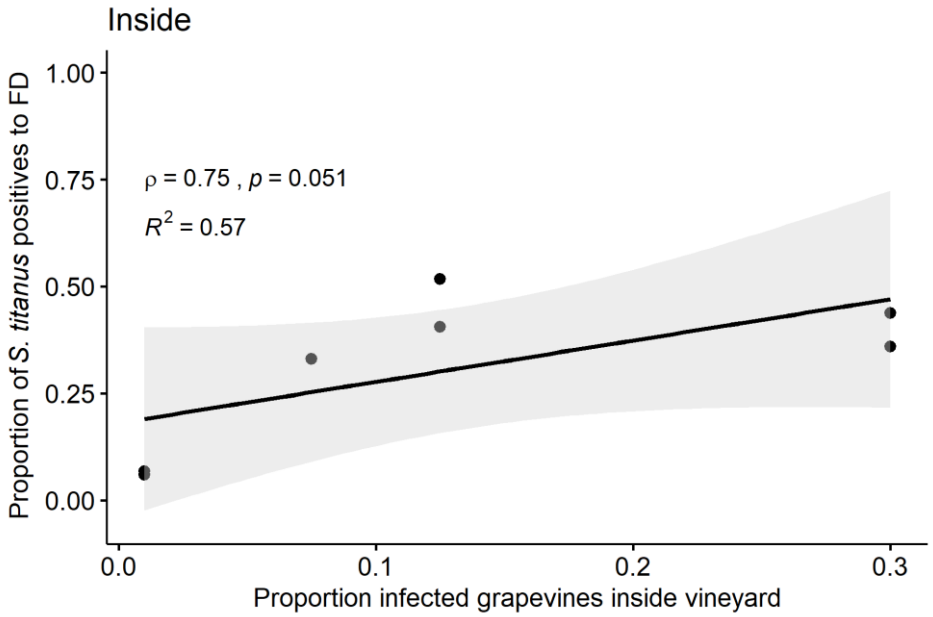

Figure 7. Correlation between proportion of FDp-positive *S. titanus* (collected inside the vineyard) and proportion of infected grapevines in the same vineyard.

4. Discussion

This study was conducted under field conditions in different viticultural areas of the Piedmont Region of Italy to describe the abundance of vector populations and to estimate vector infectivity inside the vineyards and in the wild-compartments surrounding the vineyards.

The selected sites were characterized by the presence of FD-infected cultivated *V. vinifera*, and of a wild compartment with

potential alternative host plants for the FDp and its vector. For these reasons, vector population levels and proportion of FDp-infected leafhopper do not reflect the average situation in the Piedmont Region, but rather the worst-case scenarios. We confirmed that naturalized *Vitis* may host very high populations of *S. titanus* and that vineyards close to wild vegetation (AT, CI, LM, MO, PA, PC) or not properly treated with insecticides (CR), may also host high populations of *S. titanus* adults.

The highest numbers of *S. titanus* adults were collected with YST in July (likely at the very end of July), and then captures declined more or less gradually, both within the vineyards and in the wild vegetation compartment. In most reports, *S. titanus* populations peak in the first half of August (Bosio and Rossi, 2001; Decante and Helden, 2006; Lessio *et al.*, 2009). The very warm conditions of 2015 that anticipated *S. titanus* development (Table S1) and the application of insecticides in the vineyards at the end of July may explain the abundance pattern recorded in this study. In Romania, *S. titanus* adult population peaked at the end of July in the years 2009-2011 in an abandoned vineyard close to Bucharest (Chireceanu, 2014). However, our data cannot be used to properly identify a population peak since YST were exposed in the fields for 20 day periods under our experimental conditions. The pattern of population decrease over August and beginning of September was significantly different inside and outside the vineyard (population decreased faster inside the vineyard). The faster population decrease inside the vineyards was likely due to the insecticides applied against the adults at the end of July. Very high numbers of

adults were collected from the wild vegetation compartments, where insecticide applications are forbidden by law and the only available control measure is the difficult mechanical roguing of wild *Vitis*. Based on our experience, the presence of *S. titanus* within an abandoned/wild area is highly aggregated, and therefore YST captures highly depended on their specific location within the wild vegetation area. This means that our estimate of the *S. titanus* population levels outside the vineyards suffered from some inaccuracy. Nevertheless, as very high captures were repeatedly obtained, together with the observations reported for Italian and North American vineyard agroecosystems (Beanland *et al.*, 2006; Lessio *et al.*, 2007; Pavan *et al.*, 2012), we can conclude that wild vegetation areas are very important sources of the vector for the nearby agroecosystems. So far, we have no hints to explain uneven aggregated spatial distribution of *S. titanus* in the wild compartment. This issue is very difficult to study, as wild compartments are very different among them in size, slope, orientation, plant composition. However, the presence of large surfaces of wild *Vitis* climbing on high broadleaved trees, as was the case for all the analyzed sites except CR, rather than covering the soil, is a factor that favors the presence of high *S. titanus* populations (personal observation).

Among the vineyards, the highest population levels were recorded at LM and CR. In the latter vineyard, only pyrethrins were applied against *S. titanus* and this can account for the high population of the insect. As for the LM vineyards, no specific factors (size of the vineyard and of wild vegetation area, slope, exposure) could be

evoked to account for this high *S. titanus* density which is, to some extent, unpredictable.

Our GLMM model showed that a higher proportion of infected insects was recorded for the leafhoppers collected in the wild compartment compared to those from within the vineyard. This evidence is consistent with data of Lessio et al. (2007), confirming the major role of the wild vegetation in the spread of FD. Similarly, untreated vineyards are a known source of infected *S. titanus* (Pavan *et al.*, 2012). However, at some of the sites, the proportion of FDp-carrier insects was similar in the two compartments. If we assume that the proportion of FDp-infected leafhoppers can be used as a marker of insect dispersal, we can speculate that at most of our sites (AT, CI, MO, PA and PC) there was a flow of *S. titanus* between the cultivated and wild compartments of the vineyard agroecosystem. On the contrary, at LM the two populations were apparently separated. In fact, very few insects were FDp-carriers inside the vineyards, and many were infected in the wild compartment. Interestingly, at LM the wild vegetation was present in a large area standing downhill and below the level of the vineyard, with a woodland shield protecting the vineyard from major air flows. It is then possible that leafhoppers, in the absence of ascendant air flows, are unable to fly upwards and reach the vineyard. Indeed, without prevalent wind conditions, *S. titanus* does not move far (Lessio and Alma, 2004). Where the wild vegetation surrounding the vineyard is at the same height or above the vineyard, leafhoppers may move freely between the two compartments or into the vineyard itself. Also, where the wild vegetation is below the level

of the vineyard but upward and downward air currents are present, the leafhoppers might circulate between the two compartments. The role of wind in *S. titanus* dispersal has been noticed and considered in pest risk assessment of FD (Steffek *et al.*, 2007).

Proportion of FDp-infected vectors slightly increased over the summer, in line with the data of Lessio *et al.* (2009); it is worth remembering that this proportion increased in spite of the higher mortality of FD-infected *S. titanus*, demonstrated by Bressan *et al.* (2005). Increase in the proportion of infected-insect vectors over the season is expected, since FDp circulates, multiplies and thus persists for life in the insect body. With time, chances for the insects to move and feed on an infected source plants obviously increase, and this also contributes to increasing the proportion of FDp-positive insects during the summer season. However, in July the proportion of FDp-positive adults was already high, and this may have two concurrent explanations: i) most of the insects acquired phytoplasmas at the nymphal stages and were already infected when the adult emerged ii) a number of insects collected inside the vineyard in July already came from outside the vineyard, where chances of feeding on an infected wild *Vitis* were greater. Actually, Table 1 shows that, overall, one fourth of the wild, asymptomatic *Vitis* tested at random were FDp-infected, while percentages of infected plants within the vineyards were generally lower. Therefore, the increase in FDp-positive insects may be partly explained by the increasing load of FDp in the insects due to multiplication over time. This multiplication of FDp in most insects that fed on infected source plants would overcome the detection

threshold of the PCR assay. The chosen real time PCR assay detects phytoplasmas well before the completion of their latent period (about one month, during which the vector is infected but not infectious yet). However, FDp could also be acquired by adults (Bressan *et al.*, 2006; Alma *et al.*, 2018), and this is also consistent with our observations. In previous papers, we demonstrated that infected vines have low FDp load early in the season (Roggia *et al.*, 2014) and that acquisition of FDp by *S. titanus* correlates with phytoplasma load in the plant (Galetto *et al.*, 2014). We can then speculate that, as season progresses, the likelihood of FDp acquisition by the vectors increases and this may also account for the increasing proportion of infected insects recorded during our survey. The proportion of FDp-carrier leafhoppers inside the vineyard showed a positive correlation with the proportion of infected vines at the same site, confirming that PCR detection of FDp in the vector is a good marker of disease spread/prevalence in the vineyard. However, since the R^2 of the model was equal to 0.57, presumably other factors, besides the proportion of infected leafhoppers inside the vineyard, may account for the spread of the disease within a vineyard, e.g. the susceptibility of grapevine cultivars. Actually, the vineyards were cultivated with different varieties, and these may show different levels of susceptibility to FD (Eveillard *et al.*, 2016); differential susceptibility was not taken into account in this work because only empirical observations are available for local varieties cultivated in Piedmont Region, so far. Analyses of a robust set of experimental data on the susceptibility of different vine varieties are ongoing in our laboratory.

5. Conclusions

High numbers of *S. titanus* adults were collected from the wild vegetation compartment of several sites, and vector population levels of this compartment were higher than those measured inside the corresponding vineyard. The pattern of vector population decrease over August and beginning of September was significantly different inside and outside the vineyard (population decreased faster inside the vineyard), thus confirming the effects of the insecticides applied against the adults in the vineyards. As expected, the proportion of FDp-infected vectors increased over the summer, even though the proportion of FDp-positive leafhoppers (possibly not infectious yet) was already high in July, indicating that grapevine are exposed to infectious leafhoppers for a long period of time. A higher proportion of FDp-infected leafhoppers was recorded for the insects collected in the wild compartment compared to those from the vineyard, thus indicating the important role of outside FDp sources in the epidemiology of the disease. This study provides valuable information on the role of the wild compartment in the epidemiology of Flavescence dorée disease, and represents one of the few studies conducted at the level of the vineyard agroecosystem as a whole. Further research should be devoted to the evaluation of FD spread reduction following removal of wild *Vitis* in the surroundings of vineyards.

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Supplementary Material

Supplementary Material Table S1. Climatic data of May-September 2015. Available online at http://www.mdpi.com/2075-4450/11/5/301/s1

Supplementary Material. Table S2. Numbers of *Scaphoideus titanus* adults collected by yellow sticky traps inside and outside the seven investigated vineyards. Time period: A, July 10th to 31th; B, July 31st to August 21th; C, August 21st to September 9th. Acronyms: AT, Asti; CI, Cisterna d'Asti; CR, Castel Rocchero; LM, La Morra; MO, Montà d'Alba; PA, Paderna; PC, Portacomaro. NC: not collected.

Infected/Tested (outside)			Outside			Infected/Tested (inside)			Inside			Site
C	B	A	C	B	A	C	B	A	C	B	A	
12/30	8/30	5/28	6.30 ± 5.00	55.80 ± 61.20	113.80 ± 118.6	1/2	29/54	4/47	0.75 ± 1.00	4.30 ± 2.90	11.80 ± 6.70	AT
32/33	51/74	11/49	6.60 ± 5.50	12.60 ± 14.50	23.20 ± 37.50	5/6	42/60	43/108	2.56 ± 4.45	7.00 ± 6.02	17.33 ± 17.33	CI
NC	NC	NC	NC	NC	NC	1/2	9/30	18/46	0.30 ± 0.60	21.30 ± 11.50	72.30 ± 89.40	CR
28/41	44/71	15/36	67.60 ± 56.00	90.40 ± 78.40	142.00 ± 98.20	3/43	2/27	3/48	16.80 ± 16.90	45.00 ± 32.00	74.60 ± 71.40	LM
10/19 a	16/58	31/37	1.70 ± 2.90	23.80 ± 15.40	30.20 ± 29.20	9/14	15/46a	23/56	3.33 ± 3.51	6.33 ± 5.51	35.00 ± 14.80	MO
1/24	16/68	1/19	10.80 ± 13.60	22.00 ± 26.90	23.10 ± 21.30	2/35	3/40	3/59	3.50 ± 1.70	15.00 ± 3.90	33.30 ± 5.60	PA
27/56 b	27/57 b	51/88 b	10.75 ± 13.60	22.00 ± 26.88	23.50 ± 21.30	12/26	8/32	22/38	10.00 ± 9.90	15.30 ± 8.50	25.00 ± 3.00	PC

[a]tested leafhoppers were collected both by yellow sticky traps and sweeping net; [b]tested leafhoppers were collected by sweeping net.

Chapter 2 – Grapevine susceptibility to Flavescence dorée

Article, Plant Pathology 70: 511-520

Susceptibility to Flavescence dorée of different *Vitis vinifera* genotypes from North-Western Italy

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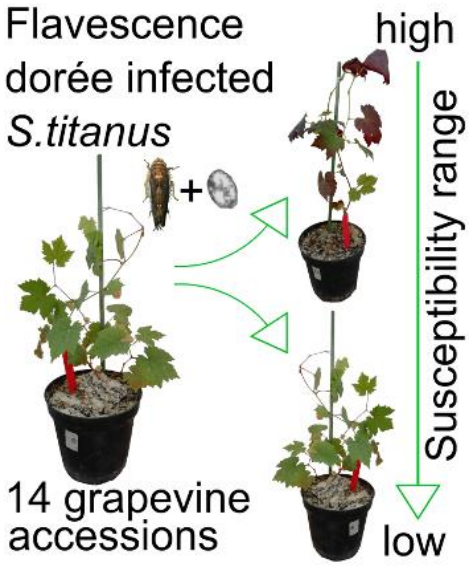
Keywords: Flavescence dorée; *Vitis vinifera*; *Scaphoideus titanus*; grapevine cultivar; micropropagated *Vitis*; susceptibility; tolerance

Abstract

Aim of the work was to evaluate the susceptibility to Flavescence dorée (FD) of 12 *Vitis vinifera* cultivars, grown in Piedmont and representative of the wine making tradition of this area. The experiments were conducted under controlled conditions to ensure constant infection pressure. Test plants were *ex-vitro* potted vines, singly inoculated with four *Scaphoideus titanus* infected by FD-C phytoplasma (FDp), under greenhouse conditions. Vines were tested for FDp at five and eight weeks post-inoculation (wpi) and the phytoplasma load was measured in leaves and roots at eight wpi. Within the 14 *V. vinifera* accessions (belonging to 12 cultivars), three susceptibility clusters were identified. Cultivars within the low susceptibility group, showed low phytoplasma loads and low percentages of infected plants, suggesting a tolerant behavior. To confirm these results, four *Vitis* cultivars, representing extremes of the FD susceptibility, from poorly to highly susceptible, were grafted onto Kober 5BB rootstocks and inoculated with lab-infected *S. titanus*, under semi-field conditions. The transmission experiments onto grafted cuttings confirmed that susceptibility to the disease depends on the scion genotype. These data indicated that none of the tested *V. vinifera* genotypes was resistant to FD, although some poorly susceptible cultivars are available, and can be explored for identifying genetic traits involved in disease tolerance/resistance. Moreover, ranking *Vitis* genotypes for their susceptibility to FD, is in itself a precious tool to support vine growers in their decision management, by helping them in choosing the most appropriate varieties according to their specific FD epidemiological contexts.



Graphical abstract

1. Introduction

Flavescence dorée is a quarantine disease of grapevine that threatens viticulture in several wine-growing areas of Europe (EFSA, 2014). The disease is caused by phytoplasmas (FDp) belonging to the 16SrV taxonomic group, showing genetic variation at several genetic loci (Arnaud *et al.*, 2007; Rossi *et al.*, 2019). In particular, genetic differences at the *vmp*A locus, encoding a putative variable membrane protein, allow prediction of the ability of the leafhopper *Scaphoideus titanus* to transmit the disease (Malembic-Maher *et al.*, 2020), as the protein is involved in specific molecular interactions with unidentified leafhopper vector proteins (Arricau-Bouvery *et al.*, 2018). The phytoplasma is transmitted in a persistent propagative manner mainly by the grapevine-feeder leafhopper *S. titanus* (Chuche and Thiéry, 2014), but some polyphagous leafhopper species may also transmit FDp from alternative hosts to grapevine (Malembic-Maher *et al.*, 2020).

Infected vines show a range of phytoplasma-specific symptoms, among which bunch shriveling causes severe yield reduction. Infected plants may die or recover (Caudwell *et al.*, 1987; Maggi *et al.*, 2017), although recovered vines are still prone to re-infections (Rossi *et al.*, 2020). The control of Flavescence dorée relies on different measures. Two to three compulsory insecticide treatments are applied in the infected areas to reduce vector population (Bosco and Mori, 2013). Roguing of infected plants and pruning of symptomatic vegetation during the vegetative season are implemented to minimize the inoculum source, and hot water-treated grafted cuttings are often employed for new plantations and to replace missing plants. Overall, these strategies are costly, impact on the health of the environment and of wine growers, and raise concerns on insecticide residues in the final product. Hence, alternative FD management strategies are currently explored (Oliveira *et al.*, 2019) to support viticulture by stimulating grapevine defences with abiotic *(Gutiérrez-Gamboa et al., 2019; Miliordos et al.*, 2017; reviewed in Oliveira *et al.*, 2019) and biotic (reviewed in Oliveira *et al.*, 2019) elicitors, and interfering with insect vector ability (Gonella *et al.*, 2019; Galetto *et al.*, 2020) or mating behavior (reviewed in Oliveira *et al.*, 2019). In North-western area of Italy (Piedmont Region), FD is widespread, together with abundant and highly infective vector populations colonizing vineyards and wild surrounding areas (Ripamonti *et al.*, 2020). As wild areas cannot be treated with insecticide for environmental concerns, a challenging landscape management is required. Therefore, FD disease is hard to control and its impact on vineyard productivity is of growing

concern. The identification of cultivars (cvs) with reduced susceptibility to the disease is a critical issue to support sustainable viticulture in Europe. Indeed, preliminary reports suggest that, under field conditions, FD incidence differs in vineyards where different cvs are grown (Morone *et al.*, 2007; Eveillard *et al.*, 2016). However, evaluating cv susceptibility under field conditions is difficult due to uncontrolled environmental conditions affecting *S. titanus* presence and abundance (e.g. presence of abandoned vines as refuges for the insect), infection pressure (presence of asymptomatic re-growth branches of naturalized *Vitis* plants in abandoned vineyards and woods), accession routes to the vineyards (main wind direction, altitude of abandoned *Vitis* groves with respect to the vineyard, vineyard slope).

Two main mechanisms of plant defense against pathogens are known: resistance (the host's ability to limit pathogen multiplication), and tolerance (the host's ability to reduce the effect of infection on its fitness regardless of the level of pathogen multiplication, Pagán and García-Arenal, 2018). The two may also coexist, and result in low susceptible genotypes. To allow the contemporaneous evaluation of the FD susceptibility of different *Vitis* genotypes, a standardized protocol has been described to inoculate vine plants with infectious *S. titanus* conditions (Eveillard *et al.*, 2016). This approach exploits *ex-vitro* potted plantlets grown in uniform, semi-controlled conditions, therefore eliminating most of the confusing environmental effects described above, and inoculated with infective insects allowed to acquire FDp under controlled conditions. Indeed, this protocol has been applied to characterize several *Vitis*

genotypes for their susceptibility to FD disease including the most common rootstocks in France (Eveillard *et al.*, 2016), but many other economically relevant cultivars grown in different viticultural areas still need to be characterized. Aim of the present work was to evaluate the susceptibility of several grapevine varieties mostly grown in Piedmont, one of the most important wine production area of Italy. Several of the most well-known local *Vitis* genotypes were analyzed, taking into consideration the traditional single varietal wine production strategy of this area. Moreover, to confirm the results obtained with this protocol, four *Vitis* cvs, representing extremes of the obtained FD susceptibility ranking, were grafted onto Kober 5BB rootstocks and their susceptibility to the disease was assessed upon inoculation with lab-infected *S. titanus*, under semi-field conditions. Two local cvs, Moscato and Brachetto, showed low susceptibility to FD in both the experimental settings, although, for the white cv Moscato, preliminary results suggested that this behavior may result from cv-specific effects on vector fitness.

2. Materials and methods

2.1. Plant material

2.1.1. Plants from in vitro culture

Woody cuttings were collected from 12 *Vitis vinifera* varieties, supporting the most economically important wine production in Piedmont. The cuttings were taken in winter from virus-free potted plants of specific clones which are the primary source of registered clones and are grown in the CE.PRE.MA.VI. screenhouse near Alba

(CN; http://www.ipsp.cnr.it/projects/ce-pre-ma-vi/?lang=en), (Table 1, Supplementary Table S1).

Table 1. Survival rate of infectious *Scaphoideus titanus* following one-week inoculation access period on either *ex vitro* plants or grafted cuttings of the different cultivars.

Accession name	Cultivar	Clone code	Survival rate [%]	
			ex vitro plants	Grafted cuttings
Cortese	Cortese	AL CO 2	47.8	-
Arneis	Arneis	AR CVT CN 32	64.8	-
Barbera 84	Barbera	BA AT 84	80.5	66.7
Barbera NC	Barbera	BA NC	74.2	-
Brachetto	Brachetto	BRA CVT 20	67.5	40.3
Dolcetto	Dolcetto	DO CVT 64	76.2	-
Erbaluce	Erbaluce	ER CVT TO 55	70.0	-
Freisa	Freisa	FR CVT 20	43.2	-
Merlot	Merlot	M VCR 489	78.6	25.0
Moscato	Moscato	MO CVT 190	17.5	22.4
Nebbiolo 71	Nebbiolo Michet	NE CVT 71	66.3	-
Nebbiolo 423	Nebbiolo Picoutener	NE CVT 423	70.8	-
Ruchè	Ruchè	RU CVT 10	67.9	-
Timorasso	Timorasso	TIM 18	73.8	-

For the cv Nebbiolo, which has a quite large genetic variability, two biotypes were collected, Michet (Nebbiolo 71) and Picoutener (Nebbiolo 423). Two additional accessions were also included: Merlot (clone VCR489), from the IPSP grapevine collection field of Grinzane Cavour (CN), and a healthy Barbera plant from an old FD-infected vineyard made of non-clonal Barbera plants (Barbera NC).

Micro-propagated grapevines of the 14 genotypes were produced. Briefly, axillary buds (obtained by forcing the woody cuttings in water) were surface-sterilized and cultivated *in vitro* on a modified Murashige and Skoog (1962) medium (Gribaudo *et al.*, 2007) without plant growth regulators; the resulting plantlets were multiplied by repeatedly sub-culturing apical cuttings (3-4 cm long) on the same medium. After a four-week rooting and acclimatization period in Jiffy-7® peat pellets, plantlets were transplanted in 14 cm pots and grown under greenhouse condition, 16:8 L:D, 24±2°C with no humidity control. Sulfur was sprayed to control powdery mildew once per month, or at the onset of the first symptoms.

2.1.2. Grafted cuttings

Cuttings of the Barbera 84, Brachetto, Merlot and Moscato (White Muscat) grafted onto the Kober 5BB rootstock were potted in 80L pots in a screenhouse made of insect-proof net in March 2018. Fungicide applications (copper- and sulfur-based treatments) followed the conventional calendar, while no insecticide treatments were applied.

2.1.3. Vicia faba and *Avena sativa*

Plants of *Vicia faba* (cv Agua-dulce Supersimonia) and *Avena sativa* were grown in pots in a greenhouse, at 24±2°C, no humidity and no photoperiod control, and used two weeks after sowing as host plants to maintain the FDp isolate (*V. faba*) or to rear healthy *E. variegatus* colonies (*A. sativa*).

2.2. Insect rearings

Scaphoideus titanus laboratory colonies were initiated from two-year-old canes collected in 2016, 2017, 2018, and 2019 (January to

February) in Piedmontese vineyards known to host high populations during the previous seasons. The collected canes were stored in the cold (6±1°C), covered with a plastic sheet to avoid egg desiccation, until use. Grapevine canes were transferred to Plexiglas cages in the greenhouse at 24±2°C and maintained damp by daily water misting. After four weeks, three-week old broad bean plants were introduced into the cage. After egg hatching, broad bean plants were replaced every three/four weeks. Nymphs were reared in these cages in the greenhouse, at 24±2°C, with no humidity and no photoperiod control. Healthy *Euscelidius variegatus* laboratory colonies were routinely maintained under controlled conditions on oat plants (Galetto et al., 2009).

2.3. FDp isolate and acquisition by *S. titanus* under controlled conditions

Flavescence dorée phytoplasmas (FD-C, Firrao *et al.*, 2013) isolated in Piedmont Region was routinely maintained on *V. faba* plants with sequential transmission by *E. variegatus*, as detailed in Galetto *et al.* (2009). For acquisition by *S. titanus,* fourth/fifth instar nymphs were isolated onto four FD-infected broad bean plants for an acquisition access period (AAP) of two weeks, and then isolated onto four healthy broad bean plants for two-week latency period (LP). A representative number of *S. titanus* adults was collected at the end of the LP to assess the presence of FDp in the leafhoppers, and the acquisition efficiency (percentage of PCR positive individuals out of the sampled ones) was measured for each experiment. Figure 1 details the experimental work-flow.

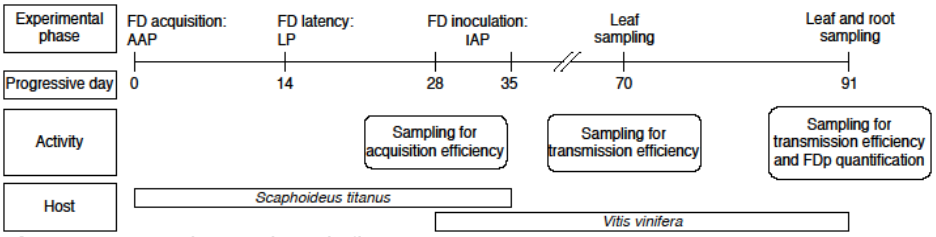


Figure 1. Experimental work-flow.

2.4. Inoculation of *Vitis* cvs with Flavescence dorée infected *S. titanus*

2.4.1. *ex vitro* plants

At the end of LP, four infectious *S. titanus* were caged on each grapevine, for one-week inoculation access period (IAP). At the end of the IAP, dead and alive insects were collected, and stored under ethanol at -20°C before total DNA extraction and phytoplasma detection. Survival rate of the infectious insects was calculated, as the percentage of alive insects on the total number of insects caged for IAP on each cultivar. Inoculated plants were maintained in a greenhouse at 24±2°C, without humidity and photoperiod control. Five weeks post inoculation (5 wpi), leaves of the inoculated grapevines were sampled (three leaves uniformly distributed in the plant) and tested for the presence of FDp. Eight weeks post inoculation (8 wpi), both leaves (five) and roots (up to one gram) were sampled and tested for the presence of FDp. When present, symptomatic leaves were preferred, while asymptomatic leaves and roots were randomly selected. For each cv, the results were expressed as percentage of infected plants, assayed on leaves (5 and 8 wpi), roots (8 wpi), and percentage of infected whole plants (plants with infected leaves and/or roots) at 8 wpi, over the

inoculated ones for each experiment. At 8 wpi, the phytoplasma was also quantified in the leaf and root samples of all infected plants of the different cvs as described below. Four experiments were run, once a year, starting from 2016. Plants of Barbera 84 were included in each inoculation experiment, as control of the different experiments. For each experiment, the inoculation efficiency of infective *S. titanus* was calculated as the percentage of infected plants of Barbera 84 over the total number of inoculated ones (same year).

2.4.2. Grafted cuttings

Four cultivars were selected for the validation of their susceptibility to FD, on the basis of their performances following the inoculation under greenhouse conditions: Brachetto, Merlot, Moscato and Barbera 84; this latter was included as control for inoculation efficiency. At the end of LP, four infectious *S. titanus* were caged onto a branch of the screenhouse grown grafted cuttings for a seven-day IAP. A total of 35 plants (10 Barbera 84, 10 Brachetto, 5 Merlot, and 10 Moscato) were used for this experiment, and were inoculated with FD-C infectious *S. titanus*, as described above. Survival rate of the infectious insects was calculated, as the percentage of alive insects on the total number of insects caged for IAP on each cultivar. Symptomatic leaves, when present, were sampled from each plant at the end of the vegetative season of the same year of the inoculation (September), and at the beginning of the following growing season (June). In the absence of symptoms, five leaves uniformly distributed in the plant were randomly

collected. Leaf samples from all plants were analyzed by Real-Time PCR to confirm their infectious status (see below).

2.5. Detection and quantification of FDp

Total DNA was extracted from midribs of five pooled leaf samples of the same plant, both from the plantlets issuing from greenhouse experiments and the grafted cuttings of the semi-field conditions, according to Pelletier et al. (2009). Real-time PCR for diagnosis of FDp on *ex vitro* plant samples was conducted with primer pairs mapFD-F/R (Pelletier *et al.*, 2009), with a modified reaction mixture consisting of SYBR® Green Master Mix (Biorad), primers (300 nmol each), template (20 ng of total DNA). Cycling condition were first denaturation at 95°C for 30 s, then 45 cycles composed by a denaturation of 5 s at 95°C followed by 10 s of annealing/extention at 60°C. A melting curve analysis was run at the end of the PCR cycles, to confirm amplicon specificity. Grafted cuttings derived samples were analyzed using a commercial kit (Detection kit *Flavescence dorée et Bois Noir*, Multiplex Real-time PCR system, IPADLAB), through a Real-time PCR-based assay. A Taq Internal Positive Control IPC (TaqMan® Exogenous Internal Positive Control, Applied Biosystems®) was added to the reaction mix, in order to confirm absence of contaminations inhibiting the amplification process. Flavescence dorée phytoplasma relative quantification was performed according to Roggia et al. (Roggia *et al.*, 2014), and the phytoplasma load was expressed as phytoplasma genome units per nanogram of plant DNA (FD GU/ng plant DNA).

2.6. Statistical analysis

All statistical analyses were conducted on the R software, version 3.6.2 (R Core Team., 2020), using multiple packages, as detailed in Supplementary File S1. Acquisition efficiencies, defined as the percentage of PCR-positive *S. titanus* at the end of the latency period, and transmission efficiencies from *S. titanus* to Barbera, defined as the percentage of positive Barbera plants at the end of the 8 wpi period, were compared among years using Fisher's exact test. p-values' multiple comparisons were adjusted with BH method (Supplementary Table S2 & S3). Survival rate of *S. titanus* on the 14 different *Vitis* genotypes at the end of the 7-day inoculation period were tested with a beta-regression model (Supplementary Table S4 & Figure S1). Comparisons among genotypes were computed with estimated marginal means (or least-squares means), followed by Tukey post-hoc test with significance level set at 0.05. Hierarchical classification was conducted on four main variables: FD percentage of infection and mean FD load, both in leaves and roots, for every analysed *Vitis* accession. Variables were standardized with Z-score method. Euclidean distance and Ward's method were applied as similarity and association methods, respectively. Principal Component Analysis was conducted on the same standardized variables, and represented cultivars were grouped according to clustering results. To test differences between the resulted groups after PCA, PERMANOVA test was utilized.

3. Results

3.1. *S. titanus* infectivity and FDp transmission to the control clone Barbera 84

To test the susceptibility of the different *Vitis* genotypes, one experiment per year was performed from 2016 to 2019 (four experiments). The acquisition efficiencies of *S. titanus* ranged between 64 and 95%, with 2019 efficiency (64%) significantly different from those of the three other experiments (Fisher's Exact Test for Count Data: p-value = 5.485e-07; Supplementary Table S2). Under these conditions, inoculation efficiencies of *S. titanus* to Barbera 84 plants were 100%, 78.6%, 83.3% in 2016, 2017, 2018, with a mean value of 87.3% (Supplementary Table S1). As the inoculation efficiency of the 2019 experiment was lower (13.3%) than those of the previous years and not comparable with them, data from this experiment were not included into further statistical analyses (Fisher's Exact Test for Count Data: p-value = 4.173e-05; Supplementary Table S3).

3.2. Insect survival rate on different *Vitis* cultivars

Survival of the infectious *S. titanus* at the end of the 7-day IAP on the different *Vitis* cvs is detailed in Table 1. Overall, more than 64% of the insects survived the 7 day IAP on most of the cultivars. In the case of Freisa and Cortese, *S. titanus* survival rates were 43 and 48%, respectively. Survival rate of the infectious insects on Moscato (17.5%) was significantly lower than that on the first group of cultivars (Supplementary Table S4, Supplementary Figure S1). Survival rates of the infectious vectors on the Barbera 84 and Brachetto grafted cuttings were higher than on Merlot and Moscato

(Table 1). Survival rate on grafted Merlot was significantly lower ($\chi 2$ = 16.017, df = 1, p-value = 6.279e-05) than that on *ex vitro* plantlets under greenhouse conditions (Table 1).

3.3. FD susceptibility of *ex vitro Vitis* genotypes

3.3.1. Phytoplasma infection

Moscato and Brachetto showed less than 30% infected plants (20 and 25%, respectively) at 8 wpi. At the same time, Freisa (36.4%) and Merlot (42.9%) showed an intermediate proportion of infected plants, while Cortese, Dolcetto, Erbaluce, and Timorasso showed about 70% of PCR-positive plants. More than 80% of the inoculated Barbera, both clone 84 and NC, both Nebbiolo 71 and 423, Ruchè, and Arneis plants were FD-infected at 8 wpi (Supplementary Table S5). In the 2019 experiment, Ruchè also showed higher infection rates compared to Barbera 84 (23.1% vs 13.3%, respectively). In the same experiment, about 15% and 7% of Merlot and Nebbiolo 423 inoculated plants were infected (Supplementary Table S1).

Supplementary Table S5 also details the numbers of FD phytoplasma-positive leaf and root samples at 8 wpi. In particular, in the case of Merlot, FDp was never detected in leaf samples, while it was detected in the root samples of the three infected plants. On the other hand, the phytoplasma was found in the leaves and roots of the infected plants of all the remaining cvs. Indeed, for most of them (Arneis, Cortese, Brachetto, Erbaluce, Freisa, Nebbiolo 71 and Barbera NC), FDp was detected more frequently in roots than in leaves. In three cvs (Barbera 84, Moscato and Ruchè), FDp was present with the same frequency in leaves and roots, while for Dolcetto, Nebbiolo 423 and Timorasso, FDp was more frequently

detected in leaves than in roots. In the 2019 experiment, FDp was also more frequently detected in leaves than in roots of Barbera 84 and Nebbiolo 423 plants (Supplementary Table S1).

Leaf samples were also collected for FDp detection at 5 wpi (Supplementary Table S5& S1). More than 40% of the inoculated plants of the Barbera 84, Nebbiolo 423 and Timorasso cvs were already positive for the presence of FDp in their canopy at 5 wpi. At the same time, 5% of the Brachetto and Moscato inoculated plants were already infected, and two of the seven inoculated Merlot plants were positive for the presence of FDp in their canopy. A similar percentage of inoculated plants was already infected for Erbaluce (30%), Barbera NC (37,5%) and Nebbiolo 71 (33,3%). In the case of Merlot, two plants showed infected canopies at 5 wpi (Figure 2).

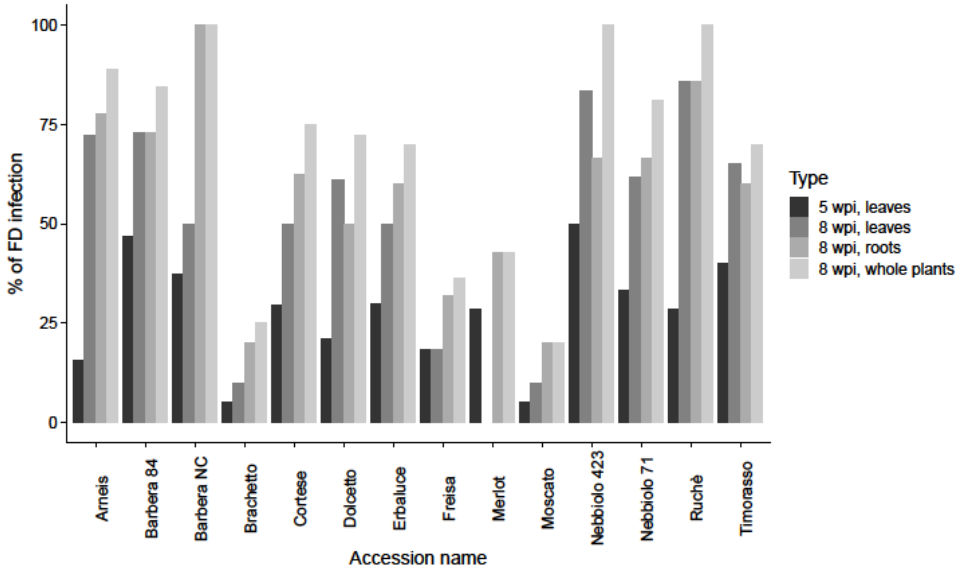


Figure 2. Percentages of FD-positive leaf samples at five weeks post infection (wpi), leaf and root samples at eight wpi, and whole infected plants (showing FDp positive root and/or canopy) at eight wpi. Percentages were calculated on plants from 2016 to 2018 experimental repeats pooled together.

3.3.2. Phytoplasma loads

Flavescence dorée phytoplasma loads were measured in both leaves and roots of the infected plants at 8 wpi. Phytoplasma load was below the quantification threshold for about 40% of the infected plants, irrespective of the cultivar. For the remaining samples, FDp load ranged from 5×10e-03 and 1.2×10e02 GU/ng plant DNA in leaves, and from 5×10e-01 and 4.18×10e02 in roots (Figure 3). Phytoplasma load was below the quantification threshold for all Erbaluce, Merlot and Moscato leaf samples of infected plants; for the remaining cvs, it ranged from 2.4×10 and 3.9×10 GU/ng plant DNA (Arneis, Barbera NC, Dolcetto, and Nebbiolo 423), and it was above 6.5×10 GU/ng plant DNA in the leaves of Barbera 84, Cortese, Freisa, Nebbiolo 71, and Timorasso. In the case of Brachetto, FDp load was measurable in one of the two infected canopies (5.6×10e-03 GU/ng plant DNA) (Figure 3, Supplementary Table S1). As for the roots of the infected plants, FDp load was below the quantification threshold in all the Merlot and Nebbiolo 423 infected plants, and it was 5.2×10e-01 in one of the four infected Moscato plants. For the other cultivars, FDp load was above 2.9×10e02 GU/ng plant DNA in Cortese and Barbera 84, and it ranged between 4.89×10 and 1.5×10e02 GU/ng plant DNA for Arneis, Barbera NC, Brachetto, Dolcetto, Erbaluce, Freisa, Nebbiolo 71, Ruchè, and Timorasso (Figure 3, Supplementary Table S1).

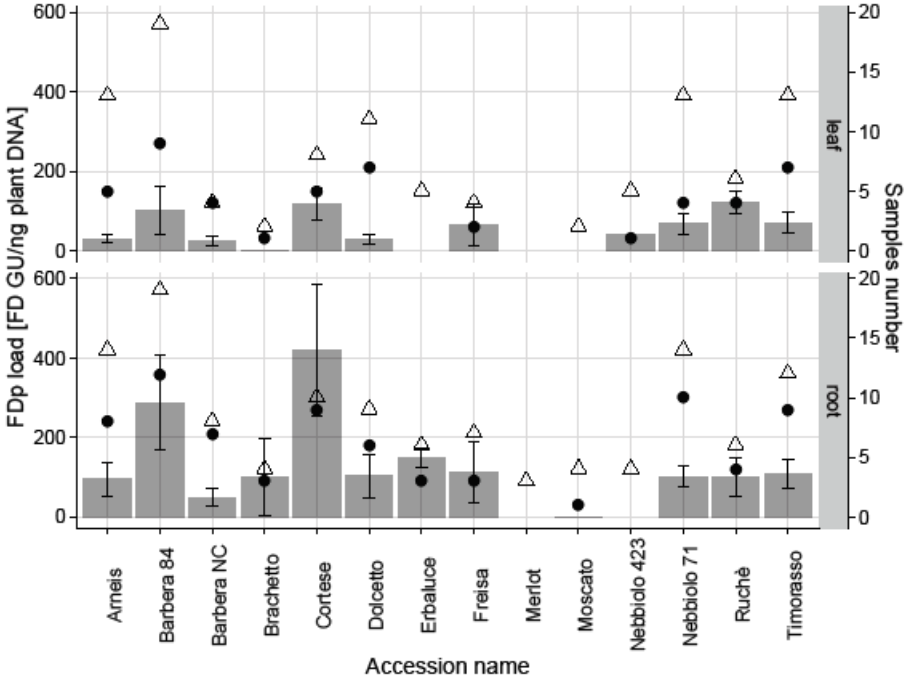


Figure 3. Mean Flavescence dorée phytoplasma load ± standard error (left axis) in leaf (upper panel) and root (lower panel) samples (grey boxes). Numbers of FD-positive samples (white triangles) and FD-quantifiable samples (black circles) are indicated (right axis).

Overall, *V. vinifera* genotypes showing low numbers of infected canopies also hosted low phytoplasma loads in their leaves, while the situation was less clear at the root level.

3.4. Hierarchical Classification and PCA

Hierarchical classification of four variables (percentage of infected leaves and roots, phytoplasma loads in leaves and roots) grouped Brachetto, Freisa, Merlot and Moscato separated from Arneis, Barbera NC, Dolcetto, Erbaluce, both Nebbiolo clones, and Timorasso. Barbera 84, Cortese and Ruchè formed a third cluster (Figure 4a). Using the clustering obtained by hierarchical

classification, standardized variables were then explored with a PCA, where the first and second components explained 61.1% and 26.6% of the variability (Figure 4b). The PCA biplot suggested strong differences between groups, validated through a PERMANOVA test (*F*-ratio 12.813, p-value 1e-04, 9999 permutations). In particular, the group including Brachetto, Freisa, Merlot and Moscato showed an indirect relation with the original variable vectors, meaning a general low susceptibility behavior. The group of Barbera 84, Cortese and Ruchè shared a general direct relation with the original variables, confirming their susceptibility to the disease. The other cultivars were in an intermediate position, with some extremes in FD percentage of infection (Barbera NC and Nebbiolo 423).

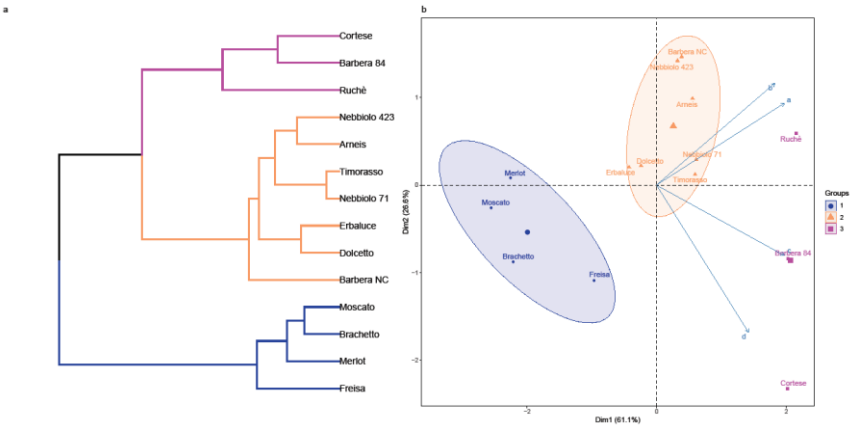


Figure 4. Hierarchical classification (a) and PCA biplot (b) of percentage of Flavescence dorée infected plants and phytoplasma loads in leaves and roots of infected grapevines of the different cultivars, at 8 weeks post inoculation. Cluster analyses identified three groups of cultivars characterized by high (purple, up), medium (orange, center), and low susceptibility (blue, bottom) (Similarity index: Euclidean distance; Association method: Ward). In panel b, clusters were grouped with ellipses and the centroid of each was represented. PCA vectors represented the original variables: mean FD percentage of infection for leaves (a) and roots (b), and mean FD load for leaves (c) and roots (d). The new condensed PCA variables explained 61.1% of variability (Dim1, x-axis) and 26.6% (Dim2, y-axis).

3.5. Susceptibility to FD of grafted cuttings under semi-field conditions

Following inoculation with FD-infective *S. titanus* on Kober 5BB-grafted vines, three out of 10 and one out of five of the inoculated Barbera 84 and Merlot plants were infected one year after inoculation, respectively. No infected plants were recorded among the 10 inoculated Brachetto and Moscato plants.

4. Discussion

Here we evaluated, under controlled conditions, susceptibility to Flavescence dorée phytoplasma (FDp) of 14 grapevine accessions. Grafted cuttings of selected cvs at the extremes of the disease susceptibility range were also inoculated with infective *S. titanus* under semi-field condition, to further confirm the results. None of the selected *Vitis* accessions was resistant to FD phytoplasma, when tested as plantlets from *in vitro* culture. In fact, under this experimental condition, all genotypes became infected upon vector inoculation, although in few instances only one to few plants were FD-positive, and a cluster of less susceptible accessions was found, including both non-coloured (Moscato) and coloured cvs (Brachetto, Merlot, and Freisa). These cvs confirmed their low susceptibility to FD upon grafting on Kober 5BB rootstock. Two of the least susceptible cvs, Moscato and Brachetto, share the presence of aromatic compounds in their leaves and berries, and are classified as ‘aromatic varieties’ (Mazza *et al.*, 2003; Pollon *et al.*, 2019). Aromatic compounds may play a role in determining tolerance to FD, although the mechanism is not clear. In the case

of Moscato, poor FD susceptibility may result from an indirect effect against the vector, as suggested by our preliminary results on the reduced vector survival during the IAP on this cv, with reduced chances to transmit the disease. In the case of Brachetto, the low susceptibility may act directly on the phytoplasma, as *S. titanus* survival on this cv during IAP was similar to that on Barbera 84 plants while phytoplasma load was low. Indeed, specific investigations evaluating *S. titanus* fitness and feeding behavior on the above-mentioned poorly susceptible cvs are ongoing, to confirm the role of the plant/vector relationship in defining the degree of FD susceptibility of different *Vitis* genotypes. The feeding behavior of *S. titanus* on Cabernet Sauvignon grapevine has been described by means of electropenetration graph analyses (Chuche *et al.*, 2017), and this technique may indeed be helpful in unveiling possible differences in feeding behavior of the vector on *Vitis* genotypes with different susceptibilities to FD. Among the cluster of poorly susceptible accessions, FDp was sporadically detected in the canopy of the Merlot plants only at 5 wpi, therefore confirming previous results based on both field and laboratory observations (Eveillard *et al.*, 2016). Nevertheless, under our experimental conditions, phytoplasmas were detected (although below the quantification threshold) in the roots of three inoculated plants, therefore challenging the hypothesis that FDp diffusion from the inoculation point may be hampered in this cv (Eveillard *et al.*, 2016). In other pathosystems involving herbaceous host plants, rapid phytoplasma movement from the inoculation point to the root has been described (Saracco *et al.*, 2005), although the root system has

been excluded from the analyses of FDp presence in different grapevine organs upon time (Prezelj *et al.*, 2013), probably due to difficulties in retrieving *Vitis* roots under field conditions. This work showed that FDp spreads rapidly to the roots, where it accumulates to higher loads than in the plant canopy. Our results confirm that poorly susceptible cvs host low phytoplasma loads (Eveillard *et al.*, 2016), at least at the leaf level. Nevertheless, this trend was less evident at the root level. Phytoplasma presence in the roots raises the question on its epidemiological role. Indeed, the mere presence of phytoplasmas in the root system does not imply that phytoplasmas actively multiply there, as they can be translocated from epigeal sites. Also, FDp routes for colonization of the aerial part of the plant from the root have never been explored, and, as the phloem runs basipetally, the roots may represent a dead end accumulation site, rather than the source of plant re-infection over time. However, phytoplasma movement towards sinks of the canopy through acropetal flow cannot be ruled out. Also, phloematic flux towards the roots may differ in *ex-vitro* plantlets compared to field grapevines. Further investigations are needed to clarify the interactions between roots and phytoplasmas in grapevine.

Medium and high susceptible cvs hosted high FDp loads, but no evident relationship with the number of infected plants was found. Susceptibility of *ex vitro* Barbera 84 and both Nebbiolo clones was similar, although Nebbiolo is reported as less susceptible to FD under field conditions (Roggia *et al.*, 2014). *Ex vitro* plantlets were already symptomatic and infected at 8 wpi (sometimes already at 5 wpi), while grafted plants in the field generally become infected one

year after the inoculation. A similar situation was described by Eveillard *et al.* (2016). These discrepancies can be due to the different physiology of herbaceous micropropagated plants and woody grafted ones, as the genotype is identical. Nevertheless, three poorly susceptible genotypes identified in this study maintained their phenotype upon inoculation as grafted rootstocks. The two tested Nebbiolo genotypes behaved similarly, both falling within the intermediate susceptible cluster, despite a genetic difference between them has been described as based on the specific functional category 'responses to pathogens' (Gambino *et al.*, 2017). The Barbera NC fell within the intermediate cluster, separated from the Barbera 84 clone, included in the high susceptible cluster. The low number of tested Barbera NC plants may prevent robust conclusions, and genetic differences between the two accessions have not been explored. Together with the work of Eveillard *et al.* (2016), different *Vitis* genotypes have been tested for FDp susceptibility, and Merlot (of different clones) was the only common genotype included in the studies, and both ranked it among the least susceptible cvs. However, different survival rates of *S. titanus* on this cv were recorded, as in our experiments survival was much higher and comparable to those on most suitable varieties. From an epidemiological perspective, it is worth noting that FDp load in infected grapevines is not a good predictor of phytoplasma spread by the vector, as infected Moscato is a poor source of inoculum for *S. titanus*, while Brachetto is a better one, thus proving that variety is an important factor, independently from the phytoplasma load (Galetto *et al.*, 2016). Since the diffusion of

the disease depends, among other factors, on the probability of a competent vector feeding on an infected plant, vineyards of poorly susceptible varieties have a limited number of infected plants, thus slowing secondary spread of FD (from vine to vine within the vineyard).

In the 2019 experiment, phytoplasma acquisition and transmission efficiencies by *S. titanus* were lower compared to the previous three years. The introduction of a control cv, Barbera 84 (as suggested by Eveillard *et al.*, 2016), allowed to separately analyze this experiment. The reasons for this low efficiency are unclear and possibly due to a low phytoplasma load in the source broad bean plants.

This work, together with the one of Eveillard *et al.* (2016), shows that none of the explored genotypes is immune to FDp, but some poorly susceptible ones are available for identifying genetic traits involved in FD tolerance/resistance. This step is crucial for successive traditional or cisgenic breeding applications, and for targeted genome editing through CRISPR-Cas9 technology (Ren *et al.*, 2019). The possibility of ranking *Vitis* genotypes for their susceptibility to this very important disease is a precious tool to support vine growers in their decision management, by helping them in choosing the most appropriate varieties according to their specific FD epidemiological contexts.

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[dataset] ‘The data that support the findings of this study are available from the corresponding author upon reasonable request.’

Supplementary Material

Supplementary File S1. R packages used for data analysis.
All data and statistical analyses were conducted on the R software, version 3.6.2 (R Core Team., 2020). The following packages were utilized: dplyr (Wickham *et al.*, 2020), fifer (Fife, 2017), emmeans (Lenth, 2020), ggplot2 (Wickham, 2016), dendextend (Galili, 2015), factoextra (Kassambara and Mundt, 2020), patchwork (Pedersen, 2019), betareg (Cribari-Neto and Zeileis, 2010), vegan (Oksanen *et al.*, 2019). Data extrapolation and manipulation from the complete database were conducted with dplyr package. Acquisition efficiencies (percentage of PCR-positive *S. titanus* at the end of the inoculation access period) and transmission efficiencies (percentage of PCR-positive Barbera 84 at the end of the 8 week period), were compared between years using Fisher's exact test on counts (fifer package). p-values' multiple comparisons were adjusted with BH method (Benjamini and Hochberg, 1995). Survival rate of *S. titanus* on the 14 different *Vitis* genotypes at the end of the 7-day inoculation period were tested with a beta-regression model (betareg package). Comparisons among genotypes were computed with estimated marginal means (or least-squares means; emmeans package), followed by Tukey post-hoc test with significance level set at 0.05. Hierarchical classification (dendextend package) was conducted on four main variables: FD percentage of infection and mean FD load, both in leaves and roots, for every analysed *Vitis* accession. Variables were standardized with Z-score method (Becker *et al.*, 1988). Euclidean distance and Ward's method were applied as similarity and association methods, respectively (Becker *et al.*, 1988; Murtagh and Legendre, 2014). Principal Component Analysis (Venables and Ripley, 2002) was conducted on the same standardized variables (factoextra package), and represented cultivars were grouped according to clustering results. To test differences between the resulted groups after PCA, PERMANOVA test was applied (Anderson, 2001) with vegan package. All figures were produced via ggplot2 package, Figure 3 & 4 subplots joined via patchwork package.

Supplementary Table S1. The full database of the Flavescence dorée inoculation experiments onto *ex-vitro* plantlets of the different cultivars can be downloaded at the following link:
https://bsppjournals.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fppa.13301&file=ppa13301-sup-0002-TableS1.csv

Supplementary Table S2. Counts and comparisons of *S. titanus* acquisition efficiencies among years. Computed with fifer package (Fife, 2017) on R software (R Core Team., 2020).
Raw data (counts):

year	St_FDpos	St_FDneg
2016	85	11
2017	121	6
2018	37	2
2019	36	20

Fisher's exact test, p-value adjust method: BH (Benjamini and Hochberg, 1995)

comparison	raw.p	adj.p	sign
2016 vs. 2017	0.076	0.113	
2016 vs. 2018	0.346	0.415	
2016 vs. 2019	0.001	0.001	***
2017 vs. 2018	1	1	
2017 vs. 2019	0	0	****
2018 vs. 2019	0	0.001	***

Supplementary Table S3. Counts and comparisons of inoculation efficiencies onto Barbera 84 (control cultivar) among years. Computed with fifer (Fife, 2017) package on R software (R Core Team., 2020).

Raw data (counts):

exp_year	neg	pos
2016	0	6
2017	3	11
2018	1	5
2019	13	2

Fisher's exact test, p-value adjust method: BH (Benjamini and Hochberg, 1995)

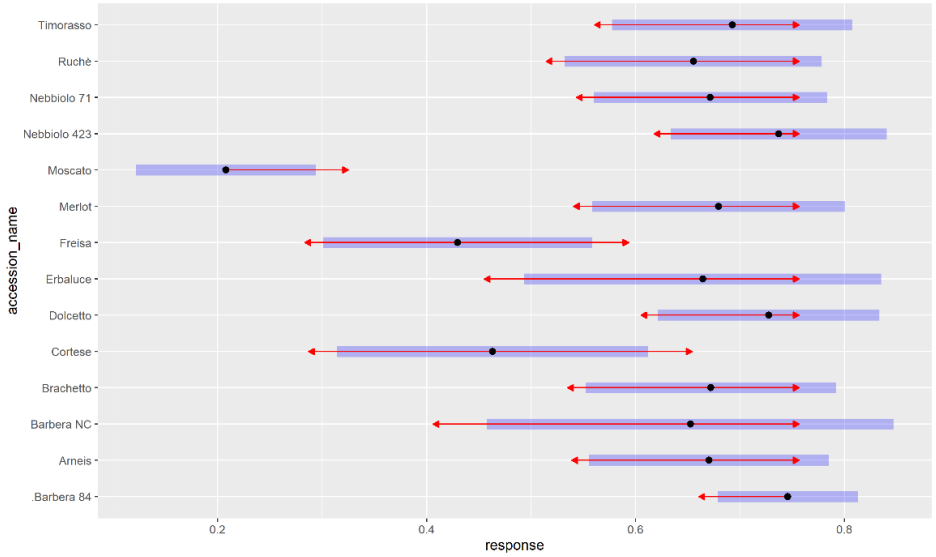
comparison	raw.p	adj.p	sign
2016 vs. 2017	0.521	0.782	
2016 vs. 2018	1	1	
2016 vs. 2019	0.001	0.002	**
2017 vs. 2018	1	1	
2017 vs. 2019	0.001	0.002	**
2018 vs. 2019	0.006	0.011	*

Supplementary Table S4. Pairwise comparisons of estimated marginal means of the beta-regression model on *Scaphoideus titanus* survival rates among the different cultivars. Computed with betareg (Cribari-Neto and Zeileis, 2010) and emmeans (Lenth, 2020) packages on R software (R Core Team., 2020).

cv	alive_St	dead_st	response	SE	df	asymp.LCL	asymp.UCL	.group
Moscato	14	66	0.208	0.0439	Inf	0.122	0.294	a
Freisa	38	50	0.43	0.0657	Inf	0.301	0.558	ab
Cortese	32	35	0.463	0.076	Inf	0.314	0.612	abc
Barbera NC	23	8	0.653	0.0994	Inf	0.458	0.847	bcd
Ruchè	54	26	0.655	0.0628	Inf	0.532	0.778	bcd
Erbaluce	28	12	0.664	0.0872	Inf	0.493	0.835	bcd
Arneis	57	31	0.67	0.0584	Inf	0.556	0.785	bcd
Nebbiolo 71	61	31	0.672	0.057	Inf	0.56	0.783	bcd
Brachetto	54	26	0.672	0.061	Inf	0.553	0.792	bcd
Merlot	52	24	0.679	0.0618	Inf	0.558	0.801	bcd
Timorasso	59	21	0.693	0.0587	Inf	0.578	0.808	bcd
Dolcetto	61	19	0.727	0.0541	Inf	0.621	0.834	cd
Nebbiolo 423	64	16	0.737	0.0527	Inf	0.634	0.84	cd
Barbera 84	146	40	0.746	0.0342	Inf	0.679	0.813	d

Confidence level used: 0.95
P value adjustment: tukey method for comparing a family of 14 estimates
significance level used: alpha = 0.05

Figure S1. Graphical comparison of estimated marginal means of the beta-regression model on *S. titanus* survival rate among the different cultivars.



Supplementary Table S5. Number of Flavescence dorée infected plants of each cultivar at five and eight week post inoculation (wpi) with infective *Scaphoideus titanus*. At five wpi leaves were sampled for the PCR assay; at eight wpi both roots and leaves were sampled and plants were considered infected when either their leaves or roots were positive at the PCR assay. n = total number of analyzed samples.

Accession name	FD positive (n)			
	5 wpi	8 wpi		
	leaves	leaves	roots	whole plants
Cortese	5 (17)	8 (16)	10 (16)	12 (16)
Arneis	3 (19)	13 (18)	14 (18)	16 (18)
Barbera 84	14 (30)	19 (26)	19 (26)	22 (26)
Barbera NC	3 (8)	4 (8)	8 (8)	8 (8)
Brachetto	1 (20)	2 (20)	4 (20)	5 (20)
Dolcetto	4 (19)	11 (18)	9 (18)	13 (18)
Erbaluce	3 (10)	5 (10)	6 (10)	7 (10)
Freisa	4 (22)	4 (22)	7 (22)	8 (22)
Merlot	2 (7)	0 (7)	3 (7)	3 (7)
Moscato	1 (20)	2 (20)	4 (20)	4 (20)
Nebbiolo 71	7 (21)	13 (21)	14 (21)	17 (21)
Nebbiolo 423	3 (6)	5 (6)	4 (6)	6 (6)
Ruchè	2 (7)	6 (7)	6 (7)	7 (7)
Timorasso	8 (20)	13 (20)	12 (20)	14 (20)

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Chapter 3 – *Scaphoideus titanus* feeding behaviour

Article, Journal of Insect Physiology, submitted

Scaphoideus titanus Ball feeding behaviour on three grapevine cultivars with different susceptibilities to Flavescence dorée

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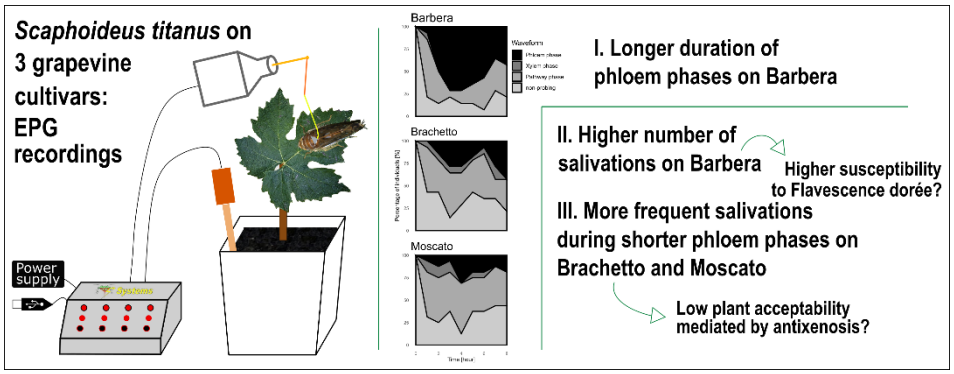
Keywords: EPG; Electrical Penetration Graph; leafhopper vector; *Vitis vinifera*; cultivar

Abstract

Scaphoideus titanus (Ball) is a grapevine-feeder leafhopper, and the most important vector of Flavescence dorée of grapevine (FD), a disease associated with phytoplasmas belonging to ribosomal subgroups 16Sr-V–C and –D. FD is a major constraint to viticulture in several European countries and, so far, its control has relied on roguing of infected plants and insecticide applications against the vector. Detailed knowledge on different levels of the multifaceted phytoplasma-plant-vector relationship is required to envisage and explore more sustainable ways to control the disease spread. In the present work, *S. titanus* feeding behaviour was described on three grapevine cultivars: Barbera (susceptible to FD), Brachetto, and Moscato (tolerant to FD) using the Electrical Penetration Graph (EPG) technique. Interestingly, no differences were highlighted in the non-phloem probing phases, thus suggesting that the tested cultivars have no major differences in the biochemical composition or structure of the leaf cuticle, epidermis or mesophyll, that can affect the first feeding phases. On the contrary, the results showed significant differences in leafhopper feeding behaviour in terms of the duration of the phloem feeding phase, longer on Barbera and shorter on Brachetto and Moscato, and of the frequency of interruption-salivation events inside the phloem, higher on Brachetto and Moscato. These findings indicate a preference for the Barbera variety, that appears a more suitable hosts for the leafhopper. *Scaphoideus titanus* feeding behaviour on Barbera correlates with an enhanced FDp transmission efficiency, thus explaining, at least in part, the higher susceptibility of this variety to

FD. The mechanisms for the non-preference for Brachetto and Moscato are discussed, and a possible antixenosis is hypothesized. We propose that breeding for resistance against FD should take into account both plant traits associated with the response to the phytoplasmas and to the vector.



Graphical abstract

1. Introduction

The leafhopper *Scaphoideus titanus* Ball is the main vector of phytoplasmas associated with the Flavescence dorée of grapevine (FD), a disease spread in most European viticultural countries (EFSA, 2020) that causes severe reduction of yield and quality of grapes, requires roguing of infected plants and leads to uneven-aged vineyards (Morone *et al.*, 2007). FD is associated with phytoplasmas belonging to the 16SrV group, subgroups –C and –D (Davis and Dally, 2001; Martini *et al.*, 2002; Lee *et al.*, 2004), and it causes severe losses to European viticulture (EFSA, 2016). Although different insect species are competent for the transmission of FD phytoplasmas (FDp), *S. titanus* is by far the most important vector, being strictly associated with *Vitis* plants and thus sustaining both primary (from wild grapevines outside the vineyards to

cultivated vines) and secondary (from vine to vine within the vineyard) disease spread (Maggi *et al.*, 2017; Ripamonti *et al.*, 2020). Control of FD relies on prophylactic measures, such as the use of healthy propagation material, as well as on compulsory measures in infected vineyards, namely roguing of infected plants, and insecticide treatments against the vector (Bosco and Mori, 2013). However, the large-scale application of insecticides is a concern to human health and environment, priming cascade ecosystem effects (Desneux *et al.*, 2007) with a strong negative impact on pollinators (Tosi *et al.*, 2018). For this reason, recent studies focused on identifying sources of resistance to FDp phytoplasmas within the grapevine germoplasm (Eveillard *et al.*, 2016; Ripamonti *et al.*, 2021), that would represent the best strategy to minimise damage and limit FD spread and insecticide applications. Grapevine tolerance to FDp may be due to a direct response of the plant against the pathogen or mediated by some resistance against the vector, or by a combination of the two. Resistance against insects occurs when plant structural or chemical traits deter herbivore feeding and thus minimize the amount of herbivore damage experienced by the plant, while tolerance occurs when plant traits reduce the negative effects of herbivore damage on crop yield (Mitchell *et al.*, 2016). As an example, it was demonstrated that resistant tea cultivars sustained lower phloem ingestions for *Empoasca vitis* (Miao *et al.*, 2014). Moscato and Brachetto are grapevine varieties tolerant to FD, as demonstrated by Ripamonti et al. (2021) using transmission experiments with *S. titanus* under controlled conditions (Ripamonti *et al.*, 2021). The

reduced *S. titanus* survival on Moscato observed by the above mentioned authors, suggest that vector-host interaction could be the pivotal factor underlying Moscato tolerance to FD. *S. titanus* is monophagous on *Vitis* species, mainly *Vitis vinifera* and naturalized rootstocks of *V. riparia* in Europe, while in North America, *V. labrusca* and *V. riparia* are reported as the preferred host plants (Chuche and Thiéry, 2014). Although the species is regarded as monophagous, it shows a good level of plasticity and can feed on plant species of different families, e.g. Vitaceae, Fabaceae, Ranunculaceae (Caudwell *et al.*, 1970; Trivellone *et al.*, 2013). Plant resistance against sap-sucking insects can be conveniently investigated by Electrical Penetration Graph (EPG), that describes the nutrition pattern of a sucking insect on a given plant genotype, by identifying possible altered nutrition on non-suitable genotypes (Backus *et al.*, 2020; Lucini *et al.*, 2021).

Here we expand the first findings on *S. titanus* behaviour on grapevines, by analyzing the vector probing behavior on three varieties with a different degree of susceptibility to FD: one susceptible, Barbera, and two tolerant, Moscato and Brachetto, through the Electrical Penetration Graph (EPG) (McLean and Kinsey, 1964; Tjallingii, 1978; Backus and Bennett, 2009).

EPG is a powerful tool to describe pierce-sucking insects' probing behaviour, previously applied to describe *S. titanus* feeding behaviour on Cabernet-Sauvignon cuttings (Chuche, Backus, *et al.*, 2017; Chuche, Sauvion, *et al.*, 2017). EPG studies on different plant cultivars/genotypes provide precious information for the

epidemiology of vector-borne plant pathogens, also permitting the identification of traits making a *Vitis* genotype unsuitable for the vector. A number of EPG studies aimed at identifying plant resistance to insect vectors have been performed on planthoppers (Kimmins, 1989), whiteflies (Jiang *et al.*, 2001; Rodríguez-López *et al.*, 2011) and aphids (Caillaud, Di Pietro, *et al.*, 1995; Caillaud, Pierre, *et al.*, 1995; Sauge *et al.*, 1998; Garzo *et al.*, 2018). Among these latter, EPG was applied to identify resistance factors involved in virus transmission inhibition (Chen *et al.*, 1997) as well as the presence of antixenosis (Kordan *et al.*, 2019). Besides those on *S. titanus* (Chuche, Backus, *et al.*, 2017; Chuche, Sauvion, *et al.*, 2017), few EPG studies have been conducted on Deltocephalinae leafhoppers (Kawabe and McLean, 1980; Lett *et al.*, 2001; Stafford and Walker, 2009; Carpane *et al.*, 2011; Trębicki *et al.*, 2012), and very few of these are relevant to phytoplasmas/mollicutes transmission (Carpane *et al.*, 2011; Chuche, Backus, *et al.*, 2017).

The aim of this study was to compare *S. titanus* probing behaviour on three different grapevine cultivars characterised by different susceptibilities to FD, in order to better characterize the mechanisms underlying varietal tolerance/susceptibility to this phytoplasma disease.

2. Materials and Methods

2.1. *S. titanus* collection and rearing

To establish a *S. titanus* laboratory colony, in January/February 2019 two-year-old grapevine canes with eggs were collected in

vineyards of the Piemonte Region. The selected sites were known to host a high population of the leafhopper in the previous summer, as estimated by yellow sticky traps captures of adults. The collected canes were stored in a cold room at 6±1°C, covered with plastic film to avoid egg desiccation, until use. When needed, grapevine canes were transferred into an insect-proof greenhouse at 24 ± 2°C and maintained damp by daily water spraying. After four weeks, canes were isolated in a cage together with a three-week-old broadbean plant as a food source for the nymphs. After egg hatching, the broadbean plant was replaced every three weeks. Nymphs were reared under controlled conditions inside a greenhouse chamber, T = 24 ± 2°C, with no humidity and photoperiod control, from the beginning of April to the end of September 2019. As FDp is not transovarically transmitted, and all the plants used for the rearing and the experiments were phytoplasma-free, all *S. titanus* used in the experiments were phytoplasma-free. For the EPG experiments, adults emerged from 7-21 days were used (modified from (Chuche, Backus, *et al.*, 2017), since in this time frame they were sexually mature, highly active and not subjected to high mortality (Mazzoni *et al.*, 2009; Bocca *et al.*, 2020).

2.2. Plant rearing

The test plants were obtained from phytoplasma-free *V. vinifera* cuttings of three different cultivars, Barbera N. - Clone I-AT 84, Brachetto N. - Clone I-CVT 20 and Moscato Bianco B. - Clone I-CVT 190 as described in Ripamonti *et al.* (2021). Grapevine cuttings were grown in a greenhouse at 24 ± 2°C, with no humidity

and photoperiod control, inside 0.9 L pots (2:2:1 topsoil, clay, perlite), and watered once a week. Cuttings were used when three- to five-months old, and periodically pruned in order to keep them within 80 cm height. Broadbean plants used for *S. titanus* rearing were seedlings maintained in a growth chamber (24 ± 2°C, with no humidity and photoperiod control) in 2.4 L topsoil, five per pot, and watered twice a week.

2.3. EPG setup and data analysis

Selected adults were collected and anesthetised with carbon dioxide for 5 seconds in a glass tube, then immobilised at the edge of a cut pipette tip connected to a vacuum pump under a stereomicroscope. A small drop of water-based silver glue (EPG Systems, Wageningen, The Netherlands) was placed on the pronotum of the insect, then a gold wire of 18 µm (previously attached with solvent-based silver glue (Ted Pella Inc., USA) to a 3 cm copper wire in turn attached to a brass nail with melted stain) was positioned on the dried drop, and covered with another small drop of silver glue. Before the EPG assay, insects were starved for a 30-minute period, during which they were attached to the electrode and hanged, inserting the nail in a polystyrene base.

The substrate voltage probe was inserted in well damped soil of a potted grapevine cutting, and *S. titanus*, attached to the assembled electrode, was connected to a probe and positioned onto the abaxial surface of a leaf. The feeding behaviour was then monitored for 8 hours with a Giga-8dd DC-EPG amplifier (EPG Systems, Wageningen, The Netherlands), inside a Faraday cage to isolate

the system from external electrical noise. Input resistance used was 1 giga Ohm, output set at 75x gain and plant voltage adjusted so that the EPG signal fitted into +5V and -5V. All recordings were done between June and August 2019, and started between 11:00 and 11:30 a.m. every day.

A total of 153 recordings were done, each day a total of 6 recording were run. Each single recording was represented by a different plant-insect combination, one male or one female on one grapevine plant. Potted plants of the three varieties were randomly arranged in the Faraday cage for every recording and discarded after use. In case of falling from the leaf, the insect was repositioned. At the end of the recording, dead insects were noted and excluded from further analyses.

2.4. EPG acquisition and marking of EPG files

Recordings were acquired and marked using Stylet+ software (v01.30, Electrical Penetration Graph Data Acquisition and Analysis, EPG Systems, Wageningen, The Netherlands). Waveform marking was conducted accordingly to Chuche *et al.* (Chuche, Backus, *et al.*, 2017) and Stafford & Walker (2009), focusing on the following waveforms: np (non-probing activity), pathway-phase (phase “C”), active ingestion (phase “G”) of mesophyll (<60 seconds) or xylem sap (>100 seconds) (see (Stafford and Walker, 2009), passive ingestion of phloem sap (phase E), interruptions during ingestion (phase N of Chuche, Backus, *et al.*, 2017). For more details, see Supplementary File S1.

Once marked, all the recordings were singly selected for the successive analysis. In particular, recordings with electrical noises, bad electric connections, or when insects fell from the plant for more than 20% of the recording time, were discarded from further analysis.

2.5. Statistical analysis

All the statistical analyses were conducted on R software v4.0.3 (R Core Team., 2020). Selected recordings were analysed through a package of the software R ad-hoc produced for the analysis on EPG recordings, called Rwaves (Chiapello, https://github.com/mchiapello/Rwaves). Rwaves conducts summary statistics on the input recordings on a set of variables of EPG analysis (Table 1), producing a table including the values of all the variables for all the input recordings. The resulting table was composed as follows: every row corresponded to a single recording (represented by the unique combination of one leafhopper and one grapevine plant), while every column represented a single EPG variable. Once obtained, the table was subjected to modifications to enhance readability (packages dplyr, tidyr, stringr: (Wickham, 2019, 2020; Wickham *et al.*, 2020), and descriptive statistics were run (Tables 3, 4, 5, 6 and Supplementary File S2). Univariate analyses were conducted starting from Generalised Linear Model (GLM) of different families specific for the nature of the variable: quasi-Poisson or negative-binomial for counts, Gamma or inverse-Gaussian for positive continuous variables, beta-regression for proportions (packages stats, betareg, MASS: Cribari-Neto and

Zeileis, 2010; Venables and Ripley, 2002). Goodness-of-fit for every model was evaluated plotting half-normal plots with simulated envelope against deviance residuals, with 95% confidence level (hnp package: Moral *et al.*, 2017). Homoscedasticity for every model was evaluated through Levene's test (car package: Fox and Weisberg, 2019). In case of rejection of the null hypothesis, heteroscedasticity-consistent standard errors (sandwich package: Zeileis *et al.*, 2020) were calculated and considered for pairwise-comparisons. Comparisons among groups were conducted with least-square means method and Tukey method for p-value adjustment, at 0.05 significance level and 95% confidence intervals (packages emmeans and multcomp: Hothorn *et al.*, 2008; Lenth, 2020). Cultivar, Sex, and their reciprocal interaction were selected as explanatory variables. If no significant effects were found for Sex and Cultivar × Sex, the GLM was run with Cultivar as the only explanatory variable. GLMs summaries were reported in Supplementary File S3 using package jtools (Long, 2020). Packages ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2020) were used to produce Figure 1, and Supplementary File S4 and S5.

A multivariate Canonical Correspondence Analysis (CCA, Legendre and Legendre, 2012) was conducted through the vegan (Oksanen *et al.*, 2019) and ggordiplots packages (Quensen, 2018), considering all the variables except multi-collinear ones, that were excluded from the analysis, based on a correlation coefficient higher than 0.95 (usdm package: Naimi *et al.*, 2014), in order to strengthen

the predictor value of the model. Starting from 25 variables, 5 variables were found to have collinearity problem, and were thus excluded from further analyses. The remaining variables were standardised (Hellinger method, Legendre and Gallagher, 2001) and subjected to CCA, with Cultivar, Sex and their interaction as explanatory variables. The CCA result was confirmed through a permutational Multivariate Analysis of Variance (perMANOVA; Anderson, 2001).

The complete R code will be made publicly available on GitHub (https://github.com/matteo-rpm).

Table 1. EPG variable selected for the study.

Variable	Abbreviation from Sarria *et al.*, 2009 (implemented in Rwaves)	Abbreviation from Backus *et al.*, 2007	Type (NS: non-sequential; S: sequential)
"Number of non-probing periods"	n_np	NWEi np	NS
"Total duration of non-probing periods [s]"	s_np	WDi np	NS
"Time from 1st np to 1st probe [s]"	s_npto1stprobe	-	S
"Duration of the 2nd non-probing period [s]"	s_2np	-	S
"Number of probes"	n_Pr	NPi	NS
"Total probing time [s]"	s_Pr	PDi	NS
"Total duration of pathway phase [s]"	s_C	WDi C	NS
"Number of active ingestion phases"	n_G	NWEi G	NS
"Total duration of active ingestion [s]"	s_G	WDi G	NS
"Number of phloem ingestions"	n_E2	NWEi E2	NS
"Number of sustained (> 600 s) phloem ingestion"	n_sE2	NWEi sE2	NS
"Total duration of phloem ingestions [s]"	s_E2	WDi E2	NS
"Mean duration of a single event of phloem ingestion [s]"	mean_E2	WDEi E2	NS
"Duration of the longest phloem ingestion [s]"	s_longestE2	-	NS
"Total duration of non-phloematic phases [s]"	s_notE	WDi C-G	NS

"Time from 1st probe to 1st phloem ingestion [s]"	t_1E2.exp	-	S
"Time from 1st probe to 1st sustained (> 600 s) phloem ingestion [s]"	t_1sE2.exp	-	S
"Time of 1st sustained phloem phase [s]"	t_1st_sE2	-	S
"Percentage of probing time spent in phloem ingestion [%]"	percprobtime_E2	-	NS
"Percentage of probing time spent in pathway-phase [%]"	percprobtime_C	-	NS
"Percentage of probing time spent in active ingestion [%]"	percprobtime_G	-	NS
"Potential E2 index [%]"	E2index	-	S
"Mean frequency of Np interruptions during phloem phase [mHz]"	mean_fr_Ninterrup	-	NS
"Percentage of time spent in Np interruption during phloem phase [%]"	percNinterrup_E2	-	NS
"Number of Np interruptions during phloem ingestion"	n_Ninterrupt_E2	NWEi Np	NS

3. Results

Number of recordings obtained from male and female *S. titanus* adults on the three grapevine varieties are summarised in Table 2. In particular, 51-cultivar specific recordings were acquired, of which

a fraction was selected for further analysis (31 for Barbera, 32 for Brachetto, 37 for Moscato), as described in Material and Methods section.

Table 2. Number of total and selected recordings of *S. titanus* feeding behaviour on three grapevine cultivars.

Cultivar	Total recordings (females, males)	Selected recordings (females, males)
Barbera	51 (25, 26)	31 (18, 13)
Brachetto	51 (25, 26)	32 (13, 19)
Moscato	51 (25, 26)	37 (16, 21)

No significant differences in acquiring successful EPG signals among cultivars, possibly caused by human errors, were found (Pearson's Chi-squared test, X-squared = 0.36811, df = 2, p-value = 0.8319). From now on, when referring to recordings, only the selected ones will be considered, unless otherwise stated.

Irrespective of the cultivar, most of the insects started probing within the first minute from their access to the leaf (median ± SE = 41 ± 21 s). Waveforms were graphically summarised in a temporal progress representation (Figure 1). An overall larger area of phloem phase was found for leafhoppers feeding on the Barbera variety.

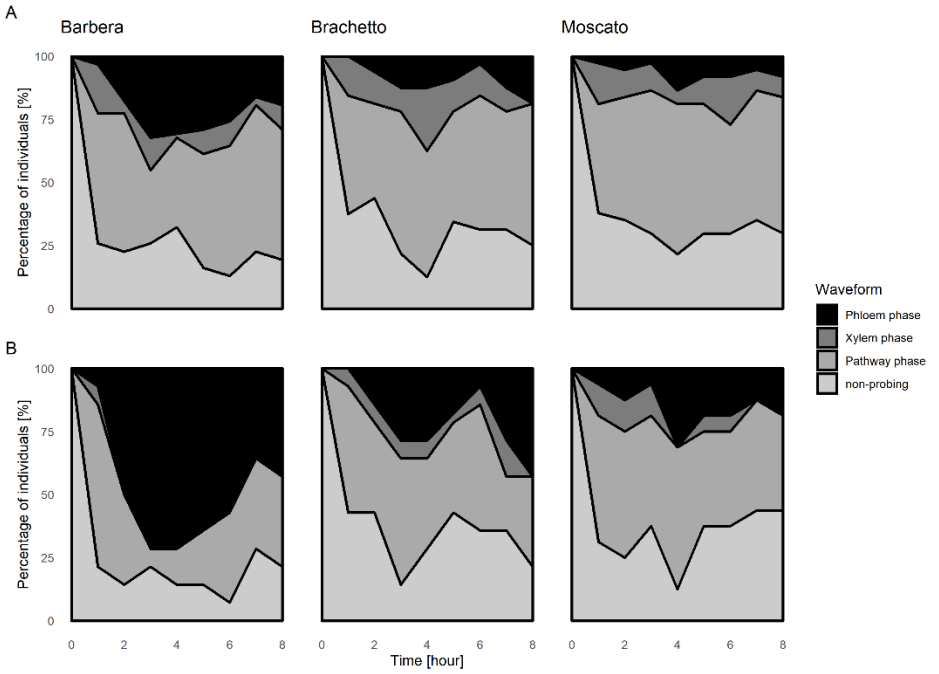


Figure 1. Temporal progress of *S. titanus* stylets activities on three grapevine cultivars during the 8-h EPG recording. Probing behaviours were represented as percentages of leafhoppers in a given phase (non-probing, pathway phase, active ingestion of xylem sap, passive ingestion of phloem sap) at 1 h intervals, starting from hour 0 (start of the recording) to hour 8 (end of the recording). a) Graphs produced considering all recordings; b) graphs produced considering only recordings where a phloem phase was present. The total number of recordings used to produce Figure 1 are reported in the third column of Supplementary File S2 (all recordings) and Table 3 (phloem recordings).

Values of non-phloem variables of all selected recordings are reported in Supplementary File S2. No differences were identified in the variables among the three grapevine varieties. The proportion of recordings with phloem phases were not significantly different among cultivars (Supplementary File S6, Pearson's Chi-squared test, X-squared = 0.026378, df = 2, p-value = 0.9869), as almost half of the recordings (45% for Barbera, 44% for Brachetto, 43% for Moscato) showed phloem phases, irrespective of the cultivar

(Supplementary File S6). No differences were highlighted among cultivars for all the non-phloem variables (Supplementary File S5), when considering the recordings without a phloem-phase. Further, the non-phloem variables were analysed for recordings with phloem phases (Table 3). Number of events and their duration for the non-phloem phases did not differ among groups (Table 3). Interestingly, the total time spent by the insect with stylets inserted in the plant tissues ("Total probing time") were similar among the three *Vitis* genotypes. Some differences were found for the related variables "Number of non-probing periods", and "Number of probes", as higher values were recorded for both variables on Brachetto, compared to Barbera. On Barbera, females showed fewer "Number of active ingestion (from mesophyll or xylem) phases" than males. No differences were observed between sexes on the other varieties. No significant differences among cultivars were found for the "Number of phloem ingestions", or for the "sustained" (longer than 10 minutes) ones (Table 4). Although the "Mean duration of a single event of phloem ingestion" did not differ significantly among cultivars, a longer duration of phloem ingestion events on Barbera was evident. Indeed, significant differences were found for "Total duration of phloem ingestions", "Duration of the longest phloem ingestion", and "Time from first probe to first sustained phloem ingestion" between Barbera and the other two grapevine varieties (Table 4). "Time from first probe to first phloem ingestion" was shorter on Barbera compared to Moscato, with an intermediate duration recorded on Brachetto (Table 5). This also suggests a preference of *S. titanus* for Barbera. For the "Total duration of non-

phloematic phases", for which an effect for the leafhopper sex was found, a difference was recorded between *S. titanus* feeding on Barbera and on Moscato, at least for females. *Scaphoideus titanus* also spent a higher percentage of time in the phloem ingestion phase on Barbera, compared to Brachetto and Moscato varieties and, consequently, less time in pathway- and active ingestion phases (Table 5). Since the presence of "Np" (typical interruption between two different passive ingestion phases) in phloem phases has been repeatedly recorded (Chuche, Backus, *et al.*, 2017; Supplementary File S1 of the present work), three variables were introduced for their description in the present work and are reported in Table 6: "Mean frequency of Np interruptions during phloem phase", "Percentage of time spent in Np interruption during phloem phase", and "Number of Np interruptions during phloem ingestion". The second and third variables showed significant differences between leafhoppers feeding on Barbera and those feeding on the other varieties, underlying different phloem feeding behaviour on the former variety.

Tables 3, 4, 5, 6. Median ± SE of non-phloem variables related to recordings presenting phloem phases. Every row reports a single combination of grapevine Cultivar and leafhopper Sex. Every column reports a specific variable. Comparisons between rows were done with a specific GLM family for every variable: quasi-Poisson or negative-binomial for counts, Gamma or inverse-Gaussian for continuous time variables, beta-regression for proportions. Cultivar, Sex and their interaction (Cultivar × Sex) effects for every variable were evaluated. In case of no effect for Sex and Cultivar × Sex, the GLM was run with only Cultivar as explanatory variable (indicated in the tables with the * sign after the specific variable name). In case of effect for Sex or Cultivar × Sex, GLM was run with all the three explanatory variables (indicated in the tables with the ** sign after the specific variable name). Post-hoc comparisons were conducted with least-square means method and Tukey method for p-value adjustment, at significance level as 0.05 and 95% confidence intervals, and represented by letters for every specific group. GLMs specific details (family, coefficients, standard errors, AIC, BIC, R2) are reported in Supplementary File S3a-b.

Table 3. Median ± SE of non-phloem variables related to recordings presenting phloem phases.

Cultivar	Barbera	Barbera	Brachetto	Brachetto	Moscato	Moscato
Sex	female	male	female	male	female	male
n	8	6	6	8	7	9
Number of non-probing periods *	9.5 ± 1.7 a	14 ± 1.9 a	21 ± 6.1 b	16 ± 2 b	16 ± 3.3 ab	18.5 ± 3.5 ab
Total duration of non-probing periods [min] *	80.8 ± 22.8 a	32.3 ± 47.7 a	164.8 ± 32.1 a	174.8 ± 20.9 a	105 ± 24.9 a	168.8 ± 28.3 a
Time from 1st np to 1st probe [s] *	33.5 ± 41.5 a	19 ± 11.8 a	48.5 ± 216.8 a	63.3 ± 80.7 a	87.6 ± 169.5 a	54.6 ± 24.2 a
Duration of the 2nd non-probing period [s] *	108 ± 143.4 a	22.1 ± 43.8 a	45.9 ± 80.9 a	42.5 ± 48.5 a	40.9 ± 96 a	22.9 ± 79.5 a
Number of probes *	9.5 ± 1.8 a	12.5 ± 1.9 a	21 ± 6.1 b	15.5 ± 2 b	16 ± 3.2 ab	17 ± 3.6 ab
Total probing time [min] *	397.2 ± 22.8 a	446.6 ± 47.5 a	313.9 ± 31.8 a	303.9 ± 20.5 a	374.5 ± 25 a	310.5 ± 28.3 a
Total duration of pathway phase [min] **	96.1 ± 18.5 b	124.9 ± 36.6 ab	175.1 ± 30.4 ab	160.3 ± 25 ab	231.5 ± 36.5 a	154.9 ± 24.7 ab
Number of active ingestion phases **	8 ± 6.9 a	42 ± 11.1 b	52 ± 23.3 b	51 ± 15.3 b	47 ± 9.9 b	33 ± 14.3 b
Total duration of active ingestion [min] *	3.2 ± 3.8 a	21.2 ± 8 a	34.3 ± 13.7 a	36.9 ± 11.6 a	26.3 ± 7.5 a	22.5 ± 19.5 a

Table 4. Median ± SE of phloem variables related to recordings presenting phloem phases.

Cultivar	Sex	n	Number of phloem ingestions *	Number of sustained (> 600 s) phloem ingestion *	Total duration of phloem ingestions [min] *	Mean duration of a single event of phloem ingestion [min] *	Duration of the longest phloem ingestion [min] *	Total duration of non-phloematic phases [min] **	Time from 1st probe to 1st phloem ingestion [min] *	Time from 1st probe to 1st sustained (> 600 s) phloem ingestion [min] *	Time of 1st sustained phloem phase [min] *
Barbera	female	8	13 ± 2.7 a	2 ± 0.4 a	301.9 ± 37.7 a	23.4 ± 11 a	240.2 ± 38.4 a	107.4 ± 20.1 b	76.7 ± 9.5 b	111.7 ± 27.3 b	114.1 ± 27.1 b
Barbera	male	6	14 ± 1.3 a	2.5 ± 0.7 a	190.3 ± 48.4 a	12.7 ± 3.1 a	158.3 ± 41.3 a	156.2 ± 37 ab	104.4 ± 32.9 b	131.1 ± 34.2 b	133.5 ± 34.1 b
Brachetto	female	6	7 ± 3.8 a	1 ± 0.6 a	83 ± 33.2 b	5.7 ± 4 a	50.2 ± 25.6 b	208.1 ± 43.2 ab	135.4 ± 15.3 ab	156.6 ± 63.7 a	157.1 ± 63.3 a
Brachetto	male	8	18 ± 5.9 a	2 ± 0.6 a	53.9 ± 26.1 b	3.5 ± 7.9 a	38 ± 13.9 b	237.6 ± 25.6 a	167.1 ± 43.4 ab	229.2 ± 49.2 a	240.5 ± 47 a
Moscato	female	7	10.5 ± 4.5 a	1 ± 0.4 a	37.6 ± 42.7 b	3.1 ± 3.5 a	20.2 ± 30.9 c	283.4 ± 44.6 a	187.1 ± 50.8 a	267.8 ± 62.3 a	270.7 ± 65.1 a
Moscato	male	9	25.5 ± 3.8 a	1 ± 0.3 a	47 ± 11.8 b	2.3 ± 0.6 a	13 ± 7.1 c	257 ± 33.8 a	107.8 ± 54.6 a	203.9 ± 55.8 a	208 ± 55.5 a

Table 5. Percentage variables related to recordings presenting phloem phases.

Cultivar	Sex	n	Percentage of probing time spent in phloem ingestion [%] *	Percentage of probing time spent in pathway-phase [%] **	Percentage of probing time spent in active ingestion [%] **	Potential E2 index [%] *
Barbera	female	8	75 ± 6.6 b	22.4 ± 6 a	0.7 ± 1.1 a	77.7 ± 8.1 a
Barbera	male	6	51.2 ± 9.4 b	38.5 ± 9.2 ab	8.3 ± 1.7 ab	66.1 ± 16 a
Brachetto	female	6	26.9 ± 9.3 a	60 ± 7.1 bc	13.4 ± 3.2 ab	29.1 ± 7.7 a
Brachetto	male	8	14.6 ± 7.4 a	54.3 ± 5.3 bc	9.5 ± 4.2 b	48.3 ± 10.2 a
Moscato	female	7	11.6 ± 10.9 a	75.9 ± 8 c	9.9 ± 2 ab	48.6 ± 14.2 a
Moscato	male	9	11.9 ± 4.4 a	55.4 ± 4.9 bc	7.5 ± 3.9 b	32.1 ± 9.5 a

Table 6. "Np" phloem-interruptions variables related to recordings presenting phloem phases.

Cultivar	Sex	n	Mean frequency of Np interruptions during phloem phase [mHz] **	Percentage of time spent in Np interruption during phloem phase [%] *	Number of Np interruptions during phloem ingestion *
Barbera	female	8	9.4 ± 0.8 b	1.4 ± 0.7 a	148.5 ± 27.2 b
Barbera	male	6	14.6 ± 1.9 ab	3 ± 1.6 a	167.5 ± 58 b
Brachetto	female	6	16.7 ± 1 ab	6.8 ± 5.8 ab	88 ± 27.8 a
Brachetto	male	8	19 ± 3.1 a	9.7 ± 7.3 ab	38.5 ± 26.2 a
Moscato	female	7	15.5 ± 3.4 a	16.2 ± 4.6 b	44 ± 20.6 a
Moscato	male	9	19.8 ± 2 a	11.8 ± 5.3 b	53 ± 16.1 a

A constrained Canonical Correspondence Analysis (CCA) was conducted to explore the comprehensive effect of the explanatory variables Cultivar, Sex, and their interaction with *S. titanus* feeding behaviour (Figure 2). The CCA is a graphical representation of the

non-multi-collinear variables more related to the different groups. In particular, considering the absence of effect for Sex and Cultivar × Sex (Table 7), ellipses were drawn containing 99% confidence intervals for the standard errors related to Cultivar variable. Again, this representation highlighted the difference between *S. titanus* feeding behaviour on Barbera, on one side, and on Brachetto and Moscato, on the other side. Moreover, CCA shows a clear correlation between phloem variables and Barbera cultivar.

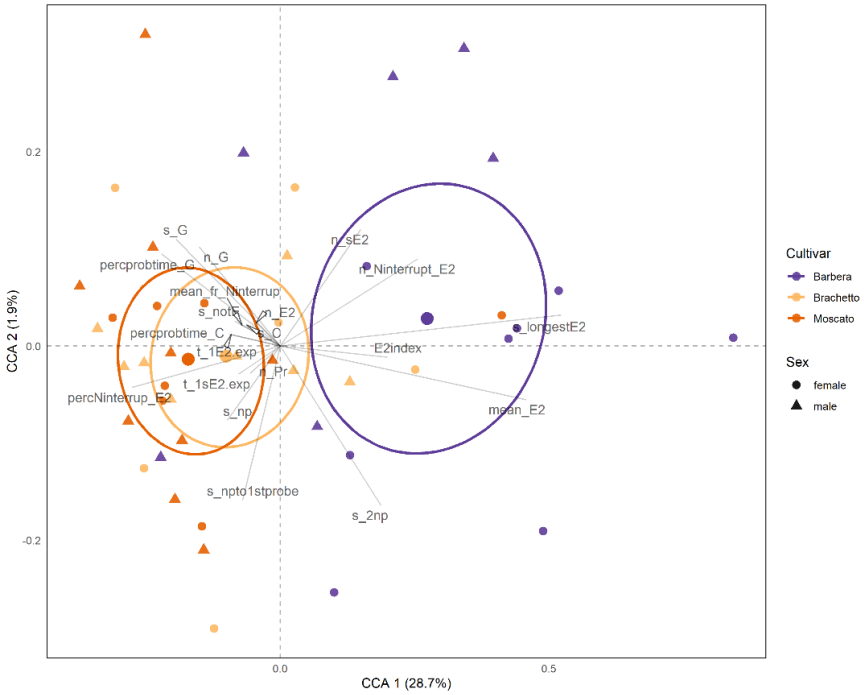


Figure 2. Canonical Correspondence Analysis (CCA) on recordings with phloem phases. The new condensed CCA variables explained 28.7% (CCA 1, x axis) and 1.9% (CCA 2, y axis) of the variability. Cultivar-specific recordings were grouped with ellipses, representing 99% confidence intervals for the standard errors, and the centroid of each was represented. Every point represents a single recording, colour refers to the grapevine cultivar and shape refers to the leafhopper sex. Original variables were plotted and reported with their acronym (Table 1 for acronym interpretation); all variables start from the intersection of the axes and are projected according to their unique composition of CCA 1 and 2.

Results of the CCA were confirmed through a perMANOVA (Table 7), which highlighted significant differences among Cultivars, while no significative differences were found for Sex or the interaction of Cultivar and Sex.

Table 7. perMANOVA results based on Bray-Curtis dissimilarities, using all the non-multi-collinear EPG variables (as described in Materials & Methods section). Df: degrees of freedom; SumOfSqs: sequential sums of squares; F: F statistics values by permutations; Pr(>F): p-values, based on 9999 permutations (the lowest possible p-value is 0.0001).

	Df	SumOfSqs	R2	F	Pr(>F)	signif
Cultivar	2	0.21459034	0.24924254	6.867267	0.0001	***
Sex	1	0.02922068	0.03393925	1.870225	0.1269	
Cultivar × Sex	2	0.02344144	0.02722678	0.750167	0.6142	
Residual	38	0.59371753	0.68959144	NA	NA	
Total	43	0.86096998	1	NA	NA	

4. Discussion

In this work, the probing behaviour of the FD leafhopper vector *S. titanus* on grapevine varieties with different susceptibility to the disease was analysed, to highlight possible differences that can account for different transmission efficiencies. As phytoplasmas are phloem-limited in the plant, vector acquisition and transmission abilities are related to phloem feeding phases, and thus a plant genotype that does not sustain efficient phloem feeding may be less prone to infection.

To understand if probing behaviour of *S. titanus* may contribute to explain tolerance/susceptibility mechanisms of grapevine genotypes, the FD highly susceptible Barbera and the FD tolerant Brachetto and Moscato varieties (Ripamonti *et al.*, 2021) were compared. Indeed, *S. titanus* showed a feeding preference for the FD highly susceptible Barbera variety. To describe *S. titanus*-grapevine interaction, total probing time was subdivided into different probing phases, mainly related to the inter/intra-cellular movements of the stylets (pathway-phase), the active ingestion of mesophyll or xylem sap, the passive ingestion of phloem-sap.

A preference of the leafhopper for Barbera was suggested at first by the overall higher proportion of *S. titanus* feeding on phloem of this variety (larger area under the phloem phase), compared to Brachetto and Moscato in the temporal progress area graph. However, it is possible that duration of phloem phases was underestimated in this study, as well as in those of Chuche et al. (Chuche, Backus, *et al.*, 2017; Chuche, Sauvion, *et al.*, 2017), and indeed longer recording times can possibly highlight longer durations of phloem ingestion, as hypothesized for *Dalbulus maidis* (Carpane *et al.*, 2011). Under our experimental setting, eight-hour recordings were long enough to allow 50% of the insects to reach the phloem phase, irrespective of the cultivar. This recording time was chosen for the experiments as it represents a widely used standard in EPG studies, and because in previous experiences, Chuche et al. (Chuche, Sauvion, *et al.*, 2017) showed that, in average, 27% of the *S. titanus* probing time was spent in phloem

feeding phases with four hour recordings. According to our results, most of the cultivar-dependent differences in *S. titanus* lies in phloem feeding behaviour. Actually, leafhoppers spent more than 50% of their probing time feeding on the Barbera phloem, while on the other two cultivars spent less than 20%. This result is in line with an enhanced possibility of acquisition and inoculation of phloem-limited agents, like FDp in the case of Barbera (Galetto *et al.*, 2014). Although the "Potential E2 index", a parameter regarded as a reliable indicator of phloem acceptability (Girma *et al.*, 1992; Alvarez *et al.*, 2006), was not significantly different among the tested cultivars, higher values were recorded for Barbera, further supporting a possible preference of the leafhopper for this cultivar. Moreover, since only half of the vectors reached the phloem phase during the 8-hour recordings, we cannot exclude that the amount of time was not sufficient to obtain a more descriptive feeding behaviour from all leafhoppers. Dramatic differences were highlighted in the "Total duration of phloem ingestions" on the different cultivars, while the "Number of phloem ingestions" and "sustained phloem ingestions" were similar. The former was actually the variable accounting for the highest differences in phloem phase among cultivars, and suggests that *S. titanus* prefers Barbera phloem to Brachetto or Moscato ones. Since no differences were recorded among cultivars in the percentage of leafhoppers reaching phloem, but "Total duration of phloem ingestion" and "Duration of the longest phloem ingestion" were higher on Barbera, it can be hypothesized that Brachetto and Moscato phloem saps contain some repellent compounds disturbing phloem feeding. During the

phloem phase, the main waveforms were related to the passive ingestion of phloem and to the interruption between two different passive ingestion phases (mainly “Np”). These interruptions were already described for *S. titanus* by Chuche et al. (Chuche, Backus, *et al.*, 2017) and for *Circulifer tenellus* by Stafford and Walker (2009), and were suggested to represent salivation events. Two are the main functions of saliva in piercing-sucking insects: i) production of stylets sheath in the inter-cellular pathway phase (sheath saliva) or ii) dilution of to-be-ingested sap and the suppression of defensive mechanism by the plant through effectors (watery saliva) (Miles, 1972; Tjallingii, 2006; Will *et al.*, 2013). According to Chuche et al. (Chuche, Backus, *et al.*, 2017) and Stafford and Walker (2009), the “Np” interruptions found during *S. titanus* ingestion of phloem sap correspond to watery-salivation events. This type of salivation is related to the inoculation of persistent-propagative agents from the insect salivary glands into the plants tissues (Hogenhout *et al.*, 2008). Therefore, the greater number of interruption-salivation events on Barbera, that are a reflection of the longer phloem phase, can explain, at least in part, the high susceptibility to FDp of this cultivar. Phytoplasma spread can be regarded as a function of insect acquisition efficiency, which is directly related to the duration of the phloem feeding phase, and of the inoculation efficiency, which is putatively related to the absolute number of watery salivation events, these latter also occuring during phloem feeding phase. According to this hypothesis, on Barbera, the vector acquires and transmits efficiently, because it feeds longer in the phloem and produces a higher number of salivation events

compared to Brachetto and Moscato. Indeed, Galetto et al. (2016) demonstrated that FDp acquisition by *S. titanus* depends on the grapevine variety, with high efficiency from the most susceptible ones. Also, on Brachetto and Moscato a high frequency of Np interruptions events was recorded, but phloem phase was much shorter, leading to a lower absolute number of salivation events. It is worth noting that, when the three grapevine varieties were exposed to equally infected leafhoppers, Brachetto and Moscato showed a strong tolerance against the infection (Ripamonti *et al.*, 2021). This is a clear indication that either the inoculation, more than acquisition, has a major impact on transmission efficiency, or plant genotype account for different susceptibilities. The high frequency of Np interruptions on the tolerant varieties can be explained by the presence of repellent compounds in the phloem saps. Brachetto and Moscato are aromatic varieties (Pollon *et al.*, 2019) and they are genetically related (Raimondi *et al.*, 2020). Their leaves contain high quantities of terpenoids (Mazza *et al.*, 2003), and this class of compounds can be transferred through the plant via the phloem flux (Zhang *et al.*, 2016) like other defence compounds (Will *et al.*, 2013). Hence, it can be speculated that *S. titanus* disturbed behaviour may be associated with the presence of aromatic compounds, that act as repellents in Brachetto and Moscato phloems. Repellent compounds can therefore act as antixenotic compounds. Antixenosis, defined as the modification of herbivore behaviour by plant factors, which results in the inability of a plant to serve as a host (Kogan and Ortman, 1978; Kordan *et al.*, 2019), is a well-known factor determining host plant resistance.

Terpenoids and other volatile compounds have well-known antixenotic activities in different plant-insect interactions (Messchendorp *et al.*, 1998; Koul, 2008; Chand *et al.*, 2017). Antixenosis may represents a valuable factor to be considered in the development of grapevine resistance against *S. titanus*, *de facto* causing a reduction in the inoculation efficiency of FDp. Indeed, leafhopper survival is reduced following a 7 day exposure to Moscato compared to Barbera (Ripamonti *et al.*, 2021). Further research is needed to clarify possible Moscato antibiosis effect on *S. titanus*.

In our study, the leafhoppers started probing within the first minute, regardless of the grapevine variety. No evident differences were highlighted in the non-phloem related variables, as well as on total probing time. These results suggest that tested cultivars have no major differences in the biochemical composition or structure of the leaf cuticle, epidermis or mesophyll, that can impact the first feeding behaviour phases. Grapevine trichomes are of the non-glandular type, subdivided in prostrate or erect (Gago *et al.*, 2016). Interestingly, Barbera has a highly dense trichomes surface in the abaxial leaf blade (OIV, 2007), suggesting a possible repellence towards piercing-sucking insects (Smith and Chuang, 2014). Nevertheless, Barbera was the most suitable variety for *S. titanus* among those tested. For leafhoppers, data on trichome density acceptability are available mainly for species of the Empoascini tribe, that are mostly insensitive to trichome density on leaves. This is the case of *Empoasca vitis* on grapevine (Pavan and Picotti,

2009), *E. terminalis* on soybean (Nasruddin *et al.*, 2014), and *E. fabae* on potato (Kaplan *et al.*, 2009). On the other hand, *E. fabae* and *Amrasca devastans* tend to avoid high trichome density when feeding on edamame (*Glycine max* (L.)) and cotton, respectively (Murugesan and Kavitha, 2010; Menger *et al.*, 2018). As for *S. titanus,* it can be concluded that a dense abaxially pubescence does not hamper nutrition on grapevine.

This work failed to identify clear differences in feeding behaviour of males and females. Although small differences between sexes were recorded for some variables, no differences were highlighted in the multivariate analysis conducted through CCA followed by perMANOVA. On the contrary, Chuche et al. (Chuche, Sauvion, *et al.*, 2017) reported that males feed more in the phloem, compared to females. Following the analysis of our EPG recordings, we conclude that no clear differences in feeding behaviour can be identified. Although unlikely, we cannot exclude that the different grapevine varieties used in the studies may explain for this difference.

Future research should focus on antixenotic compounds in *V. vinifera* genotypes, and their role in vector-associated resistance to FD. On the other hand, plant secondary metabolites involved in defense mechanisms against pathogens, such as polyphenols, particularly vein flavonols and flavanonols (Kedrina-Okutan *et al.*, 2018, 2019) may play a role in plant resistance towards the phytoplasma. All these grapevine genetic traits should be regarded

as a natural resource to be exploited to obtain tolerant genotypes for a more sustainable viticulture.

5. Conclusions

The results of the present work indicate that Barbera variety is a better food source than Brachetto and Moscato for *S. titanus*. Indeed, the leafhopper showed longer phloem ingestion, with an absolute higher number of watery-salivation events, on grapevines of the Barbera cv. This latter feature is consistent with the high susceptibility of Barbera to FDp, as watery salivation has been associated with the inoculation of persistent-propagative agents from the insect salivary glands into the plants tissues. When feeding on Brachetto and Moscato, *S. titanus* showed reduced phloem nutrition, possibly due to antixenotic factors such as terpenoids, given the aromatic nature of the two varieties.

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Supplementary Material

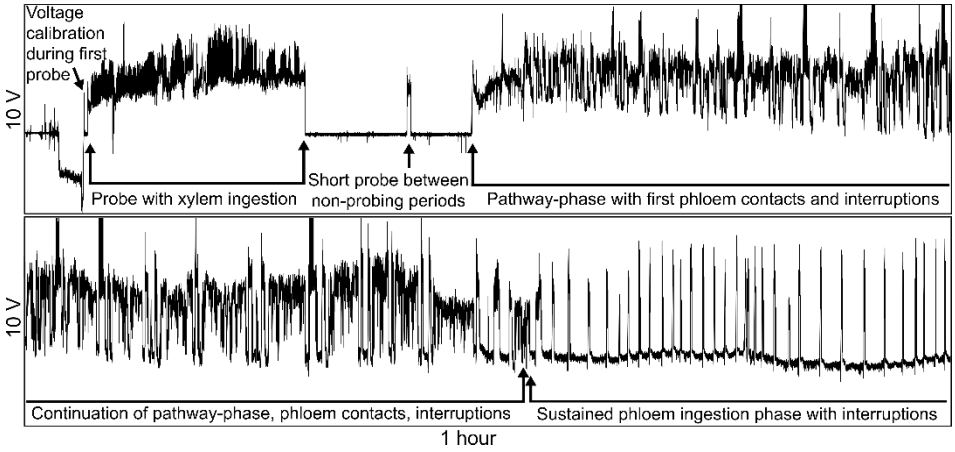
Supplementary File S1, Table S1. Summary and graphic representation of the main waveform characteristics for *Scaphoideus titanus* on grapevine.

Waveform	Putative plant tissue*	Amplitude ± SE [V]	Amplitude range (min - max) [V]	Amplitude ± SE [%] **	Amplitude range (min - max) [%] **	Frequency ± SE [Hz]	Frequency range (min - max) [Hz]	Voltage level ***
Pathway-phase	Epidermis and mesophyll	2.37 ± 0.17	0.3 - 5	23.68 ± 1.74	mar-50	Mixed	-	e/i
Active ingestion	Mesophyll (< 60 s) and xylem (> 100 s)	2.41 ± 0.16	0.8 - 6.3	24.13 ± 1.61	ago-63	4.28 ± 0.1	2.5 - 6	e
Passive ingestion	Phloem	0.33 ± 0.03	0.07 - 0.9	3.31 ± 0.28	0.7 - 9	3.69 ± 0.13	02-giu	i
"Np" interruption	Phloem	8.22 ± 0.23	4.8 - 11.3	82.18 ± 2.28	48 - 113	25.27 ± 0.34	20 - 32	e/i

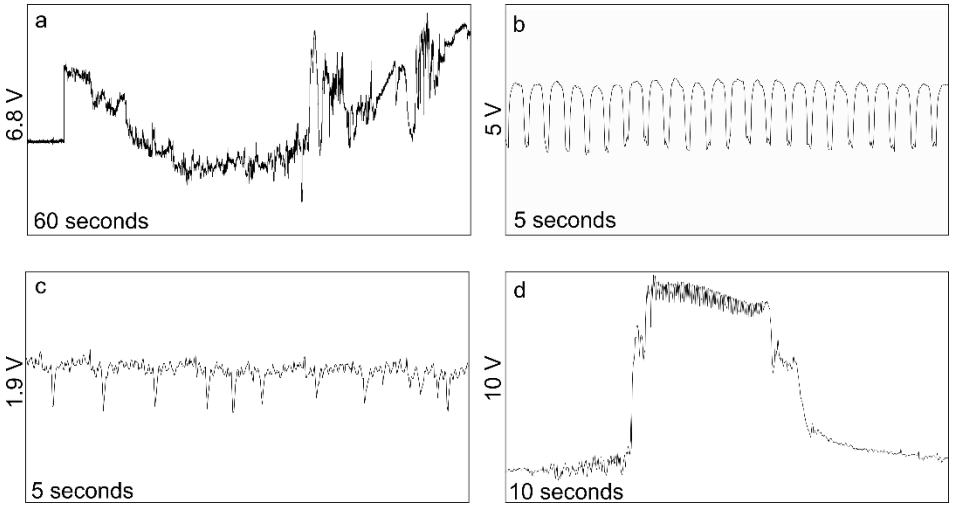
* Putative plant tissue suggested from previous studies (Chuche et al. 2017a; Stafford & Walker, 2009)
** 100% amplitude = 10 V
*** e = extracellular; i = intracellular

Values presented in Table S1 were calculated starting from thirty insect-recordings, ten per cultivar, randomly selected among recordings presenting phloem phases. For each insect, two replicates for the same waveform were randomly selected in all the 8-hour recording, resulting in a total of 60 replicates for every waveform. Examples of the waveform are graphically represented in the figures below.

Supplementary File S1, Figure I. Overall view of a 2-hour recording, presenting all the phases described in the table and in the main text.



Supplementary File S1, Figure II. Specific waveforms view: a) non-probing, followed by the beginning of a probe as pathway-phase, b) active ingestion of mesophyll or xylem sap, c) passive ingestion of phloem sap, d) "Np" interruption during phloem phases (watery salivation).

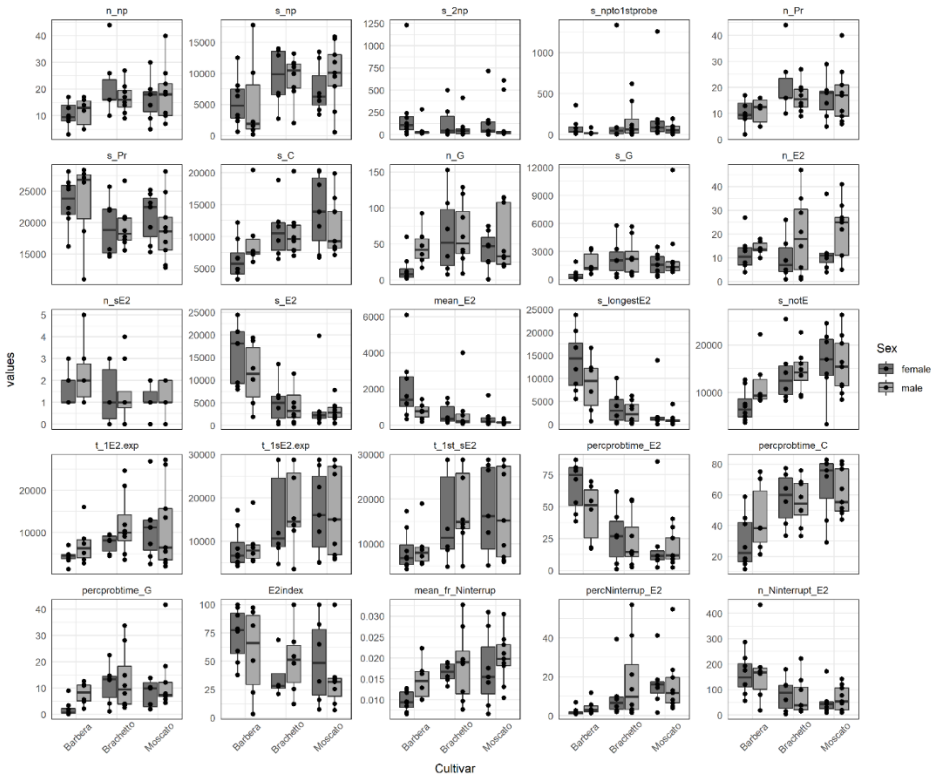


Supplementary File S2. Median ± SE of non-phloem variables related to all recordings. The Table was drawn as Table 3 (main text).

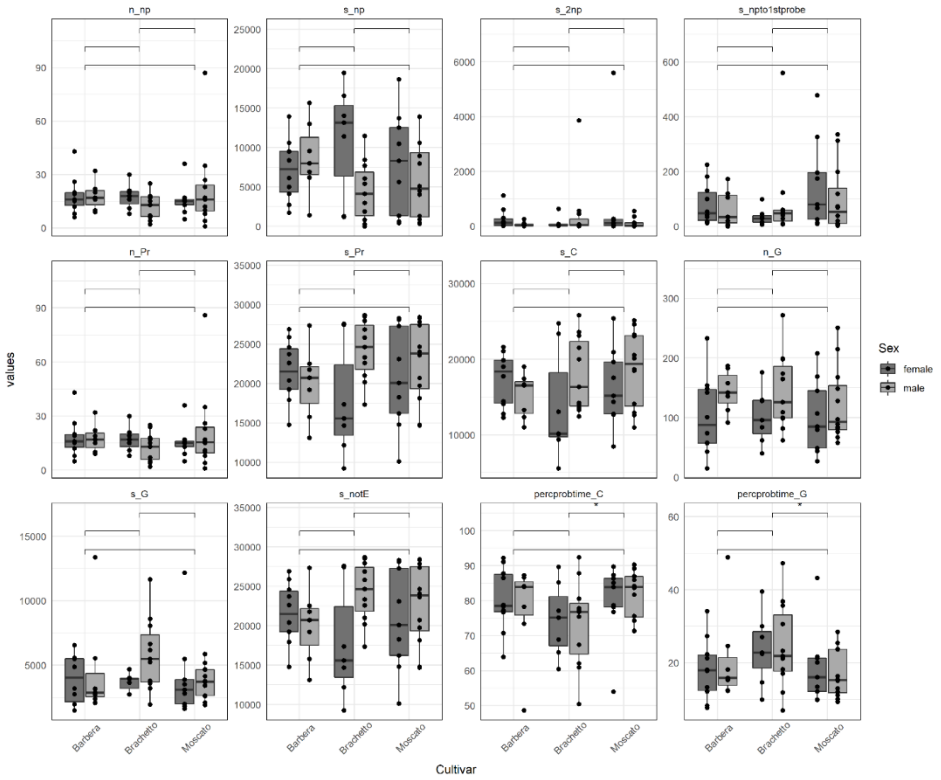
Cultivar	Sex	n	Number of non-probing periods *	Total duration of non-probing periods [min] *	Time from 1st np to 1st probe [s] *	Duration of the 2nd non-probing period [s] *	Number of probes *	Total probing time [min] *	Total duration of pathway phase [min] *	Number of active ingestion phases *	Total duration of active ingestion [min] *
Barbera	female	18	14 ± 2.4 a	104.3 ± 15.1 a	38.6 ± 22 a	111.7 ± 86.4 a	14 ± 2.4 a	374.7 ± 15.1 a	209 ± 25.4 a	50 ± 15.8 a	32.5 ± 8.9 a
Barbera	male	13	15 ± 2.1 a	115.3 ± 27 a	20.4 ± 15 a	24.7 ± 26.3 a	15 ± 2 a	363.2 ± 26.9 a	205.9 ± 22.8 a	93 ± 16.3 a	45 ± 15.2 a
Brachetto	female	13	19 ± 3 a	215.2 ± 27.6 a	27.9 ± 100.5 a	45.8 ± 57.3 a	17 ± 2.9 a	262.5 ± 27.5 a	169.7 ± 28.3 a	84 ± 14.7 a	54.4 ± 7.2 a
Brachetto	male	19	14 ± 1.7 a	119.2 ± 16.5 a	48.4 ± 43.3 a	44.3 ± 201.3 a	14.5 ± 1.8 a	350.2 ± 16.6 a	226.8 ± 22.3 a	100 ± 15.5 a	63.1 ± 11.4 a
Moscato	female	16	16 ± 2.2 a	109.1 ± 22.3 a	83.8 ± 77.8 a	100.1 ± 343.9	16 ± 2.2 a	354.8 ± 22.3 a	246.4 ± 22.4 a	69 ± 14.6 a	40.9 ± 11.6 a
Moscato	male	21	17.5 ± 4.2 a	132.8 ± 19.2 a	54.6 ± 21.9 a	17.3 ± 43.3 a	15.5 ± 4.3 a	345.5 ± 19.3 a	235.2 ± 21.3 a	84 ± 13.8 a	44 ± 8.9 a

Supplementary File S3a-b. Available on request, since the two tables do not fit into thesis formatting.

Supplementary File S4. Graphical representation of EPG variables related to recordings with phloem phases. For the variable acronyms, see Table 1 in the main text.



Supplementary File S5. Graphical representation of EPG variables related to recordings without phloem phases. Differences between group were evaluated with Wilcoxon rank sum test. For the variable acronyms, see Table 1 in the main text.



Supplementary File S6. Number of selected recordings of *S. titanus* probing behaviour with and without phloem phases on three grapevine cultivars.

Cultivar	Recordings with phloem (females, males)	Recordings without phloem (females, males)	Percentage of recordings with phloem phase
Barbera	14 (8, 6)	17 (10, 7)	45.2
Brachetto	14 (6, 8)	18 (7, 11)	43.8
Moscato	16 (7, 9)	21 (9, 12)	43.2

Chapter 4 – *Scaphoideus titanus* fitness

Article, in preparation

Scaphoideus titanus fitness parameters on grapevine varieties with different susceptibility to Flavescence dorée phytoplasma

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Keywords: *Scaphoideus titanus*; fitness; grapevine; cultivar; survival; developmental time; prolificacy; vitellogenin

1. Introduction

Flavescence dorée of grapevine (FD) is a phytoplasma-associated disease transmitted by the Nearctic leafhopper *Scaphoideus titanus* Ball (Schvester *et al.*, 1963). The vector and the disease are present in several European countries (Chuche and Thiéry, 2014; EFSA, 2020) and cause severe damages to viticulture. The control of FD largely relies on prophylactic measures, such as the use of healthy propagation material, as well as on compulsory control measures in infected vineyards, i.e. roguing of infected plants and insecticide treatments against the vector (Bosco and Mori, 2013). There is an urgent need for developing new, innovative and environmental friendly control strategies, as the current measures are costly, have side effects on non-target insects and are not effective enough, given FDp is still spreading.

The best sustainable strategy to minimize damages due to pathogen or parasites is the exploitation of plant resistance or tolerance. For arthropod-borne plant pathogens, plant resistance can exploit its activity i) against the pathogen or ii) against insect vectors. Resistance against insects occurs when plant structural or chemical traits deter herbivore feeding and thus minimize the amount of herbivore damage experienced by the plant, while tolerance occurs when plant traits reduce the negative effects of herbivore damage on crop yield (Mitchell *et al.*, 2016). Resistance against pathogens is the host's ability to limit pathogen multiplication, while tolerance is the host's ability to reduce the effect of infection on its fitness regardless of the level of pathogen

multiplication (Pagán and García-Arenal, 2018). These definitions of the same terms against two different targets largely overlap. Indeed, resistance deters (for insects) or limits (for pathogens) the presence of the unwanted host, while tolerance is the ability of the plant to live with it.

Resistance or tolerance towards pathogens are directed against viruses (Hashimoto *et al.*, 2016), fungi (powdery mildew: Riaz *et al.*, 2020); downy mildew: Yu *et al.*, 2012)), and bacteria, such as grapevine-infecting (Riaz *et al.*, 2018) and olive-infecting *Xylella fastidiosa* (D'Attoma *et al.*, 2019). Resistance or tolerance towards insects are directed against any kind of phytophagous insects, including vectors of plant pathogens. Within the Hemiptera order, that account for many of the economically significant plant pests (Koch *et al.*, 2016), studies on plant genotypes resistant or tolerant to insects were conducted, among others, on aphids (Bowling *et al.*, 1998), planthoppers (*Nilaparvata lugens*, Srinivasan *et al.*, 2015; Kang *et al.*, 2019; Yue *et al.*, 2019), splittlebugs (*Mahanarva fimbriolata*, Orozco-Restrepo *et al.*, 2017), and leafhoppers (Miao *et al.*, 2014). In the latter work, resistance of some varieties of *Camelia sinensis* against *Empoasca vitis* was associated with an altered feeding behaviour. Life history parameters, such as mortality/survival, developmental time and prolificacy, are the most common features used to evaluate phytophagous insect performances on plants under variable conditions (e.g. different plant varieties, temperatures, insecticides applications, etc.) (Akca *et al.*, 2015; Akkopru *et al.*, 2015; Xu *et al.*, 2016; Zhang *et al.*,

2018). Shorter survival is a direct indicator of impaired fitness (Jandricic *et al.*, 2010; Krechemer and Foerster, 2017; Orozco-Restrepo *et al.*, 2017). More recently, also feeding behaviour of sap-sucking insects has been widely and effectively applied to estimate plant acceptability, by comparing insect probing behaviour on susceptible and resistant genotypes (Miao *et al.*, 2014; Yorozuya, 2017; Baldin *et al.*, 2018; Kordan *et al.*, 2019).

With the aim of identifying sources of resistance or tolerance to FDp phytoplasmas within the grapevine germoplasm, two research works have been conducted in France and Italy (Eveillard *et al.*, 2016; Ripamonti *et al.*, 2021). However, only very few information on the resistance/tolerance of the tested grapevine genotypes towards the vector insect *Scaphoideus titanus* are provided in the cited papers. In the course of the experiments, Eveillard *et al.* (2016) observed lower survival rates of *S. titanus* on Merlot, a tolerant variety, when compared to Cabernet Sauvignon, a susceptible one. Similarly, Ripamonti *et al.* (2021) observed a lower survival rate of *S. titanus* on Moscato FD tolerant variety. These preliminary hints suggest that the impact of grapevine genotype on the vector fitness is worthy of investigation with the aim of understanding if the mechanism underlying the tolerance to FDp acts against FDp or against the vector. Therefore, in order to gain information on the resistance/tolerance mechanism and to test the hypothesis that the different susceptibility to FDp of the cultivars might be vector-mediated, a study on the fitness of the vector on the selected varieties was conducted. Three grapevine varieties

were chosen among the extremes of the tolerance range (Ripamonti *et al.*, 2021), considering both their tolerance to Flavescence dorée phytoplasma and the impact on *S. titanus* short-term survival. In particular, Barbera was chosen because it is highly susceptible to FD and the leafhopper showed high survival on this variety. Brachetto was chosen as a tolerant variety for FDp with none/little effects on *S. titanus* short-term longevity. Moscato was selected as tolerant to FD with possible negative effects on insect survival (Ripamonti *et al.*, 2021). Some key fitness parameters, such as development time, longevity (= survival), and fecundity can be regarded as markers of host plant acceptability by the insect. A description of longevity and fecundity of S*. titanus* on Kober 5BB, a hybrid of *Vitis riparia* that is considered its most preferred natural plant host, has been recently published (Bocca *et al.*, 2020) and can serve for comparative analyses of *S. titanus* fitness on different cultivars. Besides life-cycle and demographic parameters, the feeding behaviour of *S. titanus* on different grapevine genotype can have a major consequence on its FDp transmission ability/efficiency. This last feature has been investigated in a separate paper (Ripamonti *et al.*, submitted to Journal of Insect Physiology), using the electropenetrography (EPG) technique. Results have shown a significant decrease in phloem activities in leafhopper feeding on the two FDp tolerant varieties, Brachetto and Moscato, thus supporting the hypothesis that some vector-mediated tolerance factors to FDp occur in grapevine cultivars.

Hence, in the present work we describe three key fitness parameters of *S. titanus*: nymphal developmental time, adult longevity, and female prolificacy, in association with the expression of vitellogenin mRNA, on three grapevine varieties and we discussed the possible implications of these data on grapevine tolerance to Flavescence dorée.

2. Materials and Methods

2.1. *S. titanus* collection and plant rearing

S. titanus colony was initiated in greenhouse condition, starting from eggs, as described in Ripamonti et al. (2021). Dormant wood with eggs were collected in winter in vineyards of the Piedmont Region where a high number of adult *S. titanus* was captured by yellow sticky traps during the previous summer.

To obtain *S. titanus* nymphs from eggs, broadbean plants were sown and maintained in an insect-proof greenhouse in 2.4 L topsoil, five per pot, watered twice a week. *Vitis vinifera* plants of three different cultivars, Barbera N. - Clone I-AT 84, Brachetto N. - Clone I-CVT 20 and Moscato Bianco B. - Clone I-CVT 190 (Ripamonti *et al.*, 2021), grafted on Kober 5BB, were used for fitness experiments with *S. titanus*. Grapevines were grown in an insect-proof screenhouse, under natural photoperiod, potted in 9.5 L soil (3:1 clay-soil, perlite), watered once a week, regularly sprayed with copper- and sulphur-based fungicides to prevent downy and powdery mildew. One week before the scheduled beginning of

every replicate of the tests, one potted grafted cutting per variety was moved to greenhouse for acclimation (T = 24 ± 2°C, photoperiod 16:8 L:D).

2.2. Fitness tests

All tests were conducted in greenhouse condition (T = 24 ± 2°C, photoperiod 16:8 L:D). Insects' rearing conditions were the same for all the tests, and consisted in a Plexiglas-net cage (36 x 36 x 50 cm) per test per cultivar. In every cage one well-developed branch of a single cultivar was enclosed (Figure 1).

Figure 1. Fitness test isolators.

2.2.1. Developmental time test

Two groups (60 and 99) of *S. titanus* first instar nymphs were collected from the main rearing, in 2019, one at the beginning of July and one at the beginning of September. The nymphs were randomly subdivided and equally assigned to each cultivar-

treatment. Nymphs were left growing undisturbed and checked every day for the presence of newly emerged adults. As soon as they emerged, new adults were collected, their sex and day of emergence recorded. The total number of nymphs exposed to each cultivar-treatment was the same (53 nymphs).

2.2.2. Adult longevity test

Two batches (33 and 70) of fourth/fifth instar nymphs *S. titanus* were collected from the main rearing, one in summer 2018 and one in summer 2019, and maintained in a separate cage on broadbean until adult emergence. Newly emerged adults were collected twice, the day of the first emergences (day 0) and two days later (day 2), subdivided per sex, randomly assigned to one cultivar-treatment; the same ratio of males/females was caged on the three cultivars. Survival status was recorded every day, from the beginning of the test up to the death of the last insect. Dead insects were removed from the cage and discarded.

2.2.3. Prolificacy test

Two groups (210 and 210) of first/second instar nymphs *S. titanus* were collected from the main rearing, in 2020, one at the end of May and one at mid-August. The nymphs were randomly subdivided and assigned to one cultivar-treatment. They were left developing undisturbed until they reached the adult stage. Adults emerged from the same cultivar were grouped per day of emergence on the same cultivar, on a different branch, using a net cage (30 x 10Ø cm). Sex ratio was maintained at 1:1 in every net cage. In case of absence of males due to protandry (Chuche and Thiéry, 2012) for the ‘cultivar-

day of emergence' combination, adult males were taken from the main rearing. Insects' survival status was recorded every Tuesday and Friday, during both nymphal and adult stages. At 14, 25, or 35 days post emergence, an equal number of females were taken, their abdomen dissected and eggs counted. Adults were left undisturbed until the scheduled day of dissection. Different set of data were obtained in this experiment on the three cultivars, besides the one on insect prolificacy: nymphal mortality, developmental time of nymphs and adult survival. In describing this latter, females that were taken from the rearing for abdomen dissection and egg counting were defined as "censored" and so considered in the analyses. Dissections were conducted under a stereomicroscope (Leica S9E, Deutschland), females were CO_2 anaesthetised and then the abdomen dissected with two entomological needles in a drop of 50 µl of PBS 1X. Eggs were considered mature when elongated and with a curved tapering apex, as explained in Bocca *et al.* (2020). After eggs counting, the single dissected female was collected, transferred a 1.5 ml Eppendorf tube with the same buffer, and stored at -80°C until RNA extraction.

2.3. RNA extraction and gene expression

Total RNAs were extracted from single *S. titanus* females following dissection and eggs count, with Direct-zol RNA Mini Prep kit (Zymo Research), following manufacturer's protocol and including the optional DNAse treatment step. Concentration, purity, and quality of extracted RNA samples were analysed in a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Quantitative RT-PCR (qRT-PCR) was used to quantify the possible effect of the cultivar on the expression of females vitellogenin mRNA (Table 1), in order to correlate the vitellogenin expression level with eggs count. The vitellogenin sequences were retrieved from *S. titanus* transcriptome (Ottati *et al.*, 2020). For each sample, cDNA was synthesized from total RNA (250 ng) with random hexamers using a High Capacity cDNA reverse transcription kit (Applied Biosystems). The resulting cDNA was used as a template (1 µl) for qPCR in a 10 µL volume mix, containing 1× iTaq Universal Sybr Green Supermix (Bio-Rad) and 300 nM of each primer. All the primer pairs used for qRT-PCR are listed in Table 1.

Table 1. List of primers used in the work.

Application	Primer name	5′-3′ sequences	Organism and target gene	Product size	qPCR efficiency	R^2	Melt peak	Reference
qPCR target gene	St_Vitel_F	AAGAGGAACATGCGCTCCTA	*S. titanus* vitellogenin	98 nt	96.2%	0.998	78.0 °C	Galetto et al., unpublished
	St_Vitel_R	TTGCTGGGCAGGAAACTATC						
qPCR reference genes	St_EF1-α_F215	CCATTGACATTGCCCTGTGG	*S. titanus* elongation factor-1α	110 nt	98.0%	0.999	77.5 °C	Galetto et al., (submitted to Journal of Pest Science)
	St_EF1-α_R325	CCTGAGAAGTTCCAGTAATCATG						
	St_GST1_F257	CTAAGGATGCCCAGAAACGA	*S. titanus* glutathione S-transferase	113 nt	94.5%	0.998	79.0 °C	
	GST1_R369	TGGCGCTCCTCCAAACATCA						

Samples were run in triplicate in a CFX Connect Real-Time PCR Detection System (Bio-Rad). Cycling conditions were: 95 °C for 3 min, and 40 cycles at 95 °C for 30 s and 60 °C for 60 s of annealing/extension step. The specificity of the PCR products was verified by a post-amplification melting curve analysis for all samples. No-template controls (water devoid of cDNA) were included in the plates. Primers targeting glutathione S-transferase and elongation factor-1α were used as housekeeping genes to normalize the cDNA among samples (Table 1). Relative normalized expression levels of the target gene for each sample was calculated by CFXMaestro™ Software (Bio-Rad). The stability of the expression of reference genes was validated in a multiplate gene

study using the M-value (Vandesompele *et al.*, 2002) furnished by the above mentioned software (Supplementary File S1).

2.4. Statistical analysis

All the statistical analyses were conducted on R software v 4.0.3 (R Core Team., 2020). Raw data were subjected to modifications to enhance readability (packages dplyr, tidyr, stringr: Wickham, 2019, 2020; Wickham *et al.*, 2020), and descriptive statistics were run (Tables 2, 3, 4, 5, 6). Survival curves were applied to distinguish differences among treatments in every experiment. Developmental time curves (Figure 2, 4) were obtained through Kaplan-Meier estimates (Kaplan and Meier, 1958) with an inverse transformation function (f(y) = 1- y), in order to emphasizes the reaching of adult stage. Survival curves for longevity test (Figure 3) were obtained with Kaplan-Meier estimator (1958), while survival curves for females for the prolificacy test were obtained with Aalen-Johansen estimator (Aalen and Johansen, 1978), due to censored data (Figure 5). Supplementary Files S2-S3 were obtained from Cox Proportional Hazard Model (Cox, 1972), in order to statistically distinguish significant differences among cultivars or insect sex in development (Supplementary File S2) or survival (Supplementary File S3). Log-Rank test (Mantel, 1966) for pairwise comparisons, with Benjamini and Hochberg p-value adjustment (Benjamini and Hochberg, 1995), was applied on data belonging to the longevity test (Figure 2) and to the developmental time inside the prolificacy test (Figure 4), producing Supplementary Files S4-5. All the different estimates conducted in the work measured the probability

of survival or developmental time from the beginning of the experiment to the verification of the event of interest (death for survival estimates, adult emergence for developmental time). Individual females were censored (sampled when still alive) only in the prolificacy test, but the Aalen-Johansen model taken that possibility into account. Figure 6 boxplots was produced based on raw data of mature eggs, resulted from counting, plus the relative normalized expression level of vitellogenin mRNA, resulted after qRT-PCR in the CFX Maestro software. R packages utilized for analyses and production of figures were survival (Therneau, 2021), survminer (Kassambara *et al.*, 2020), ggplot2 (Wickham, 2016), and patchwork (Pedersen, 2019).

3. Results

Data presented here are preliminary, as further replicates are ongoing in this 2021 season.

3.1. Developmental time test

No significant differences were found in first-instar-to-adult developmental time of *S. titanus* on the three cultivars (Figure 2, Table 2, Supp. File S2). However, the presence of protandry in *S. titanus* (Chuche and Thiéry, 2012; Bocca *et al.*, 2020) is confirmed by our results (Figure 2, Supp. File S2). Males emerged before females (protandry), with a hazard ratio to emergence more than two times higher compared to females (p-value < 0.001, Cox proportional hazard regression model, Supp. File S2). As for the mortality, 9, 8 and 10 nymphs died during development on Barbera,

Brachetto, and Moscato, respectively. Therefore, mortality rate of nymphs did not differ among grapevine varieties in this experiment.

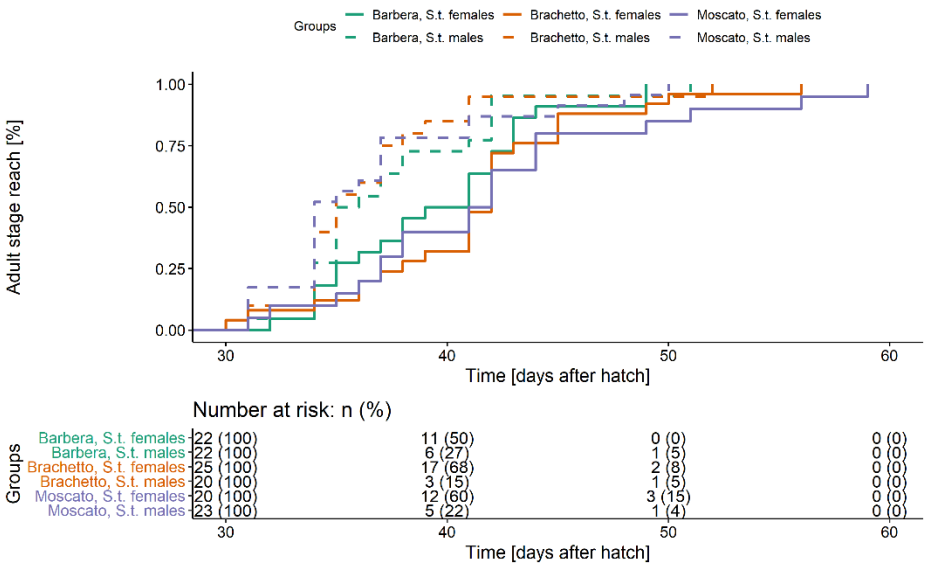

Figure 2. Developmental time curves (Kaplan-Meier estimates) for *Scaphoideus titanus* nymphs reared on three grapevine cultivars. *S. titanus* sex was represented by line type (solid for females, dashed for males), while grapevine cultivar was represented by line colour (green for Barbera, orange for Brachetto, purple for Moscato). Risk table was also reported, with number of residual nymphs in absolute number and percentage in brackets.

Table 2. Summary statistics for *S. titanus* developmental time test on three grapevine cultivars. IQR: inter-quartile range; Q1: first quartile (25th percentile); Q3: third quartile (75th percentile).

Cultivar	Sex	n	Mean [days]	Median [days]	IQR [days]	Q1 [days]	Q3 [days]
Barbera	female	22	39.5	40	7.5	35.25	42.75
Barbera	male	22	37.4	35.5	6	34.25	40.25
Brachetto	female	25	41.2	42	5	38	43
Brachetto	male	20	36.5	35	3.25	34	37.25
Moscato	female	20	42.0	41.5	7	37	44
Moscato	male	23	36.5	34	3	34	37

3.2. Adult longevity test

Longevity test analysis showed some differences in the hazard ratio for *S. titanus* reared on the three different cultivars (Figure 3). In particular, males reared on Moscato and Brachetto apparently survived for a shorter time compared to i) Barbera males and ii) females reared on all the three cultivars (Figure 3, Table 3). The result is statistically confirmed only for Moscato males (X-squared = 30.5, df = 5, p-value = 1e-05; Pairwise comparisons Log-Rank test, p-value adjustment BH, Supp. File S4). Statistical tests show that Moscato males' survival probability is statistically different from all the other combinations of cultivar-insect sex, except for Brachetto males.

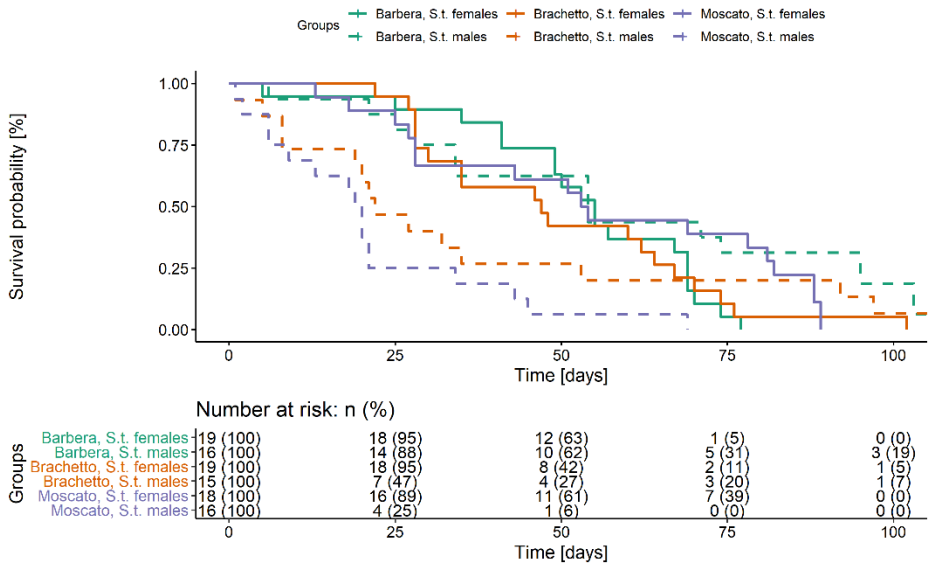

Figure 3. Survival curves (Kaplan-Meier estimates) for adult *Scaphoideus titanus* reared on three grapevine cultivars. *S. titanus* sex was represented by line type (solid for females, dashed for males), while grapevine cultivar was represented by line colour (green for Barbera, orange for Brachetto, purple for Moscato). Risk table was also reported, with number of residual alive adults in absolute number and percentage in brackets.

Table 3. Summary statistics for *S. titanus* survival on three grapevine cultivars. IQR: inter-quartile range; Q1: first quartile (25th percentile); Q3: third quartile (75th percentile).

Cultivar	Sex	n	Mean [days]	Median [days]	IQR [days]	Q1 [days]	Q3 [days]
Barbera	female	19	53.2	55	24	45	69
Barbera	male	16	60.1	54	62.5	32.5	95
Brachetto	female	19	49.9	47	36.5	29	65.5
Brachetto	male	15	36.7	22	30.5	13.5	44
Moscato	female	18	55.8	53.5	53.75	28	81.75
Moscato	male	16	21.7	19.5	16	8.25	24.25

3.3. Prolificacy test and vitellogenin gene expression

Prolificacy test was conducted in two different replicates. Total number of emerged adults and nymph mortality are summarized in Table 4. In the first replicate (repA), a significant difference was found between the number of dead nymphs on Barbera and Brachetto (X-squared = 4.7574, df = 1, p-value = 0.02917). In the second replicate (repB), the number of dead nymphs was different among all cultivars (X-squared = 31.437, df = 2, p-value = 1.491e-07). However, since the number of dead nymph was not different between the replicates of our reference variety (Barbera) (X-squared = 0.86768, df = 1, p-value = 0.3516), the two replicates were pooled together for further analyses.

Table 4. Number of total isolated *S. titanus* nymphs for the prolificacy experiment, subdivided by replicate. Mortality during nymphal stages was reported.

Cultivar	repA	Total emerged adults	Nymph mortality [%]	repB	Total emerged adults	Nymph mortality [%]
Barbera	70	34	51.4	70	46	34.3
Brachetto	70	15	78.6	70	26	62.9
Moscato	70	22	68.6	70	2	97.1

Developmental time test analysis conducted on the pooled replicates of the prolificacy experiment showed differences in the time needed to reach the adult stage (Figure 4, Table 5, Supp. File S5). In particular, the presence of protandry (Chuche and Thiéry, 2012; Bocca *et al.*, 2020) was confirmed again, since males tended to reach the adult stage in a significant lower amount of time (Figure 4, Table 5, Supp. File S5). Moreover, *S. titanus* females reared on Moscato showed an increased amount of time to emerge as adults compared to females reared on the other two varieties (Supp. File S5).

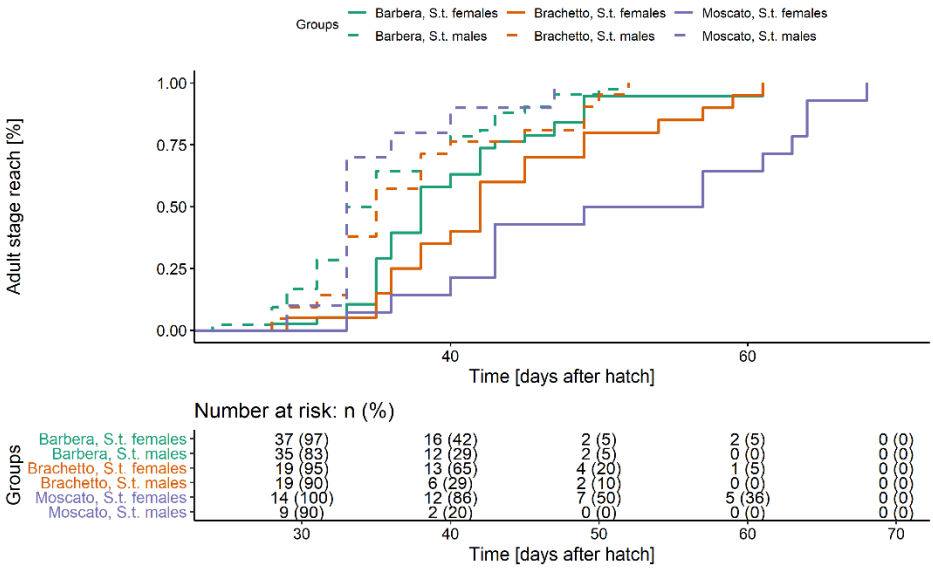

Figure 4. Developmental time curves (Kaplan-Meier estimates) for *Scaphoideus titanus* nymphs reared on three grapevine cultivars, belonging to the female fertility test. *S. titanus* sex was represented by line type (solid for females, dashed for males), while grapevine cultivar was represented by line colour (green for Barbera, orange for Brachetto, purple for Moscato). Risk table was also reported, with number of residual nymphs in absolute number and percentage in brackets.

Table 5. Summary statistics for *S. titanus* developmental time on three grapevine cultivars, belonging to the female fertility test. IQR: inter-quartile range; Q1: first quartile (25th percentile); Q3: third quartile (75th percentile).

Cultivar	Sex	n	Mean [days]	Median [days]	IQR [days]	Q1 [days]	Q3 [days]
Barbera	female	38	40.2	38	7.75	35	42.75
Barbera	male	42	35.8	34	9	31	40
Brachetto	female	20	43.7	42	11.5	37.5	49
Brachetto	male	21	37.7	35	7	33	40
Moscato	female	14	51.5	53	19.5	43	62.5
Moscato	male	10	35.0	33	2.25	33	35.25

Longevity test analysis conducted on females of the prolificacy experiment showed a significantly lower survival probability for female *S. titanus* reared on Moscato, compared to the other two cultivars (Figure 5, Table 6, Supp. File S3).

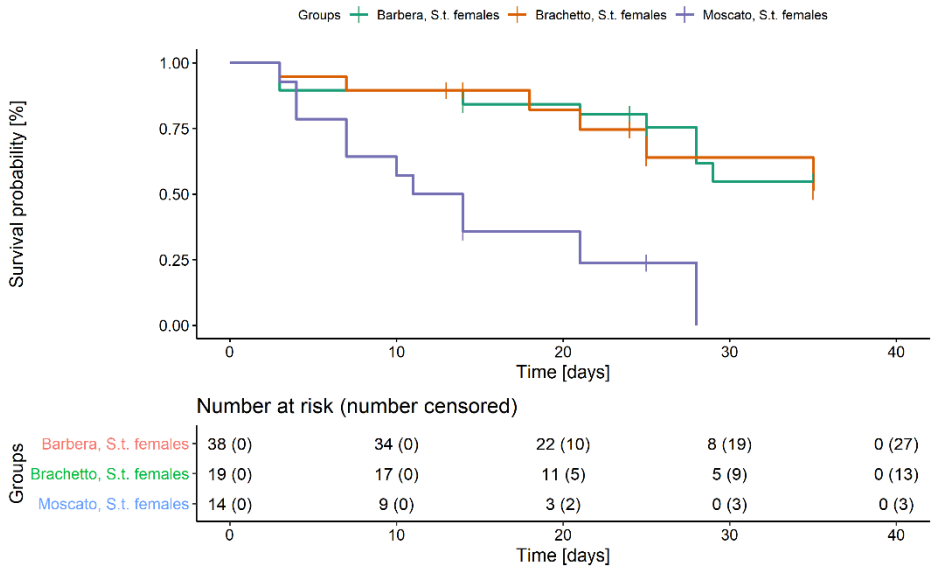

Figure 5. Survival curves (Aalen-Johansen estimator for censored data) for *Scaphoideus titanus* female adults reared on three grapevine cultivars (prolificacy experiment). Grapevine cultivar is represented by line type (green for Barbera, orange for Brachetto, purple for Moscato). Risk table is also reported, with number of residual adults in absolute number and percentage in brackets. Censored females, sampled for eggs count and RNA extraction, were represented by vertical lines.

Table 6. Summary statistics for *S. titanus* dead female survival on three grapevine cultivars (prolificacy experiment). IQR: inter-quartile range; Q1: first quartile (25th percentile); Q3: third quartile (75th percentile). Censored females were excluded from the table.

Cultivar	Sex	n	Mean [days]	Median [days]	IQR [days]	Q1 [days]	Q3 [days]
Barbera	female	11	15.5	14	23.5	3	26.5
Brachetto	female	6	18.2	19.5	14.25	9.75	24
Moscato	female	11	11.2	10	8.5	5.5	14

Alive females at defined interval post emergence were dissected and mature eggs were counted (Table 7). The high nymphal mortality of *S. titanus* reared on Brachetto and Moscato, followed, on Moscato, by a high mortality of adults, resulted in a reduced number of females that could be used for eggs count (Table 7).

Table 7. Number of total dissected *S. titanus* females for fertility test, subdivided per cultivar.

	Dissection day		
Cultivar	14	25	35
Barbera	10	9	8
Brachetto	5	5	5
Moscato	2	1	NA

Eggs count derived from the dissected females (Table 7) is reported in Figure 6, paired with the level of vitellogenin mRNA expressed in the same samples. Since the number of biological replicates was limited, a strong statistical procedure could not be applied. Shifting to a more observational point-of-view, some differences are still quite sizable. In particular, it was possible to appreciate a higher number of eggs counted in Barbera-females at 14 day post emergence (dpe), compared to females reared on the other two varieties. The same response was found for vitellogenin expression at 14 dpe. At 25 dpe, the number of mature eggs was found similar among females on the three varieties. However, 25 dpe vitellogenin expression in Barbera-females seemed reduced compared to its 14 dpe level, while for Brachetto and Moscato-reared females was expressed at a higher lever compared to the one measured at 14 dpe. Finally, at 35 dpe, the egg number in females reared on

Barbera and Brachetto was similar to the one at 25 dpe and did not differ between the females from the two varieties. At 35 dpe, the vitellogenin expression level was reduced in the females reared on both Barbera and Brachetto (while it was not possible to estimate this parameter on Moscato, as no female survived until 35 dpe). Summarising, it is possible to appreciate a shift in the eggs amount, paired with vitellogenin expression, in the two FD tolerant varieties. In particular, eggs load in Barbera-reared females at 14 dpe was higher compared to the females reared on the other two cultivars. Moreover, the level of vitellogenin expression in Barbera-reared females peaked at 14 dpe. For Brachetto-reared females, the maximum level of vitellogenin expression was measured at 25 dpe, followed by a decline at 35 dpe.

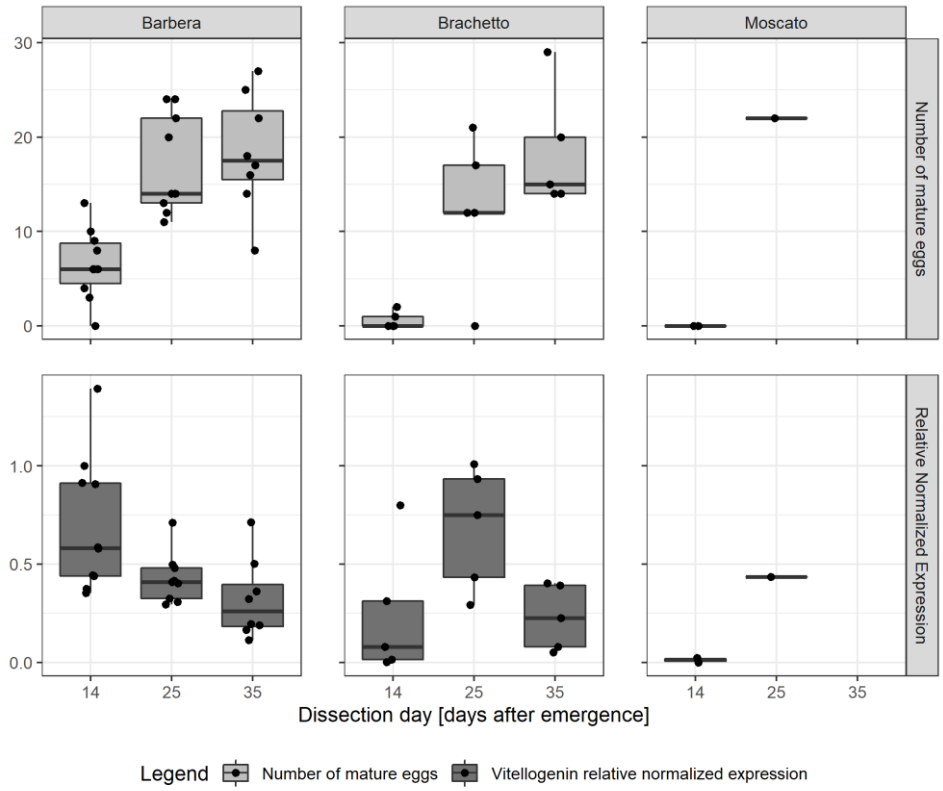

Figure 6. *Scaphoideus titanus* female prolificacy on three grapevine varieties. Mature eggs found in ovaries after dissection (upper row, light grey boxplots), vitellogenin gene expression (lower row, dark grey boxplots), subdivided based on the cultivar in which females were reared (columns). The total number of dissected females (and prepared for RNA extraction) is reported in Table 7.

4. Discussion

Life history and prolificacy parameters have been used to estimate grapevine variety suitability for the FDp vector *S. titanus*. A different adaptation of the vector to a plant genotype may be a clue of preference/non preference of the vector for different plant genotypes, that can in turn explain, at least in part, susceptibility/resistance to the disease spread by the vector.

Actually, susceptibility/resistance of a plant genotype to an arthropod-borne pathogen can be due to a response of the plant to the pathogen or to the vector, that mediates the transmission. Based on previous studies (Ripamonti *et al.*, 2021), Barbera was identified as reference susceptible cv, while Brachetto and Moscato showed the higher level of resistance among the tested varieties. The fitness parameters selected for the study were nymphal developmental time (measured as n° of days from first instar nymphs-to adult emergence), adult survival (measured as survival probability of males and females at different time post-emergence), and female prolificacy (measured as mature ovarian eggs and expression level of vitellogenin).

In our work, a general better fitness performance of *S. titanus* on the Barbera variety was suggested throughout all the conducted experiments.

In this work, the survival rate of *S. titanus* adults on Moscato was impaired. In particular, the two replicates conducted ad-hoc for the survival analysis showed a lower survival rate for males reared on Moscato. Moreover, the females reared on Moscato in the prolificacy experiments survived for a shorter time on this variety compared to Barbera one. Adult survival on Brachetto was intermediate between Barbera and Moscato, and the absence of statistical differences does not allow to conclude on the suitability of this variety for *S. titanus*. Our results of *S. titanus* survival on its most suitable host, Barbera, are in line with those recently reported by Bocca and co-authors (2020). These authors proved that survival

of the adult leafhoppers was longer than already thought, with an IQR approximately between 44 and 80 days for females, between 22 and 64 days for males. Minor differences between our results and those of Bocca *et al*. (2020) can be ascribed to the different *Vitis* species (American hybrids *versus* European grapevine varieties) and rearing conditions (detached shoots in small cages *versus* grafted cuttings inside larger cages).

The developmental time experiment did not reveal differences among leafhoppers that developed on the different cultivars. Males developed faster than females, thus proving that protandry occurs in this species, as already noticed by Chuche and Thiery (2012) and Bocca *et al.* (2020). However, during the preliminary phases of the female prolificacy experiment, nymphs have shown high mortality when reared on Moscato, with a dramatic reduction in survival in one of the replicates. Egg-to-adult developmental time is considered one of the parameters that can be used to estimate plant acceptability by the phytophagous insect. As an example, *Lobesia botrana* reared on non-preferred grapevine varieties showed a delayed development (Moreau *et al.*, 2006). Therefore, slowing of the development is an index of negatively impacted fitness, although in few cases the non-preferred varieties may induce a faster development, like in the case of the spittlebug *Mahanarva fimbriolata* on sugarcane (Orozco-Restrepo *et al.*, 2017). Although in our experiments we did not record an impact of the grapevine cultivar on nymph development time, considering also the survival

of nymphs on the three cultivars, the results suggest an overall reduced fitness of Moscato-reared *S. titanus.*

Female prolificacy tests were heavily affected by the mortality experienced by nymph and adult mortality of *S. titanus*, mainly on Moscato cv; as a consequence, a further experiment is planned for this 2021 season. However, if we restrict the comparative analysis to female prolificacy on Barbera and Brachetto we can still draw some interesting conclusions. Barbera-reared *S. titanus* showed a higher number of mature eggs, compared to Brachetto at the first sampling time, 14 days post emergence. At the same time, the level of vitellogenin transcripts was found at its highest level on females reared on Barbera, a level that was considerably higher than the one measured in females reared on Brachetto and Moscato. In *S. titanus* females reared on Barbera, the number of mature eggs increased with time, while vitellogenin transcript level decreased from 14 to 35 dpe. Females on Brachetto showed the same trend for mature eggs, although starting from a low number of eggs at 14 dpe. Consistently, vitellogenin transcript level showed a similar delay as for egg maturation, reaching its higher value at 25 dpe. These results suggest that females reared on Brachetto show a physiological delay in egg maturation and vitellogenin expression.

Despite the different experimental design, our results are quite consistent with those of Bocca *et al*. (2020). We found that, at 14 dpe, Barbera-reared females were already carrying mature eggs. Similarly, Bocca *et al.* (2020) found that the median time for the start of oviposition was approximately 14 dpe. Moreover, in this work,

covering only a window of 21 days for oviposition, we estimated a median total load of about 30 to 40 eggs per female. Consistently, Bocca *et al*. (2020) estimated an average of more than 60 eggs per female over a median oviposition period of about 45 days.

Vitellogenin gene expression was used to estimate female fitness in a number of works (e.g. Liu *et al.*, 2015; Ge *et al.*, 2017). This protein is by far the most abundant yolk protein precursor (YPP) in oocites of most insect species, and, like other YPPs, plays a major role for the sustenance of the developing embryo (Sappington and Raikhel, 1998). Hence, a decrease in vitellogenin mRNA level should lead to a reduction in its bioavailability, unless, when the mRNA level drops, the protein shelf life rises. In our experiment, the reduced amount of expressed vitellogenin mRNAs in Brachetto-reared females at 14 dpe pairs with the reduced amount of eggs found in the same females. The gap between Brachetto and Barbera reared females was then filled at 25 dpe. The temporal shift of high vitellogenin expressed mRNAs in Brachetto-females may represent an index of decreased fitness for young female performance on this cultivar. Liu *et al.* (2015) described the expression profile of vitellogenin mRNA in *Chrysopa septempunctata*, showing that the maximum transcript accumulation occurred at 10 dpe. The subsequent depletion of vitellogenin transcripts led to a significant reduction in egg-laying and a decrease in egg hatching rate. Similarly, in our work we found a maximum accumulation of vitellogenin mRNA at 14 dpe in Barbera. A delayed production of egg, not compensated by a longer

survival or even coupled with reduced longevity, results in a lower population rate of increase (Birch, 1948). The results suggest that *S. titanus* fitness is impaired on Brachetto and Moscato. In particular, Moscato seems to affect *S. titanus* survival and development time, while Brachetto seems to cause a delay in female sexual maturity. Further replicates of the experiments will better clarify the role of grapevine cultivars on *S. titanus* fitness.

In conclusion, if confirmed by further experiments, the lower suitability of Moscato and Brachetto compared to Barbera for *S. titanus* may reflect a non-preference of this vector for these tolerant varieties. A different probing behaviour on the three grapevine varieties has been demonstrated (Ripamonti *et al.*, submitted to Journal of Insect Physiology): Barbera sustains much longer phloem ingestion phases of *S. titanus*, together with a higher number of salivations. All these data consistently indicate that Barbera, which is a very susceptible host of FDp, is a preferred host for the vector, while, among the tolerant varieties, at least Moscato is a non-preferred host. These findings allow to hypothesize that resistance/tolerance to FDp may be associated not only with a direct plant response to the phytoplasma, but also to a non-preference for the vector. A lower number of visiting insect vectors, feeding less efficiently in the phloem, may explain the lower incidence of this phloem-limited pathogen in some grapevine varieties. The identification of the genetic trait that underline this non-preference should be included in programs of breeding for resistance to this major grapevine pathogen.

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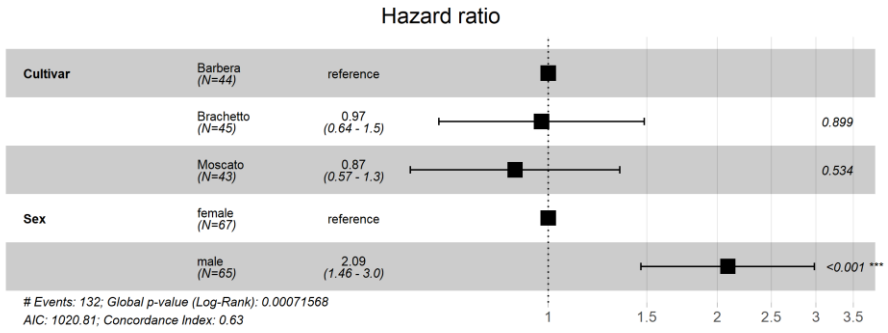
Supplementary Material

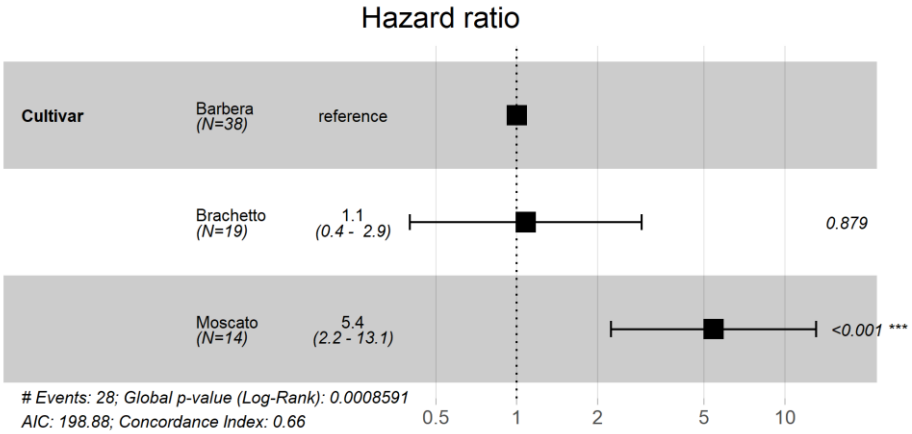
Supplementary File S1. Acceptability of the qRT-PCR multiplate study, based on the M-value, as resulting from the CFX Maestro Software (Bio-Rad).

M Value Grid:					
Order	Gene Name	Evaluation	Avg M Value	Stability (Ln(1/AvgM))	# Samples
1	GST1	Acceptable	0.674149686	0.394303107	58
2	EF1alpha	Acceptable	0.674149686	0.394303107	58

Supplementary File S2. Hazard ratio for developmental time test resulting from Cox proportional hazards regression model, considering cultivar and sex as predictor variables (developmental time test).



Supplementary File S3. Hazard ratio for female survival resulting from Cox proportional hazards regression model, considering cultivar as predictor variables (prolificacy test).



Supplementary File S4. Log-Rank test pairwise comparisons for *S. titanus* survival on three grapevine cultivars of the longevity test (p-value adjustement method BH). Numbers reported in the table are BH adjusted p-values, every cell comparing a specific combination of cultivar and sex.

	Cultivar=Barbera, Sex=female	Cultivar=Barbera, Sex=male	Cultivar=Brachetto, Sex=female	Cultivar=Brachetto, Sex=male	Cultivar=Moscato, Sex=female
Cultivar=Barbera, Sex=male	0.23310	-	-	-	-
Cultivar=Brachetto, Sex=female	0.81437	0.23310	-	-	-
Cultivar=Brachetto, Sex=male	0.56899	0.24892	0.56628	-	-
Cultivar=Moscato, Sex=female	0.23310	0.24892	0.44081	0.69062	-
Cultivar=Moscato, Sex=male	0.00049	0.00049	0.00056	0.23310	0.00049

Supplementary File S5. Log-Rank test pairwise comparisons for *S. titanus* developmental time on three grapevine cultivars of the prolificacy test (p-value adjustement method BH). Numbers reported in the table are BH adjusted p-values, every cell comparing a specific combination of cultivar and sex. Considered data belongs to the female fertility test.

	Cultivar=Barbera, Sex=female	Cultivar=Barbera, Sex=male	Cultivar=Brachetto, Sex=female	Cultivar=Brachetto, Sex=male	Cultivar=Moscato, Sex=female
Cultivar=Barbera, Sex=male	0.02108	-	-	-	-
Cultivar=Brachetto, Sex=female	0.24130	0.00206	-	-	-
Cultivar=Brachetto, Sex=male	0.31728	0.31728	0.02659	-	-
Cultivar=Moscato, Sex=female	0.00190	0.00008	0.02108	0.00121	-
Cultivar=Moscato, Sex=male	0.01767	0.71698	0.00355	0.24130	0.00078

Conclusions

Grapevine Flavescence dorée is a severe threat to European viticulture, and its spread is bound to rise, also due to climate change. In fact, warmer conditions may facilitate the settlement of both FDp and its natural vector *Scaphoideus titanus* in northernmost viticultural areas of Europe that are still FD-free (EFSA, 2016). Environmental and human health risks, coupled to the recent ban on several insecticides in agriculture, including viticulture, may represents the turning point for new strategies to fight this disease. Thus, it is crucial to find new, effective methodologies to counteract the presence and spread of vector pests. Viticulture is an important asset of the European economy, but it is also an important cultural, historical and sociological pillar, besides a valuable tool for the development and repopulation of agricultural areas otherwise neglected because of the absence of rural/industrial activities.

In this thesis, several poorly explored aspects of the epidemiology of FD were tackled. When needed, laboratory experiments (*ex-vitro* vines, EPG, insect fitness parameters) were run to dissect the complex interactions among the three partners of the FD epidemiological cycle, and the results were confirmed under semi-field conditions (potted grafted vines). Field experiments were also conducted to identify important aspects of the interaction between the cultivated and the feral compartments in the epidemiology of the disease in the vineyard, under the current control strategies.

The different approaches of this thesis proved i) a substantial role of primary transmission events in FD spread, irrespective of the current compulsory insecticide strategy applied to control the disease, suggesting that *S. titanus* continuously acquires the phytoplasma by feeding on feral *Vitis* plants during the season, ii) the existence of variability in cultivar susceptibility to FD within traditional grapevine genotypes of the Piemonte viticulture, iii) the preference of *S. titanus* for some very susceptible genotypes, explored and confirmed with several experimental approaches.

The importance of primary infections of grapevines, even in the presence of correct application of the compulsory insecticide strategy to control vector populations, clearly indicates that feral areas hosting gone-wild *Vitis* plants nearby cultivated areas should be destroyed, or at least contained. This is an important advice to agriculture policy makers for the phytosanitary management of vines in areas where the agricultural landscape features small vineyards surrounded by uncultivated areas at different stages of naturalization. Moreover, a continuous active acquisition of FDp during the season, supported by the increase of the percentage of infected *S. titanus*, suggests that primary infections should be especially controlled during late summer and fall, dangerously close to harvest with increasing risk of insecticide residues on the grapes. Although FD infection reduces longevity of *S. titanus* (Bressan et al., 2005a, 2005b), and the correct application of insecticides effectively controls the vector population within the vineyard, *S. titanus* lifespan under feral conditions is longer than previously

thought, with alive and active leafhoppers found until the end of October (Bocca *et al.*, 2020). Within this frame of knowledge, our results strongly recommend including the high risk of late primary infections in management plans of FD, after careful evaluation of the vineyard topology.

Flavescence dorée prevalence differs in vineyards with different grapevine cultivars (Morone *et al.*, 2007; Eveillard *et al.*, 2016). Our results confirmed this observation, even in the presence of comparable infection pressure by vineyard-resident *S. titanus*, suggesting that the *Vitis* genotype must be taken into account for the efficient management of the disease. It is reasonable to state that susceptible varieties can sustain higher prevalence of disease. Indeed, Merlot and its parental Magdeleine Noire des Charentes genotypes are listed as FD resistant, while Cabernet Sauvignon ranks among the most susceptible French cultivar (Eveillard *et al.*, 2016), both upon field observations on the disease prevalence and severity, and laboratory conditions with controlled infection pressure. Although an evaluation of cultivar susceptibility is available based on complex intersection of the numbers of replaced plants following FD infection for each cultivar and the prevalence of the disease in single variety vineyards (Morone *et al.*, 2007), ranking of the FD susceptibility under identical infection pressure of local *Vitis* genotypes, of paramount importance for the diversity and the economy of viticulture in the north-western part of Italy, was missing. Indeed, the 14 screened grapevine accessions could be ascribed to three susceptibility groups, spanning from the most

susceptible ones, comprising Barbera, Cortese, and Ruchè, to the least susceptible ones, including Brachetto, Moscato, and Freisa. Grapevines within the former group showed high percentage of infected plants, and high phytoplasma loads; conversely, plants within the latter group shared low percentages of infected plants, and low phytoplasma loads. The remaining accessions showed intermediate FD prevalence and phytoplasma loads. Grafting of accessions selected within the extremes of the susceptibility range (Barbera as susceptible, Brachetto and Moscato as tolerant) on the same rootstock did not alter the performance of the analysed genotypes. The availability of an FD-susceptibility list for the most representative *Vitis* varieties is a precious tool for vine-growers in the decision process of establishing a new vineyard, allowing them to support their choices through the input of a wide array of phytosanitary/epidemiological data, including the susceptibility of the plant genotype to the disease.

The genetic mechanism underlying grapevine tolerance to FD is so far unknown, despite description of different aspects with several metabolic, molecular (Gambino *et al.*, 2013; Margaria *et al.*, 2013, 2014), physiological (Vitali *et al.*, 2013), and transcriptomic (Bertazzon *et al.*, 2019; Pagliarani *et al.*, 2020) tools. The complexity of the issue is due to the fact that FD tolerance may also be explained by some native immunity-like mechanisms of the plant lowering insect vector attractiveness. Considering this aspect, three different scenarios were found in this work: i) high FD susceptibility and high *S. titanus* survival (Barbera), ii) FD tolerance and high *S.*

titanus survival (Brachetto), iii) FD tolerance and poor *S. titanus* survival (Moscato). The different situations recorded for Barbera and Brachetto suggests that tolerance to the disease could be directed against the phytoplasma, but some immunity-like mechanisms, negatively impacting vector performance on this variety, must be foreseen in the case of Moscato.

Decreased insect fitness (Srinivasan *et al.*, 2015; Orozco-Restrepo *et al.*, 2017; Yue *et al.*, 2019), as well as the impairment of feeding behavior on resistant cultivars (Miao *et al.*, 2014; Kordan *et al.*, 2019) are known, but so far unexplored within the grapevine-FD pathosystem. Indeed, *S. titanus* showed a clear preference for Barbera, feeding for longer periods in its phloem, with less frequent disturbance-related behaviors, such as interruption-salivation events, and an overall higher number of phloem salivation events compared to both tolerant varieties. These characteristics inevitably provide grounds for an enhanced ability to inoculate phytoplasmas to Barbera and justify the high FD susceptibility of this genotype. A parallel evaluation of several *S. titanus* fitness parameters on grapevine genotypes with different FD susceptibilities showed that insects reared on Brachetto and Moscato behave differently than those reared on Barbera. In particular, when reared on both FD tolerant varieties, *S. titanus* took longer to reach its maximum fertility, and the result was mirrored by the expression of the fitness indicator vitellogenin gene (Liu *et al.*, 2015; Ge *et al.*, 2017). Despite the requirement of more experimental replicates, *S. titanus* longevity was also negatively impacted when reared on Moscato as

adult, especially if male. This work proved that, indeed, plant-mediated effects on the insect vector combine with a direct immune response of the plant against the phytoplasma. Hence, the necessity of disentangling this complex pathosystem for appropriate design of environmental-friendly control strategies.

The results of this research thesis provide important indications to support the request for more sustainable control of FD, addressing both the plant genetics and vector containment. Recent development in 'new genomic techniques' (NGTs) legislation in EU hints a possible future utilization of NGTs crops, even in the European area (European Commission, 2021a, 2021b), allowing genetic modification through the most recent and reliable strategies to contrast established and emerging plant diseases. In that scenario, novel cis-genesis techniques will benefit from knowledge generated by this thesis, and by more extensive studies on the nature of the compounds negatively affecting *S. titanus*. Further research should clarify the biochemical and molecular nature of those compounds, as well as identify pivot plant genes involved in the resistant phenotype, for future application of this knowledge towards a more sustainable viticulture.

References (Introduction and Conclusions sections)

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A special thanks to my expanded family for having supported me in this passage of time. Even far, I know I have you by my side.

Since it is almost sure that I have forgot someone and I am very sorry, you have a beer ticket on me.

A special mention to my wife ‘Luci’, my enzymatic stone, my pillar of wisdom, my *‘Sehnsucht’*.

So long, and thanks for all the fish!

So di non sapere (Έτσι, δεν γνωρίζω)

Socrates

Appendix A

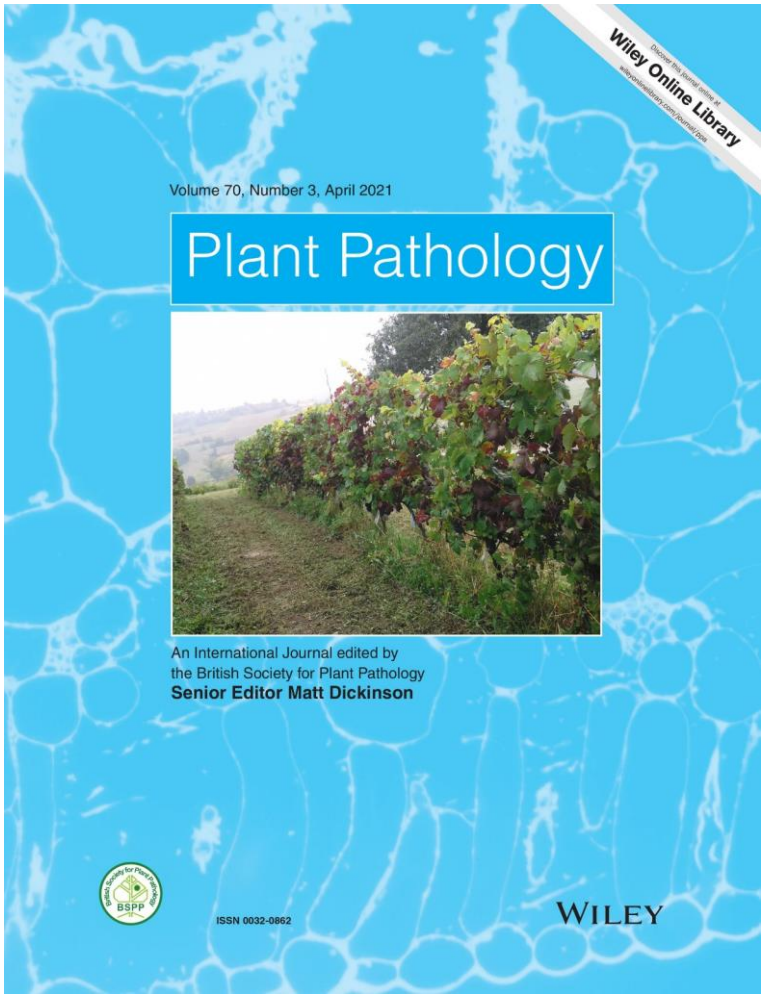
Reference for Chapter 1

Ripamonti M, Pegoraro M, Rossi M, Bodino N, Beal D, Panero L, Marzachì C, Bosco D. Prevalence of Flavescence Dorée Phytoplasma-Infected *Scaphoideus titanus* in Different Vineyard Agroecosystems of Northwestern Italy. *Insects*. 2020; 11(5):301. https://doi.org/10.3390/insects11050301

Reference for Chapter 2

Ripamonti, M, Pegoraro, M, Morabito, C, Gribaudo, I, Schubert, A, Bosco, D, Marzachì, C. Susceptibility to flavescence dorée of different *Vitis vinifera* genotypes from north-western Italy. *Plant Pathology*. 2021; 70: 511– 520. https://doi.org/10.1111/ppa.13301

Front cover: Mid-summer symptoms of flavescence dorée on *Vitis vinifera* plants of the Barbera variety. See **70**, 511–520.



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Ripamonti, M, Maron, F, Cornara, D, Marzachì, C, Fereres, A, Bosco, D. *Scaphoideus titanus* Ball feeding behaviour on three grapevine cultivars with different susceptibilities to Flavescence dorée. Submitted to *Journal of Insect Physiology.*

Appendix B

Matteo Ripamonti biographical sketch

2021 – ongoing: **Research fellow in insect vectors of phytoplasmas**, *Institute for Sustainable Plant Protection (Turin), National Research Council of Italy (IPSP-CNR).*

2017 – 2021: **PhD student in Agricultural entomology**, *University of Turin, Department of Agricultural, Forest and Food Sciences; association with IPSP-CNR.*

11/2016 – 09/2017: **Applied research grant winner,** *Enocontrol S.C.A.R.L., Alba (Italy), funded by Lagrange Project, CRT Foundation.*

10/2014 – 07/2016: **Master's Degree in Environmental Biology**, *University of Turin, Department of Life Sciences and Systems Biology. Graduated cum laude.*

10/2011 – 11/2014: **Bachelor's Degree in Biological Sciences**, *University of Milano-Bicocca, Department of Biotechnology and Biosciences.*

Publications

Original research papers

- Galetto L, **Ripamonti M**, Abbà S, Rossi M, Manfredi M, Bosco D, Marzachì C (2021) Silencing of ATP synthase β induces female sterility in a leafhopper phytoplasma vector. *Entomologia Generalis*. DOI: 10.1127/entomologia/2021/1267
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- **Ripamonti M**, Pacifico D, Roggia C, Palmano S, Rossi M, Bodino N, Marzachì C, Bosco D, Galetto L (2020) Recovery from grapevine Flavescence dorée in areas of high infection pressure. *Agronomy* 10, 10: 1479. DOI: 10.3390/agronomy10101479.
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Extended abstract

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- Plazio E, Bodino N, **Ripamonti M**, Demichelis S, Isetta D, Bosco D (2016) “From macro to micro”: phenology and ecology of Philaenus spumarius, vector of Xylella fastidiosa, at different scales. European PhD Network in Insect Science Annual Meeting. La Colle sur Loup, France, October 27-29, 2016
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Con una sola mano sgrano l'uva americana
chicco per chicco come la vecchia sgrana
il rosario. A differenza sua, la mia preghiera
è un bestiario. Ad ogni acino che non s'eleva
in vino chiedo che il tempo spezzi la trama
tra il timore e il credo. Che non mi spinga alla fede
nei giorni prossimi all'addio ma soltanto verso
la paura. Come un animale che cede alla natura.

Paolo Agrati, *Tecniche di Seduzione Animale* (2020)