UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente

Corso di laurea in Scienze e Tecnologie Viticole ed Enologiche

PRESENCE OF THE FLAVESCENCE DORÉ PHYTOPLASMA ON WOODY ORGANS OF SYMPTOMATIC VINES AND RISK OF TRANSMISSION THROUGH VEGETATIVE PROPAGATION

PRESENZA DEL FITOPLASMA DELLA FLAVESCENZA DORATA SU ORGANI LEGNOSI DI VITI SINTOMATICHE E RISCHIO DI TRASMISSIONE TRAMITE PROPAGAZIONE VEGETATIVA

Relatore/Supervisor

Prof. Rita Musetti

Correlatore/Co-supervisor

Dott.ssa Nadia Bertazzon

Laureando /Submitted by

Giovanni Helm

Matricola n./Student n.

2000033

ANNO ACCADEMICO/ACADEMIC YEAR 2022-2023

1. INTRODUCTION

1.1 The Flavescence dorée

Flavescence dorée (FD) is a grapevine disease caused by a phytoplasma, a parasitic microorganism of plants that spreads through the sieve elements of the grapevine. This disease can cause a reduction in the quality and quantity of the crop and hence, it can have a significant impact on grape production. It is important to monitor and prevent the spread of this disease to ensure plant health and crop quality. In highly susceptible cultivars, if left unchecked, the disease can lead to the death of infected plants.

Symptoms of (FD) include yellow, red, or purple leaves, red or purple streaks on vine shoots, and loss of grape quantity and quality. This disease is transmitted by an insect vector, a small insect known as the "grapevine leafhopper", i. e. *Scaphoideus titanus*. Insect vectors feed on the sieve elements of the grapevine and can transmit the FD phytoplasma (FDp) from one plant to another.

To prevent the spread of FD, farmers should use control methods against *S. titanus*, such as using insecticides and removing infected plants. In some countries, it is mandatory to monitor and report the presence of FD to the competent authorities to prevent the spread of the disease.

FD was first studied in the 1950s in France by Dr. Jules Emile Planchon, when it was first observed in a vineyard in the Champagne region. Planchon named the disease "Flavescence dorée" (yellowing gold) because of the characteristic yellow color of the leaves of infected plants. Initially, the disease was identified as a form of virosis but was later found to be caused by a parasitic microorganism, phytoplasma, transmitted by an insect vector.

FD is a pathology that falls into the category of grapevine yellows, a series of diseases with very similar or identical symptoms caused by phytoplasmas and therefore called phytoplasmosis.

At the time of their discovery, phytoplasmosis, such as the Bois noir (BN) discovered in France or the Vergilbungskrankheit found by Gartel in Germany in 1959, were all considered diseases of viral origin since the existence of phytoplasmas was not known until they were discovered in 1967 by Japanese researchers (Doy et al., 1967).

Over the years, the disease has spread to many winegrowing regions in Europe and elsewhere, causing significant economic losses for farmers. Nowadays, research on the epidemiology, diagnosis and control of FD continues to be studied by researchers and agronomists worldwide.

The FD manifested itself for the first time in Italy in 1973 (Lombardy) and then spread to all the northern regions while its presence was already reported between the 1950s and 1960s in France.

1.1.1 Symptomatology

The disease manifests itself through a series of symptoms that can vary according to the grapevine cultivar, the phase of development of the plant and the environmental conditions. The main symptoms of FD on grapevine are described below:

Foliage Disorder: The leaves of infected plants show a pale yellow or yellowish-green coloration in white grape vines and red or yellowish red coloration in red grape vines. In some cases, the leaves may turn pink or reddish along the margins or on the main veins. Leaf coloration can be uniform or patchy and may appear first on the lower part of the grapevine. Leaves may be smaller than those of healthy plants and drop prematurely. In addition, the leaves often assume a triangular shape by rolling their margins downward due to an accumulation of starch in these organs.

Cane Disorder: The canes of infected plants may be misshapen or streaked with red or purple. In some cases, the vines may be thinner than those of healthy plants, with a leathery texture. It is very common that the branches lignify less or not at all at the end of the growth season when it starts to get cold, causing the branches to turn black due to the low temperatures.

Reduced Growth: Infected plants show reduced growth compared to healthy ones and may appear less vigorous. Also, the shoots may be shorter and thinner than those of healthy plants.

Wood necrosis: Infected plants may have wood necrosis, which is manifested by a greyish or dark brown discoloration of the wood and the appearance of cracks or fractures on the surface. Wood necrosis can cause a reduction in productivity and lower product quality.

Cluster alteration: In spring, the flowers of infected plants can wither and fall, the grape clusters of infected plants can be smaller and have problems reaching ripeness. In addition, these grape clusters may have an anomalous flavor, with high acidity and a less intense aroma, which can negatively impact the quality and value of wine products. Moreover, in the late season, the grape clusters may also wither and shrivel, further reducing the yield and quality of wine.

It is important to note that these symptoms are identical to other grapevine phytoplasma diseases and that the definitive diagnosis of FD requires the analysis of plant tissue samples using specific laboratory techniques. In case of suspicion of infection, it is important to consult an expert in the field for the diagnosis and prevention of the disease.

Symptoms at the end of the season on Trebbiano leaves with yellowing affecting the entire leaf blade including the veins.

Pinot noir leaves showing redness between the veins with curling down.

Chardonnay leaves showing the typical triangular curling.

Dark pustules with an oily appearance appear at the base of the non-lignified shoots.

Dried inflorescence of Garganega in mid-June.

Symptoms on a bunch of a white grape cultivar in mid-August with withering of some berries. Wilting may affect all or part of the bunch.

Symptoms on the Garganega bunch in mid-August. The bunch shows a general wilting.

1.1.2 Susceptibility and incidence

The susceptibility of a grapevine variety to FD could depend on its ability to resist the pathogen and its ability to tolerate the effects of the disease, but also on its ability to repel or be less attractive to the vector insect of the phytoplasma, as above reported.

Although certain grapevine types may exhibit symptoms of infection, they may also be able to withstand disease without suffering serious harm to grape quality or yield. Other grapevine varieties may be particularly vulnerable and exhibit severe infection-related symptoms, including leaf deformation, tissue necrosis, slowed growth, and decreased yield.

Some studies suggest that *Vitis vinifera*, the most widely cultivated species of grapevine, is particularly susceptible to FD, while other species such as American and hybrid varieties are more resistant (Pavan et al., 2012). However, wide differences in susceptibility to FD, have been reported for *V. vinifera* varieties both from field observations and in controlled inoculation conditions (Borgo, 2016; Eveillard et al., 2016; Ripamonti et al., 2021). Field experiences revealed that some varieties, such as Chardonnay, Pinot gris, Barbera and Cabernet sauvignon show very serious damage from FD, while when some others, such as Tocai friulano, Bonarda or Moscato bianco, are subjected to the same disease pressure, only a few plants display the symptoms, usually in a less severe form (Borgo, 2016). Similarly, insect-mediated transmission of FDp allowed the classification of different genotypes into three distinct categories: i) accessions with high FD titres and high number of infected plants (i.e. Sauvignon and Cabernet Sauvignon); ii) accessions with intermediate FDp titres and and high number of infected plants (i.e Grenache, Chardonnay, Cabernet franc); iii) accessions with intermediate/low FDp titre and few infected plants (Syrah, Magdeleine, Merlot) (Eveillard et al., 2016). Recently, a similar work, performed on the most important *V. vinifera* varieties grown in Piedmont, identified three susceptibility clusters, one of which included Moscato and Brachetto showing low FDp titres and low percentage of infected plants (Ripamonti et al., 2021). Another study evaluated the susceptibility of different grapevine varieties to FD in a field trial (Jarausch et., 2021). The researchers inoculated various grapevine varieties with the FDp and monitored their infection rates. They found that some varieties, such as Muscat Ottonel, Gewürztraminer,

and Sylvaner, showed a lower infection rate and milder symptoms compared to other varieties such as P. gris and Pinot blanc, which showed a higher infection rate and more severe symptoms.

Overall, these studies suggest that different grapevine varieties have varying levels of susceptibility to FD, and some varieties may be more tolerant than others. This information can be valuable for grapevine growers in choosing the most appropriate varieties for their vineyards and implementing disease management strategies. Research efforts are underway to identify the most disease resistant vine varieties and to develop effective disease management techniques.

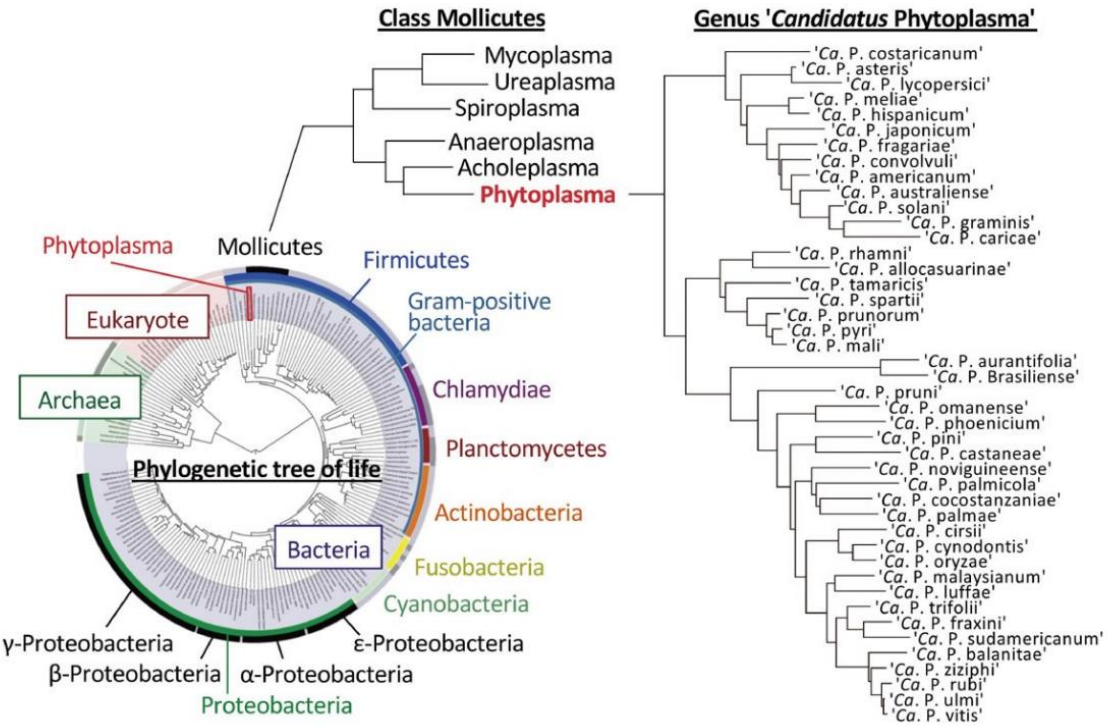
1.2 Phytoplasmas

Phytoplasmas are pathogenic bacteria that infect plants and cause a wide range of plant diseases, known as 'phytoplasmosis'. Phytoplasmas are bacteria with a missing cell wall that replicate inside the sieve elements of the phloem. They are transmitted by insect vectors such as plant hoppers and leafhoppers, which feed on the phloem sap of infected plants and transmit the bacterium from one plant to another.

Phytoplasmas are very different from common pathogenic bacteria, such as *Escherichia coli* and Salmonella, because they do not have rigid cell walls and are unable to survive outside plant cells. This makes their *in vitro* cultivation and molecular analysis difficult (Contaldo et al., 2016). However, DNA sequencing technology has made it possible to identify and classify phytoplasmas according to their genome.

The causative agent underlying FD is the FDp, for which the name of 'Candidatus Phytoplasma vitis' (IRPCM 2004) has been proposed. Because of their impossibility to be cultivated and therefore studied in all phases of their cycle, phytoplasmas are usually described within the provisional genus 'Candidatus Phytoplasma' and classified on the basis of 16S rRNA phylogeny. The specific strain to which FD-associated phytoplasma belongs is 16SrV, which in turn comprises two taxonomic subgroups: the 16SV-C and the 16SV-D (Rossi et al., 2019). FD-D is considered widespread, and it was reported in France, Spain, Portugal, Switzerland and

northern Italy regions, while FD-C is present in some Italian areas and in Slovenia, Croatia, Austria, Hungary and Switzerland (Angelini et al., 2001).



The location of the genus 'Candidatus phytoplasma' within the phylogenetic tree of life (Shigetu Namba, 2019).

1.2.1 Leaf diagnostics

Experts often conduct a visual inspection to identify grapevine yellows on leaves. Especially when they manifest during the growing season, the disease can typically be recognized by their specific symptoms.

As above specified yellowing and a red or purple tinge between the veins are symptoms of FD on leaves. Additionally, the edges of the leaves may curl downward. Later in the growing season, the leaves may turn dry and brittle and prematurely drop off the plant. However, symptoms caused by FD and BN, another disease caused by phytoplasma and carried by leafhoppers, are exactly the same

and the only way to distinguish FD from BN and have an exact diagnosis is through molecular analysis. Many laboratory procedures, such as DNA extraction followed by polymerase chain reaction (PCR), nested PCR, real-time PCR, and restriction fragment length polymorphism (RFLP), are available to establish the existence of the phytoplasma causing the disease. Early detection and identification of phytoplasmas in grapevines, which are crucial for the control and management of grapevine yellows, could be obtained collecting leaves with suspicious symptoms and the analysis trough DNA extraction and real-time PCR assay for the identification of phytoplasmas (Angelini et al.,2007). The real-time PCR assay is a rapid and sensitive method for detecting and quantifying specific DNA sequences in a sample. These methods enable the identification and measurement of the bacterium in plant leaf tissues and ensures that the growers can take the correct management measures, which are different in the case of FD rather than BN.

1.2.2 Diagnostics on wood

Some works have shown that the diagnosis of phytoplasmas in tissues other than leaves allowed the detection of a low proportion of positive samples. In grapevine the phytoplasma DNA detection from cane vascular tissues, cordons and trunks was reported for '*Candidatus* Phytoplasma australiense' (16SrXII-B) and Tomato big bud phytoplasma (16SrII-D), associated with Australian Grapevine Yellows (Constable et al., 2003), and also for BNp and FDp (Prezelj et al 2013; Terlizzi et al., 2007). In these works, the phytoplasmas were detected in various grapevine tissues throughout the year but were not always detected in all replicates. In general, the explanation of these results was attributed to the uneven distribution of phytoplasmas, or to the seasonal influence on the efficiency of detection, and/or to the low titre of phytoplasma. Recently, a reliable FDp detection in the secondary phloem of 1-year-old canes, arms and trunks of infected plants was obtained with in an optimized procedure for the extraction of high-quality RNA from woody tissues (Casarin et al., 2023).

1.3 Transmission by vector

The grapevine leafhopper (*Scaphoideus titanus Ball*) is the primary insect vector responsible for the transmission of the phytoplasma that causes FD.

When the leafhopper feeds on an infected grapevine the bacterium invades the salivary glands of the leafhopper, where it replicates and multiplies until the leafhopper feeds on a healthy grapevine and the bacteria are injected into the plant, causing the disease.

The bacterium is able to survive and multiply in the insect's salivary glands because it has adapted to the unique environment of the insect. The bacterium has developed mechanisms to evade the insect's immune system and to manipulate its behavior, making it more likely that the insect will feed on infected plants and spread the bacterium to healthy ones (Bressan et al., 2019).

The transmission of the bacterium through the insect vector is a complex process that involves a series of interactions between the bacterium, the insect, and the plant. The precise mechanisms involved in these interactions are still being studied, but it is known that the bacterium is able to manipulate the insect's behavior, making it more attracted to infected plants and less attracted to healthy ones (Chuche et al., 2016). Additionally, the bacterium can modify the plant's physiology in a way that makes it more hospitable to the insect, facilitating its colonization and reproduction (Bertazzon et al., 2019).

This leafhopper has a life cycle that includes egg, nymph, and adult stages. Eggs are laid in the bark of grapevine trunks in late summer or early fall, and they hatch in the following spring. Nymphs feed on grapevine leaves and buds and go through several molts, usually five stages, before reaching adulthood. The adult leafhoppers feed on grapevine phloem and are capable of transmitting the FD phytoplasma to healthy vines. The infectivity of the leafhoppers varies depending on their developmental stage, with adults being the most infective. (Pirola et al., 2013).

The grapevine leafhopper is a highly mobile insect that can move over great distances and feed from numerous grapevines over the course of its lifetime. As a result, it serves as an important vector for the dissemination of FD in vines. The danger of transmission is greatest during the summer months when the bug is most active.

An adult specimen of *S. titanus*

1.3.1 Agronomic management and vector control

Preventing the spread of FD requires effective control of the grapevine leafhopper, *S. titanus*. Several control methods have been developed to reduce the populations of grapevine leafhoppers and prevent the transmission of the bacterium, furthermore, the Italian government has now implemented mandatory measures to control the FD spread. These measures include mandatory treatment of vineyards against *S. titanus* which are necessary to prevent the spread of FD and to maintain the health of grapevine crops. The national law is the Legislative Decree No. 214 of 2017, which establishes measures for the control of the grapevine disease, including the obligation to carry out treatments against the insect vector. Additionally, each Italian region can establish its own measures for the control of FD, and some regions have established additional rules on mandatory treatment against *S. titanus*. For example, in the Veneto region, the Regional Law No. 2 of 2014 established mandatory treatments against the insect vector. Failure to comply with these measures will result in substantial fines and penalties for vineyard owners.

One common control method is the use of insecticides. Insecticides can be applied to grapevines to kill grapevine leafhoppers and reduce their populations. However, this method can be expensive and may have negative environmental impacts.

Several insecticides are effective against *S. titanus*, and they belong to different chemical groups. Some of the most commonly used insecticides are neonicotinoids, pyrethroids, and spinosyns. Neonicotinoids such as imidacloprid and thiamethoxam work by binding to and activating nicotinic acetylcholine receptors in the insect's nervous system, leading to paralysis and death. Pyrethroids such as deltamethrin and lambda-cyhalothrin act by prolonging the activity of sodium channels in the insect's nervous system, leading to hyperexcitation and paralysis. Spinosyns such as spinosad and spinetoram work by disrupting the activity of nicotinic acetylcholine receptors and gamma-aminobutyric acid receptors in the insect's nervous system, leading to hyperexcitation and paralysis. Insecticides can be applied by foliar spraying or soil drenching, depending on the stage of the insect and the timing of the treatment (www.regione.veneto.it) It is important to use insecticides responsibly, following the label instructions and local regulations, to minimize the risk of environmental contamination and development of insecticide resistance, the choice of insecticide and the time of application will depend on various factors such as the severity of the infestation, the stage of the insect's life cycle, and the local regulations governing their use.

The timing of insecticide applications against *S. titanus* is crucial for effective control of the vector. In general, it is recommended to treat vineyards during the nymphal stage of the insect, which occurs from May to June, and during the pre-harvest period in August to September. During the nymphal stage, the insects are more vulnerable to insecticides and have not yet acquired the FD phytoplasma. Treating the vineyards during this stage can reduce the number of insects that become infective and, consequently, reduce the spread of FD. The pre-harvest period is also a critical time to control *S. titanus* as the insect is highly active and can transmit the pathogen to healthy grapevines. Proper timing of insecticide applications is crucial for effective control of *S. titanus* and the spread of FD (www.regione.veneto.it).

The European policies aimed at protecting human health, biodiversity and sustainability have led to the revision of agrochemicals used in viticulture. Some highly effective but toxic insecticides have been banned, while the remaining ones are less effective and have lower toxicity. Therefore, precision timing and application of treatments, as well as proper agronomic practices, have become crucial for effective pest control. The number of insecticide treatments in

integrated viticulture has doubled, and the use of precision techniques has become important for both integrated and organic viticulture.

Controlling the population of grapevine leafhoppers can also be done through cultural activities. Infected grapevines should be removed, resistant grapevine cultivars should be planted, and nitrogen fertilization should be decreased. Additionally, minimizing the use of pesticides and avoiding overpruning can aid in the promotion of the grapevine leafhopper's natural predators.

Biological control agents, such as parasitic wasps and spiders, can also be used to control *S. titanus* populations. These agents can be introduced into vineyards to prey on grapevine leafhoppers and reduce their populations.

1.4 Transmission by grafting

Grafting is a common practice in viticulture that involves the transfer of plant tissues from one individual to another in order to propagate and maintain desirable grapevine cultivars. However, the practice of grafting also presents a risk for the transmission of systemic plant diseases, including FD. The transmission of FD through grafting can occur when scion and rootstock material is harvested from infected plants and then used to propagate new plants. Even if the scion and rootstock materials appear healthy, they can still carry the phytoplasma and infect the new plants. This poses a serious risk for the spread of FD, particularly in areas where the disease is already present. Therefore, it is essential for grapevine nurseries to carefully monitor their plant material and take measures to prevent the transmission of FD through grafting, such as testing for the presence of the phytoplasma and using clean, disease-free plant material for propagation. Additionally, it is important for grape growers and nursery managers to be aware of the risks associated with grafting and to take precautions to prevent the spread of FD within their vineyards.

1.4.1 Management in nursery

Grapevine nurseries play a critical role in the spread of Flavescence dorée (FD) and other grapevine yellows (GY) diseases. These diseases, caused by phytoplasmas, pose a significant threat to viticulture in various European countries. It is essential for grape growers and nursery operators to be vigilant and take necessary

precautions to prevent the transmission of FD. The occurrence of FD and Bois noir (BN) in nurseries and young vineyards has been investigated, and it was found that the presence of infected grapevines is relatively low in nurseries. However, as the plants grow older in vineyards, the incidence of infection increases significantly.

To minimize the transmission of FD in grapevine nurseries, several measures should be taken. First and foremost, strict sanitary controls must be implemented to identify and eliminate infected plants. Visual inspections and molecular analyses should be conducted regularly to detect symptomatic grapevines. Infected plants should be promptly removed to prevent further spread. Additionally, it is crucial to monitor the insect vectors responsible for transmitting FD, such as the leafhopper Scaphoideus titanus and the planthopper Hyalesthes obsoletus. Regular monitoring of these vectors in nurseries, vineyards, and nearby areas can help identify their presence and take appropriate measures to mitigate their impact. In particular, attention should be given to the presence of BN-infected spontaneous plants, as they can act as additional sources of transmission.

Strict adherence to traceability protocols is vital in grapevine nurseries. It is essential to keep track of grapevine materials from the nursery to the vineyard, ensuring that infected materials are not sold or used for new plantations. By implementing proper tracking systems, it becomes possible to identify the sources of infection and prevent their introduction into new vineyards.

Furthermore, nursery operators and grape growers should prioritize the use of healthy grapevine materials obtained from reliable sources. Regular inspections and testing of vine multiplication materials are essential to ensure their health and prevent the spread of FD. In case infected materials are detected, they should be isolated, treated, or destroyed to prevent further transmission.

In conclusion, the management of FD in grapevine nurseries requires vigilance and proactive measures. Strict sanitary controls, monitoring of insect vectors, traceability protocols, and the use of healthy grapevine materials are key strategies to minimize the transmission of FD and protect vineyards from this devastating disease. By implementing these practices, grape growers can safeguard their vineyards and contribute to the overall health and sustainability of the viticulture industry.

1.4.2 New rules for the healing of cuttings

New European rules outline the management requirements for wood material used for planting *Vitis* L. plants (excluding seeds) to prevent the spread of FDp. According to these rules, plants for planting must meet specific criteria:

(a) They must originate from an area known to be free from FDp.

(b) Alternatively, if the plants originate from a production site, the following conditions must be met:

(i) No symptoms of FDp have been observed on *Vitis* L. plants at the production site or within a surrounding zone of 20 meters during the last complete cycle of vegetation. In the case of plants used for *Vitis* L. propagation, no symptoms of FDp have been observed at the site of production or within a surrounding zone of either 20 meters for scion production sites or 40 meters for rootstock production sites during the last two complete cycles of vegetation.

(ii) Monitoring of the vectors (insects that transmit the phytoplasma) is conducted, and appropriate treatments are carried out in areas where the vectors are present to control their populations.

(iii) Any abandoned *Vitis* L. plants within a 20-meter radius of the production site must be removed (rogued out).

(c) Alternatively, the plants must have undergone hot water treatment in accordance with international standards.

These rules aim to ensure that plants for planting are sourced from disease-free areas or production sites with proper vector monitoring and control measures in place. Hot water treatment is another approved method to eliminate potential pathogens from the plants. Compliance with these regulations is essential to prevent the introduction and spread of FDp and protect vineyards from this devastating disease.

2 PURPOSE OF THE THESIS

The aim of this thesis is to investigate the presence and the titre of the FDp in woody organs of Glera and Pinot gris grapevines. FD is a destructive grapevine disease caused by phytoplasma that affects both the yield and quality of grapes. The disease spreads through leafhopper vectors, but it can also be transmitted through infected plant material. Hence, studying the presence and amount of phytoplasma in woody organs can help understand the disease's progression and devise efficient control measures. Glera and P. gris grapevines were chosen as they are widely cultivated in the Veneto region, and FD has been reported in both varieties.

The present work focuses on the diagnosis of FDp in different woody organs of grapevine, an innovative aspect in FD research. The quantity of FDp in different parts of the plant, such as the trunk, the cordon, 2-year-old arms and the annual growth canes is investigated and the FDp spread within symptomatic plants of Glera and P. gris is compared. The focus on the woody organs of the vines will allow for a more comprehensive understanding of the disease's progression and spread in two grapevine cultivars. Furthermore, the risk of FDp-transmission through the graft is investigated.

The findings of this study can be used to develop effective control measures to prevent the spread of FD disease, providing new insight in the management of symptomatic vines in vineyards and limiting the production of infected propagation material by the nurseries. Finally, the present work will contribute to the sustainability and profitability of grapevine cultivation.

3 MATERIALS AND METHODS

3.1 Grapevine materials and sampling

Two vineyards located in Cessalto (TV), cultivated with Glera and P. gris, had been selected based on their high rate of FD-infection (88 and 31% for Glera and P. gris in 2022, respectively). The Glera vineyard had been planted in 2012 while the P. gris in 2005. Both vineyards were grown vertically with sylvoz pruning, with three fruiting arms, and had similar climate, soil, growing techniques, and other factors that made them ideal for comparison.

The two vineyards were monitored in 2021 and 2022 and symptomatic plants were identified, mapped and labelled during the years. The monitoring of the two vineyards was an essential aspect of the study as it enabled us to track the disease's presence and to compare the plants' health throughout the two fields. The FD-infected symptom evaluation was based on the plant's subdivision in the following three classes of infection: (1) plants with only one symptomatic fruiting arm; (2) plants with two symptomatic fruiting arms; (3) plants with all 2-year-old arms with FD symptomatic canes.

In September 2022, 40 plants, 20 for each cultivar, which were asymptomatic in 2021 and showing a different severity of FD symptoms in 2022, were selected and symptomatic and asymptomatic annual growth canes were marked. Regarding Glera, the lignified canes were distinguished between those showing symptomatic leaves and those with all asymptomatic leaves. In February 2023, secondary phloem of permanent structures (trunk, cordon), fruiting arms and 1-year-old lignified canes were collected, and some samples were obtained by longitudinally cortical scraping (10 cm), after bark removal, according to the scheme depicted in figure 1. The sampling took place in vineyard using liquid nitrogen to ensure tissue preservation until the storing at -80°C. After that, plants have been uprooted and roots fragments (length 10 cm, diameter 3-5 mm) were collected, washed with water and, after cork removal, they were freeze dried and then kept at -80°C until RNA extraction.

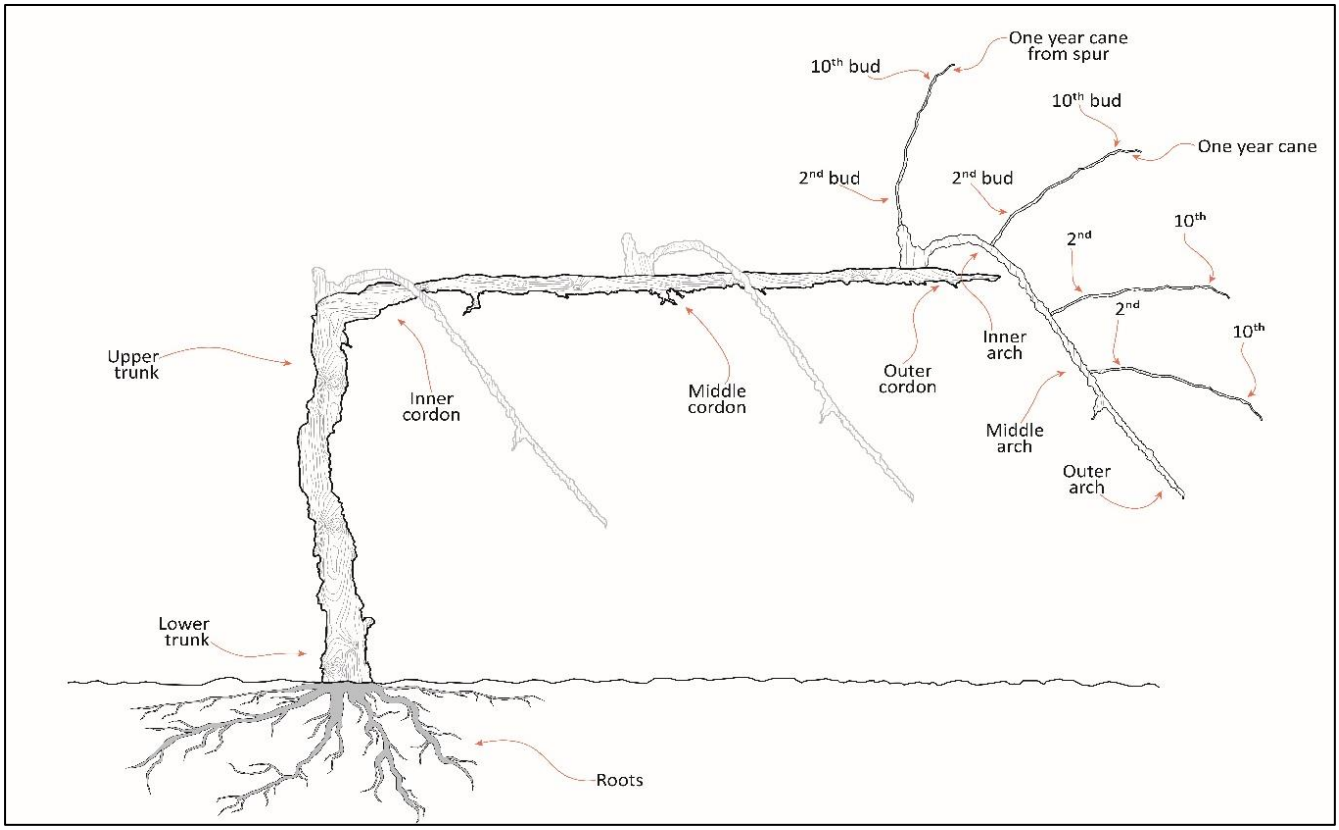


Figure 1: schematic representation of sampling portions collected in FD-infected Glera and Pinot gris plants.

Among the sampled grapevines, six plants per variety, three plants with severe symptoms of FD (Figure 2) and three plants with milder symptoms (Figure 3), were selected to study the distribution of FDp.

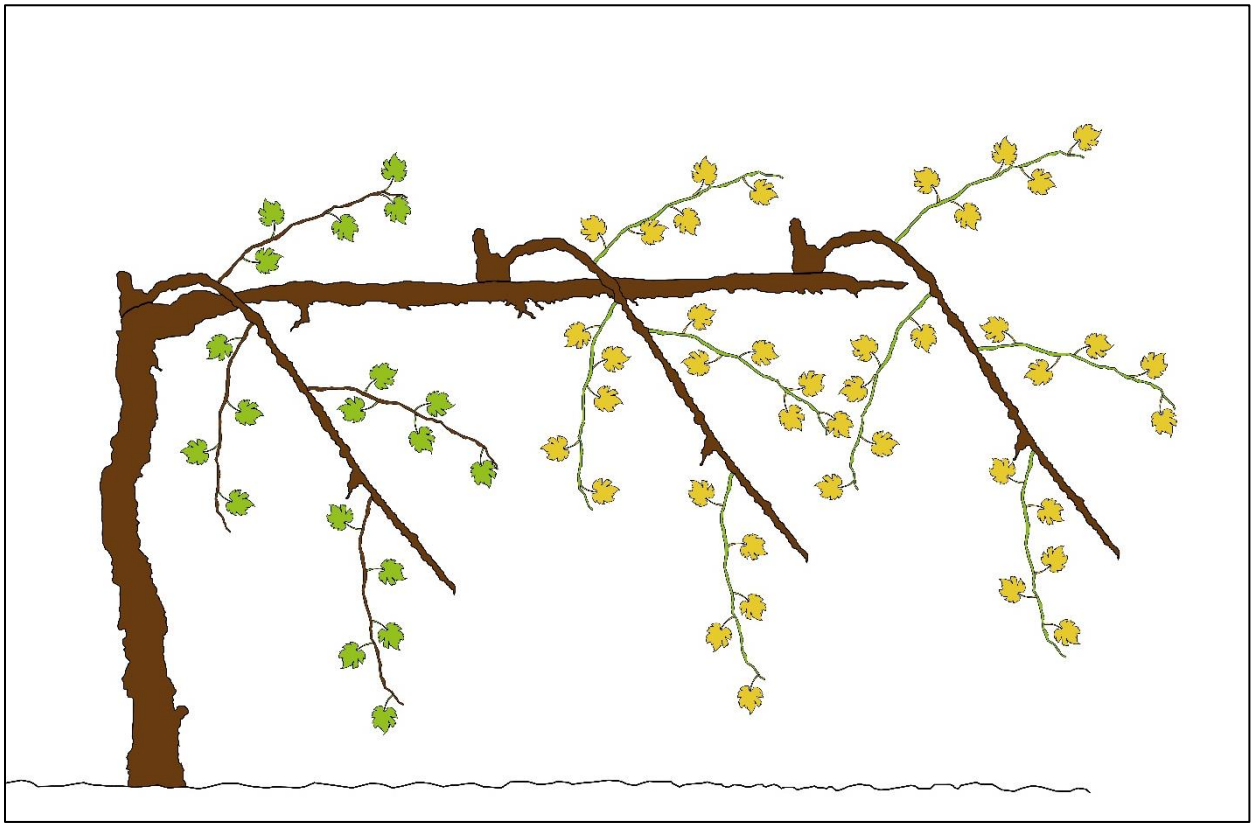


Figure 2: schematic representation of plants showing severe FD-symptoms selected during the survey performed in September. Symptomatic portions, indicated with yellow leaves and green canes, usually involved two or more fruiting arms.

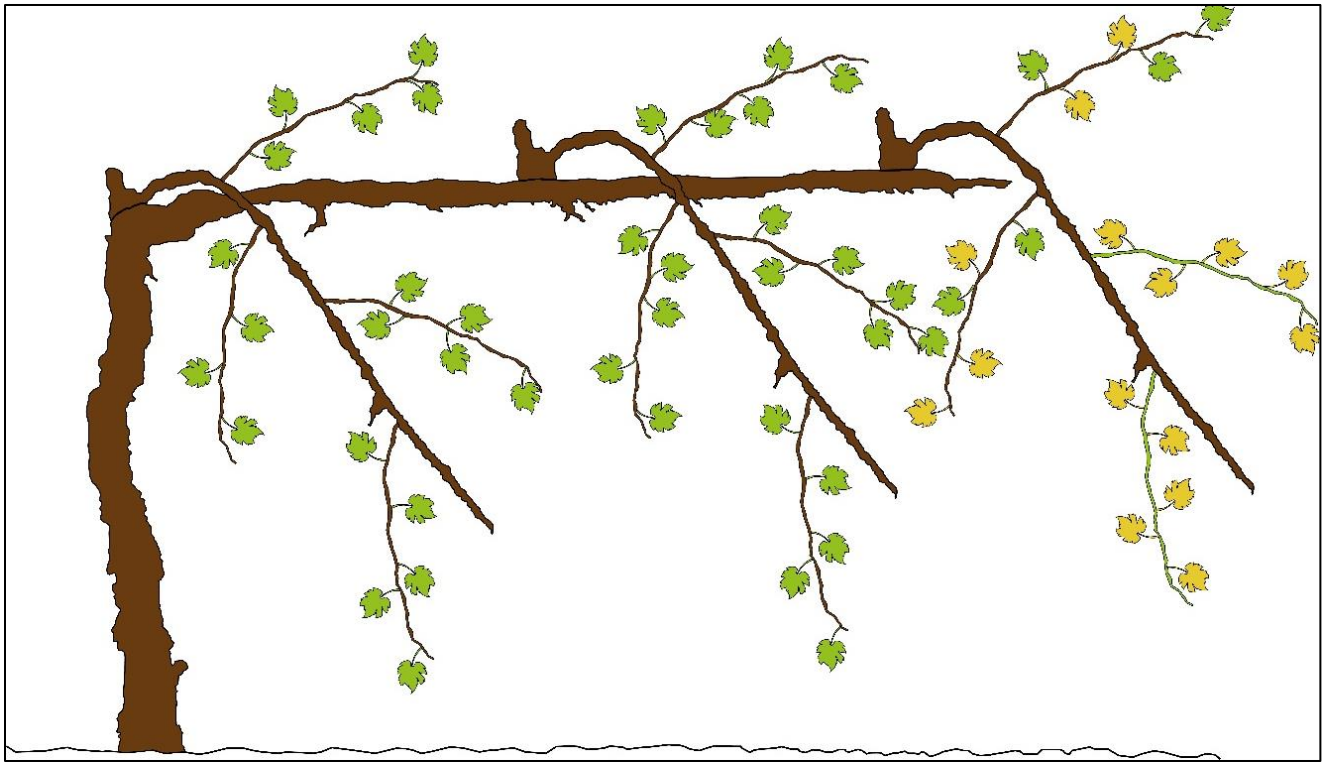


Figure 3: schematic representation of plants showing mild FD-symptoms selected during the survey performed in September. Symptomatic portions, indicated with yellow leaves and green canes, usually involved only one fruiting arm.

Among the longest 1-year-old lignified canes grown from symptomatic plants, five were selected for P. gris and ten were selected for cultivar Glera, comprising five asymptomatic canes and five lignified canes which previously showed symptomatic leaves. Phloem samples were collected from approximately the second, eighth, twelfth and the twentieth buds.

In September 2022 nine plants with yellowing symptoms were marked in the rootstock ampelographic collection of the Research Centre for Viticulture and Enology of Susegana (Northeast Italy) and later tested positive for the presence of FDp on leaves. They all belonged to nine different rootstock cultivars: Riparia Martin, Cosmo 4, 16.3 Couderc, 2413 Grimaldi, 1321 Paulsen, 8 Richter, 110

Richter, 205 Ruggeri. In February 2023 from each of these plants, parts of the trunk and all the lignified canes were collected and sampled as described above.

3.2 RNA extraction from secondary phloem of woody organs

The extraction of RNA from woody organs of grapevines was performed using the RNeasy Plant Kit (Qiagen), with a modified protocol optimized for the extraction of high-quality RNA from woody tissues, which are rich in polysaccharides and polyphenolic compounds, to avoid inhibition of PCR reaction (Mackenzie et al., 1997). The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. In the first step 100 mg of the frozen sample were homogenized using a mortar and pestle, and the resulting powder was transferred to a tube. The extraction of RNA was performed according to the following protocol:

1. Add 1 ml of extraction buffer, containing guanidinium thiocyanate, PVP, sodium acetate, EDTA, and metabisulfite, to each sample.
2. Add 200 µl of sarcosyl to each sample to help denature the cells.
3. Vortex and spin the samples briefly before incubating them at 70°C for 10 min. Use tubes with safe-lock lids to avoid leaks and shake the tubes after 5 min to ensure homogenization.
4. Carefully transfer 650 µl of the resulting liquid to the purple columns provided in the kit and centrifuge them for 2 min at 14,000 rpm.
5. Add 450 µl of the resulting liquid, along with 225 µl of pure ethanol, to the pink columns to help bind the RNA to the column filter. Centrifuge the columns for 5 min at 10,000 rpm.
6. Wash the columns using the kit-provided wash solutions, once with RW1 solution and twice with RPE solution making sure to empty the resulting liquid every time.
7. Add 50µl of Rnase-free water twice, spin the columns, after which the columns should contain the desired RNA and be placed at -80°C for preservation until use.

The quality and quantity of the extracted RNA were evaluated by absorbance reading using Nanodrop one (Thermo scientific).

3.3 RNA extraction from roots

The following is a modified protocol for RNA extraction from lyophilized roots that have been ground to dust using the TissueLyser II (Qiagen), using the Spectrum Plant Total RNA Kit by Sigma:

1. Begin by washing, cleaning, and removing the bark from the roots. Lyophilize the cleaned roots to obtain lyophilized samples.
2. Using a tissue lyser, grind the lyophilized root samples twice for two min at 30 Hz to obtain a fine powder.
3. Take 50 mg of the ground root sample and add it to a tube.
4. Add 500 µl of lysis solution to the tube containing the root sample.
5. Vortex the tube for 30 sec to ensure thorough mixing of the sample with the lysis solution.
6. Incubate the tube at 56°C for 5 min to allow for effective lysis of the cells.
7. Spin the tube at 14,000 rpm for 3 min to separate the lysate from any debris or insoluble material.
8. Transfer the clear lysate to a filtration column and spin it at 14,000 rpm for 1 min to collect the filtrate.
9. Transfer the collected supernatant (filtrate) to a new 1.5 ml tube.
10. Add 750 µl of binding solution to the tube containing the lysate and mix thoroughly.
11. Transfer 500 µl of the lysate mixture to the binding column and spin it at 14,000 rpm for 1 min. Discard the flow-through.
12. Repeat step 11 for the remaining lysate mixture, transferring 500 µl at a time to the binding column and spinning it at 14,000 rpm for 1 min. Discard the flow-through each time.

13. Wash the binding column twice with 500 µl of wash solution 1, spinning at 14,000 rpm for 1 min each time. Discard the flow-through after each wash.

14. Wash the binding column four times with 500 µl of wash solution 2, spinning at 14,000 rpm for 30 sec each time. Discard the flow-through after each wash.

15. After the final wash, spin the binding column at 14,000 rpm for 1 min to remove any residual wash solution and dry the column.

16. Transfer the binding column to a new 1.5 ml collection tube.

17. Add 40 µl of elution solution to the binding column and incubate for 2 min to elute the RNA.

18. Spin the binding column at 14,000 rpm for 1 min to collect the eluted RNA in the collection tube.

At this point, the eluted RNA is ready for further downstream applications or storage as per your experimental requirements.

The quality and quantity of the extracted RNA were evaluated by absorbance reading using Nanodrop one (Thermo scientific).

3.4 Transcription

RNA is a valuable molecule in many biological processes, but for certain applications, it is necessary to convert it into DNA. This process, known as reverse transcription, is commonly used in molecular biology and genetic research. Reverse transcription involves the use of a reverse transcriptase enzyme, which synthesizes a complementary DNA (cDNA) strand from an RNA template. The resulting cDNA can be used in a variety of downstream applications, including PCR, cloning, and sequencing.

The process of reverse transcription typically involves two main phases: annealing and extension. To initiate the reaction, 18 µl of extracted RNA is combined with 2 µl of random hexamer primers (0.5 µg/µl). The mixture is then heated in a thermocycler, such as the BIO-RAD iCycler, at 95 °C for 5 minutes to facilitate denaturation. After denaturation, a reaction mix is prepared by adding 10 µl of buffer (5x), 2.5 µl of dNTPs (2.5 mM), 2 µl of DTT (0.1 M), and 0.5 µl of M-MLV

Reverse Transcriptase (200 U/µl) (Invitrogen) to the sample. The reaction mix is thoroughly mixed and incubated at 42 °C for 50 minutes to allow for annealing.

Following the annealing step, the complementary DNA (cDNA) generated during the reverse transcription process is preserved by storing it at -20 °C until further amplification. It is important to note that these specific quantities and steps are provided as an example and may vary depending on the experimental protocol and specific requirements.

3.5 DNA extraction

DNA extraction is a fundamental step in molecular biology and genetic research, allowing scientists to isolate and purify DNA from different sources for further analysis. The DNeasy Plant Mini Kit by Qiagen is a widely used and reliable kit specifically designed for extracting DNA from plant tissues. This kit utilizes silica membrane-based purification technology to efficiently bind DNA, while removing contaminants such as proteins, polysaccharides, and other cellular components.

Here is the DNA extraction protocol using the DNeasy Plant Mini Kit by Qiagen:

1. Disrupt samples of woody tissue using the TissueRuptor®, the TissueLyser II, or a mortar and pestle with liquid nitrogen.
2. Add 400 µl of Buffer AP1 and 4 µl of RNase A to 50mg of the disrupted samples. Vortex the mixture and incubate for 10 min at 65°C. Invert the tube 2-3 times during incubation. Note: Do not mix Buffer AP1 and RNase A before use.
3. Add 130 µl of Buffer P3 to the mixture. Mix well and incubate for 5 min on ice.
4. If desired, centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm).
5. Transfer the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x g.
6. Carefully transfer the flow-through into a new tube without disturbing any pellet if present. Add 1.5 volumes of Buffer AW1 and mix by pipetting.
7. Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 x g (≥8000 rpm). Discard the flow-through. Repeat this step with the remaining sample.

8. Place the spin column into a new 2 ml collection tube. Add 500 µl of Buffer AW2 and centrifuge for 1 min at ≥6000 x g. Discard the flow-through.
9. Add another 500 µl of Buffer AW2 and centrifuge for 2 min at 20,000 x g. Note: Carefully remove the spin column from the collection tube to avoid contact with the flow-through.
10. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
11. Add 100 µl of Buffer AE for elution. Incubate for 5 min at room temperature (15-25°C). Centrifuge for 1 min at ≥6000 x g.
12. Repeat step 11 to further elute the DNA.

Following these steps will enable the extraction of DNA from the woody plant material, yielding purified DNA that can be used for various downstream applications.

3.6 Real time PCR

Real-time PCR is a highly sensitive and accurate technique used to detect and quantify DNA in real-time during amplification. In this research, a real-time PCR approach was used to detect the presence of FDp DNA obtained from transcription of the RNA found in the woody organs of grapevines. A specific FD DNA primer designed for the phytoplasma was used. Additionally, a control primer targeting the plant cell cytochrome oxidase DNA was used for normalization and accurate quantification of the results.

The real-time PCR process involves three stages: denaturation, annealing, and extension. During denaturation, the DNA template is heated to a high temperature to separate the DNA strands. Then, during annealing, the primers bind to their complementary sequences on the DNA template. In this study, specific primers were designed for the FD DNA and the plant cell cytochrome oxidase DNA. The final stage is the extension, where the polymerase enzyme binds to the primers and copies the DNA template. The length of the extension phase depends on the size of the DNA fragment being amplified.

The real-time PCR technique allows for the detection of DNA in real-time by monitoring the fluorescence emitted by a fluorescent marker, such as SYBR Green, during amplification. This fluorescence is proportional to the amount of double-stranded DNA present in the reaction mixture, enabling quantification of the DNA. The amplification curves generated from the real-time PCR were used to quantify the amount of FD DNA present in the grapevine samples' woody organs.

The amplification of FD DNA was compared to the amplification of the plant cell cytochrome oxidase DNA, which served as a reference gene. The reference gene was chosen because its expression remains constant under all plant conditions. This comparison allowed for data normalization and accurate quantification of the FD DNA in the grapevine samples.

Overall, real-time PCR provides a sensitive, accurate, and rapid method for detecting and quantifying FD DNA in the woody organs of grapevines. The use of a fluorescent marker, such as SYBR Green, in the reaction buffer enables real-time detection and quantification of the DNA. This technique is valuable for the detection and quantification of FD in grapevines.

In this study, the real-time PCR analysis was performed using a Bio-Rad thermocycler (model CFX96) with 96-well plates. PCR reactions were conducted in duplicate, with a total volume of 10 µl. The reaction mixture consisted of 1 µl of cDNA, 5 µl of 2x Platinum SYBR Green qPCR Supermix-UDG (Invitrogen), and 0.5 µl of each primer (6 µM). The thermal protocol included an initial decontamination step at 50 °C for 3 min to activate the UDG (Uracil DNA Glycosylase) enzyme, followed by a step at 95 °C for 3 min to activate the Taq polymerase, inactivate the UDG, and denature the DNA double strand. Subsequently, 50 cycles of a two-step protocol were performed: denaturation at 95 °C for 5 sec and primer annealing and extension at 60 °C for 30 sec. The specificity of the amplification was verified by constructing a melting curve, gradually heating the reaction from 50 °C to 95 °C.

For the diagnosis of FD, a primer pair designed for the 16S rRNA gene was used (forward primer: 5’- AAGTCGAACGGAGACCCTTC -3’, reverse primer: 5’- TAGCAACCGTTTCCGATTGT -3’) (Angelini et al., 2007). The citochrome oxidase (COX) gene of grapevine was used as the reference gene for normalizing the FD DNA titre (forward primer: 5’- CGTCGCATTCCAGATTATCCA -3’, reverse primer: 5’- CAACTACGGATATATAAGAGCCAAAACTG -3’) (Bertazzon et al., 2012).

3.7 Nursery operations

To evaluate the risk of transmission of FDp through vegetative propagation, a trial was conducted with different scion-rootstock combinations, some healthy and some probably infected.

On the 27th of April, the grafting procedures were carried out in a specialized nursery using an Omega grafting machine. In order to prevent contamination, the healthy theses were grafted first, followed by the infected ones. This was done to avoid any potential spread of infection to the healthy grafts. The Omega grafting machine is known for its precision and efficiency in grafting and is widely used in the agricultural industry. With this machine, the process of grafting is made easier and faster, which allows for a greater number of grafts to be completed in a shorter amount of time. The use of this machine in conjunction with proper hygiene protocols helps to ensure that the newly grafted plants are healthy and disease-free.

Several grafting combinations were performed as described below and the number of graftings is reported in table 1:

1-FD-infected P. gris and Glera scions on SO4:

The first thesis involves grafting P. gris and Glera scions from infected plants on to healthy SO4 rootstocks. The mother plants that were used to harvest the scions were selected carefully and canes were harvested from plants that showed symptoms in two out of three arches, this was done to ensure the wood was of good quality but still originating from a very badly infected plant (fig. 2). Three different types of canes were considered for Glera: a) Lignified canes which previously showed symptomatic leaves (thesis 1), b) Lignified canes with asymptomatic leaves (thesis 2), c) Lignified canes that tested positive to FDp (thesis 3). Two different types of canes were considered for P. gris: a) Lignified canes with asymptomatic leaves (thesis 4), b) Lignified canes that tested positive to FDp (thesis 5).

2-Healthy P. gris and Glera scions on SO4:

Healthy Glera and P. gris was also grafted on to healthy SO4 to have as a control (theses 6 and 7, for Glera and P. gris respectively).

3- FD-infected P. gris and Glera scions grafted on healthy P. gris and Glera as rootstocks*:*

This thesis required grafting infected P. gris and Glera scions on healthy P. gris and Glera rootstocks to understand if the grafting union itself has a healing effect on the scion (theses 8 and 9, for Glera and P. gris respectively).

4- FD-infected cuttings of Glera and P. gris:

In the fourth thesis the infected canes were kept as cuttings to be planted as is to determine the effect of grafting on the manifestation of symptoms. In detail, lignified cuttings of Glera which previously showed symptomatic leaves (thesis 10), lignified cuttings of Glera with asymptomatic leaves (thesis 11) and lignified cuttings of P. gris with asymptomatic leaves (thesis 12).

5-FD-infected rootstocks on P. gris:

In the last thesis nine different American rootstocks were used for grafting with healthy P. gris, the rootstocks were harvested from infected mother plants. The purpose of this last thesis is to see if infected rootstocks, which are known to be more resistant to FD, can transmit the disease to the scion (theses from 13 to 21).

Thesis	rootstock	scion	Quantity
1	SO4	GLERA-Lignified canes with symptomatic leaves	330
2	SO4	Glera-Lignified canes with asymptomatic leaves	520
3	SO4	Glera-Lignified canes that tested positive to FDp	100
4	SO4	P. gris-Lignified canes with asymptomatic leaves	520
5	SO4	P. gris-Lignified canes that tested positive to FDp	100
6	SO4	Glera-Healthy	220
7	SO4	P.gris-Healthy	83
8	Healthy Glera	Glera-Lignified canes with asymptomatic leaves	150
9	Healthy p.grigio	P. gris-Lignified canes with asymptomatic leaves	190
10	---------	GLERA-Lignified cuttings with symptomatic leaves	115
11	---------	Glera-Lignified cuttings with asymptomatic leaves	200
12	---------	P. gris-Lignified cuttingswith asymptomatic leaves	200
13	16.3 Couderc	Pinot gris	24
14	16-109 Prosperi	Pinot gris	16
15	8 Richter	Pinot gris	17
16	Riparia Martin	Pinot gris	13
17	Cosmo 4	Pinot gris	26
18	205 Ruggeri	Pinot gris	28
19	1321 Paulsen	Pinot gris	12
20	110 Richter	Pinot gris	27
21	2413 Grimaldi	Pinot gris	11

Table 1: for each experimental thesis the combination of rootstock/scion and the numbers of carried out grafting are indicated.

4 Results

4.1 FDp Detection in Different Woody Organs of Glera and P. gris symptomatic plants.

To investigate the presence of FDp in various woody organs, we analyzed six plants per cultivar, with three plants representing each of the two symptom classes (Fig 2 and 3). Results reporting the presence and the titre of FDp are displayed in the schematic representation of the plants in figure 4 and 5, for Glera and P. gris, respectively. In detail, results on different woody organs are described below.

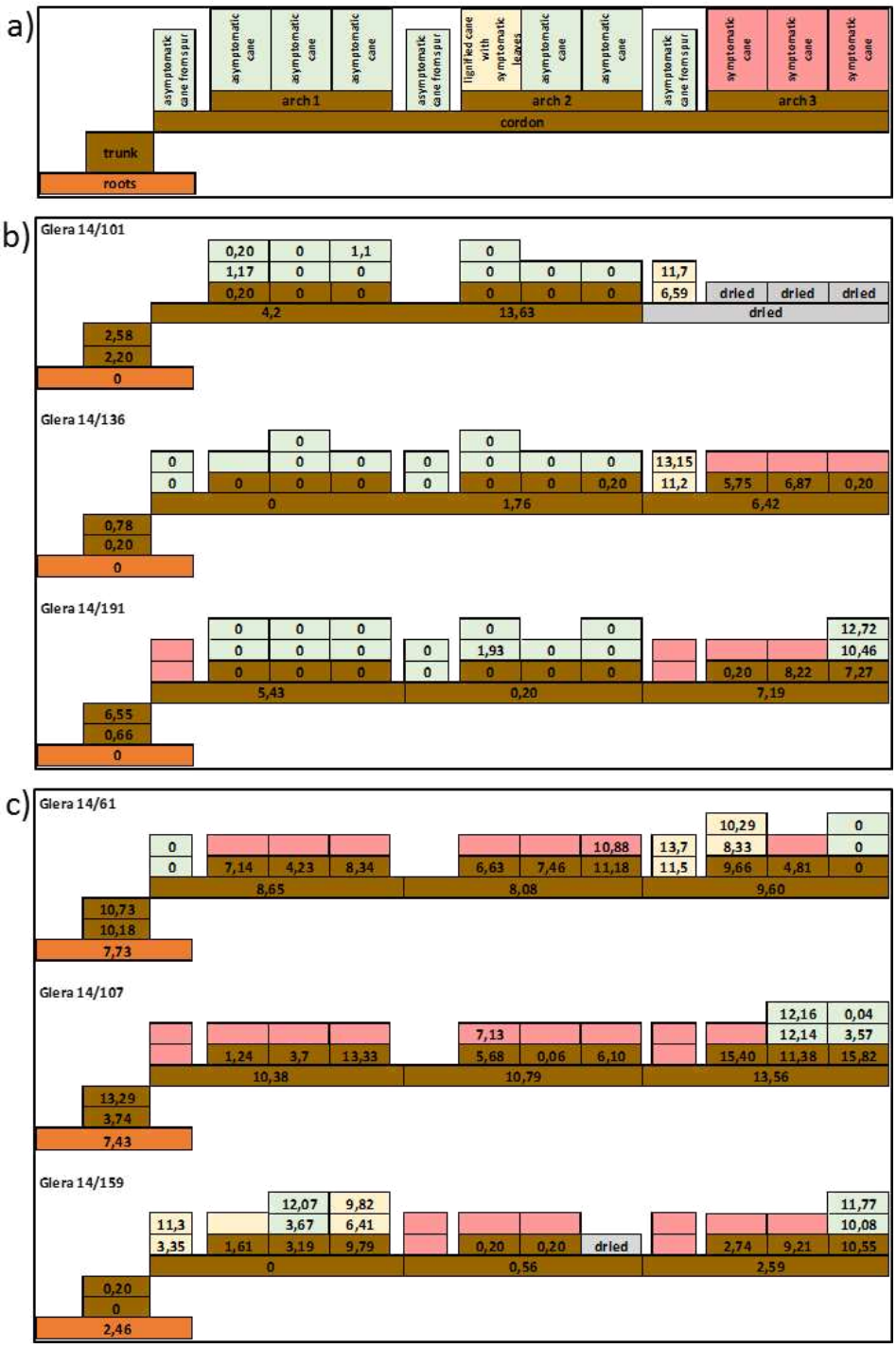


Fig. 4: a) schematic representation of different woody parts of symptomatic plants; b) titre of FD phytoplasma, expressed as arbitrary units (log2), in different woody parts of plants of cultivar Glera showing mild FD symptoms; c) titre of FD phytoplasma, expressed as arbitrary units (log2), in different woody parts of plants of cultivar Glera showing severe FD symptoms.

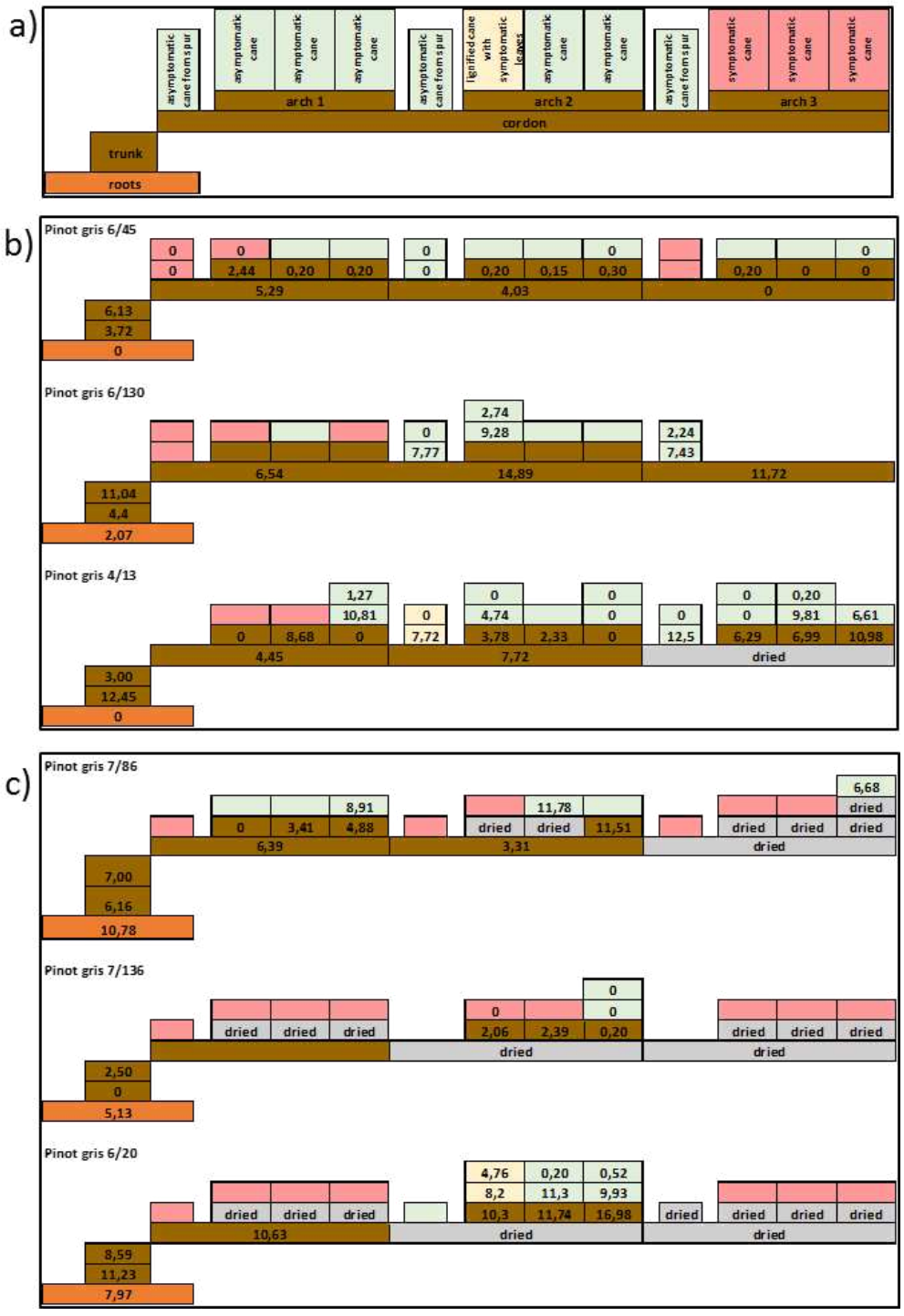


Fig. 5: a) schematic representation of different woody parts of symptomatic plants; b) titre of FD phytoplasma, expressed as arbitrary units (log2), in different woody parts of plants of cultivar Pinot gris showing mild FD symptoms; c)) titre of FD phytoplasma, expressed as arbitrary units (log2), in different woody parts of plants of cultivar Pinot gris showing severe FD symptoms.

Roots

Analysis of the roots revealed that 58.3% of the plants tested positive for FDp. In both Glera and P. gris, all three plants from the highly infected thesis showed positive results. However, among the plants belonging to the less infected thesis, only one out of three P. gris plants tested positive, while all were negative in Glera. The average FDp titre was higher in Glera than P. gris, showing 5,87 and 7,96 arbitrary units, respectively. However, statistical analysis indicated that the difference was not significant (p value=0,39).

Trunk

FDp was detected in at least one portion of the trunk in all plants. Figure 6 demonstrates that the titre of FDp in the trunk of Glera was relatively lower than P. gris. Interestingly, a significant difference (p=0,04) was observed in the upper trunk where P. gris exhibited a higher concentration of FDp compared to Glera. To further investigate, we extended the analysis to 16 additional symptomatic plants (8 per cultivar). The results, shown in figure 7, displayed a high variability in FDp concentrations among plants and consequently no statistically significant differences were observed. However, it was confirmed that Glera tended to have lower FDp concentrations in the trunk, especially in the lower part.

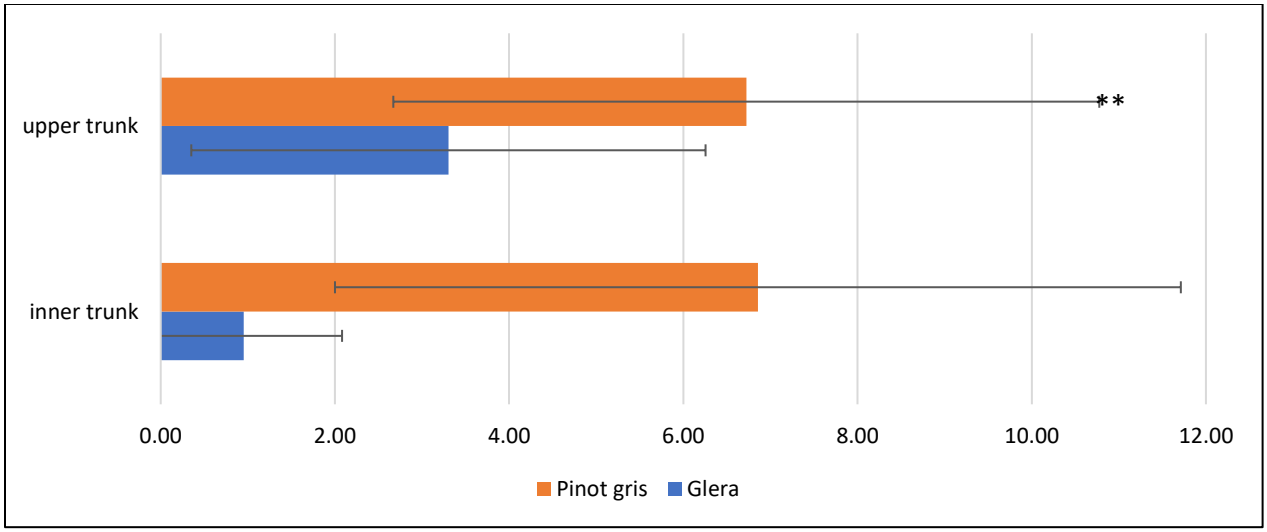


Fig. 6: FDp titre, expressed as arbitrary units (log2), in two trunk portions (inner and upper) of symptomatic plants of cultivars Glera and Pinot gris. Significant differences are indicated by asterisks, as determined by the Student-Newman-Keuls test ($p \leq 0,05$).

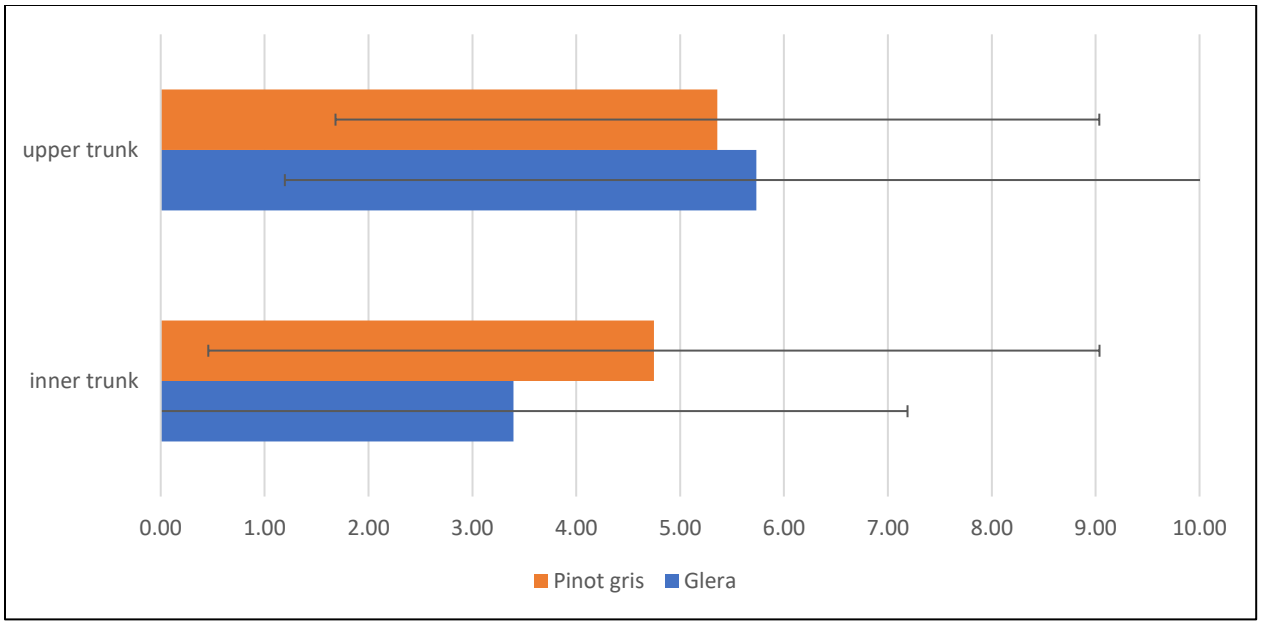


Fig. 7: FDp titre, expressed as arbitrary units (log2), in two trunk portions (inner and upper) of another 16 symptomatic plants of cultivars Glera and Pinot gris

Cordon

FDp was consistently detected in the cordons of all plants. Notably, Glera consistently exhibited a higher concentration of FDp in the outer part of the cordon, with an average of 7,89 arbitrary units, compared to an average of 4,77 in the inner part.

Two-Year-Old Arches

In asymptomatic arches of Glera, FDp presence was minimal to none, while P. gris exhibited higher FDp presence. However, in partially or completely symptomatic arches, FDp was almost always present. In Glera, the titre of FDp was higher in the outer part of the arch (average of 9,38) compared to the inner part (average of 5,58). On the other hand, desiccation was common in the worst plants of both arches and the cordon in P. gris, with six out of nine arches experiencing desiccation.

One-Year-Old Canes

1. Analysis of lignified canes from selected plants:

In Glera, canes from asymptomatic arches showed a small amount of FDp in only three out of seventeen canes (82,4% were negative). Canes from partially symptomatic arches had seven out of eight canes testing positive for FDp. In P. gris, canes from asymptomatic arches had five out of nine canes testing positive (44,5% were negative), while canes from partially symptomatic arches had six out of seven canes testing positive.

Comparison of FDp titre between the second and tenth buds showed a significant difference in both cultivars. Glera exhibited a higher concentration of FDp in the tenth bud, while P. gris had a higher concentration in the second bud (figure 8).

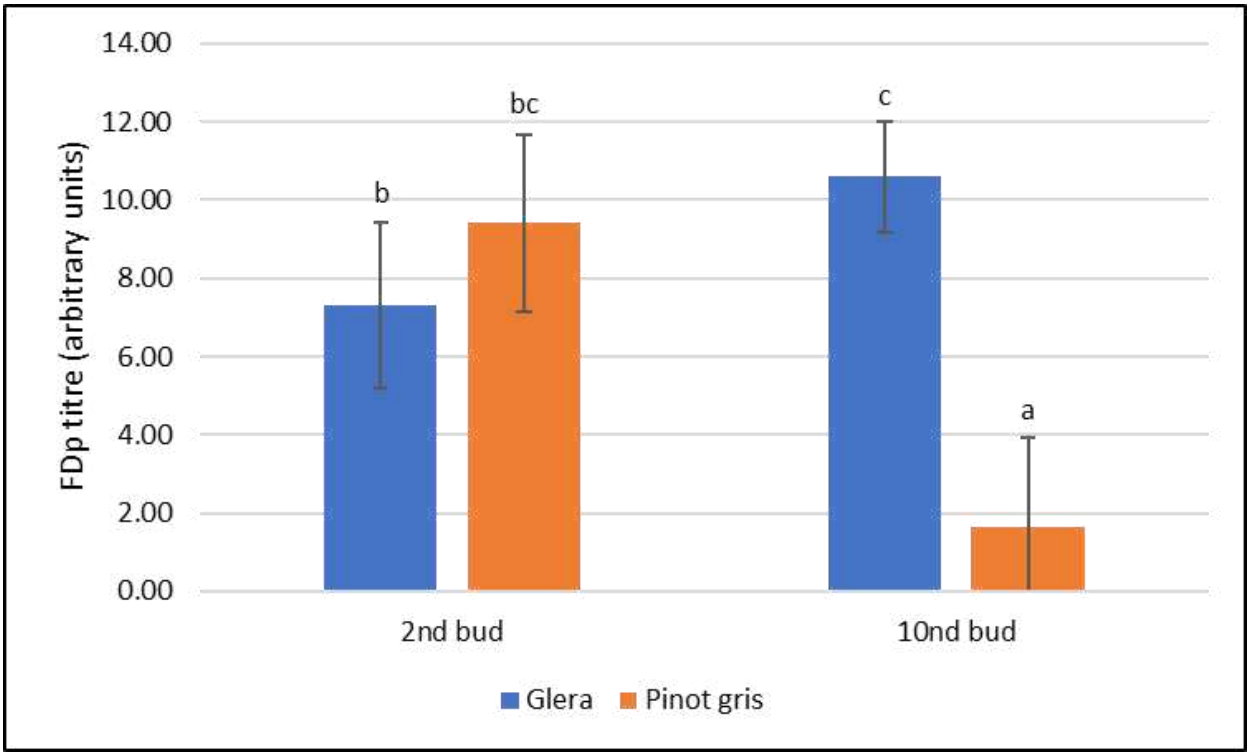


Fig. 8: FDp titre, expressed as arbitrary units (log2), measured in the 2nd and 10nd buds of 1-year-old lignified canes growth from symptomatic plants of Glera and Pinot gris. Each value represents the mean ± SE of 11 biological replicates. Significant differences are indicated by different letters, as determined by the Student-Newman-Keuls test ($p \leq 0,05$).

2. Extension of analysis to additional canes:

The analysis was extended to forty other canes collected from symptomatic plants of Glera and P. gris. The ratio between the tenth and second bud was significantly higher in Glera than P. gris (p=0,048) (figure 9).

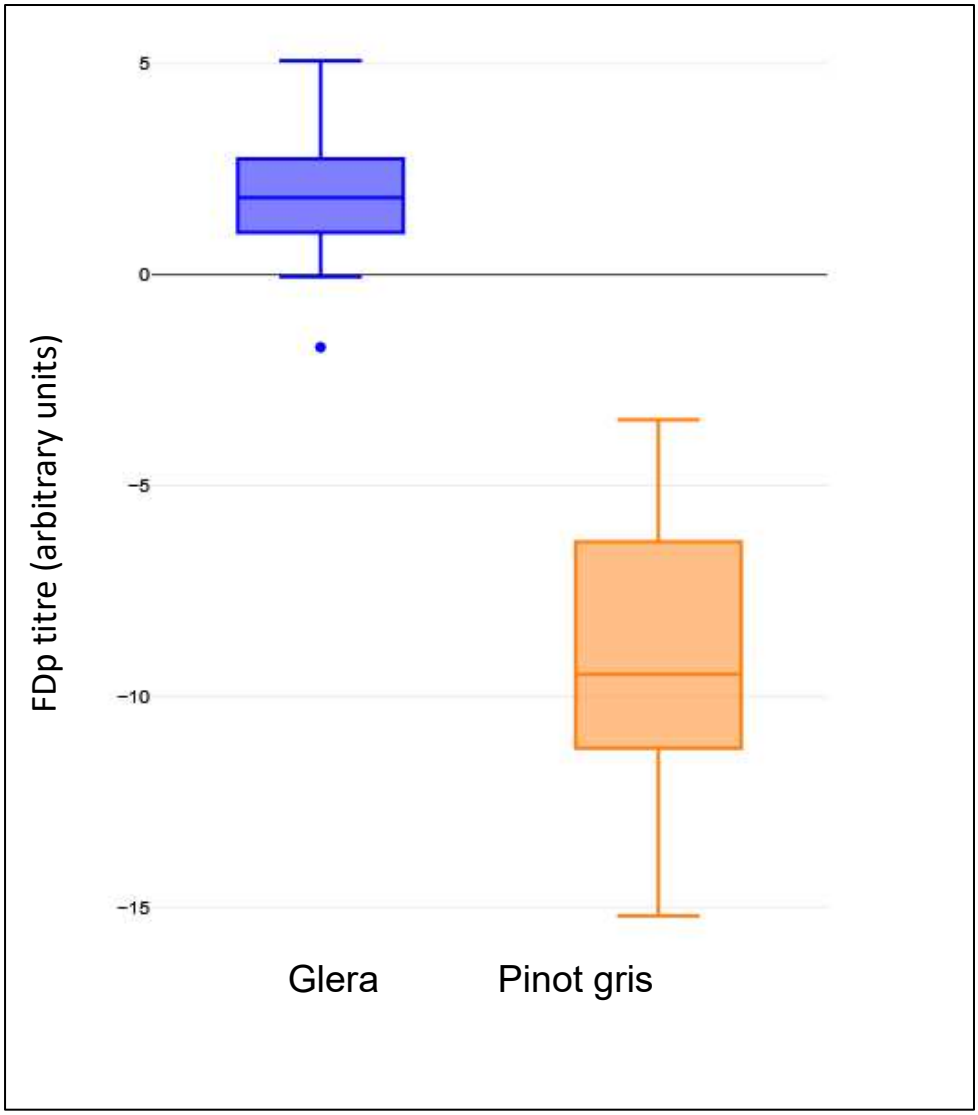


Fig. 9: FDp titre ratio, expressed as arbitrary units (log2), between the 2nd and 10nd buds of 1-year-old lignified canes growth from symptomatic plants of Glera and Pinot gris.

3. Distribution and titre of FDp along the lignified canes:

The analysis focused on ten long canes of Glera, comprising five asymptomatic canes and five lignified canes which showed symptomatic leaves (figure 10), as well as five asymptomatic longer canes of P. gris (figure 11). The results confirmed the acropetal distribution of FDp in Glera and the basipetal distribution in P. gris.

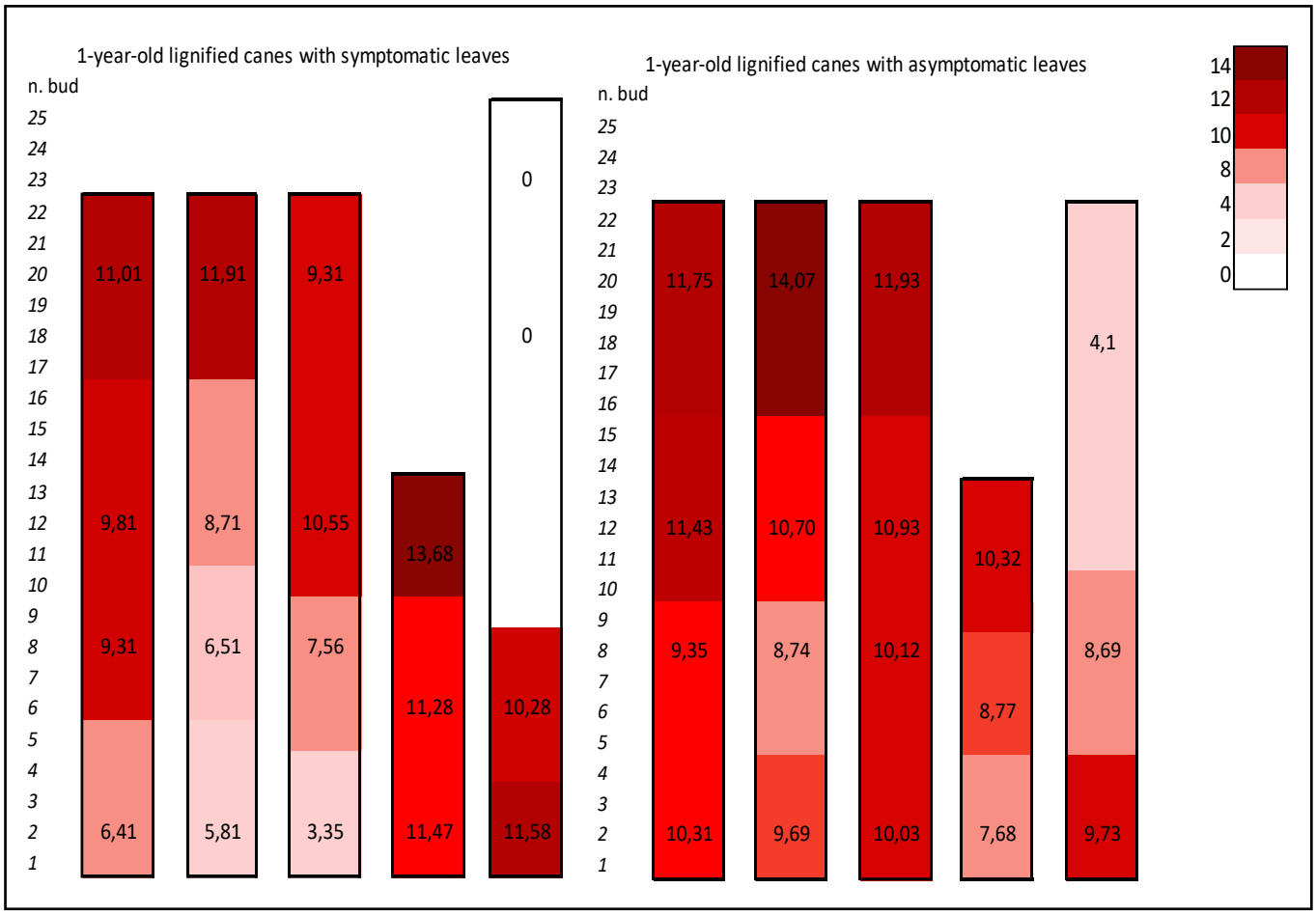


Fig. 10: FDp titre measured in different buds of 1-year-old a) symptomatic and b) asymptomatic lignified canes growth from symptomatic plants of Glera. A color scale was used to show the FDp titre, expressed as arbitrary units (log2).

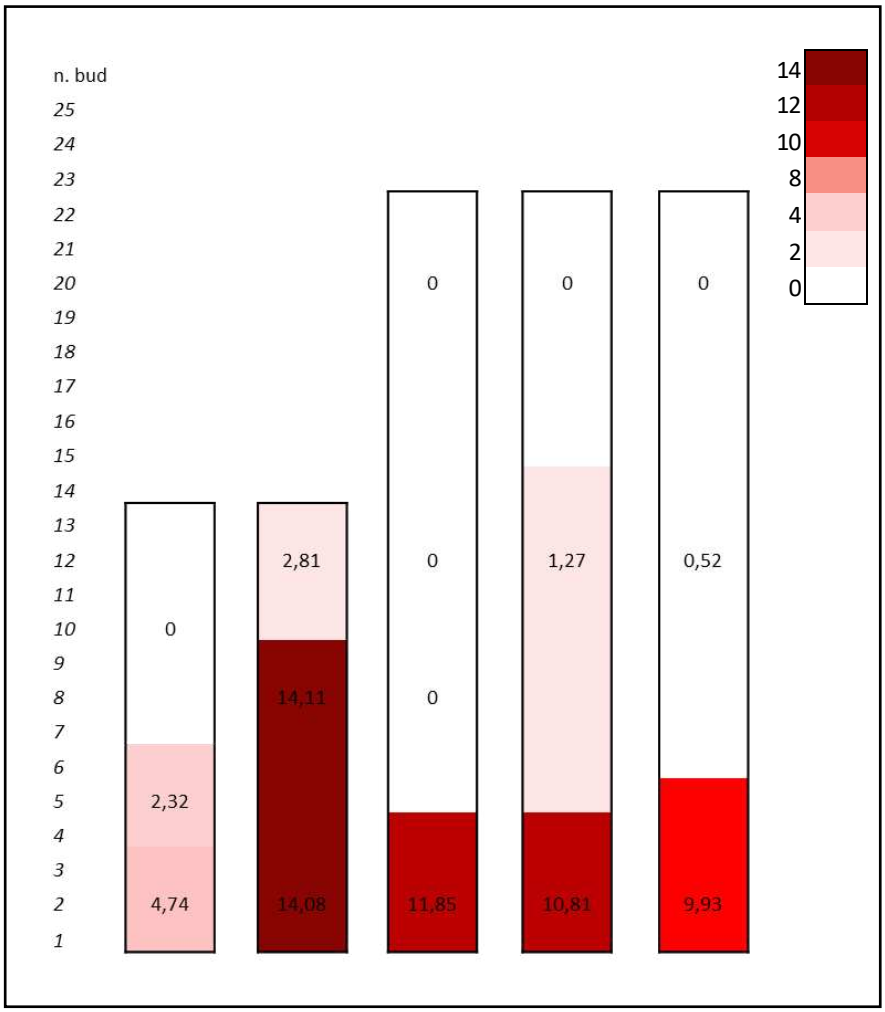


Fig. 11: FDp titre measured in different buds of 1-year-old asymptomatic lignified canes growth from symptomatic plants of Pinot gris. A color scale was used to show the FDp titre, expressed as arbitrary units (log2).

4.2 FDp detection in trunks and 1-year-old canes of symptomatic rootstocks.

Nine rootstock plants from a collection vineyard were chosen due to the presence of grapevine yellowing symptoms on certain canes and confirmed FDp positivity in the leaves. In the trunks, five out of nine plants tested positive for FDp (figure 12). Moreover, all nine plants had at least one lignified cane that tested positive for FDp.

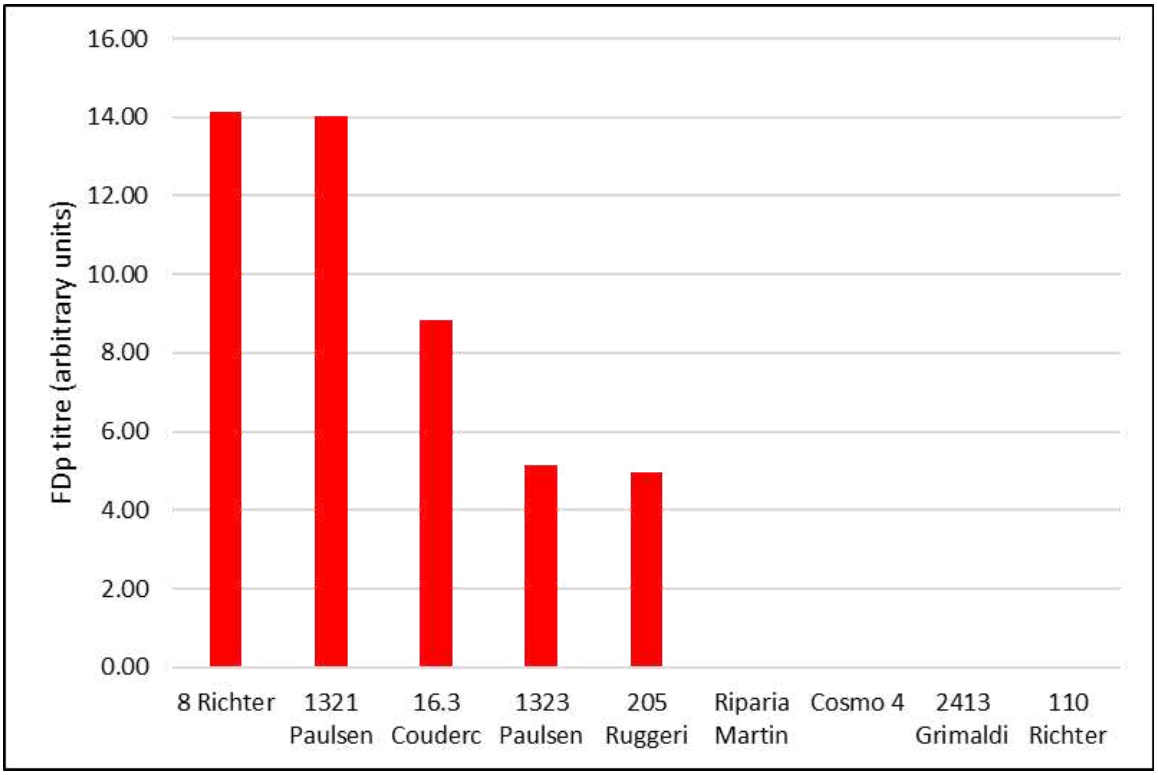


Fig. 12: FDp titre, expressed as arbitrary units (log2), measured in trunks of symptomatic rootstocks.

4.3 FDp detection from woody materials: RNA vs DNA

Fifteen samples, extracted from 1-year old canes or 2-year old canes and showing large variability in FDp titre, were further analyzed with DNA extraction in order to compare the results obtained between RNA and DNA extraction methods.

Following DNA extraction, we used SYBR Green-based assays to analyze the presence of FDp (Table 2). The results showed that 80% of samples obtained from the DNA extraction resulted positive for FDp.

n. sample	cDNA	DNA
1	31,05	31,71
2	23,08	25,05
3	22,99	NA
4	20,55	NA
5	26,22	26,88
6	25,9	26,75
7	29,92	28,15
8	22,77	30,04
9	23,49	23,16
10	21,65	19,52
11	30,38	NA
12	34,77/NA	31,75/NA
13	18,1	20,2
14	22,26	22,7
15	18,33	24,13
n. positive	15	12

Table 2: quantification cycles (C_q) obtained from real-time PCR performed on cDNA and DNA samples.

4.4 Risk of transmission by grafting with FD-infected propagation materials

Use of FD-infected scions

To evaluate the risk of transmission of FDp through vegetative propagation, a trial was conducted with different scion-rootstock combinations, some healthy and some probably infected. Preliminary results have been obtained through the survey carried out two weeks after the planting in nursery conditions.

Among the theses involving the cultivar Glera, the higher percentage of not sprouted graftings (89.5%) was obtained when FD-infected canes, previously tested for the presence of FDp, were used as scions (theses 3) (figure 13). Moreover, low rooting of plants (34% on average) was achieved when lignified canes, which previously showed symptomatic leaves, were both grafted on to SO4 or planted as cuttings (theses 1 and 10). Interestingly, no significant differences were observed on the percentage of not sprouted plantlets between the healthy thesis (number 6) and those obtained using putative infected canes of Glera (theses 2, 8 and 11). The only detected difference is concern the vigor of sprouted plants which was higher for healthy grafted plants.

Among the theses involving the cultivar P. gris, similar percentages of not sprouted plants (52 and 59%) were detected in graftings performed with asymptomatic lignified canes collected from symptomatic plants, previously tested or not for the presence of FDp (theses 4 and 5) (figure 14). Surprisingly, higher rooting rate (69%) was achieved with cuttings (thesis 10).

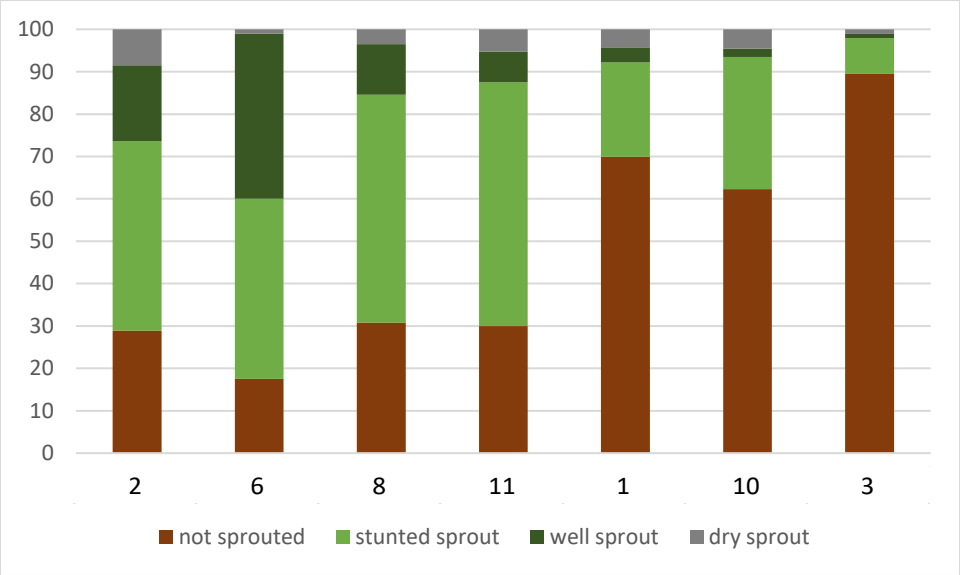


Figure 13: results of the survey performed two weeks after planting of plantlets belonging to different theses of cultivar Glera: 1) lignified canes which previously showed symptomatic leaves grafted on to healthy SO4; 2) lignified canes with asymptomatic leaves collected from FD-infected plants grafted on to healthy SO4; 3) lignified canes that tested positive to FDp grafted on to healthy SO4; 6) healthy Glera grafted on to healthy SO4; 8) lignified canes with asymptomatic leaves collected from FD-infected plants grafted on healthy Glera rootstocks; 10) lignified cuttings of Glera which previously showed symptomatic leaves; 11) healthy Glera grafted on to healthy SO4. For each thesis the percentage fractions of plantlets not sprouted, showing stunted or well sprouts, and that of plantlets with dry sprouts are reported.

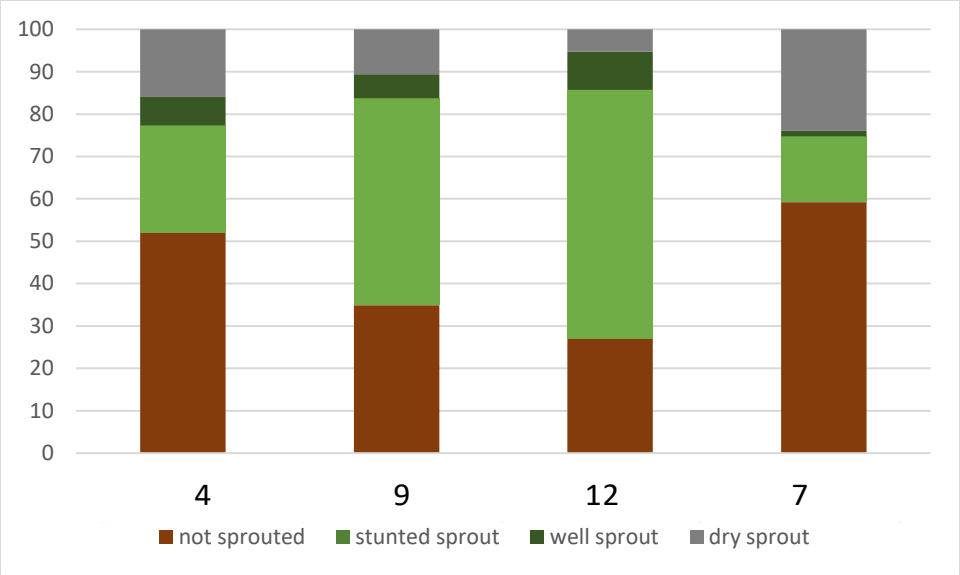


Figure 14: results of the survey performed two weeks after planting of plantlets belonging to different theses of cultivar Pinot gris: 4) lignified canes with asymptomatic leaves collected from FD-infected plants grafted on to healthy SO4; 5) lignified canes that tested positive to FDp grafted on to healthy SO4; 9) lignified canes with asymptomatic leaves collected from FD-infected plants grafted on healthy Pinot gris rootstocks; 10) lignified cuttings of Pinot gris with asymptomatic leaves. For each thesis the percentage fractions of plantlets not sprouted, showing stunted or well sprouts, and that of plantlets with dry sprouts are reported.

Use of FD-infected rootstocks

The risk of transmission by grafting with FD-infected rootstocks was evaluated using nine different American rootstocks, collected from FD-infected mother plants, grafted with healthy P. gris. Preliminary results have been obtained through the survey carried out two weeks after the planting in nursery conditions (figure 15). The number of not sprouted plants was very high (90%) for three rootstock varieties: 16.3 Couderc, 1321 Paulsen and Riparia Martin. On the opposite, the higher percentage of rooted plants was reached with 2413 Grimaldi.

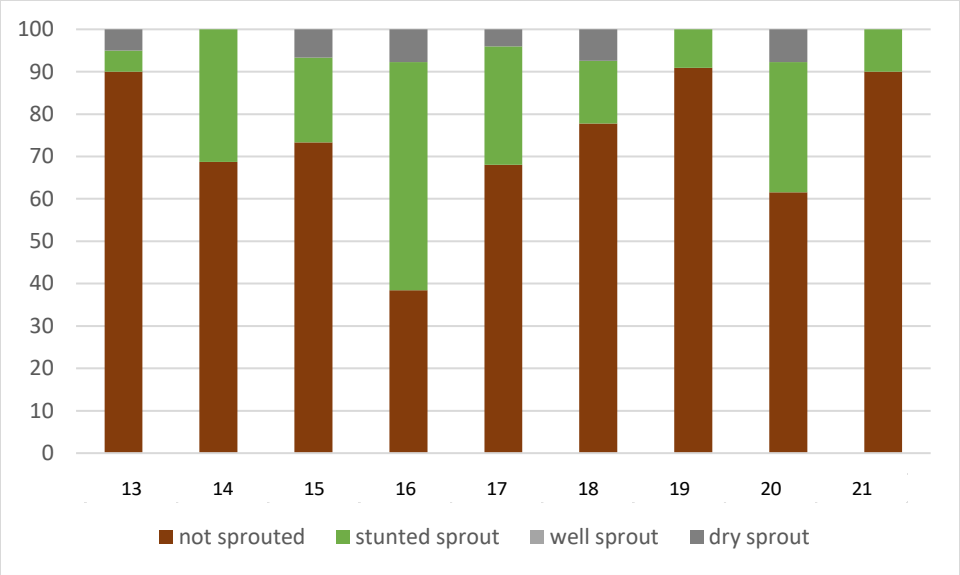


Figure 15: results of the survey performed two weeks after planting of plantlets obtained by grafting of infected rootstocks and healthy P. gris. For each thesis the percentage fractions of plantlets not sprouted, showing stunted or well sprouts, and that of plantlets with dry sprouts are reported.

The three types of cases found in the field after planting the grafted plantlets, from left to right: Well sprouted, stented sprout and not sprouted.

5 DISCUSSION

The main aim of the present thesis was to study the presence and the distribution of FDp in various woody organs of grapevines. The results obtained from the real-time PCR analysis using specific primers for FDp provided valuable insights into the presence and concentration of FDp in different plant parts. The implications of the results, their significance in understanding the disease progression, and their potential impact on grapevine health and management strategies are discussed below. Additionally, the observed similarities between RNA and DNA extraction methods and their implications for future research and diagnostic approaches are addressed. The present study contributes to the broader understanding of FDp infection dynamics and provide insights for the development of effective control measures to mitigate the impact of FD on grapevine cultivation.

In our study, we made an intriguing observation regarding the presence of FDp in the roots of symptomatic plants. Our findings align with a previous study on '*Candidatus* Phytoplasma solani' associated to *Bois noir* disease (BNp), where researchers reported BNp presence in at least 50% of the samples, a percentage similar to our study. Moreover, the authors suggested that plants with long-term infections tend to accumulate BNp, indicating that the titre of phytoplasma in the roots serves as an indicator of infection severity (Landi et al., 2019). Interestingly, in our research, we detected FDp in the roots even in the first year of infection, highlighting the early establishment of the pathogen in this organ. Furthermore, we observed a strong correlation between the severity of symptoms and the titre of FDp in the roots. Indeed, all the plants showing severe FD-symptoms harbored FDp in their roots, while it was detected only into one out of three P. gris grapevines manifesting mild FD-disease and in none of Glera plants. These findings underscore the potential of root tissue sampling as a reliable indicator of FDp presence and infection severity.

Further support for the idea that sampling of wood tissue in roots could serve as a valuable indicator for the presence of FDp and potentially offer insights into the severity of the infection within the plant has been provided in the experiment conducted by Ripamonti and colleagues (2020). The study focusing specifically on FDp investigated *ex vitro* plants with controlled inoculation of FDp (Ripamonti et al., 2020). The researchers placed four infected *S. titanus* per plant for one week and conducted sampling after five weeks, collecting three leaves, and after eight weeks, collecting five leaves and roots. Interestingly, the study revealed that FDp

was more frequently detected in the roots than in the canopy. For instance, in the case of Merlot, FDp was never detected in leaf samples but was consistently found in the root samples of the three infected plants. In contrast, the phytoplasma was detected in both leaves and roots of infected plants from other cultivars. Notably, in most cultivars such as Arneis, Cortese, Brachetto, Erbaluce, Freisa, Nebbiolo 71, and Barbera NC, FDp was detected more frequently in roots than in leaves. However, three cultivars, namely Barbera 84, Moscato, and Ruchè, exhibited equal frequencies of FDp presence in both leaves and roots. Furthermore, for Dolcetto, Nebbiolo 423, and Timorasso, FDp was more frequently detected in leaves than in roots.

Taken together, our findings, along with data from previous studies on BNp and FDp, emphasize the significance of root tissue sampling for assessing phytoplasma presence and infection severity in grapevines. The root system plays a vital role in the overall health and vitality of the plant, and our observations underscore its importance as a site for phytoplasma colonization and accumulation.

Throughout this research, we sought to determine the most suitable woody organ of the grapevine for detecting FDp during the winter when leaves are absent, and symptoms are challenging to find. Our results demonstrated that FDp was consistently present in the trunk and cordon, albeit in varying proportions depending on the grape cultivar. In our study, we found that in the Glera cultivar, the upper trunk and outer cordon were the optimal sampling sites, while for P. gris, the lower trunk and inner cordon yielded better results. A similar study conducted from February to October suggested different sampling strategies throughout the growing season (Prezelj et al., 2012). For instance, in the early season, sampling flowers was recommended for reliable detection, while as the season progressed, sampling grapes and leaves became more effective due to their increasing phytoplasma concentration. On the opposite, on the 2-year-old canes (arches) the FDp was not present consistently, in agreement to our findings in Glera where the FDp was almost exclusively present in the symptomatic arches. Notably, the presence of FDp in the trunk was not explored in the study of Prezelj and colleagues.

These findings highlight the disadvantage of solely relying on yearly growth testing during the active season and emphasize the importance of sampling woody organs to enable year-round monitoring, even during winter. Another research study emphasized the significance of understanding the distribution of phytoplasma

across various plant organs to gain a better understanding of phytoplasma-plant host interactions (Landi et al., 2019). Typically, FDp diagnosis in grapevines is limited to a specific seasonal period, from June to September, when phytoplasma symptoms are evident in the leaf tissue. However, the ability to test roots and successfully detect phytoplasma expands the testing timeframe.

Furthermore, by conducting RNA analysis, we confirmed that FDp remains active and alive in the plant even during the winter season, a comparison was also done between some samples using both DNA and RNA extractions, and the results suggest that RNA extraction method was more effective in detecting FDp in the woody samples than DNA method. The results further validate the reliability and consistency of our experimental procedures, highlighting the robustness of the SYBR Green assay for the detection of FDp in both RNA and DNA samples although the advantage of RNA presence is that it indicates the active genetic expression and biological activity of FDp in the infected plant.

Phytoplasma detection are usually performed in the central leaf veins where they are mostly abundant and their impact in plant physiology seems greater. By colonizing phloem sieve cells, phytoplasmas act as an additional sink for photosynthesized carbohydrates and block sugar transport from leaves to sink tissues because of the plant's response to the infection (Maust et al., 2003; Bernardini et al., 2022). Therefore, soluble sugars and starch start accumulating in the infected source leaves and are depleted in roots (Lepka et al., 1999; Choi et al., 2004). Since phytoplasmas lack the enzymes needed to use sucrose (Oshima et al., 2004) they might use fructose or glucose as a source of energy. Indeed, after phytoplasma infection, plants undergo several changes in the transcription of genes involved in carbohydrate metabolism (Hren et al., 2009).

Interestingly, during winter, the starch reservoir accumulated in the trunk and cordon begins to break down into sugars in preparation for the upcoming season. Consequently, starch concentrations are lowest during winter, while soluble sugar concentrations increase by 30-40% (Fregoni, 2013). The reduction in starch and the increase in sugar concentrations in the cane happen after the shedding of the leaves and they reach, in the coldest time of the year, the lowest and the highest concentrations, respectively. During the winter the canes appear to be dormant, when in fact there is a notable increase in sugars not only because of their migration from other organs, but also because there is transformation of hemicellulose in the cane, a reserve substance of comparable importance to starch.

Together these considerations support results obtained in the present study, in fact: i) RNA transcripts of FDp are abundant in woody organs of infected plants; ii) FDp needs simple sugars; iii) during dormancy woody organs accumulate simple sugars.

Overall, our research highlights the importance of considering woody organs for FDp detection, especially during the winter season when symptoms may not be visible. By expanding the sampling scope to include roots and utilizing RNA analysis, we gain valuable insights into the persistence and activity of FDp throughout the year. This knowledge enhances our understanding of FDp dynamics in grapevines and provides a broader timeframe for effective phytoplasma testing and management.

Another study, focused on Tocai Friulano, investigated the presence of FDp by analyzing woody organs and the defence strategy against FD infection (Casarin et al., 2023). The study also compared T. friulano with P. gris to assess their different responses. In T. friulano, FD symptoms remained localized near the initial affected area throughout the entire vegetative season. Interestingly, analyses of the secondary phloem in the trunk revealed a complete absence of FDp, and in 2-year-old arms, the phytoplasma disappeared from July to November. This pattern differed from the highly susceptible P. gris variety. Moreover, molecular analysis of 1-year-old canes from FD-infected T. friulano plants showed diverse modulations of defense genes and the accumulation of metabolites, depending on the plant's health status. In symptomatic cane portions, there was a significant activation of both jasmonate- and salicylate-mediated responses, along with a substantial accumulation of resveratrol. Asymptomatic cane portions adjacent to the symptomatic ones exhibited an activation of the jasmonate-mediated response and a high content of ε-viniferin. Our research corroborates these observations and confirms the different behaviors exhibited by T. friulano and P. gris. T. friulano demonstrated a tendency to confine FDp within the canes, limiting its spread. In contrast, P. gris, being highly susceptible, displayed a more systemic distribution of FDp. Notably, we detected the presence of FDp in all the trunks of both P. gris and Glera cultivars, emphasizing the systemic nature of the infection in P. gris and its prevalence throughout the grapevine. These findings shed light on the contrasting responses of different grapevine cultivars to FD infection and provide insights into their defense mechanisms.

The presence of FDp has been observed in the trunks of symptomatic plants of P. gris and Glera. Interestingly, it was found to be present in lower quantities in the lower trunk of the less infected plants of Glera, while completely absent in the roots. This led to the question of whether these less infected plants could be recovered through trunk renewal. An interesting study was previously conducted to investigate the effectiveness of trunk renewal as a means of recovery in Glera vines (Forte et al., 2016). The results of this study shed light on the success of this practice on this particular cultivar. The study aimed to determine the best approach for executing the trunk renewal technique and its impact on the recovery process. By comparing different methods of wood cutting, both qualitatively and quantitatively, the researchers assessed the presence or absence of symptoms, the severity of symptoms, and other parameters such as grape yield and residual wood weight. The initial situation of the 8 selected vine rows (848 vines) was challenging, with only 25% of the plants in good health, 47% showing symptoms of FD, and the remaining 28% deceased. The infected vines displayed severe symptoms, with varying intensities. Over the course of three years following the pruning-trunk renewal intervention, the percentage of dead or missing vines did not increase. However, the proportion of symptomatic vines decreased to 12.5% in the first year, and 6% and 7% after two and three years, respectively. The severity of symptoms also decreased over time. The study demonstrated that both trunk renewal and precise pruning can lead to grapevine recovery, although in different quantities. The most successful technique was trunk renewal the trunk at 30 cm from the graft, achieving a recovery rate of 96.8% in the first year and 97.9% in the following two years. Trunk renewal the trunk at 100 cm also yielded statistically similar results, but only after two years, recovering 80.8% of the vines in 2012, 88.9% in 2013, and 89.9% in 2014. It is worth noting that the pruning technique, carried out in 2013, might have contributed to improving the outcome of the 100 cm trunk renewal. Although the precise pruning method induced recovery in a significant number of vines, it yielded statistically inferior results compared to the other two methods. This can be attributed, in part, to new infections, as observed in the control group (pruning on symptom-free vines), where 5% of the vines fell ill over the three-year period. Furthermore, the study highlighted the importance of proper pruning, which involves removing all non-productive woody parts showing symptoms of FD, such as incomplete lignification and withering. This type of pruning is essential for removing infected shoots from the vineyard, which act as dangerous sources of pathogen transmission and increase the possibility of the vector insect acquiring the FDp. The study also noted that Glera, characterized by moderate susceptibility

to FD and medium-high productivity, exhibited comparable yield quantity and quality in topped vines after three to four years, as the plant regains its typical vegetative age dimensions within a short time. Finally, it should be considered that the high effectiveness of trunk renewal in this study was achieved thanks to other essential factors for vine recovery. The conversion of the vineyard from organic to conventional farming significantly and rapidly reduced the vector insect population, thereby increasing the incidence of recovery in FD affected vines. Additionally, the spread of the phytoplasma in the area had considerably decreased compared to the epidemic situation 20 years ago when it was at its peak. Consequently, the present situation might be more suitable for implementing a strategy focused on recovery, rather than just containment or eradication (Forte et al., 2016). It is clear that our findings should explain the success of trunk renewal of the Glera vines since the FDp, as we will discuss more later, in this cultivar tends to migrate towards the younger parts of the plant therefore indicating a much lower or absent concentration of FDp in the older and lower parts of the vine, unfortunately this technique is not suitable for application to P. gris vines since this cultivar is more susceptible and tends to have a high titre of FDp in the trunk.

One of the key observations during our research pertained to the distribution of FDp along the canes of the two cultivars, Glera and P. gris. We noticed that in Glera, the distribution appeared to be acropetal, moving from the base towards the tip of the cane, while in P. gris, it showed a basipetal distribution, moving from the tip towards the base of the cane. Intrigued by this observation, we further investigated it by testing 40 additional canes from similar plants of P. gris and Glera, which confirmed our hypothesis. To gain more insights, we extended our research to very long canes of both cultivars and sampled around multiple buds along the cane. The results revealed a concentration gradient of FDp from the base to the tip of the cane, but interestingly, the direction of the gradient was opposite in the two cultivars, further confirming our hypothesis. Detailed data illustrating this gradient can be found in Figure 10 and 11 in the results section.

These findings led us to consider whether the gradient of FDp along the canes corresponds to the concentration of sugars in the same regions. This question aligns with a previous study that suggested phytoplasma infection could interfere with host plant sugar metabolism, resulting in higher levels of reducing sugars and sucrose in the source leaves of infected plants. It was hypothesized that phytoplasmas might follow sugar gradients within the sieve elements, growing and proliferating according to the availability of nutrients. However, it remains

unknown whether the higher FDp titre in tissues with increased sugar concentration is due to the phytoplasma-induced transport or synthesis of sugars within the tissues expressing symptoms, or if it is a result of plant responses to the infection (Prezelj et al., 2012).

In our investigation of the cane gradient, we analyzed growth data related to the cultivars and observed a correlation between the growth pattern of a cultivar and the gradient of FDp. Glera, being a highly vigorous cultivar, exhibits lower fertility in the basal buds and prefers apical growth. Consequently, the shoots of Glera canes serve as significant sinks for sugars and nutrients during the growth season. On the other hand, P. gris, with lower vigor and higher fertility in the basal buds, tends to attract fewer nutrients to the shoots compared to the basal parts of the canes and arches. This difference in sugar and nutrient movement and concentration between the two cultivars can potentially explain the contrasting concentration gradients of FDp observed along the canes and other woody organs.

To address the question raised by Prezelj in his study, it seems plausible that FDp migrates and follows the gradient of sugars and nutrients within the host plant. During the growth season, the shoots predominantly attract sugars and nutrients from the leaves. However, after flowering, the shoots receive nourishment from only a few nearby leaves, while other leaves synthesize sugars and transport them to the clusters. Throughout the summer, nutrients migrate from the leaves to the clusters, canes, trunk, and roots, as part of the dynamic nutrient movement within the grapevine (Fregoni, 2013). This understanding supports the possibility that FDp exploits the sugar and nutrient gradients within the plant to establish its distribution pattern.

Furthermore, another crucial observation we made relates to the lignified canes. The collected lignified canes exhibited varying levels of symptoms. Some canes displayed asymptomatic leaves, while others, particularly in the Glera cultivar, showed clear foliar symptoms, indicating an evident infection in those canes. However, despite the infection, these canes managed to lignify after the growing season, rendering them indistinguishable as infected during the winter. This poses a potential risk when collecting woody canes for vegetative propagation, as the seemingly healthy woody material may be mistakenly used for grafting, unintentionally spreading FDp to new plants.

Moreover, the risk becomes even more significant and perilous when it comes to rootstock mother plants. Our research revealed the presence of FDp in asymptomatic canes of rootstock plants within the experimental vineyard collection in Susegana (Northeast Italy). In fact, all the analyzed rootstocks harbored FDp in at least one of the examined canes, and in 55% of the cases, FDp was also detected in the trunk. Consequently, we consider these rootstock cultivars to be tolerant rather than resistant to FDp. Although these plants can resist most of the symptoms associated with FD, they are unable to effectively control the presence of FDp, resulting in a relatively high concentration of FDp in their organs. Notably, our research identified the most severe cases of FDp presence in the rootstock cultivars 1321 Paulsen and 8 Richter, see figure 12 in the results section for the detailed data.

In contrast, cultivars with successful resistance strategies, such as T. Friulano, which is able to suppress most of the symptoms and confine the FDp to certain parts of the canes (Casarin et al., 2023), should be able to somewhat block the symptoms of FD and fight the systemic spread of FDp in the rest of the grapevine.

These observations regarding lignified canes and rootstock plants underscore the importance of accurate identification and careful selection of disease-free materials for propagation purposes. Ignoring the presence of FDp in seemingly healthy canes or utilizing infected rootstock mother plants can have detrimental consequences, contributing to the further dissemination of FDp in vineyards. Therefore, implementing rigorous screening and certification procedures for grapevine propagation materials is crucial for preventing the inadvertent spread of FDp and ensuring the long-term health and productivity of vineyard ecosystems.

6 CONCLUSIONS

In this study, our findings highlight the presence of FDp in woody organs, even in plants infected in their first year. The consistent and high concentrations of FDp throughout the year emphasize the significance of wood analysis as a valuable diagnostic tool for FD. Moreover, our investigation identifies the specific woody organs that yield the best results for FD diagnosis in the studied cultivars, Glera and P. gris. Notably, we observed a distinction in FDp behavior between these two cultivars, with Glera showing a localized infection in the upper parts of the plant. This suggests that targeted pruning techniques and agronomic management may potentially facilitate the recovery of infected Glera plants. In contrast, P. gris exhibited a more systemic infection, rendering recovery virtually impossible. These insights prompt further exploration of the relationship between FDp distribution and specific physiological parameters, with the aim of developing novel vineyard management approaches to mitigate the FD epidemic. Additionally, even though the grafting theses will have to be monitored for a longer period of time to assess the effect of grafting over time, our study highlights the potential risk of transmitting FDp through vegetative propagation using asymptomatic scion and rootstock material, emphasizing the need for long-term monitoring of grafting practices to assess their impact over time.

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