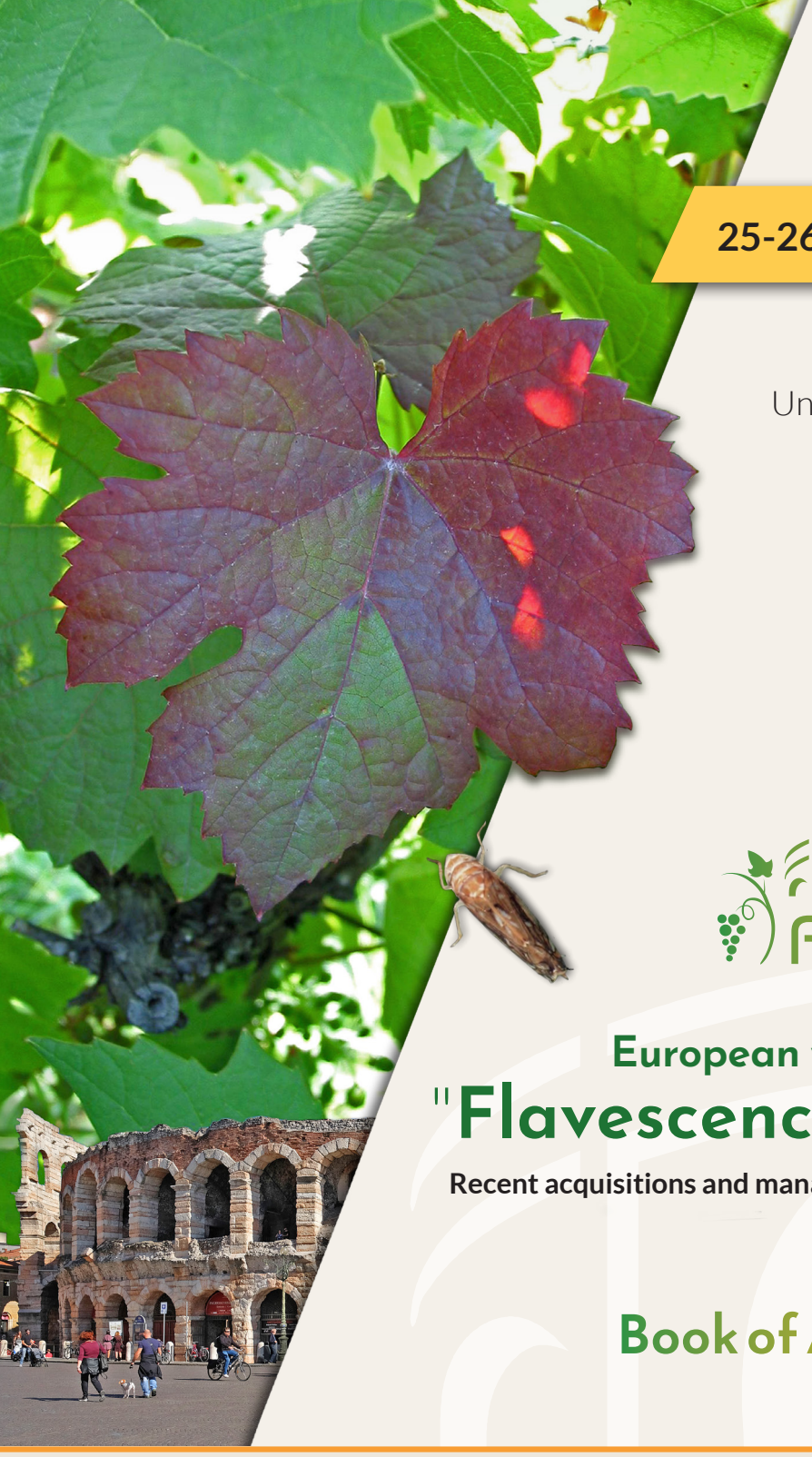

25-26 January 2024

Verona, Italy

University of Verona
Polo Zanotto

FD Verona, Italy 2024

European workshop on

"Flavescence dorée"

Recent acquisitions and management strategies

Book of Abstracts

International Phytoplasmologist Working Group

crea Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria

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Welcome Message

We are warmly welcoming the participants to the European workshop on "Flavescence dorée": recent acquisitions and management strategies held in Verona, Italy. “Flavescence dorée” in Europe is an historical quarantine disease strongly limiting factor and productivity of grapevine. Losses due to this phytoplasma-associated disease vary, but under the favorable conditions it always leads to severe economic consequences to farming communities and to the whole vine industry.

During the workshop held on 25-26 January 2024, with an expected attendance of more than 100 colleagues from 8 among the most affected countries in Europe, keynote lectures will be given by Sandrine Eveillard from France on Review in plant-“flavescence dorée” phytoplasma interactions by Domenico Bosco from Italy on Leafhopper vectors and epidemiology of “flavescence dorée”: complexity and knowledge gaps hamper efficient control. The workshop is organized in two sections on grapevine-“flavescence dorée” phytoplasmas and insect vectors, respectively. Recent achievements on phytoplasma strains/disease detection and identification, and epidemiology enclosing environmentally friendly control methods will be reported in 26 oral presentations.

We are sure that the content of this workshop will represent the basis for expanding and integrating the different fields of European research on “flavescence dorée” to help all the components of the grapevine sector to manage the disease in the best environmentally friendly manner. All the abstracts in this book have been reviewed by the Scientific Committee and will also be available online at the IPWG (International Phytoplasmologist Working Group) website: www.ipwgnet.org

We want to thank the contributors for their diligence and timely submissions of abstracts. We apologize for errors that could have arisen during the editing process despite our careful attention. We want to thank all the workshop participants, the scientific committee members and Fabio Montanari for their essential support.

We welcome everyone attending the European workshop on "Flavescence dorée" and wish them a comfortable stay and meaningful interactions with colleagues in romantic Verona where wine is accompanying the love story between Juliet and Romeo and Arena concerts just support friendship and peace…

Assunta Bertaccini, Nicola Mori, Elisa Angelini and Annalisa Polverari

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Verona, Italy
FD 2024

Review in plant-"flavescence dorée" phytoplasma interactions

Sandrine Eveillard

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"Flavescence dorée" (FD) phytoplasma is responsible for an epidemic quarantine disease of grapevine in Europe and control methods are mandatory. Nevertheless, the disease still progress, and alternative control methods are needed implying a better knowledge of the plant-FD phytoplasma interactions. It is known that grapevine have various susceptibilities to "flavescence dorée" in terms of percentage of infected plants, symptoms severity and phytoplasma titers, with all the *Vitis vinifera* being susceptible although at different degrees. This was shown in vineyard and in controlled conditions, for example for Tocai friulano, Nebbiolo and Merlot (Ripamonti *et al.*, 2021; Eveillard *et al.*, 2016).

Omics studies were then undertaken to determine the mechanism of response of the plant to the FD phytoplasma infection. Highly and poorly susceptible varieties were studied to identify resistance or susceptibility factors. Indeed, transcriptomic changes were observed in infected plants for several genes in various pathways (Casarin *et al.*, 2023; Bodin *et al.*, 2023). Metabolomic changes were also observed between healthy and infected grapevine, but also between highly and poorly susceptible cultivars (Deborde *et al.*, 2023; Teixeira *et al.*, 2020, 2023; Davosir *et al.*, 2023). Moreover, these changes could be linked to genes overexpression or repression. In addition to poorly and highly susceptible cultivars, transcriptomic analysis were also done on recovered grapevine showing specificities (Pacifico *et al.*, 2018). In parallel to these researches concerning the plant response, effectors secreted by phytoplasmas have been shown to interfere with host plant physiology, presumably in favor of phytoplasma multiplication or dissemination. Most of the recent studies of phytoplasma effectors have focused on a few proteins that are not encoded in the "flavescence dorée" genome but one (Debonneville *et al.*, 2023). These researches aim to identify grapevine genes, metabolites or pathways associated to FD resistance or susceptibility that could be used as biomarkers or targets to inactivate or enhanced to help the plant fighting against the disease.

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Past and present genetic diversity of "flavescence dorée" phytoplasma strains explored in grapevine samples collected in Veneto and Friuli Venezia Giulia (Italy)

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Recurrent epidemics of "flavescence dorée" (FD) in Veneto and Friuli Venezia Giulia (FVG) regions raised questions about the genetic variability of the associated phytoplasmas. In a survey conducted during the 2016-2021 vegetative seasons, approximately 130 FD phytoplasma strains from grapevine samples belonging to different areas of FVG and Veneto were typed by nested PCR/RFLP analyses and/or sequencing of 16S rRNA, *map* and *vmpA* genes. Samples were collected from vineyards with a strong ongoing epidemic and from vineyards where FD occurrence was sporadic and stable since several years. Moreover, the FD strains were compared with 25 selected FD strains obtained during the years 1999-2005 from the same areas.

PCR/RFLP analyses of samples collected from 2016 to 2021 produced two restriction profiles: 16SrV-D/map-FD2 and 16SrV-C/map-FD3. Two *map*-genotypes were found: M54 (100% nt sequence identity with strain VF00-SP5) and M3 (100% nt identity with strain VI04-C28) (Arnauld *et al.*, 2007; Malembic-Maher *et al.*, 2020), with M54 strains being much more widespread than M3 strains, and the last being present only in the neighbouring provinces of Treviso (Veneto) and Pordenone (FVG). In Veneto, no genetic differences have been found in phytoplasma strains from grapevines of epidemic or non-epidemic vineyards (16S rRNA and *map* genes). Finally, there were no genetic differences comparing these to the "flavescence dorée" strains from infected grapevines collected in 1999-2003 in Veneto (16S rRNA and

map genes) and in 2004-2005 in FVG (*map* and *vmpA* genes), suggesting that no new FD strains distinguishable with the used markers are spreading in the investigated areas.

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Acknowledgements

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Recent findings on "flavescence dorée" in Franciacorta (North Italy): prevalence of associated phytoplasma genotypes in symptomatic grapevines and in additional plant and insect hosts within and around vineyards

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This study aimed to survey the spread and incidence of "flavescence dorée" (FD) in Franciacorta vineyards (North Italy) and study the diffusion of the associated phytoplasmas not only in symptomatic grapevines but also in the vineyard agroecosystem. The activities were carried out in 2021 and 2022 in 20 representative vineyards. Average grapevine yellows (GY) incidence in vineyards was around 6% in both 2021 and 2022. Molecular analyses, conducted on 1,250 symptomatic grapevines, identified 16SrV phytoplasmas in 31% (2021) and 34% (2022) of the plants, localized in 14 out of 20 vineyards. In these vineyards, 17 (2021) and 37 (2022) species of known or potential "flavescence dorée" insect vectors were captured and grouped into 1,600 pools for molecular analysis. 16SrV phytoplasmas were identified in 22% (2021) and 6% (2022) of the insect pools. Seven species (*S. titanus*, *Allygidius* spp., *Dictyophara europaea*, *Neoaliturus fenestratus*, *Orientus ishidae*, *Phogotettix cyclops*, *Psammotettix* spp.) were found to be infected both in 2021 and 2022. Moreover, molecular analyses revealed the presence of 16SrV phytoplasmas in 25 out of 45 wild plant species collected around vineyards. Sequence analyses of *map* gene identified (i) "flavescence dorée" genotype M54 in symptomatic grapevines (78% and 100% of 16SrV phytoplasma-infected grapevines in 2021 and 2022, respectively), in *S. titanus*, *N. fenestratus*, *Psammotettix* spp. (2021 and 2022), and *D. europaea* (2021), and in seven wild plant species; (ii) "flavescence dorée" genotype M51 in symptomatic grapevines (22% of 16SrV phytoplasma-infected grapevines in 2021), in *D. europaea*, *N. fenestratus*, and

P. cyclops (2021 and 2022), and in 22 wild plant species; (iii) "flavescence dorée" genotypes M12, M50, and M122 in other insects. So far, "flavescence dorée" genotype M54, prevalent in northern Italy, is believed to be strictly associated with the grapevine - *S. titanus* pathosystem, while the spread of "flavescence dorée" genotype M51 includes additional host plants and vectors (*D. europaea* and *O. ishidae*). Obtained results reinforced recent evidence of an increasing FD epidemiological complexity, suggesting that also the prevalent "flavescence dorée" genotype M54, at least in the examined area, can be related to an open pathosystem, involving additional plant hosts and insect vectors.

Acknowledgements

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"Flavescence dorée" strains in Tuscany, Emilia-Romagna, Veneto and Trentino Alto Adige regions of Italy

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During the past 30 years "flavescence dorée" (FD) phytoplasmas belonging to 16SrV-C and -D subgroups (Martini *et al*., 1999) were detected in several viticultural areas of the majority of grapevine growing regions located in north Italy. Sequencing *secY*, *map* and *rpsC* genes further allowed the identification of variants within the two FD ribosomal subgroups of phytoplasma populations (Martini *et al*., 2002; Bertaccini *et al*., 2023). To verify and update the presence and distribution of FD strains grapevine samples were collected from symptomatic plants in 11 provinces of four Italian regions: Treviso, Belluno, Verona (Veneto), Trento, Bolzano (Trentino Alto Adige), Modena, Reggio Emilia, Forli-Cesena (Emilia Romagna) Florence, Massa Carrara and Prato (Tuscany). FD phytoplasmas enclosed in both FD-D and FD-C subgroups were detected (Martini *et al*., 1999). FD-C strains were prevalent in Treviso and Tuscany provinces, while FD-D strains were detected in Verona, Modena and Forlì-Cesena provinces; both FD-C and FD-D were detected in Trentino-Alto Adige region. Multilocus strain typing analysis on *secY-map*, *secY* and *rpsC* genes on Chardonnay, Sangiovese, Trebbiano, Teroldego, Lambrusco, Glera, Garganega and Pinot noir varieties FD-infected allowed the identification of several genetic variants. In particular variant M54 was identified in FD-D phytoplasmas collected in Modena, Reggio Emilia, Forli, Bolzano, Verona and Belluno provinces. Among FD-C strain, the variant M3 was found in Belluno, the variant M51 in Bolzano, variants M50 and M12 in Tuscany and variant M54 in Prato. The latter is for the first time identified

in grapevine samples infected by FD-C. Sequencing selected strains on the *secY* gene showed the presence of variability among FD-D phytoplasmas, with 3 clusters enclosing samples from Veneto and Emilia Romagna identical to others collected in 2012 that confirm the long-time presence of variants in FD-D phytoplasmas. In particular it was detected the presence of the same SNP in samples of cultivars Teroldego and Trebbiano collected in Verona province and in cultivar Sangiovese located in Forlì-Cesena province. On the other hand, among the FD-C phytoplasma strains the highest variability was detected in *rpsC* gene, with 5 restriction profiles identified after RFLP analyses in samples from cultivar Glera collected in Treviso and Belluno provinces. The epidemiology of the FD disease must be further monitored, especially considering the presence of alternative insect vectors/plant hosts species possibly connected with the emergence of FD genetic variants. The continuous and capillary monitoring of new and genetically homogeneous FD strains associated with the disease is useful for the early application of appropriate and stringent focused control measures aimed to mitigate the epidemic spread of virulent FD strains.

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Comparative genomics analysis of “flavescence dorée” phytoplasma strains from Chardonnay and Pinot gris cultivars

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“Flavescence dorée" (FD) poses a severe threat to grapevine cultivation in Europe, with phytoplasmas as associated agents residing in phloem vessels of infected plants and transmitted by leafhopper vectors. Various “flavescence dorée” phytoplasma strains have been detected in infected vineyards across Europe. Over the past decade, determining whole genome sequences for multiple phytoplasma strains has improved the understanding of their phylogenetic positions and their biology. The increased availability of genomic sequences suggests that comparing whole bacterial genomes is highly valuable in microbial phylogenetic research. Thus, comparative studies utilizing phytoplasma genome sequences are relevant for phylogeny, taxonomy, and functional genomics which not only enhances the knowledge of the molecular interplay between grapevines and phytoplasmas, but also facilitates the detection of key pathogenic mechanisms (Endo and Oshima, 2023).

In this study, the complete genomes of two “flavescence dorée” strains from Chardonnay and Pinot gris infected cultivars collected from different areas of the Trentino region using the Oxford Nanopore (ONT) long-read sequencing approach were determined. Both strains belong to the 16SrV-C group (FD2 cluster), based on 16S rRNA and *map* genes (Pierro *et al.*, 2023). A comparative genomics analysis among these strains and the available phytoplasma genomes from the NCBI database was performed, by exploiting several state-of-the-art

methods. The analysis encompassed genome assembly, comparison, functional annotation, and identification of "flavescence dorée" genetic clusters.

The *de novo* assembly of Chardonnay and Pinot gris strains resulted in single scaffolds of 654,174 bp (619 coding sequences) and 653,714 bp (754 coding sequences), respectively. Comparative analysis of all the available complete phytoplasmas genomes enabled to (i) get information related to gene content dynamics (presence/absence of orthologs), (ii) classify the strains into clusters with highly similar gene content, and (iii) build phylogenetic trees describing the relationships of the studied strains, based on a long concatemer of core genes.

This work offers a comprehensive assembly of two "flavescence dorée" phytoplasmas, providing novel insights into the genomics of Italian strains and phytoplasmas in general. The findings highlight both conserved features among phytoplasmas and specific characteristics unique to the "flavescence dorée" phytoplasmas.

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Towards the identification of genetic resistance traits against "flavescence dorée"

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Grapevine genotypes show different susceptibility to "flavescence dorée" (FD) in the field, but complete resistance has not been confirmed under controlled conditions. Nevertheless, several *Vitis* cultivars have been ranked from susceptible to tolerant to FD infection. This natural tolerance needs to be further explored, extending investigations into the high genetic variability of grapevine, to highlight plant FD-resistance traits, in cultivars never ranked before, as a starting point for breeding programs.

Some *Vitis* accessions, including some rootstocks and *V. vinifera* subsp. *sylvestris*, are partially resistant/tolerant to FD. Among these, are Brachetto and Moscato bianco (Ripamonti *et al*., 2021), although both show a lower palatability for *Scaphoideus titanus* than that of the susceptible Barbera. In addition, the insect vector has reduced fitness when feeding on these tolerant cultivars (Ripamonti *et al*., 2022). As grapevine challenge under controlled conditions is expensive and time consuming, a modified protocol was developed, based on mass inoculation of plants derived from *in vitro* culture exposed to *S. titanus* infected under experimental conditions. Two selection criteria were explored. *Vitis* varieties associated with the progenitor Moscato bianco were selected in an attempt to identify accessions that disjunctively exhibit pathogen resistance and vector repellency. The second criterion for choosing the cultivated varieties under analysis was the frequency of genome introgression of genetic elements from *V. vinifera* subsp. *sylvestris*. This information was available for many Piedmontese grapevine varieties, thanks to preliminary results (Anna Schneider *et al*., unpublished). Vector feeding

behavior on the selected varieties, explored by electropenetrography, was also described to disentangle pathogen resistance due to the plant's response to phytoplasmas rather than to repellency/antibiosis toward the insect vector.

Finally, a protocol was developed to identify putative genes related to FD susceptibility in the model plant *Arabidopsis thaliana*, using bioinformatic analyses coupled to reverse genetics. Three mutants were characterized as less susceptible to FD infection compared to Col0 wild type control. The identified genes are good candidates as potential targets for disruption of grapevine susceptibility to the disease.

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A regulatory SNP upstream of the *GST25* gene could be putatively associated with "flavescence dorée" susceptibility in grapevine

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"Flavescence dorée" (FD) occurs in many European viticulture regions, and it is considered one of the most destructive grapevine diseases. The only means available to control this disease are uprooting symptomatic plants and using insecticides. Understanding the genetic control of FD susceptibility is a crucial step in managing the disease. Identification of single nucleotide polymorphism (SNP) markers linked to FD resistance can be a powerful tool for the introgression of valuable genes needed to develop resistant varieties. SNP markers are gaining popularity among grapevine breeders because they are particularly abundant in the grapevine genome and their detection is efficient and accurate. Observations performed in many experimental vineyards revealed differences in resistance to FD among varieties, that appear to have a genetic basis. The cultivar Tocai friulano is a variety less susceptible to FD, and its ability to compartmentalize FD phytoplasmas in the symptomatic portions of the plant was recently demonstrated (Casarin *et al.*, 2023). Moreover, comparing transcriptional changes between Tocai friulano and Chardonnay (highly susceptible) highlighted passive defence strategies in response to the insect vector and/or the phytoplasma infection (Bertazzon *et al.*, 2019). The aim of this study was the genetic dissection of FD resistance revealed by Tocai friulano through the adoption of molecular approaches such as

genotyping-by-sequencing (GBS), qPCR allelic discrimination, and RNAseq data (Bertazzon *et al.,* 2019). GBS was carried out on the F_1 population derived by crossing Tocai friulano and Chardonnay. The discovered SNPs through GBS were then examined to assess the functionality of gene transcription factors binding motifs in regulatory regions of genes differentially expressed between the two parents at the constitutive level. GBS results allowed the identification of a single SNP in the upstream regulatory genetic sequence of a *glutathione S-transferase 25* gene (GST25), and qPCR gene expression confirmed a significantly higher expression of its transcript in Chardonnay than in Tocai friulano. The selected SNP was subsequently confirmed as present by rhAmp allelic discrimination also in the whole F_1 progeny, and the GST25 transcript abundance was tested in a part of the F_1 progeny. This study led to the identification of a regulatory SNP marker that could be associated with FD susceptibility in grapevine.

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Dissecting the phloem-specific responses of different grapevine cultivars to "flavescence dorée" phytoplasma

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"Flavescence dorée" (FD) is associated with phloem-restricted phytoplasmas, whose management relies mainly on the preventive control of the insect vectors using compulsory insecticide application. Knowledge of the complex mechanisms underlying the plant-pathogen relationships could give opportunity to actuate more efficient and sustainable control strategies. In this context, the study of phytoplasma interaction with the host plants at the ultrastructural, biochemical and molecular level could provide important information about the plant immune response. In grapevine, disruption of the phloem mass flow, following phytoplasma infection, is one of the first traits of the immune defense (Schulz, 1998; van Bel, 2003). In fact, the rapid sieve-element occlusion due to callose and protein accumulation (Furch *et al*., 2007) might form a physical barrier to stop uncontrolled lack of phloem sap, to restrict pathogen invasion and to accumulate defense and signal molecules. Thus, cytological analyses were carried out to compare the ultrastructural features of the phloem tissue of different grapevine cultivars (*i.e*., Glera, Friulano, Refosco), which have shown different degrees of susceptibility to FD infection (Bertazzon *et al*., 2019). Microscopical observations revealed remarkable differences in phloem ultrastructure of infected susceptible Glera plants in comparison with the less susceptible Friulano and Refosco plants (Bertazzon *et al*., 2019). In infected Glera, large phloem areas presented collapsed cells or cells with deformed, thick walls. The lumen of sieve elements was often filled with starch, callose and proteinaceous material. Such alterations were present in restricted areas of the phloem in infected Refosco and Friulano plants. Analyses performed in uninfected control plants demonstrated that Refosco and Friulano grapevines had relevant constitutive accumulations of vacuolar

phenolics in the phloem parenchyma cells, giving a possible reason for the efficient response of these cultivars against infection. Using FD-infected and uninfected grapevines grown under controlled conditions, expression analysis of genes coding phloem-resident proteins at different time points is in progress to check the reactions of different cultivars to phytoplasma infection and the possible emergence of defense mechanisms at the site of infection.

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Differentiation of the "flavescence dorée" phytoplasma genetic clusters by multiplex quantitative PCR assay targeting the *map* gene

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Over the past several decades "flavescence dorée" phytoplasma and its insect vector have been a great threat to European viticulture and winemaking. These important European socioeconomic sectors faced substantial costs due to yield losses and reduction in the quality of grapevines caused by this grapevine disease. Moreover, side effects of insecticide treatments used to control the disease spread, challenge the environmental sustainability of traditional wine production (Morone *et al.*, 2007; EFSA, 2016).

Because of the epidemic spread, the control of the FD disease is especially difficult. Management strategies previously focused exclusively on compulsory insecticide treatments and eradication of grapevines are now aimed more at coexisting with the disease (Morone *et al.*, 2007). For this reason, better understanding of the insect-phytoplasma-plant interactions through analysis of "flavescence dorée" phytoplasma genetic markers that could improve knowledge of the epidemiological cycle can lead to promising tools against "flavescence dorée" phytoplasmas.

Previous research of *map* gene sequences identified three genetic clusters of "flavescence dorée" phytoplasma – Map-FD1, -FD2 and -FD3. Each cluster is affiliated with different geographical distribution and characterized by different genetic variability and epidemiological properties (Arnaud *et al.*, 2007). Those clusters are identified through laborious and time-consuming nested PCR-RFLP or sequencing and phylogenetic analysis. Here, a multiplex quantitative PCR assay was developed for rapid and sensitive detection of the three "flavescence dorée" phytoplasma genetic clusters by using a single primer pair and three distinct BHQ probes labelled with different fluorescent

dyes. The assay was evaluated using "flavescence dorée" phytoplasma reference strains, phytoplasma strains belonging to different phytoplasma groups and field collected "flavescence dorée" phytoplasma strains. The assay was found to be highly sensitive and reliably detected and discriminated all three Map-FD genetic clusters within the 16SrV group. Early detection of Map-FD genetic clusters and thus phytoplasma genotypes which are less likely to cause epidemic outbreaks due to different transmission pathways, can significantly accelerate "flavescence dorée" phytoplasma epidemiological studies and facilitate the efforts to control the FD disease.

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PhenoTruck^{AI}: mobile laboratory for hyperspectral and molecular detection of "flavescence dorée"

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The quarantine "flavescence dorée (FD)", associated with 16SrV-C and -D phytoplasmas, is threatening the wine growing areas of Germany which is so far regarded as FD-free. However, one single FD-infected grapevine plant has been detected in 2020 (Jarausch *et al.*, 2021) which was immediately eradicated. This case as well as the new EU regulations highlighted the need for a large scale screening of FD. This is hampered by the widespread presence of "bois noir (BN)", associated with '*Candidatus* Phytoplasma solani', which induces similar symptoms in grapevine like FD. Therefore, fast and reliable detection methods for FD monitoring in the field have to be developed. The concept of the PhenoTruck^{AI} is based on three axes: large-scale screening of vineyards using remote sensing by drones (UASs), hyperspectral screening of leaf samples for phytoplasma infections and molecular identification of FD in a mobile laboratory. The mobile laboratory is a special vehicle with 4-wheel drive which allows autonomous laboratory work direct at the field. Drone image data will be automatically processed, and sample strategies developed. One compartment of the mobile laboratory is equipped with a dual hyperspectral camera system (VNIR+SWIR, wavelength range from 400 – 2500 nm). The spectra of leaf samples will be automatically analyzed for phytoplasma symptom presence. Ongoing research focus on the spectral discrimination of FD- and BN-infected leaves based on machine learning technologies. Rapid molecular identification of FD-infections will be achieved by LAMP assays.

A case study with a first prototype of the forthcoming PhenoTruck^{AI} was conducted in Trentino and South Tyrol in summer 2023. A total of 430 either FD- or BN-infected as well as asymptomatic leaf samples of the cultivars Chardonnay and Pinot Gris were analyzed with the hyperspectral camera system in a mobile laboratory. The same samples were extracted in the molecular compartment for identification of FD and BN by PCR. Later on, spectral data were processed and segmented leaf data were analyzed patch-wise by machine learning techniques with a leave-n-out cross validation. Phytoplasma infections were identified in VNIR spectra with 95% accuracy and in SWIR spectra with 98% accuracy compared to healthy leaves. Discrimination between FD- and BN-infected leaves was more challenging. Nevertheless, machine learning approaches achieved an accuracy of FD/BN distinction of about 80% in VNIR spectra. Further work is needed to improve the FD detection.

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Leaf disk processing technique to enhance DNA extraction and sample storage for "flavescence dorée" phytoplasma detection by real-time LAMP assay

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"Flavescence dorée" (FD) is the most damaging grapevine yellows disease in many of the European grapevine producing countries. In the absence of effective control methods, early detection of the infection is essential to avoid and/or contain further pathogen spread. To this aim, the *in situ* diagnostic method real-time LAMP can play an important role in efficiently reducing the inoculum presence by rapid detection of infected grapevines to be uprooted, and also reducing the risk of accidentally moving infected insect vectors to pathogen-free viticultural areas while allowing a high number of samples to be processed in a relatively short time. This work aimed at improving the DNA extraction method for the LAMP assay, while enhancing the long-term DNA preservation, using leaf disks instead of leaf macerates. The study was conducted in an infected vineyard of Arezzo province (Tuscany, Italy), carrying out monthly surveys and analyses at the vineyard, between July and October 2023 on the cultivars Sangiovese, Merlot and Cabernet. Leaf macerates (5 leaves/sample) and leaf discs (5 vein discs) were prepared for DNA extraction from symptomatic and asymptomatic leaves and processed by LAMP following the protocol of a commercial kit (Enbiotech, Italy). Part of the same samples was sent to the Regional Phytosanitary Service of Tuscany (Italy) for parallel testing

by qPCR. Phytoplasma detection by LAMP was also achieved by qPCR for cv. Sangiovese throughout the sampling period. Conversely, positive samples of cv. Merlot were only detected by LAMP. In both cultivars, no difference was observed between very symptomatic and slightly symptomatic leaves collected from the same shoot, especially at the end of the season. All cv. Cabernet symptomatic samples were negative using both techniques. Even though the two extraction techniques showed the same results by LAMP, the leaf disc proved to be faster, easier and with less interferences. The Eppendorf cup was used for dissecting the leaf discs and DNA extraction was carried out in the same tube. This method allows the sample to be safely moved before analysis and has proven effective in long-term storage of leaf discs in the same tube used for the LAMP assay.

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Using hyperspectral data to early detect "flavescence dorée" in Tuscany vineyards

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"Flavescence dorée" (FD) is the most threatening grapevine yellows disease in Europe, with severe FD outbreaks reported in major viticulture areas, including Tuscany (Central Italy) (Rizzo *et al.*, 2018). High-throughput techniques for early and accurate detection and monitoring of the presence of this detrimental disease are necessary. Vegetation spectroscopy (and especially hyperspectral data) has emerged as a promising tool, being a non-destructive, rapid, and relatively low-cost technique to monitor the phytopathological status of plants (Mahlein *et al.*, 2018). This study examined the capability of hyperspectral data to early detect FD symptoms presence in a Tuscany vineyard (Sangiovese) located in Lucolena, Greve in Chianti (Florence). Leaf level reflectance measurements (350-2500 nm) were monthly collected from the beginning of July to October 2023. Since the first collections, a widespread presence of FD phytoplasmas was confirmed by standard molecular tests (*i.e.*, qPCR), whereas FD symptoms firstly occurred in September (leaf discoloration and reddening) and developed becoming harsher and more evident in October (also with downward curling of leaves). Interestingly, analyzing hyperspectral signatures collected in August on asymptomatic leaves, it was possible to accurately discriminate the plants tested positive to FD from the negative ones (overall accuracy of around 85%). This accuracy lacked in July, likely because of a lower pressure of the disease, as well as later in September and October, likely because of the presence of visible symptoms which interfered with the

sensitivity of hyperspectral data to FD infection (as well as also because of the scarcity of negative samples). However, in September and October, it was possible to accurately discriminate leaves with different FD symptoms, determined with four classes, *i.e.*, 0 (no symptoms), 1-20, 21-50, and >50% of symptomatic leaf area. The overall accuracy was around 70%, with some misclassifications only between the most symptomatic classes. Furthermore, variations of spectral vegetation indices and leaf functional traits derived from spectra by partial least squares regression-models allowed to elucidate the effects of FD to leaf physiology and biochemistry. Overall, the present study highlights the potential of using hyperspectral data to detect FD in a timely and cost-effective manner.

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Study of mixed infection of uncultured grapevine pathogens in the Russian wine region

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Viticulture is a promising segment of the Russian agro-industrial complex. The area of grapevine plantings in Russia in 2019 amounted to 95.9 thousand hectares (including fruit-bearing ones - 77.3 thousand hectares). The application of modern agricultural technologies ensures a grapevine yield level in the Russian Federation in 2020 in farms of all categories amounts to 95.9 c/ha, or 114.3% of the average level for 2014-2019, while the gross harvest amounted to 678 thousand tons, in 2022 – 746 thousand tons. Grapevines are one of the most valuable, important and key perennial crops, having a high and important international socio-economic significance. The Russian wine industry is facing difficulties due to the lack of nurseries with highly productive and certified planting material, free from viruses and other pathogens (FAOLEX, 2016, 2023). Since the share of imported planting material is large, it is necessary to ensure its quality, first of all, in terms of phytosanitary standards. Today, the complex of pathogens, which is mandatory for control, refers only to quarantine diseases, for example, "flavescence dorée", Raspberry ringspot virus, Tobacco ringspot nepovirus, Tomato ringspot nepovirus, Peach rosette mosaic nepovirus. At the same time, a wide range of infectious agents associated with grapevines (Fuchs, 2020) cause more damage and do not have a phytosanitary status. For example, '*Candidatus* Phytoplasma solani', Grapevine leafroll-associated viruses, Grapevine fanleaf virus, Grapevine fleck virus are found in industrial vineyards in some regions of Russia, but there is no full testing of propagation materials upon import. In 2020-2023 a monitoring of vineyards was carried out in the Derbensky district (Republic of Dagestan). Imported grapevine plants aged from 15 to 25 years were sampled. The collected material was used to determine the presence of a number of

symptoms typical for viral infections (yellowing of leaves, chlorosis, necrosis, variegation, wilting and drying out of bunches), the material of which was analyzed by reverse transcription PCR. In addition to the target virus species GLRaV-1 and GFLV, GLRaV-3 and GFkV were determined. In some samples, a mixed infection of several viruses was detected. Moreover, in a number of samples, the presence of '*Ca.* P. solani', which was previously detected by the authors in the Republic of Dagestan, was confirmed.

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Leafhopper vectors and epidemiology of "flavescence dorée": complexity and knowledge gaps hamper efficient control

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"Flavescence dorée" (FD) is a well-known grapevine disease associated with phytoplasmas of the 16SrV-C and –D subgroups, transmitted by leafhopper vectors in a persistent propagative manner. The main vector is *Scaphoideus titanus* Ball but, over the years, other insect vector species have been identified, although their role in spreading the disease is still not fully understood. Transmission of FD phytoplasmas can occur from grapevine-to-grapevine within the vineyard (secondary spread) or from alternative hosts (wild *Vitis*, broadleaved trees, shrubs) to cultivated grapevines (primary spread). Therefore, epidemiological cycles are complex, as a consequence of the presence of multiple hosts and insect vectors (Malembic-Maher *et al*., 2020). Moreover, because of the genetic variability of FD phytoplasmas, the insect vectors may carry different FD phytoplasma genotypes and genetic typing may reveal routes of FD spread at the vineyard scale (Rossi *et al*., 2019).

Following the epidemics of the disease, compulsory control has been enforced in the vineyards, but, in spite of these efforts, the disease spread did not stop and nowadays FD is a re-emerging disease. In this complex picture, there is a paradigm that did not change over time: secondary infections (grapevine-to-grapevine) are sustained by *S. titanus*. Indeed, FD is defined as the disease associated with phytoplasmas of the 16SrV group transmitted by *S. titanus* (EFSA, 2014). According to this paradigm, alternative vectors are involved in primary infections only, sustained by infected insects coming from outside the vineyard. However, the role of these vectors is highly dependent on a) vegetation composition of vineyard agroecosystem and landscape; b) host-range, host-shifting and dispersal behavior; c) population dynamics and abundance; and d) FD phytoplasma transmission efficiency. Moreover, a major issue on primary spread of FD, is the capability of *S. titanus* to acquire

FD phytoplasmas outside the vineyard from feral *Vitis* (mainly American rootstocks).

The above mentioned points will be discussed, also in the light of recent studies, to evaluate if there are hints for a paradigm change or for new interpretations of FD epidemiology. Finally, control tools (including new perspectives in control strategies) will be critically revised in their potential to counteract the spread of FD.

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"Flavescence dorée" phytoplasma uses its adhesin VmpA, the insect surface protein Uk1_LRR and clathrin to enter into its vector host cell

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The "flavescence dorée" (FD) phytoplasma completes a propagative and circulative cycle into its insect vector before to be inoculated to grapevine. To achieve this cycle, the FD phytoplasma invades different types of insect cells, especially midgut and salivary gland cells. Adhesion of bacteria to eukaryotic cells is the first step of signal transduction that leads to endocytosis of the bacteria by the host cell. Adhesion of FD phytoplasma to midgut epithelium cells is partially mediated by the variable membrane protein A (VmpA), an adhesin which shows lectin properties. To decipher the molecular mechanisms implicated in cellular infection by the FD phytoplasma, it was looked for *Euscelidius variegatus* cell proteins interacting with recombinant VmpA-His_6 and different drogues to inhibit various ways of bacterial entry into cells. The *E. variegatus* proteins interacting with VmpA were identified by far Western blot followed by mass spectrometry analysis. It was selected 13 candidate proteins possessing potential N-glycosylation sites and predicted transmembrane domains. Their impact in VmpA binding was assessed by reducing their expression in *E. variegatus* cells in culture with dsRNA-mediated RNAi. The reduced expression of an unknown transmembrane protein with leucine rich repeat domains (Uk1_LRR) was correlated with the decreased adhesion of VmpA-His_6-coated beads to the insect cells. In non-infected *E. variegatus*, the uk1_LRR was more expressed in digestive tubes than in salivary glands. In infected *E. variegatus*, the expression of uk1_LRR tended towards increase in the early steps of the infection and then decreased. By infection assays of *Drosophila* S2 cells

using chlorpromazine, cytochalasin D and nystatin that inhibit endocytosis, it was shown that phytoplasma entry into cells is clathrin-dependent. In *E. variegatus* fed on infected plants, reduced expression of clathrin heavy chain gene induced by RNAi correlated to a decrease of midgut and salivary gland cells colonization by the phytoplasma. In conclusion, the protein uk1_LRR seems to be implicated in the binding with phytoplasmas via VmpA in the early stages of insect infection following phytoplasmas ingestion, and clathrin is important for the FD phytoplasma to enter insect cells and colonize its insect vector.

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Status of *Scaphoideus titanus* in Serbian vineyards two decades later

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"Flavescence dorée" (FD) is a European quarantine disease affecting grapevines, associated with the "flavescence dorée" phytoplasma and transmitted by the North American leafhopper, *Scaphoideus titanus*. This pathogen has a significant impact on the major viticultural areas across Europe, as well as all wine-producing regions in Serbia. The occurrence of *S. titanus* was initially recorded in Serbian vineyards in 2002 (Magud and Toševski, 2004). However, the high population density observed at that time suggests that the vector's presence likely dates back to the 1990s (Ivo Toševski, unpublished data). Despite the extensive measures to contain the pathogen, the encompassing disease and vector surveillance, the mandatory insecticidal treatments against *S. titanus*, and the utilization of certified phytoplasma-free planting material, the disease continues to spread. Presently, FD affects vineyards in all districts of Serbia (Krstić *et al*., 2022). A recent two-year survey (2022-2023) on *S. titanus* distribution in both wild and cultivated areas within vineyard ecosystems revealed widespread prevalence of the vector across all grapevine-growing regions in Serbia. High population densities were observed in districts where Map-FD3 genotypes are common. These include grape-producing regions all over Serbia where the autochthonous M51 genotype is dominant, hosted by grapevine, along with reservoir plants *Clematis vitalba* and *Ailanthus altissima.* Additionally, substantial *S. titanus* populations were registered in northern Serbia, where multiple outbreaks of the M12 genotype occurred, and central Serbia, where five new genotypes (M150-M154) were detected in grapevines, indicating a high endemic potential for new outbreaks in these regions. Finally, significant populations of the insect vector were discovered in riparian habitats near rivers in Podunavlje and Mačva, where two "flavescence dorée" endemic

genotypes of the Map-FD2 (M89 and M148) were found in grapevines (Krstić *et al*., 2022). The current phytosanitary situation in Serbian vineyards provides evidence that, despite measures implemented over the past two decades, there has been no substantial reduction in the population density and spread of *S. titanus*. Due to the high complexity of the "flavescence dorée" epidemiological cycle caused by natural reservoir plants and native or introduced insect vectors, linking natural habitats with adjacent vineyard ecosystems, managing ongoing epidemics and preventing new outbreaks in the Balkans remains a challenge.

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Genetic diversity of 16SrV phytoplasma strains occurring in grapevines, host plants and insects in the Veneto region (Northeastern Italy)

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A survey on "flavescence dorée" (FD) and related phytoplasmas was carried out in 40 infected vineyards located in the Veneto region (northeastern Italy) during 2020-2022, aimed to identify and characterize phytoplasma strains occurring in the vineyard agroecosystem. Molecular analyses were performed on 16Sr and *map* genes from roughly 200 grapevine samples, 500 other plant species and 1,200 leafhoppers and planthoppers, selected among known or potential hosts/vectors of 16SrV phytoplasmas. Phytoplasmas belonging to 16SrV-A, -C and -D subgroups were identified. In the grapevine samples, two map genotypes were found: M3 in three vineyards and M54 in the others. On the opposite, the genetic diversity of phytoplasma strains occurring in spontaneous plants and insects collected in the same vineyards was much higher. Six out of the 37 analysed plant species hosted phytoplasmas of the 16SrV group. *Ailanthus altissima, Alnus glutinosa, Clematis vitalba,* and *Corylus avellana* were infected by map genotypes M6, M38, M50, M51, or M130 (16SrV-C); a spontaneous *Vitis* sp. sample was infected by M54 (16SrV-D), and in *Ulmus minor* '*Candidatus* Phytoplasma ulmi' (16SrV-A) was identified. Among the insects, *Allygidius atomarius, Allygus modestus, Dictyophara europaea, Euscelidius variegatus, Hishimonus hamatus, Neoaliturus fenestratus, Phlogotettix cyclops,* and *Orientus ishidae* revealed the presence of M3, M6, M12, M38, M43, M50, M51, M118, M143, or other SNPs variants. *Scaphoideus titanus,* conversely, was always infected with M3 or M54 genotypes, the same detected in symptomatic grapevines.

The results showed that the epidemiological cycle FD phytoplasma - *S. titanus* always fitted with map genotypes, while the correspondence between

phytoplasma strains identified in host plants and insects in the same vineyards was sometimes not present, meaning that other plants and/or insects are involved in spreading these phytoplasma strains.

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Studies on alternative insect vectors for the spread of "flavescence dorée"-related phytoplasmas in Germany

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"Flavescence dorée" (FD) is a threatening disease of grapevine associated with phytoplasmas belonging to the 16SrV ribosomal group. The pathogen is efficiently transmitted within vineyards by the Nearctic leafhopper *Scaphoideus titanus*. However, the "flavescence dorée" phytoplasmas are of European origin and are widespread in natural habitats like alder growes. It was demonstrated that autochthonous *Allygus* spp. and the invasive leafhopper *Orientus ishidae* were capable of transmitting phytoplasma strains from alder to *Vicia faba*, which could then be further transmitted by *S. titanus* to grapevine (Malembic-Maher *et al.*, 2020). In Germany, a single grapevine infected by "flavescence dorée" phytoplasmas was found in the vicinity of an alder stand although *S. titanus* was absent (Jarausch *et al*., 2021) indicating that the phytoplasmas were transmitted from alder to grapevine by alternative insect vectors. Extensive transmission trials were conducted to evaluate the role of *Allygus* spp. and *O. ishidae* in the transmission of "flavescence dorée" phytoplasmas from alder to grapevine. The experiments were analyzed in terms of survival probability, transmission success and phytoplasma load in the insects, measured by quantitative PCR. A minimum inoculation titer (MIT) required for successful transmission to alder was defined for both *Allygus spp*. and *O. ishidae*. Although both species were capable of infecting alder seedlings, only *O. ishidae* was able to transmit 16SrV phytoplasmas directly to grapevine. Thus, *O. ishidae* likely poses a higher risk for FD transmission from alder to grapevine, albeit at a very low level. *Allygus* spp. acquired the phytoplasmas only as adults when feeding on alder and became infective only towards the end of the season. Contrary, *O. ishidae* adults with high phytoplasma loads were captured already

at the beginning of the season indicating that they developed on infected alder. These adults were highly infective with high transmission rates to alder. It is assumed that *O. ishidae* spreads "flavescence dorée" phytoplasmas from alder to alder and, thus, increases the risk of FD transmission to grapevine. As every single infected grapevine poses a risk of FD outbreaks in the presence of the vector *S. titanus*, specific monitoring strategies of vineyards adjacent to alder stands need to be established in FD-free regions to prevent FD outbreaks.

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Study of insects of the suborder Auchenorrhyncha known as grapevine phytoplasma vectors

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The lack of pesticides to control phytoplasmas requires the use of methods aimed at different aspects of the spread of infection. This includes phytosanitary control of vineyards, restricting the movement of infected planting material, as well as controlling the number of insect vectors. The main common and harmful phytoplasma diseases in European vineyards are "flavescence dorée" (FD), associated with the "flavescence dorée" phytoplasma, and "bois noir" (BN), associated with '*Candidatus* Phytoplasma solani'. The latter phytoplasma has a fairly wide distribution area and it was detected in almost all European countries, including Russia. It was also identified in Asia, America (Chile), and Africa (Nigeria). The main vector of this phytoplasma is a representative of the Auchenorrhyncha suborder, *Hyalesthes obsoletus* Signoret, which is a native species in Russia. In addition to *H. obsoletus*, the following insects were found in the Russian Federation *Dictyophara europaea*, *Aphrodes makarovi*, *Psammotettix alienus*, *Laodelphax striatellus*, *Philaenus spumarius*, which may be alternative vectors of '*Ca*. P. solani' (Quaglino *et al*., 2019). Most of these species are oligophagous or polyphagous, which poses the risk of establishing pathogen reservoirs in natural agroecosystems. The FD agent has not been detected in the Russian Federation. However, the insect vector of this phytoplasma, *Scaphoideus titanus*, was first identified in 2012. In 2018, the species was registered in the Republic of Adygea, in 2021 in the Krasnodar Territory in the vineyard of the Isabella and Riesling varieties, respectively (Gnezdilov *et al*., 2022).

According to the identification by molecular methods carried out during the 2019-2021 study, the presence of the following Auchenorrhyncha species in the vineyards was confirmed: *Empoasca vitis*, *Arboridia kakogawana*, *S. titanus*, *Synophropsis lauri*, *Aphrodes makarovi*, *Psammotettix confinis*, *Fieberiella florii*,

Agalmatium bilobum. Currently, research is being carried out on the possibility of transmission by these species not only of phytoplasmas, but also of other grapevine pathogens.

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Scaphoideus titanus and *Orientus ishidae* on gone-wild grapevines share phytoplasma genotypes linked to the "flavescence dorée" epidemics in cultivated vineyards

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"Flavescence dorée" (FD) is a grapevine disease associated with phytoplasmas, which may lead to important agronomic losses. Its epidemic spread is mediated by the Nearctic leafhopper *Scaphoideus titanus*, which acts as the main vector. Lately, several alternative insects (*e.g.*, *Orientus ishidae*, *Dictyophara europaea*) and host plants (*e.g.*, *Alnus glutinosa*, *Clematis vitalba*) species have been identified as possible additional actors in the maintenance of the epidemics, especially in the wild compartment surrounding cultivated vineyards. In Switzerland, *S. titanus* and FD were first found in the Southern Alps in 1967 and 2004, respectively. Although the disease incidence always remained at manageable levels (*i.e.*, estimated to <1%) thanks to the prompt and systematic application of the mandatory control measures in cultivated vineyards, FD spread over almost the entire winegrowing area of Canton Ticino in the following years. Therefore, the need to better understand and investigate the potential role of the landscape surrounding cultivated vineyards became urgent (Casati *et al.*, 2017). In this respect, gone-wild grapevines and abandoned vineyards have been repeatedly mentioned as possible reservoirs of "flavescence dorée" phytoplasma inoculum and as uncontrolled habitat for insect vectors (Oggier *et al.*, 2023). However, such compartments are usually not properly considered when investigating the FD pathosystem. Gone-wild grapevines became a prominent study subject after identifying several FD-infected grapevines and finding important populations of both *S. titanus* and the best-candidate alternative vector *O. ishidae* on gone-wild grapevines in the forest. Subsequent molecular analysis revealed radical regional differences

of FD occurrence at landscape scale, opening new insights into the potential dynamics of past, present and future FD spread. Sequencing of the genes *map*, *vmpA* and *malG* in FD-infected *S. titanus*, *O. ishidae* and *Vitis* spp. strongly suggests that the landscape, in general, and gone-wild grapevines, in particular, are crucial components of the "flavescence dorée" phytoplasma epidemiological cycle, at least locally. Implications on the FD epidemics and possible habitat management strategies aiming at lowering the risk of "flavescence dorée" phytoplasma flow between compartments will be presented.

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Does *Orientus ishidae* constitute a risk for "flavescence dorée" epidemics in Veneto region (Northeastern Italy) vineyards?

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Orientus ishidae (Matsumura) has been demonstrated to be able to transmit several phytoplasma strains belonging to 16SrV ribosomal group, including those associated to grapevine "flavescence dorée" (FD) disease (Lessio *et al*., 2016; Malembic-Maher *et al*., 2020). To understand the risk of disease dissemination associated with *O. ishidae* presence and its potential role in FD epidemics spreading in Veneto region (Northeastern Italy), the work aimed to: i) evaluate the presence, distribution, and population density of this insect vector in vineyards where FD is spreading; ii) estimate the occurrence of individuals infected by 16SrV phytoplasma strains; iii) identify and characterize the detected phytoplasmas (16S rRNA and *map* genes) in insects and symptomatic grapevines.

Approximately 7,000 individuals were captured in more than 40 vineyards in summer 2021 and 2022. The insect was present in the 85% of the investigated sites. Most of the captures (roughly 75%) were in the woods and hedges of the vineyards. The molecular characterization, carried out on almost 1,000 specimens, showed that 72 *O. ishidae* were infected with phytoplasmas belonging to the 16SrV-C subgroup. The map genotypes identified were M3, M6, M12, M38, M43, M50, M118 and a few SNPs variants. Some of these strains (M3, M38, M50) are known to be transmitted also by *Scaphoideus titanus*, the main vector of FD from grapevine to grapevine, and also occur in grapevine in Europe. In Veneto region only M3 genotype has been found in symptomatic grapevine so far. The results showed that *O. ishidae* could be a risk for the FD epidemics only in specific environments.

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Insect vectors of "flavescence dorée" and related phytoplasmas in natural areas of riparian habitats in Serbia

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"Flavescence dorée" (FD) outbreaks in Serbian vineyards first emerged in the early 2000s and were directly correlated with dense populations of the North American insect vector *Scaphoideus titanus*. The FD phytoplasma associated with the disease was first treated as alien and introduced, like it happens for its insect vector. However, the genetic peculiarities of FD phytoplasma strains from Serbia, as well as their occurrence in native alder trees in riparian areas and clematis plants in vineyard surroundings and in natural habitats, proved otherwise. Molecular data on the ecological properties of epidemiological cycles driving the epidemic outbreaks of FD in Serbia today indicated the importance of alders as reservoir plants for the epidemics (Krstić *et al.*, 2022). Riparian habitats hosting alder trees, alongside wild *Vitis*, *Salix* sp., clematis, tree of heaven, and other FD phytoplasmas reservoir plants, were screened for native leafhopper vectors as well as for *Orientus ishidae,* a newly established vector of Asian origin in Serbia (Cvrković *et al.*, 2021). Leafhopper *Allygus modestus* and *A. mixtus* were commonly found in association with alders in many sites, with the former being the dominant species. An established population of *O. ishidae* feeding on alders was found in a single location of riparian habitat in eastern Serbia. Among the analyzed leafhopper specimens, using epidemiologically informative *map* gene typing, nearly all *A. modestus* individuals were found carrying FD or -related phytoplasmas, while nearly 50% of *O. ishidae* were found infected at the same location. However, *A. modestus* specimens had a lower phytoplasma load (Cq > 25) than *O. ishidae* (Cq < 20 in 50% of infected individuals), even though they were collected in the same period of the year. When comparing the *map* genotypes carried by each of the putative vector species, *A. modestus* most often carried AldY

genotypes, while *O. ishidae* was dominantly found harboring FD phytoplasma genotypes of both Map-FD1 and FD2 clusters. The experimental verification of the transmission capability and role in FD phytoplasma epidemiology for each of the leafhopper species is under study.

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Grapevine yellows epidemiology in presence of "flavescence dorée" under different agroecological conditions

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Phytoplasma presence is reported in all the major grapevine-growing regions of the world, where a number of molecularly differentiable phytoplasmas were identified (Dermastia *et al*., 2017). Surveys conducted in Prosecco Valdobbiadene growing areas in Veneto region (Northern Italy) allow the identification of FD plus other phytoplasmas in vineyards (Zambon *et al*., 2018). To verify if the diverse agroecological conditions can influence the management of phytoplasmas infecting grapevine in areas in which FD was historically present, three vineyards in 2018 and two in 2019 were surveyed. All the vineyards were of cultivar Glera, they were organic managed and characterized by the presence of at least 30 meters of weeds from the last row where pesticide treatments were applied. To evaluate the possible influence of plant and insect biodiversity on the phytoplasma spreading, the weed and insects presence and abundance were verified in the five vineyards. Representative samplings of spontaneous plants, insect vectors and grapevines were done, and samples were analyzed for phytoplasma presence and identity. Overall, 727 insects belonging to 37 species were collected with the five main species being *Scaphoideus titanus*, *Orientus ishidae*, *Anoplotettix fuscovenosus*, *Neoaliturs fenestratus* and *Hyalesthes obsoletus*. In particular, in vineyards with little plant biodiversity the predominant insect species were *S. titanus,* the main FD vector, and *A. fuscovenosus*. On the other hands, in the vineyards where there was a large number of diverse weed species the entomophauna enclosing potential insect vectors was more variable.

In these insects phytoplasmas enclosed in groups 16SrI, 16SrII, 16SrIII; 16SrV, 16SrVI, 16SrVII and 16SrXII were detected, however the FD phytoplasmas were only detected scattered in *S. titanus* and '*Candidatus* Phytoplasma solani' was only identified in *H obsoletus*. In the 23 grapevine samples collected in the five vineyards it was detected the presence of FD (16SrV-C) (Martini *et al*., 1999), 16SrXII-A and 16SrIII phytoplasmas. The sampled weed species ranged from 2 to 17 and were all asymptomatic and scattered positive to phytoplasmas belonging to 16SrI and 16SrXII groups. The expanding presence of phytoplasma-associated symptoms in Glera grapevines do not always correspond with the presence of FD phytoplasmas suggesting that other phytoplasmas present in the agroenvironment of the vineyards in both insect and weeds are playing a relevant role in the symptomatology presence associated with reduced production.

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Process of eradication of "flavescence dorée" in Northeast Spain

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The key aspects of the eradication of the "flavescence dorée" phytoplasma in the region of Catalonia, in the northeast of Spain are summarized. The different outbreaks and their eradication over time are analyzed. In addition, the phytosanitary regulations and guidelines established for the eradication are addressed.

The first outbreak was in 1996 in Girona province, and for its eradication a close collaboration was established with the authorities of south of France. As a result of this collaboration, joint protocols were established. A first regional regulation was published that officially declared that the pathogen was present in Catalonia and determined a mandatory collective actions to eradicate it. These measures include insect vector control through aerial treatments carried out by the Generalitat de Catalunya, destruction of infected plant material, control of vineyards and nurseries, and emission of phytosanitary advisors.

A second outbreak was confirmed in 2006 in another area of Girona province. As a result of this new outbreak, Order AAR/18/2007 was published, which follows the guidelines for insect vector control and elimination of infected plant material. Since 2014, no infected plots have been detected. The official measures published in 2007 (Order AAR/18/2007) were modified twice. The first amendment (2015) concerns vineyards fields and the second (2020) concerns nurseries to reduce the number of compulsory phytosanitary treatments.

In 2021, a new outbreak was detected in Girona province. Recently, because of the new Regulation (EU) 2022/1630 establishing measures for the containment of grapevine "flavescence dorée" phytoplasma in certain demarcated areas, the

Spanish National Contingency Plan, and to update the control measures and the subsidies given to farmers, a new Catalan legislation (ORDRE ACC/231/2023) was published.

The key points for the eradication of the pathogen are through early detection, destruction of infected plant material, monitoring and control of the insect vector, and their practical application will be reported.

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Control of grapevine "flavescence dorée“ in Slovenia

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The “flavescence dorée” phytoplasma (16SrV group), which is listed as a quarantine pathogen in the European Union, was detected for the first time in Slovenia in 2005 in Primorska wine-growing region during an official survey.

In the following years, the presence of “flavescence dorée” phytoplasma was confirmed also in other Slovenian wine-growing regions. It was observed a significant spread of the disease after the ban of use of the active ingredient thiametoxam (in 2019), which was very effective for the control of *Scaphoideus titanus*, the most important vector of the disease, which is widespread in Slovenia.

For the efficient control of the disease, a timely implementation of measures is important, such as immediate destruction of infected *Vitis* plants as well as control of vector *S. titanus* with effective insecticides. To determine proper terms for pesticide treatments against *S. titanus*, monitoring of its development and the presence of adults is carried out in Slovenia every year. Furthermore, a specific module was developed which considers the calculation of degree days above a certain temperature threshold and the data on phenological development stages of *Vitis*.

To effectively prevent the spread of “flavescence dorée” phytoplasma, a thorough understanding of its epidemiology is essential, so the genetic diversity of 16SrV phytoplasmas in grapevine samples collected from different Slovenian vineyards between 2017 and 2023 was also investigated. Nucleotide sequence analysis was based on the *map* gene. The *map* genotypes detected in grapevines were compared with those found in alternative hosts and insect vectors. Based on these comparisons, possible transmission routes of this

phytoplasma in Slovenia were outlined and the importance of alternative host species and insect vectors was evaluated.

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A project to control "flavescence dorée" outbreaks in hilly areas of the Treviso district (north-eastern Italy)

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During 2019–2020, a large outbreak of "flavescence dorée" (FD), associated with an increase in *Scaphoideus titanus* population densities, was observed in hilly areas of the Treviso district. Given the serious damage inflicted on the Glera vineyards, technicians and growers asked to understand the causes of this phenomenon and identify solutions. For this purpose, a project was funded from several organizations and a number of farms. Among factors involved in FD outbreaks, the occurrence of virulent phytoplasma strains, the limited effectiveness of insecticides against *S. titanus* and the role of groves as a source of infectious insect vectors were considered. FD phytoplasma strains in symptomatic vineyards were not different from those previously reported (Arnaud *et al*., 2007). Insecticide trials showed that the most used active ingredients in the two years 2019-2020 (*i.e*., acetamiprid and flupyradifuron) were less effective than those recently removed from the market (organophosphates and thiamethoxam). Later, these results were confirmed by additional studies where some pyrethroids proved to be the most effective insecticides (Prazaru *et al*., 2023). Moreover, acetamiprid and flupyradifurone were frequently applied following inaccurate timing. The groves bordering the vineyards can be sources of infectious *S. titanus* when American grapevines grow inside them (Pavan *et al*., 2012). In addition, the binomials *Orientus ishidae*/some broadleaf species and *Dictyophara europaea*/*Clematis vitalba* are now suspected of favoring FD infection in vineyards (Filippin *et al*., 2009; Lessio *et al*., 2016). However, the spatial distribution of both *S. titanus* and FD symptomatic grapevines showed that groves were not responsible for FD recrudescence when they did not host wild grapevines. In contrast,

FD-infected vineyards represented a risk for neighboring vineyards, highlighting that the FD control strategies must be conducted following an area-wide approach. After intensive use of pyrethroids in vineyards, the monitoring of *S. titanus* populations and mapping of new symptomatic grapevines showed a reduction in both vector populations and FD incidence.

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Foliar treatment of grapevine plantlets with an experimental biocomplex reduces "flavescence dorée" phytoplasma infection and inoculation by insect vectors

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Current control of "flavescence dorée" (FD) phytoplasmas is highly dependent on management of its major vector *Scaphoideus titanus*; however nowadays insecticide sprays are often insufficient to prevent from disease outbreaks. The search for methods that directly target the phytoplasma in the plant is a crucial step to implement an integrated strategy to counteract FD outbreaks. An experimental biocomplex, containing trace elements and soluble sugars, with potential systemic spread and antimicrobial activity, was tested to explore its capability to perform an anti-phytoplasma activity into the plant, as well as in the vectors (Gonella *et al.*, 2024). Two distinct phytoplasma strains, corresponding to the map genotypes M50 (16SrV-C) and M54 (16SrV-D) were used in separate experimental trials. Grapevine plantlets, obtained through micropropagation, were sprayed with the biocomplex either before or after being inoculated with M50 or M54 phytoplasmas, to test the effect of short-time and mid-time preventive application as well as of curative application. Additionally, the capability of the biocomplex to be ingested by the insect vectors was assessed along with the resulting effect on the transmission efficiency by *S. titanus*. Phytoplasma transmission trials with sequential inoculation steps were set up, by successively offering untreated and biocomplex-treated grapevine plantlets to infectious leafhoppers. A significant reduction of phytoplasma infection was recorded in biocomplex-treated grapevines, regardless of the FD genotype. Both preventive and curative treatments resulted in decreased plant FD infection, indicating an antibacterial activity in the plant tissues. Moreover, after preventive treatments the biocomplex was effectively ingested by vectors, and a reduction of infection rate in *S. titanus* was observed. Accordingly, a decrease of *S. titanus* inoculation rates was found after the vector was exposed to a biocomplex-treated plant. The suggested reduction of transmission efficiency

was higher using the M50 rather than M54 genotype. These results indicate the tested biocomplex as a promising tool to support vector management in FD containment.

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RNA interference as innovative strategy to deal with "flavescence dorée" phytoplasmas

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RNA interference (RNAi) regulates the gene expression in eukaryotes and can be exploited in crop protection against pests by exogenous applications of double-stranded RNAs (dsRNAs), although delivery of dsRNAs to sap-sucking insects is still a major challenge for field application. Two phytoplasma vectors, *Euscelidius variegatus* and *Scaphoideus titanus* (Hemiptera: Cicadellidae), were used to identify putative candidate target genes to be silenced and verify whether RNAi can be triggered in leafhoppers by plant-mediated delivery methods. In particular, *S. titanus* is the main insect vector of "flavescence dorée" phytoplasma to grapevine, whereas *E. variegatus* is commonly used as laboratory vector. Previous works indicated that recombinant immunodominant membrane protein (Imp) of "flavescence dorée" phytoplasma selectively interacts with proteins from vector species rather than those from non-vectors (Trivellone *et al*., 2019), as well as silencing ATP synthase beta of vectors reduces phytoplasma multiplication and induces female sterility (Ripamonti *et al*., 2022). Selection of target genes was driven by this previous knowledge. Insect gut proteins interacting with His-tagged "flavescence dorée" phytoplasma Imps in pull-down assays were isolated and identified by mass spectrometry on protein dataset from *S. titanus* and *E. variegatus* transcriptomes. Three *S. titanus* and five *E. variegatus* proteins interacting with Imp were further characterized by measuring expression of their corresponding transcripts in different insect tissues and in healthy *versus* infected insects, as well as by analysing expression of the cognate proteins upon microinjection of specific dsRNAs. Silencing two insect genes interacting with "flavescence dorée" phytoplasma Imp reduced the phytoplasma multiplication in insect bodies.

A plant-mediated method to deliver dsRNAs was then optimized for a lab-scale application of RNAi, by silencing two gut-specific and one systemically expressed insect genes in both species. Insects fed on dsRNA-treated leaves by petiole absorption showed a significant reduction of target genes activity, demonstrating that plant uptake of dsRNAs can silence specific transcripts of phloem-feeders fed on those plants. This work paves the way towards innovative alternative control strategies that can potentially replace/integrate insecticide application against sap-feeding vector species. The method will be also useful in functional genomic studies to decipher the roles of genes of insect vectors in the transmission of plant pathogens.

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Workshop Programme

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14:00	**Welcome addresses**
14:30-16:00	**Session 1: "Flavescence doreé" and plants I**
16:00	Coffee break
16:30-18:00	**Session 2: "Flavescence doreé" and plants II**
20:00	Social dinner

Friday 26 January 2024	
09:00-10:30	**Session 3: "Flavescence doreé" and insect vectors I**
10:30-11:00	Coffee break
11:00-12:30	**Session 4: "Flavescence doreé" and insect vectors II**
12:30-13:00	Concluding remarks
13:00	Light lunch

THURSDAY 25th January 2024

13:00-14:00	Registration of participants
14:00-14:30	Opening and welcoming remarks

Session 1 "Flavescence dorée" and plants I

Chairs: Assunta Bertaccini & Piero Attilio Bianco

14:30-15:00 Sandrine Eveillard	**Keynote lecture** Review in plant-"flavescence dorée" phytoplasma interactions
15:00-15:15 Marta Martini	Past and present genetic diversity of "flavescence dorée" phytoplasma strains in grapevine samples collected in Veneto and Friuli Venezia Giulia (Italy)
15:15-15:30 Fabio Quaglino	Recent findings on "flavescence dorée" in Franciacorta (North Italy): prevalence of associated phytoplasma genotypes in symptomatic grapevines and in additional plant and insect hosts within and around vineyards
15:30-15-45 Francesco Pacini	"Flavescence dorée" strains detected in Tuscany, Emilia-Romagna, Veneto and Trentino Alto Adige regions of Italy
15:45-16:00 Zahra Golestani Hotkani	Comparative genomics analysis of "flavescence dorée" phytoplasma strains from Chardonnay and Pinot gris cultivars
16:00-16:30	Coffee break

THURSDAY 25th January 2024

Session 2 "Flavescence dorée" and plants II

Chairs: Xavier Foissac & Martina Šeruga Musić

16:30-16:45 Cristina Marzachì	Towards the identification of genetic resistance traits against "flavescence dorée"
16:45-17:00 Sofia Casarin	A regulatory SNP located upstream of the GST25 gene could be putatively associated with "flavescence dorée" susceptibility in grapevine
17:00-17:10 Ottone C. Viscardo	Dissecting the phloem-specific responses of different grapevine cultivars to "flavescence dorée" phytoplasma
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17:20-17:35 Wolfgang Jarausch	PhenoTruck^{AI}: mobile laboratory for hyperspectral and molecular detection of "flavescence dorée"
17:35-17:45 Rocco Caracciolo	Leaf disk processing technique to enhance DNA extraction and sample storage for "flavescence dorée" phytoplasma detection by real-time LAMP assay
17:45-17:55 Marco Carli	Using hyperspectral data to early detect "flavescence dorée" in Tuscany vineyards
17:55-18:00 Galina Bondarenko	Study of mixed infection of uncultivated grapevine phytopathogens in the Russian wine region
20:00	Social dinner

FRIDAY 26th January 2024

Session 3	"Flavescence dorée" and insect vectors I
	Chairs: Jelena Jović & Wolfgang Jarausch
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09:55-10:10 Elisa Angelini	Genetic diversity of 16SrV phytoplasma strains occurring in grapevines, host plants and insects in the Veneto region (Northeastern Italy)
10:10-10:25 Barbara Jarausch	Studies on alternative insect vectors for the spread of "flavescence dorée"-related phytoplasmas in Germany
10:25-10:30 Bairta Khamaeva	Study of insects of the suborder Auchenorrhyncha, noted in the transfer of grapevine phytoplasmas
10:30-11:00	Coffee break

FRIDAY 26th January 2024

Session 4 "Flavescence dorée" and insect vectors II

Chairs: Magda Rak Cizej & Alberto Alma

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11:10-11:15 Enea Guerrieri	Does *Orientus ishidae* constitute a risk for "flavescence dorée" epidemics in Veneto region?
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11:50-12:00 Carlo Duso	A project to control "flavescence dorée" outbreaks in hilly areas of the Treviso district (north-eastern Italy)
12:05-12:15 Elena Gonella	Foliar treatment of grapevine plantlets with an experimental biocomplex reduces "flavescence dorée" phytoplasma infection and inoculation by vectors
12:15-12:30 Luciana Galetto	RNA interference as innovative strategy to deal with "flavescence dorée" phytoplasma
12:30	Concluding remarks and light lunch

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