ICVG
International Council for the Study of Virus and Virus-Like Diseases of the Grapevine

Proceedings of the 20th Congress of the International Council for the Study of Virus & Virus-like Diseases of the Grapevine

25-29 September 2023 Thessaloniki, Greece

The 20th Congress of the International Council for the Study of Virus and Virus-like diseases of the Grapevine (ICVG)

Porto Palace Hotel, Thessaloniki, Greece, 25-29 September 2023

Welcome

We have the honor to host and welcome you to the 20th ICVG congress in Thessaloniki, five years after the 19th ICVG meeting held in Santiago, Chile and almost 60 years after the first ICVG meeting in Changins, Switzerland. This is the second meeting held in Greece, after the 10th ICVG meeting in Volos 33 years ago. The Covid-19 pandemic delayed the present congress but now that life has regained its normal pace, we are more than happy to be able to interact in person with all of you here.

We hope that you will enjoy the meeting, during which scientists from all over the world will present their research progress in grapevine viruses, viroids and phytoplasmas. Also, you will have the chance to discover and enjoy Thessaloniki, a vibrant modern city with a long history of 2300 years. And of course, last but not least, we hope that everyone will appreciate the company of colleagues and friends.

Finally, we would like to express our gratitude to all the sponsors for their kind support, as well as to all the students of the Plant Pathology Laboratory of the Aristotle University of Thessaloniki for their help in the preparation and organization of the 20th ICVG congress.

Kind regards,

On behalf of the organizing committee

Varvara Maliogka and Nikolaos Katis

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ICVG Meetings

1. Changins (Switzerland) 17-20 August 1964
2. Davis (California) 7-11 September 1965
3. Bernkastel Kues (W. Germany) September 1967
4. Colmar (France) 16-18 June 1970
5. Salice Terme (Italy) 16-19 September 1973
6. Cordoba-Madrid (Spain) 12-17 September 1973
7. Niagara Falls (Canada) 7-12 September 1980
8. Bari (Italy) 2-7 September 1984
9. Kiryat Anavim (Israel) 6-11 September 1987
10. Volos (Greece) 3-7 September 1990
11. Montreux (Switzerland) 5-10 September 1993
12. Lisbon (Portugal) 28 September - 2 October 1997
13. Adelaide (South Australia) 12-17March 2000
14. Locorotondo (Italy) 12-17 September 2003
15. Stellenbosch (South Africa) 3-7 April 2006
16. Dijon (France) 31 Aug - 4 Sep 2009
17. Davis (California) 7-14 October 2012
18. Ankara (Turkey) 7-11 September 2015
19. Santiago (Chile) 9-12 April 2018
20. Thessaloniki (Greece) 25-29 September 2023

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Dedication

These Proceedings are dedicated to the memory of Prof. Giovanni Martelli, one of the Founding Fathers and a long-lasting President of ICVG.

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Congress Program

CONGRESS VENUE PORTO PALACE HOTEL

MONDAY, SEPTEMBER 25

19:00-20:00 **Registration**
20:00-22:30 **Welcome Reception**

TUESDAY, SEPTEMBER 26

08:00-09:00 Registration and Poster Set-Up

09:00-09:30 Welcome Address

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- **Session 1 11:15-13:00 Disease Etiology-Epidemiology**

Chair: Marc Fuchs and Robin MacDiarmid

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- **Session 2 14:00-16:15 Diagnostics**

Chair: Hans Maree and Eva Varallyay

Keynote Speaker

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WEDNESDAY, SEPTEMBER 27

- **Session 3 08:30-10:45 Certification and other management approaches**

Chair: Maher Al Rwahnih and Giorgio Gambino

Keynote Speaker

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Chair: Baozhong Meng and Emmanuelle Vigne

Keynote Speaker

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Keynote Presentations Abstracts

K1. Grapevine virology highlights: 2018-2023

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INTRODUCTION

There has been tremendous research progress on virus biology and ecology since the last ICVG conference in Santiago, Chile in 2018, illustrating the vibrancy of grapevine virology as a discipline. Hitherto undescribed viruses were identified, diagnostic tools were improved, disease epidemiological features were characterized, new insights into virus-vector-host interactions were gained, revised certification standards have been adopted, and enhanced disease management strategies were implemented. Progress in these focal areas was primarily driven by interdisciplinary and cross-institutional research efforts, the adoption of innovative technologies, and the creation of new strategic spaces for impactful engagement with grower communities, extension educators, policy makers, and regulators. This article summarizes some major milestones since the last ICVG conference.

A MYRIAD OF VIRUSES

A total of 101 viruses have been identified in various *Vitis* germplasm worldwide thus far. These viruses have single-stranded or double-stranded RNA or DNA genomes and belong to 21 different families (*Alphaflexiviridae*, *Betaflexiviridae*, *Bromoviridae*, *Caulimoviridae*, *Closteroviridae*, *Endornaviridae*, *Fimoviridae, Geminiviridae*, *Luteoviridae*, *Mayoviridae*, *Nanoviridae*, *Partitiviridae*, *Peribunyaviridae, Phenuiviridae*, *Potyviridae*, *Reoviridae*, *Rhabdoviridae*, *Secoviridae*, *Solemoviridae*, *Tombusviridae*, *Tymoviridae*, *Virgaviridae*), or correspond to unclassified plant satellite viruses (Dahan et al. 2023, Diaz-Lara et al. 2023, Fuchs 2020, Katsarou et al. 2023, Roy and Fuchs 2023, Selda Rivarez et al. 2022, Shvets et al. 2022). All newly recognized viruses were identified by applying high-throughput sequencing (HTS) technologies. Noticeably, viruses identified via HTS often lack biological context and little is generally known on their effect on grapevine health.

DIAGNOSTICS

New diagnostic tools including nanobody-based serological assays (Orlov et al. 2020), and nucleic acid assays such as RT-qPCR (Diaz-Lara et al. 2018, Diaz-Lara et al. 2020, Morán et al. 2023, Ren et al. 2020, Vigne et al. 2018), qPCR (Jagunic et al. 2022a, Morán et al. 2020, Setiono et al. 2018), recombinase polymerase amplification (Kishan et al. 2023), loop-mediated isothermal amplification (Romero Romero et al. 2019), and CRISP-Cas (Li et al. 2019) were developed for several grapevine viruses. HTS approaches were improved, resulting in the elaboration of robust nucleic acid-based diagnostic assays, increasing testing accuracy and speed (Javaran et al. 2021, Massart et al. 2022, Navrostskaya et al. 2021, Read et al. 2022, Selda Rivarez et al. 2022, Shvets et al. 2022, Soltani et al. 2021, Tamisier et al. 2021, Velasco and Padilla 2021). In addition, spectroscopy (Bendel et al. 2020, Gao et al. 2020, Mandrile et al. 2022, Nguyen et al. 2021, Romero Galvan et al. 2023, Rubambiza et al. 2023, Sawyer et al. 2023, Wang et al. 2022, Wang et al. 2023) and sensors (Rizzato et al. 2023) have been explored with encouraging diagnostic potential in vineyards for fanleaf and leafroll diseases. Other ongoing diagnostic research efforts rely on the training and deployment of canines for an olfactory detection of vines infected with grapevine leafroll-associated virus 3 (GLRaV3) or infested with the vine mealybug (*Planococcus ficus*) in vineyards (Bolton 2023).

VIRUS BIOLOGY

The causative role of grapevine red blotch virus (GRBV) (Yepes et al. 2018) and grapevine berry inner necrosis virus (Fan et al. 2020) in red blotch disease and leaf chlorotic mottling and ringspot disease,

respectively, was demonstrated using *Agrobacterium tumefaciens*-mediated inoculations of full-length virus infectious clones. A similar approach was used to elucidate the etiology of leaf mottling and deformation disease by using infectious clones of grapevine Pinot gris virus (GPGV) (Tarquini et al. 2019a).

Metagenomic analyses shed light on a genomic region of nepoviruses potentially involved in host range (Hily et al. 2021a). Virus-host interaction research through transcriptomic, approaches and/or metabolomic revealed specific host biochemical pathways (Buoso et al. 2020, Martin et al. 2021, Roy et al. 2023) and host protein candidates (Osterbaan et al. 2021, Roy et al. 2023) for disease symptom development, as well as physiological changes and alterations of foliar (Wallis 2022) and berry composition (Ghaffari et al. 2020, Rienth et al. 2021, Rumbaugh et al. 2021, Rumbaugh et al. 2022, Song et al. 2021, Song et al. 2022, Zhang et al. 2023), as well as of oxidative stress parameters (Hancevic et al. 2023). Reverse genetics studies documented grapevine fanleaf virus (GFLV) determinants of symptom expression (Martin et al. 2018, Osterbaan et al. 2019). Virus diagnosis with regards to grapevine physiology was studied (Crespo-Martinez et al. 2023). Rootstocks were shown to influence vine responses to GLRaV3 (Cabaleiro et al. 2021) and to several GLRaVs with a dysregulation of pathways involved in pathogen detection, abscisic acid signaling, phenylpropanoid biosynthesis, and cytoskeleton remodeling (Vondras et al. 2021). Interestingly, genetic variants of GLRaV3 were documented to differentially affect disease symptom expression in red-berried grapevine cultivars (Chooi et al. 2022), and to accumulate to statistically supported distinct levels in single infections, while dysregulating the expression of certain microRNAs (Aldrich et al. 2019). Protein suppressors of RNA silencing were identified for GPGV (Tarquini et al. 2021a) and GFLV (Choi et al. 2023), and the suppressor of GLRV2 was functionally characterized (Zhang et al. 2022). Proteins encoded by GRBV were detected *in planta* (Buchs et al. 2018), and interactions among GLRaV3 proteins *in vitro* provided new insights into virion assembly (Moster et al. 2023).

The population structure of GFLV (Panno et al. 2021, Kubina et al. 2022) and GLRaV4 (Adiputra et al. 2019) was determined in diseased vineyards. The coat protein of GFLV was explored for its potential to express heterologous epitopes for vaccine production (Yazdani et al. 2022). Interestingly, the genomic RNAs of GFLV, including encapsidated RNAs, were shown to be mono-uridylated at their poly(A) tails, illustrating a novel type of viral genomic RNA extremity (Joly et al. 2023). In addition, GFLV was shown to be occasionally associated with improved tolerance to fungal infections (Gilardi et al. 2020) and a moderate water stress in diseased vines (Jez-Krebelj et al. 2022), while GRBV was shown to reduce cold hardiness (Bowen et al. 2020). GRBV was speculated to have emerged from wild *Vitis* latent virus 1 (Thompson 2022), a closely related virus that is prevalent in free-living grapevines in northern California (Cieniewicz et al. 2018a). Similarly, the evolutionary history of GPGV was studied (Tarquini et al. 2019b, Vu et al. 2023a) and elucidated through metagenomic analysis (Hily et al. 2020). Reverse genetics experiments revealed that the C-terminus of the GPGV movement protein influences virus titer, accumulation of siRNAs, and host genes involved in boron transporters, and to some extent, disease symptom expression (Tarquini et al. 2021b). In addition, GPGV dysregulates boron homeostasis, particularly in boron-deficiency conditions (Buoso et al. 2020), fueling the debate on the etiology of leaf mottling and deformation disease (Buoso et al. 2020, Cieniewicz et al. 2020, Tarquini et al. 2023).

Virus-vector interaction studies revealed new determinants of GFLV transmission by the ectoparasitic dagger nematode *Xiphinema index* (Belval et al. 2019), and a circulative transmission mode of GRBV by the three-cornered alfalfa hopper (*Spissistilus festinus*) (Flasco et al. 2021). Transmission of GLRaV1 and grapevine virus A by scale insects (*Parthenolecanium corni* and *Pulvinaria vitis*) was characterized in the vineyard (Hommay et al. 2020) and laboratory (Hommay et al. 2021), and the transmission of grapevine badnavius 1, grapevine virus G, grapevine virus H by the vine mealybug

(Jagunic et al. 2021, Jagunic et al. 2022b,) was reported, as well as the transmission of grapevine vein clearing virus (GVCV) by the grape aphid (*Aphis illinoisensis*) (Petersen et al. 2019). GLRaV3 was detected in *Pseudococcus calceolariae* up to 16 days on non-*Vitis* plant hosts following acquisition on infected grapevines (McGreal et al. 2021), and a single specimen of the grape mealybug (*Pseudococcus maritimus*) was shown to be sufficient for the transmission of GLRaV3 (O'Hearn and Walsch 2021). Furthermore, a lectin was documented to disrupt transmission of GLRaV3 by the vine mealybug (Prator and Almeida 2020).

DISEASE EPIDEMIOLOGY

Spatiotemporal spread patterns of GRBV (Cieniewicz et al. 2019, Dalton et al. 2019, KC et al. 2022; Flasco et al. 2023a) were characterized in diseased vineyards. Similarly, spread attributes of GLRaV3 in vineyards of own rooted wine grape cultivars were shown to be identical to those reported in vineyards of grafted wine grape cultivars (Donda et al. 2023). Significant contributions by both mealybug vector abundance and GLRaV-3 incidence to the frequency of newly diseased vines in vineyards was ascertained (Cooper et al. 2018). Spread events of GPGV were documented in vineyards (Bertazzon et al. 2020) or inferred by sequence data mining (Hily et al. 2021b, Hily et al. 2021c). Insect vector candidates of GRBV were identified in diseased vineyards (Cieniewicz et al. 2018b, Wilson et al. 2022) and the three-cornered alfalfa hopper was shown as a GRBV vector of epidemiological relevance (Flasco et al. 2023b).

Surveys of free-living *Vitis* sp. and related species confirmed earlier findings on their role as reservoirs of viruses (Cieniewicz et al. 2018a; Diaz-Lara et al. 2021, Fajardo et al. 2018, Petersen et al. 2019, Reynard et al. 2020, Sabella et al. 2018, Schoelz et al. 2021, Thompson et al. 2021, Uhls et al 2021) and viroids (Abe and Nabeshima 2021). In addition, GRBV transmission to and from free-living *Vitis* sp. was achieved by the three-cornered alfalfa hoper (Hoyle et al. 2022). Similarly, the transmission of GVCV via the grape aphid from free-living vines to cultivated vines was reported (Petersen et al. 2019). Furthermore, weeds and shrubs proximal to vineyards were described as reservoirs of GPGV (Demian et al. 2022) and as refuge of the scarlet mealybug (*Pseudococcus calceolariae*) (McGreal et al. 2021).

DISEASE MANAGEMENT

Biological indexing of GLRaV3 was reported *in vitro* (Hao et al. 2021). A droplet vitrification cryotherapy method was applied for the elimination of GLRaV3 (Bi et al. 2018) and a similar droplet vitrification cryopreservation protocol was developed from shoot tips of *in vitro*-grown plants for the long-term maintenance of *Vitis* germplasm (Bettoni et al. 2019). Viticultural practices such as cluster thinning (Copp et al. 2022) and deficit irrigation (Levin and KV 2020) had limited success at mitigating the impact of GRBV with regards to enhanced fruit quality. Some arbuscular mycorrhizal fungi were shown to enhance photosynthesis of virus-infected grapevines in the greenhouse but their potential at mitigating the effect of viruses in vineyards remains unknown (Gasi et al. 2023). Area-wide roguing and mealybug control efforts were essential for leafroll management in diseased vineyards (Hobbs et al. 2022, Cooper et al. 2018).

Certification standards were revised and adopted in several states or countries to facilitate the production of clean planting stocks. For example, emerging viruses with documented detrimental impact on grapevine health, such as GRBV in North America and GVCV in Missouri, were added to existing standards. In addition, the traditional woody indexing was relinquished in favor of more robust diagnostic technologies such as HTS and PCR-based assays. Furthermore, more stringent, and more frequent testing of grapevines in increase vineyard blocks at nurseries were implemented. These new measures have substantially improved the cleanliness of the planting stocks (Arnold et al. 2019). In the European Union, new plant health regulations came into effect, resulting in the issuing of a single

certification label/European plant health passport (Juncker 2019). The economic impact of the production and adoption of certified vines free of GLRaV3 was estimated at more than US $96 million in northern California (Fuller et al. 2019). Analyzing the economic impact of public investments in Foundation Plant Services at the University of California-Davis, a clean plant center that produces virus-tested planting stocks and distributes virus-tested scion budwood and virus-tested rootstock cuttings, suggested a benefit-cost ratio of 96:1, justifying a continued support of clean plant centers (Li et al. 2022).

Factors influencing the adoption of management practices for leafroll and red blotch diseases by growers were evaluated (Hobbs et al. 2022), and stressed the need for behavioral and technological changes, as well as cooperative approaches from multiple sectors and reliable educational resources to be effective (Fuchs 2022, Hobbs et al. 2023). Community efforts for a wide adoption of mealybug control and roguing were essential for the management of leafroll disease (Bell et al. 2018, Bell et al. 2021, Bolton, 2020, MacDonald et al. 2019). Interestingly, the removal of diseased vines and two adjacent within-row neighboring vines, an approach referred to as spatial roguing, reduced leafroll disease incidence and spread (Hesler et al. 2022).

Sources of resistance were identified for GVCV in a *Vitis vinifera* x *V. aestivalis* hybrid (Qiu et al. 2020) and for GFLV in a *V. vinifera* accession (Djennane et al. 2021). The *V. pseudoreticulata* accession 'Hunan-1' was found to be less susceptible to GLRaV3 than *V. vinifera* cultivars (Hao et al. 2022). For insect vectors of grapevine viruses, resistance to *X. index* in muscadine-derived rootstocks was shown to be durable (Nguyen et al. 2020) and to be effective against *X. diversicaudatum* (Banora et al. 2022). Resistance to the vine mealybug was described in some rootstocks (Naegele et al. 2020). Furthermore, an increased mortality of grape mealybugs via RNA interference using a cocktail of dsRNAs targeting the aquaporin and sucrase genes that are expressed in the insect gut and mediate the body fluid osmoregulatory upon feeding on sugar-rich phloem sap was described (Arora et al. 2020). Finally, resistance to GLRaV-3 was achieved in grapevine plantlets by RNA-targeting CRISPR mechanisms, creating new disease management opportunities (Jiao et al. 2022). It will be interesting to see whether follow-up work in the vineyard will validate these encouraging findings.

CONCLUDING REMARKS

Great strides in advancing our knowledge of grapevine viruses were made from 2018 to 2023 through interdisciplinary and cross-institutional research. Numerous new viruses were identified in various *Vitis* germplasm repositories and commercial vineyards. Virus diagnosis in grapevine tissue has never been so robust. Distinct disease epidemiological dynamics were characterized in vineyards, new insights into virus-vector-host interactions were gained, and potentially transformative disease management strategies were explored. The wealth of knowledge on grapevine viruses was disseminated to grower communities through educational events such as on-site visits, grower conventions, meetings of technical groups, and workshops, among others. The success of such positive interactions often required sharpened and inspirational communication skills. Nonetheless, it is my opinion that the most impactful changes experienced since the last ICVG conference in Chile in 2018 centered on the implementation of revised certification standards for a timely delivery of clean propagation material to nurseries and for an increased level of cleanliness of the planting stocks. It should be noted that such revised certification standards were strategically adopted through sustained collaborative efforts, and a continuous dialogue among scientists, grower communities, extension educators, policy makers, and regulators.

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K2. The state of viticulture in Greece: an updated report of 'grape routes' national program

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INTRODUCTION

The national research program "Grape Roots", funded by the General Secretariat of Research and Innovation, is a research and technological innovation plan to support the uniqueness of Greek wine products and strengthen their competitiveness. Given the importance of local wine-making varieties for the sector, highlighting the quality characteristics of Greek varieties was the main objective of the project in order to provide Greek winegrowers and winemakers with the necessary basic knowledge to explore their full potential. The program was divided into three distinct sub-projects: (1) Genetic and Genomic Analysis, (2) Chemical-organoleptic characterization, and (3) vineyard pest control and microbiome.

RESULTS AND DISCUSSION

(1) Genetic and Genomic Analysis

Genomic analysis and application of next-generation -omic technologies were used to describe the genetic identity of indigenous grapevine varieties (sequencing emblematic grapevine varieties and creating databases containing the genetic, genomic and transcriptomic information). Whole Genome Sequencing (WGS), Whole Transcriptome Sequencing (RNA sequencing – RNA-seq) and metagenomics analyzes were applied. The goal, among others was the creation of reference genomes for the future identification of varieties and clones of each variety.

The work resulted in a digital database with:

- The complete genomes of indigenous varieties
- The transcriptomes of indigenous varieties
- The molecular marker profile of each variety
- The polymorphisms along the entire genome of each variety

By increasing our knowledge on the genetic and genomic background of Greek varieties we provided scientific background on key performance characteristics (resistance to drought and climate change) of Greek varieties. Official ampelographic collections were checked and updated and conditions were created for the production of high-quality propagation material for native grape varieties.

(2) Chemical-organoleptic characterization and vinification

Many of the chemical components associated with the aroma and flavor of wines derive from the grapes and are genetically determined, thus differentiating the wines of the various varieties. Thereby, the aim of the second sub-project was the study of the secondary metabolites of the grapes and wines of the iconic native winegrape varieties, which shape the color, aroma and taste of their wines. At the same time, the detection, based on a detailed descriptive organoleptic examination, and the identification of wine aroma components that contribute to the typicality of each variety, as well as the development of methods for the quantitative determination of these components, resulted in the creation of the special profiles of the emblematic varieties, giving winemakers a great commercial advantage to promote them in thee international markets. At the same time, the knowledge of their oenological potential will contribute to the development and implementation of appropriate viticulture and winemaking practices that will aim to highlight and optimize the "varietal" character of the wines produced.

(3) vineyard pest control and microbiome

The vine is susceptible to a multitude of viruses, some of which cause significant qualitative and quantitative losses in production, while also reducing the productive life of the canes. The infestation by viruses often occurs already at the beginning of planting due to the lack of tools to control the propagation material. Also, knowledge, tools and utilization of pest and disease resistant varieties were limited for the Greek varieties which limited their appeal to growers. The development of tools to control the plant health of the propagating material and the modernization of plant protection of the vine to enhance the expansion of the use of indigenous grapes was therefore the object of the last sub-project.

Also, the thorough analysis of the microbiome of emblematic grape varieties, with the aim of its utilization by the wine industry and also by biotechnology companies, to highlight and strengthen the "terroir" character of Greek wines.

The modern molecular diagnostic/analysis tools that resulted from this work, can facilitate phytosanitary control of propagating material of the vine, modernize vine pest control and protection, while new pioneering diagnostic tools were developed. Lastly, standardization of native microflora by biotechnology companies as winemaking inoculums can assist in valorizing particular characteristics of the native varieties from which it was isolated.

CONCLUSION

The creation of the network contributed to the successful collaboration, development and transfer of know-how between academic and research institutions, developing and applying the most modern analytical methods using state-of-the-art equipment while activating scientists from various disciplines at the service of the wine sector. The main objective of the project is the dissemination of knowledge through the development of a digital data library open to all participants in the vitivinicultural sector and the future expansion of the network mainly through the entry of industry partners to increase implementation of research results in practice.

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K3. The consequences and responsibilities of HTS virus discovery

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The use of high-throughput sequencing (HTS) as a tool for disease etiology resolution and virus discovery has proven highly successful, identifying viruses and other agents in various agricultural crops and natural environments. In the last decade the rate of novel virus discovery increased dramatically almost exclusively due to the application of HTS. In grapevine this has led to the discovery of economically important viruses, by solving the disease etiology of diseases observed in commercial vineyards, most notably grapevine Pinot gris virus (GPGV) (Giampetruzzi et al. 2012) and grapevine red blotch virus (GRBV) (Krenz et al. 2012). The largest number of novel viruses has however been found in symptomatic vines as part of mixed infections with known disease-causing viruses or in asymptomatic vines resulting in an unclear view of disease potential. A clear example of this would be the vitiviruses. After the initial discovery of grapevine virus A, B and D which are associated with rugose diseases, no clear association with any disease symptom could be established for GVE and F by 2017 (Minafra et al. 2017). At this point the thought was that we are close to saturation with the grapevine vitiviruses, however this was only the start and many more have since been discovered and are recognised by the ICTV: GVG (Blouin et al. 2018a), GVH (Candresse et al. 2018), GVI (Blouin et al. 2018b), GVJ (Diaz-Lara et al. 2018), GVL (Debat et al. 2019). GVK was shown to be a variant of GVD (Maree et al. 2020). There are also three more species described that are yet to be officially recognized: GVM (Alabi et al. 2019), GVN (Read et al. 2022), and GVO (Read et al. 2022). The rate of new discoveries has increased in the past 5 years that leaves one to speculate if we will approach a plateau soon.

However, the increasing number of new species also prompts consideration of their biological impact on vines and grape quality, specifically their potential contribution to disease pathology. This raises the crucial question of whether these viruses should be subject to regulation. With limited experimentally proven biological information available, the approach to regulation becomes complex. Should these viruses be regulated conservatively until their impact is confirmed, or should they be considered latent with limited effects until proven otherwise?

The successful adoption of HTS for routine virus detection undoubtedly has the potential to detect known and potentially even more novel vitivirus species. The challenge lies in determining how to handle this information. To address these concerns effectively, it is imperative to engage in a thorough debate on the regulation of vitiviruses, guided by robust biological data, while simultaneously minimizing risks to the industry. The goal is to equip regulators with comprehensive information to construct a decision model for managing these viruses at a regulatory level without compromising the industry's safety.

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K4. development, validation, and regulatory adoption of revised diagnostic protocol for quarantine and certification of Vitis germplasm at foundation plant services in Davis, California, USA

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Foundation Plant Services (FPS), located in Davis, California, USA, is a source of elite propagation materials of *Vitis* scion and rootstock cultivars as part of the California Department of Food and Agriculture's (CDFA) Grapevine Registration and Certification (R&C) Program. The R&C Program targets the elimination of specific viruses that are spread through grafting or propagation. FPS also facilitates the introduction, quarantine, and release of imported grapes in the US under a Controlled Import Permit (P588) from the United States Department of Agriculture, Animal Plant Health Inspection Service, Plant Protection and Quarantine (USDA APHIS PPQ). Both CDFA and USDA APHIS PPQ regulations have required the use of conventional disease detection methods, including biological indexing, ELISA and RT-PCR.

There are drawbacks to these conventional disease detection methods. Biological indexing is time consuming, does not detect all pathogenic viruses, suffers from unreliable performance of plants, cannot specifically identify pathogenic viruses, and demands large amounts of space and labor. Conventional molecular tests like ELISA and RT-PCR are limiting because they require prior knowledge of the pathogen and are incapable of detecting variants. HTS provides an advantage because it gives a comprehensive picture of the entire microbial profile in a sample. To demonstrate the advantages of HTS over biological indexing, we first ran a side-by-side comparison (Al Rwahnih et al. 2015), which indicated that HTS was more sensitive, reproducible, and accurate than biological indexing. We next sought to validate a diagnostic protocol for replicable HTS analysis and qPCR confirmation. Through extensive in-house validation, we evaluated the performance of our TNA HTS assay to detect grapevine viruses/viroids, and concluded that testing two different plant tissues at two timepoints resulted in the highest (100%) sensitivity (Soltani et al. 2021).

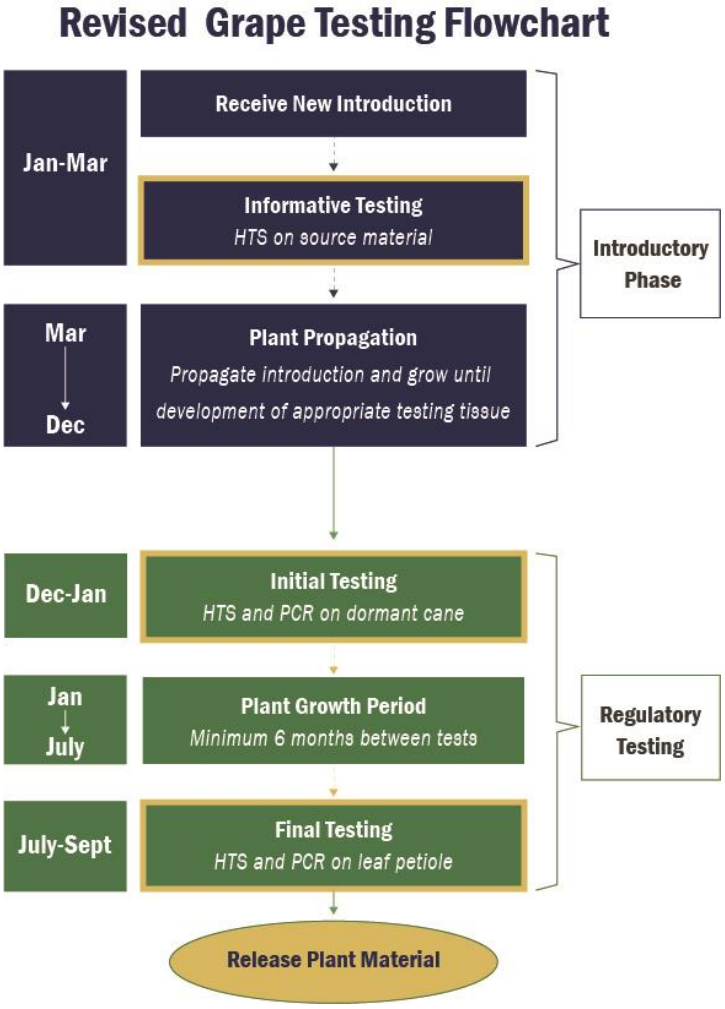

Figure 1 Flowchart showing grape HTS/PCR testing timeline at FPS under optimal conditions. Specific timelines may vary per plant growth and horticultural practices. The length of time from introduction to release of material is approximately 18-24 months.

The in-house validation was then expanded to inter-laboratory protocol validation in a joint project between FPS, USDA APHIS PPQ S&T, Center for Plant Health Science & Technology (CPHST) Lab and PPQ Field Operations, Plant Germplasm Quarantine Program (PGQP). CPHST is responsible for developing, adapting, validating, and implementing advanced molecular methods for the detection of high consequence plant pathogens in regulated plant material in the US. The goal of the project was to validate our HTS protocol (TruSeq Stranded Total RNA, Ribo-depleted) using the NextSeq Illumina. Randomized blind healthy and infected samples were distributed by CPHST to participating laboratories. All laboratories successfully detected the targeted viruses (Abrahamian et al., unpublished data).

FPS integrated the validation research knowledge into a protocol that employs the most reliable and efficient techniques using HTS and RT-qPCR/qPCR (Figure 1). The protocol requires informative testing on source material, followed by initial and final testing of two types of tissue, separated by at least six months. If negative for regulated pathogens in initial testing, the plant may be eligible for provisional release pending final testing. The USDA-APHIS-PPQ and CDFA have approved the use of this revised diagnostic testing protocol to replace biological indexing with a combination of HTS and PCR testing for release of plant material.

In addition to detecting known, regulated pathogens, HTS can detect previously unknown pathogens of unknown biological significance, and a systematic approach is required for their assessment (Fontdevila Pareta et al. 2023). Using HTS, FPS has discovered several novel viral agents in grapes. Prior to regulating any novel agent, FPS's approach is that biological significance is determined by performing graft transmission, spread and distribution studies, assessing symptoms for agronomic significance, and, if possible, applying Koch's postulates. Of the viral agents discovered at FPS, grapevine red blotch virus (Al Rwahnih et al., 2013) has been deemed biologically significant and added to the list of regulated viruses in certification programs in California and other US states.

FPS is working with other research programs to develop harmonized protocols, and work has been done to develop guidelines and considerations for implementation of HTS (Lebas et al., 2022, Massart et al., 2022). To our knowledge, the US is the first country to adopt HTS for release of plant material from quarantine. With the continued work of other scientists around the world to validate HTS protocols, soon this tool could be employed in pathogen-regulating programs worldwide.

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K5. Recent advances in grapevine phytoplasma research

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INTRODUCTION

Grapevine yellows are phytoplasma-associated diseases mainly characterized by leaf discoloration, shriveling of grapes and non-lignification of infected canes. These diseases have been reported in all grapevine growing areas and are associated with different phytoplasmas depending on the continent. The epidemiology of phytoplasmoses is dictated by the biology and ecology of the insect vector. If the vector carries out its life cycle on the infected crop, the outbreaks are characterized by the aggregation of diseased plants which are all the more abundant as the vector population is high. This is particularly the case for the grapevine Flavescence dorée (FD), which is transmitted in Europe by the ampelophagous leafhopper *Scaphoideus titanus.* Since the sequencing of phytoplasma genomes in the 2000s, genomics data provided genetic markers that have greatly facilitated the understanding of phytoplasma epidemiological cycles unveiling for instance the unexpected complexity of Bois noir phytoplasma ecology. The discovery of phytoplasma surface proteins implicated in the adaptation of phytoplasmas to different insect vectors allowed to distinguish epidemic and non-epidemic strains of FD phytoplasmas. The development of phenotyping protocols and comparative field trials is opening new perspectives in the identification of grapevine genetic determinants governing contrasted susceptibility of grapevine cultivars to FD.

NEW EPIDEMIOLOGICAL CYCLES INVESTIGATED

Multilocus sequencing strategies have facilitated to deciphering of different propagation pathways involving different plant reservoirs and insect vectors. Two "*Ca.* P. solani" genetic markers encoding surface proteins submitted to strong positive selections serve has powerful markers for the molecular epidemiology of "*Candidatus* Phytoplasma solani", the agent of grapevine Bois noir. For instance, new epidemiological cycles have recently been discovered that involves the planthopper *Hyalesthes obsoletus* propagating the phytoplasma from *Crepis faetida* (Kosovac et al., 2019). In addition to *Hyalesthes obsoletus* and *Reptalus panzeri, Dictyophara europaea* was also shown to experimentally transmit "*Ca.* P. solani" to grapevine (Curkovic et al., 2022). The use of genetic markers associated to large scale ecological surveys and vector transmission also allowed to decipher the origin of FD. Since the first outbreaks in Southwestern France, FD epidemics had been associated to the introduction of the North American leafhopper vector *Scaphoideus titanus*. It was recently demonstrated that FD phytoplasma originated from European alders and that epidemics in grapevine are restricted to some genetic variants pre-existing in alders. The compatibility of these variants to the introduced *S. titanus* insect vector resulted from the adaptation of phytoplasma variable membrane proteins Vmps to leafhoppers of the same subfamily living on alders. Therefore, alternative vectors and new reservoir plants have been identified (Malembic-Maher et al., 2020).

INTERACTION WITH INSECT VECTORS

The process of insect cells colonization requires adhesion and internalization steps. It was shown that the membrane protein VmpA of the FD phytoplasma acts as an adhesin able to interact with cells of its leafhopper vector *Euscelidius variegatus* and *S. titanus* (Arricau-Bouvery et al., 2018). VmpA binds insect glycoproteins via a lectin activity (Arricau-Bouvery et al., 2021) and the FD phytoplasma enters insect vector cells via a clathrin-mediated endocytosis (Arricau-Bouvery et al., 2023). Phytoplasma surface proteins like AMP or the IMP interacts with the insect vector actin microfilaments, a phenomenon certainly involved in the intracellular trafficking of phytoplasmas.

GENETIC BASIS OF GRAPEVINE SUSCEPTIBILITY TO PHYTOPLASMA

Upon surveys cultivar Cabernet Sauvignon was found highly susceptible to FD, with high proportion of symptomatic branches and high phytoplasma titers, in contrast to Merlot. Localized insect transmissions and grafting showed that phytoplasma circulate in the whole plant in Cabernet Sauvignon, while in Merlot they are restricted to the transmission point. Phenotyping using insect-mediated transmission allowed to classify 28 Vitis accessions into three distinct categories, according to the percentage of infected plants and their phytoplasma titers. Reduced symptoms in the *Vitis vinifera* cultivars was associated to low phytoplasma titers and low percentages of infected plants. The low susceptibility of Merlot was observed for one of its parents, i.e., Magdeleine Noire des Charentes (Eveillard et al., 2016). The same confinement of symptoms and FD phytoplasma was observed in cultivar Tocai Friulani and correlated with high activation of both jasmonate- and salicylate-mediated responses, together with a great accumulation of resveratrol (Casarin et al., 2023). In conclusion, cultivars carrying traits of reduced susceptibility to FD have been identified and might be used as future genitors and provide genetic markers to select cultivars with reduced susceptibility to FD.

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K6. Blazing a new trail to elucidate the molecular and cellular biology of GLRaV3

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is the chief agent associated with grapevine leafroll disease (GLRD), one of the most destructive diseases of grapevine. It is a member of the genus *Ampelovirus*, family *Closteroviridae* (Maree et al. 2013). GLRaV-3 has a (+)ssRNA genome of ~18.5 kb in size and contains 12-13 ORFs, depending on the phylogroup. Like other members of the *Closteroviridae*, the GLRaV-3 genome is divided into the replicative gene block (RGB) comprising ORF1a/1b and the quintuple gene block (QGB) containing ORFs 3-7. Genes of the RGB encode two polyproteins pp1a (ORF1a) and pp1ab (ORF1a/b) involved in genome replication and transcription. Based on homology to counterparts in the well-studied *Beet yellows virus* (Dolja 2003), it is likely that GLRaV-3 RQB genes encode proteins involved in virion assembly and cell-to-cell movement.

GLRaV-3 is unique in multiple respects. **1)** It has one of the largest RNA genomes among plant viruses. **2)** The polyprotein encoded by ORF1a contains both conserved [Pro (protease), MTR (methyltransferase), and HEL (helicase)] but also a novel alkylation B (AlkB) domain. **3)** Besides RGB and QGB, the GLRaV-3 genome encodes five ORFs toward its 3' end that are absent in other closteroviruses, which we designate as the unique gene block (UGB). The function of these ORFs is largely unknown except ORF10, which is a suppressor of RNA silencing. 4) Its genome contains unusually long non-coding regions: 5' UTR, and an intergenic region downstream of ORF1b. Lastly, the 5' UTR contains tandem repeats each of 65 nts in length, such repeats were detected in all phylogroups of GLRaV-3. The biogenesis and function of these sequence repeats are yet to be elucidated. Recent research suggests that a large part of the sequence in the 5' UTR is dispensable when assayed in *N. benthamiana* (Jarugula et al. 2018).

Like other (+)ssRNA viruses, GLRaV-3 must associate with a cellular membrane to form VRCs where genome replication and transcription occur. However, the source of the cellular membrane or viral proteins involved in VRC formation have not been elucidated. An early study observed vesiculating mitochondria grapevine phloem infected with GLRaV-3, suggesting that GLRaV-3 may target mitochondrial outer membrane to form VRCs (Faoro 1997). However, as the source material were from field-grown vines, it cannot be ascertained that these vesicular structures were caused by GLRaV-3 given that grapevines are often mix-infected with multiple viruses. Even if GLRaV-3 is responsible for these structures, it remains unknown which of the proteins or domains encoded by GLRaV-3 is responsible for mitochondrial targeting and membrane association.

Much work is needed to advance our understanding of the molecular and cell biology of GLRaV-3 as well as mechanisms that govern its replication and pathogenesis. In recent years, we have focused our research on several aspects of GLRaV-3 with the hope to establish GLRaV-3 as a model system. In this talk, I will highlight some of the recent results we have obtained.

Infectious clones, infectivity systems and fulfilment of Koch's postulates. Using a multi-stage strategy involving seven genome fragments, a full-length cDNA clone and EGFP-tagged variants were constructed by using pCB301.3 as the backbone plasmid (Fig. 1A). We have shown that both the wildtype and the GFP-tagged viral clones were infectious in *N. benthamiana* upon agro-infiltration as judged by fluorescence microscopy and Western blotting (data not shown). Furthermore, we have optimized a vacuum-based agro-infiltration system to launch infection from infectious viral clones in virus-free grapevine plantlets derived from tissue culture. We tested numerous factors and found that the age of plantlet, the OD of agrobacterial suspension, as well as humidity are important factors that

influence the survival of plantlets after infiltration. We further demonstrate that co-infiltration with a suppressor of RNA silencing is essential for infectivity and that dormancy treatment enhances symptom development. Infectivity was confirmed by RT-qPCR (Fig. 1B) and western blot for viral CP (Fig. 1C). Importantly, we have shown that the infectious clones induced foliar symptoms that are typical of GLRD (Fig. 1D). Therefore, we have fulfilled Koch's postulates and conclude that GLRaV-3 is the causal agent of the disease. We propose here that the name of the virus is changed from GLRaV-3 to GLRV-3.

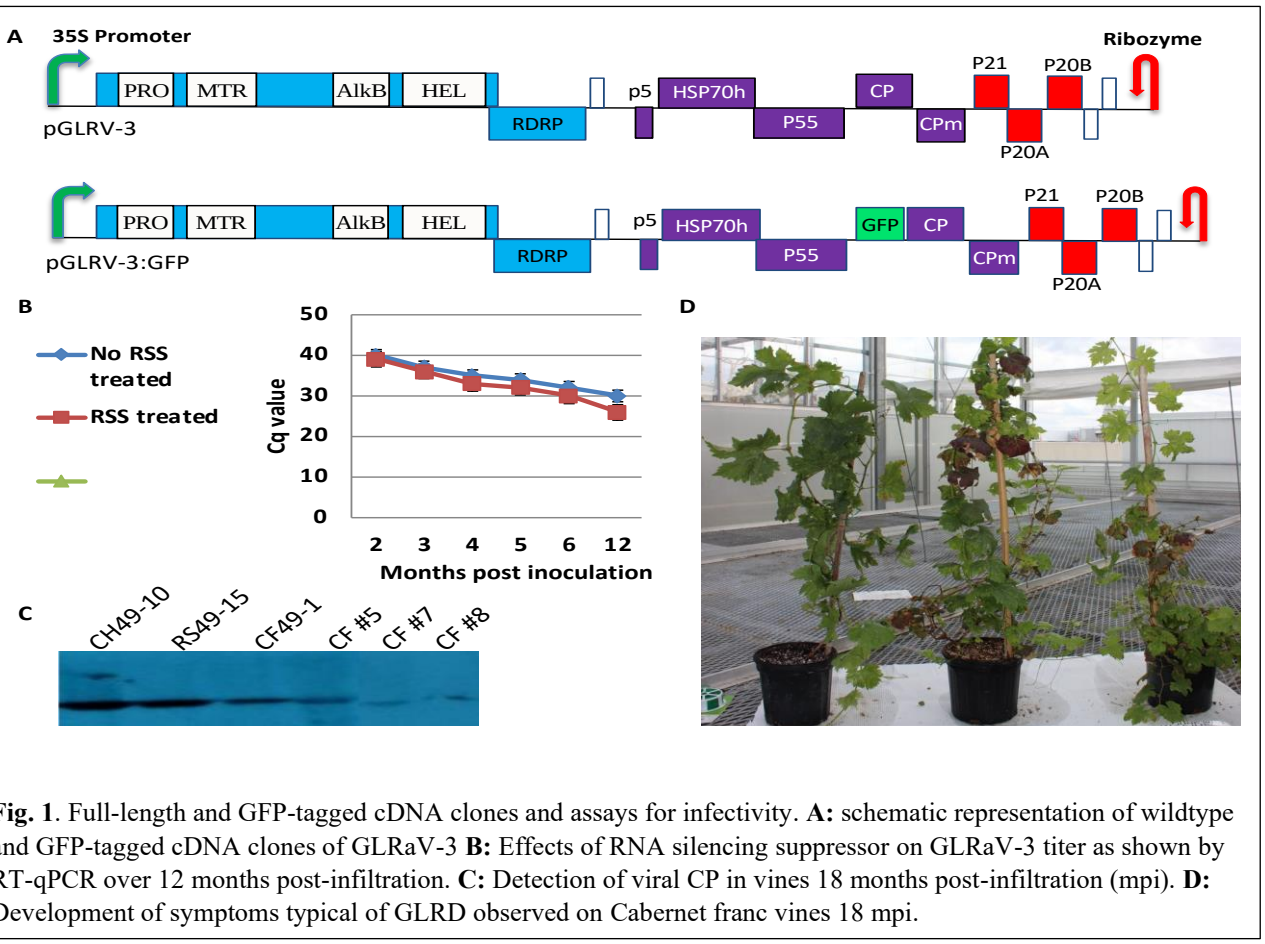

Fig. 1. Full-length and GFP-tagged cDNA clones and assays for infectivity. **A:** schematic representation of wildtype and GFP-tagged cDNA clones of GLRaV-3 **B:** Effects of RNA silencing suppressor on GLRaV-3 titer as shown by RT-qPCR over 12 months post-infiltration. **C:** Detection of viral CP in vines 18 months post-infiltration (mpi). **D:** Development of symptoms typical of GLRD observed on Cabernet franc vines 18 mpi.

Construction of a mini-replicon of GLRaV-3 and assays for biological activity. To find out the minimal GLRV-3 sequences required for genome replication and expression, a mini-replicon was constructed based on pCB301.3. pMiniGLRV-3:GFP contains only ORF1A and ORF1B flanked by the 5' and 3' UTR. To enable direct monitoring of its biological activities, EGFP sequence was introduced into the mini-replicon downstream of ORF1b. EGFP transcription is driven by the putative CP promoter sequence that was introduced upstream of EGFP (Fig. 2A). To assay for activity, pMiniGLRV-3:GFP was introduced, alone or together with agrobacteria containing a construct expressing HC-Pro, into *N. benthamiana* leaves via agro-infiltration. GFP expression was observed with fluorescence microscopy at 4, 6, and 8 days post infiltration (dpi). Green fluorescence was never observed in *N. benthamiana* leaves that were infiltrated with pMiniGLRV-3:GFP alone at any of the time points. For the plants co-infiltrated with the mini-replicon and HC-Pro construct, weak green fluorescence was first observed at 4 dpi in a small number of cells of the infiltrated leaves (Fig. 2B, left column), increased in intensity at 6 dpi and peaked at 8 dpi (Fig. 2B, middle and right columns). These results suggest that ORF1A/1B encoding the replicase proteins and the UTR at both ends of the viral genome constitute the minimal genetic elements that are required for genome replication and transcription. This finding is in line with those reported by Jarugula et al. (2018). It is worth noting that co-expression of an RNA silencing suppressor is necessary for the activity of the mini-replicon.

We will test if the inclusion of ORF10 would serve a similar function as ORF10 was shown to encode a suppressor of RNA silencing (Gouveia et al. 2012).

Subcellular localization, membrane association, and organelle targeting. A few RNA viruses establish VRCs in association with mitochondrial membrane (Miller & Alquist 2002; Rubino et al. 2001). We predict that the signal responsible for GLRaV-3 targeting to mitochondria is contained in the polyprotein pp1a. To this end, full-length ORF1a and several truncation mutants were cloned, transiently expressed in *N. benthamiana* leaves as EGFP fusions, followed by fluorescence microscopy to study their localization and putative organelle targeting. These constructs produced small, uniform, and punctate structures in the cell cytoplasm which resemble mitochondria (Fig. 3). On the other hand, the large, globular bodies are likely the nuclei due to the diffusion of the C-terminal cleavage product of the expressed fusion protein containing EGFP. We further show that the region of the replicase polyprotein containing the amphipathic helix and the two transmembrane domains (TMD) may be responsible for mitochondrial targeting and membrane association.

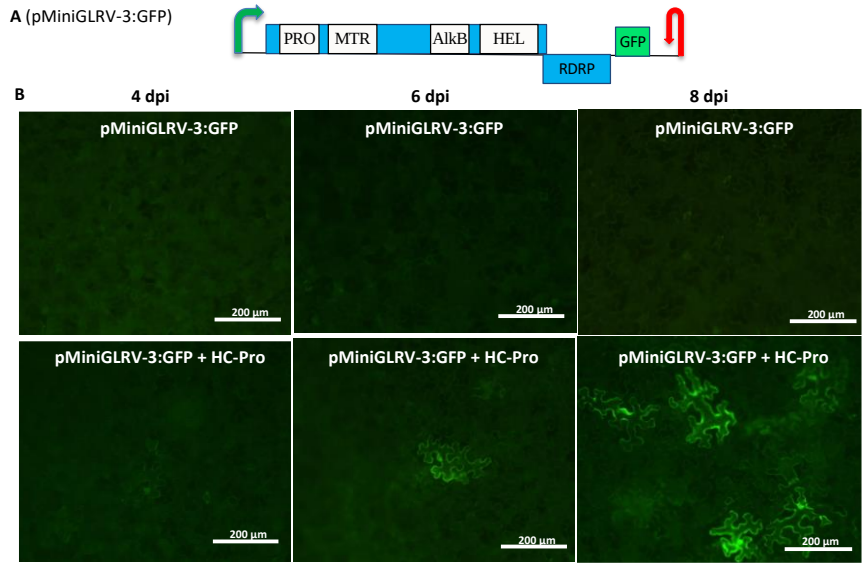

Fig. 2. GFP-tagged mini-replicon of GLRV-3 and assays of GFP expression. **A:** Mini-replicon of GLRV-3 tagged with GFP. **B:** Time course GFP expression in *N. benthamiana* leaves infiltrated with pMiniGLRV-3:GFP alone (upper panels) or co-infiltrated with a construct expressing HC-Pro (bottom panels) at 4, 6, and 8 dpi as indicated.

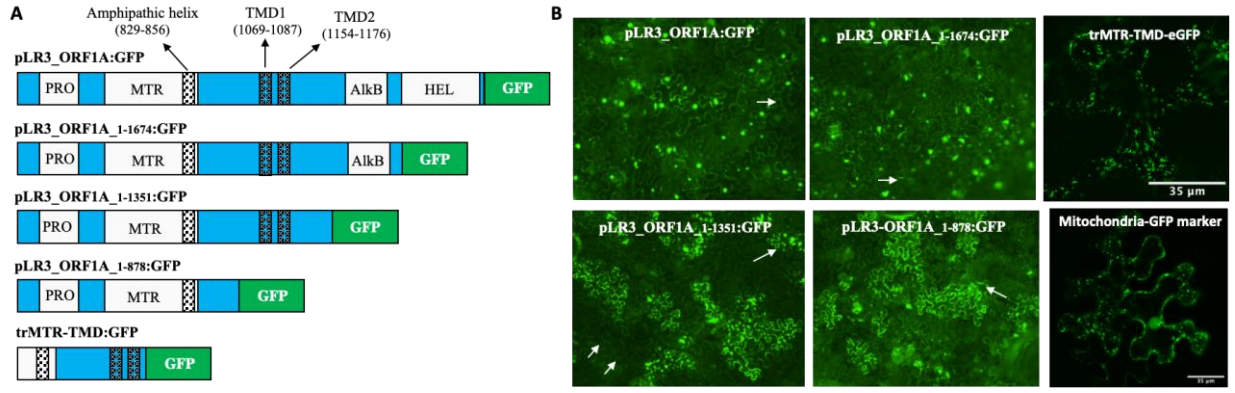

Fig. 3. Subcellular localization of full-length and truncated versions of pp1a encoded by GLRV-3 ORF1a. **A:** DNA constructs expressing full-length and C-terminal truncations of pp1a fused to EGFP. **B:** Representative images of cells expressing these gene constructs at 2 dpi. The two images in the right column were from CLSM.

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Oral Presentations Abstracts

SESSION 1

DISEASE ETIOLOGY - EPIDEMIOLOGY

Chair: Marc Fuchs and Robin MacDiarmid

O1. The prevalence of grapevine red blotch virus (GRBV) in a historical germplasm collection in Australia

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INTRODUCTION

The recent discovery of grapevine red blotch virus (GRBV; genus *Grablovirus*, family *Geminiviridae*) in several grape-producing regions of Australia (EPPO, 2023) has caused concern due to its potential to cause substantial economic losses within the industry. GRBV can delay fruit ripening and decrease yields overseas and may reduce the lifespan of vineyards (Poojari et al., 2013; Reynard et al., 2018; Sudarshana et al., 2015). In Australia, surveillance is being undertaken to understand the broader distribution of GRBV in different grape-growing regions to inform control measures. This study is investigating the prevalence and potential risk of the spread of GRBV in an Australian germplasm collection to contribute to efforts to mitigate the consequences of this virus on Australian grape production.

MATERIALS AND METHODS

The CSIRO germplasm collection comprising numerous rootstocks and dried, table and wine grape varieties, was originally established in the 1960s (Dry et al., 2022) and is located in the Sunraysia region, Victoria, Australia. During the 2022-23 growing season, it was tested for the presence of GRBV from samples comprising the basal portions of four shoots or canes (including leaves if present) were initially collected from each of the 111 grapevine varieties. The grapevines were sampled in different seasons including spring (November) in 2022 and summer (February) in 2023. As overseas research suggests that GRBV detection is most reliable in autumn (DeShields, 2023; Setiono et al., 2018), a more comprehensive sampling effort was performed in autumn (May) 2023. For this timepoint, a total of 2823 grapevines were sampled from the same germplasm collection, including the 111 that had previously been sampled. DNA was extracted individually from each of the 111 samples collected in spring and summer using a DNeasy Plant Minikit with a modified lysis buffer (Green et al., 1999). With the autumn samples, DNA was extracted using the same method from 312 samples each comprising pooled tissues of up to nine individual grapevines, representing up to three grapevine varieties or clones. If pooled samples tested positive for GRBV, DNA was re-extracted from individual varieties comprising of up to three grapevines and tested separately. GRBV detection was carried out using an in-house designed endpoint nested PCR assay, which uses the CP-Forward and CP-Reverse primers (Krenz et al., 2014) for the nested step, followed by analysis using gel electrophoresis. Amplicons of the expected size were Sanger sequenced bi-directionally to confirm detection.

RESULTS AND DISCUSSION

GRBV was detected in 12/111 grapevines sampled in spring, but only seven of the same positive grapevines sampled in summer. Analysis of the autumn sampling is currently underway. To date, four samples collected from the same 12 grapevines that were positive for GRBV in spring have also tested positive in autumn sampling. The GRBV-positive varieties include two clones of Perle de Csaba, one

clone of Kandahar and one clone of Opuzensia Rana. The seasonal surveillance contributes to improving GRBV diagnostics by identifying the most reliable time for GRBV detection in Australian environmental conditions. The broader surveillance done in autumn will determine the prevalence and spatial distribution of GRBV in the germplasm collection. This will inform the risk of spread in the region and support the implementation of control measures to contain or eradicate GRBV in Australia. It is intended to undertake phylogenetic analyses of GRBV sequences from positive grapevines to support tracing within Australia and overseas detections. The study greatly benefits the Australian grapevine industry by providing crucial information and tools to effectively manage and control GRBV, reducing its impact on grapevine health and productivity.

ACKNOWLEDGEMENTS

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O2. Red blotch disease spread dynamics are influenced by infected planting materials and *Spissistilus festinus*

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INTRODUCTION

Red blotch disease was first recognized as a serious threat to grape production in the United States in the mid-2000s (Cieniewicz et al., 2017b). This disease is caused by grapevine red blotch virus (GRBV, *Grablovirus, Geminiviridae*) (Yepes et al., 2018). GRBV isolates are grouped into two distinct phylogenetic clades, both of which are implicated in disease etiology (Yepes et al., 2018). While infected planting material has resulted in the widespread presence of GRBV across the United States (Cieniewicz et al., 2017b), secondary spread has been observed in Northern California (Cieniewicz et al., 2017a; Cieniewicz et al., 2019) and Southern Oregon (Dalton et al., 2019; KC et al., 2022). The three-cornered alfalfa hopper, *Spissistilus festinus,* has been shown to transmit GRBV in a circulative, non-propagative manner in the greenhouse (Flasco et al., 2021) and in the vineyard (Flasco et al., 2023). Little work has been done to understand GRBV spread dynamics in vineyards as it relates to both vector presence and infected planting material. In this work, we disentangle these two aspects of disease spread through yearly surveys to assess the spatiotemporal distribution of infected vines and GRBV clade occurrence in three vineyards in California, USA.

MATERIALS AND METHODS

Three neighboring vineyards in Napa County, California, USA with some vines showing red blotch disease symptoms and GRBV presence confirmed via PCR were selected for this study. A 2-ha *V. vinifera* 'Cabernet franc' vineyard planted in 2008 and a 1-ha *V. vinifera* 'Merlot' vineyard were chosen due to their proximity to a riparian area. The 'Merlot' block was planted in 2015 and provided the opportunity to monitor disease incidence from planting. A 1.5- ha *V. vinifera* 'Cabernet Sauvignon' vineyard was chosen for this study based on previous epidemiological observations (Cieniewicz et al., 2019). The block was planted in 2008 using vines of clones 4 (CS4) and 169 (CS169) on the southern and northern ends, respectively. Further interest in this vineyard was due to GRBV presence in almost all CS4 vines one year after planting (Cieniewicz et al., 2019) and its distance from a riparian area.

Every vine in each of the described blocks was visually surveyed for typical red blotch disease symptoms in October of 2015-2019, 2021, and 2022. Six leaves and petioles from newly symptomatic vines were collected at the base of the vine canopy in close proximity to the trunk to confirm GRBV presence via multiplex PCR (Flasco et al., 2021). A restriction digest assay of PCR amplicons corresponding to the replication associated gene was performed to assess the phylogenetic clade of the GRBV isolates (Flasco et al., 2023). Ordinary runs analyses were conducted to determine if the spatial distribution pattern of symptomatic vines was aggregated or random in a single dimension.

To assess the GRBV inoculum surrounding the 'Merlot' and 'Cabernet Sauvignon' vineyards, tissue was collected from free-living vines in the surrounding riparian area, as well as from vines from a another 'Merlot' block exhibiting foliar reddening. Samples were similarly collected and tested for GRBV and clade determination.

RESULTS AND DISCUSSION

Over nine years, GRBV incidence increased by 26% in the 'Cabernet franc' vineyard, while the initial aggregation near the riparian area saw a 72% increase. Previous work found that the infected vines in initial aggregation contained identical GRBV clade 2 isolates, implicating infected rootstock material as the initial infection source due to a 4-year delay in disease onset (Cieniewicz et al., 2017a). The presence of clade 1 isolates indicated *S. festinus* carrying GRBV clade 1 isolates visited the vineyard.

In the 'Merlot' block, it took three years post-planting for disease symptoms to be observed but disease incidence remained low at 1.6% in 2022. Diseased vines were aggregated in the first 24 rows in which 74% of diseased vines were found. These vines were primarily infected with clade 1 isolates. The presence of both GRBV clades in isolated, diseased vines in rows 24-90 implicated *S. festinus*-mediated spread. All surrounding free-living vines and the neighboring 'Merlot' block contained GRBV clade 2 isolates. A delayed onset of red blotch symptoms and the prevalence of a nondominant clade indicate the rootstock was likely infected at the time of planting.

The majority of CS4 vines in the 'Cabernet Sauvignon' vineyard were infected one year post planting, suggesting infected scion material as the initial infection source. A subset of CS4 vines indicated the majority of them (81%) contained GRBV clade 1 isolates, while 19% contained clade 2 isolates, supporting initial findings (Cieniewicz et al., 2019). Despite this large region of infected vines, disease incidence in 2022 in CS169 vines was only 1.4%. We hypothesize the low infection rate of CS169 vines to be due to a tenfold lower *S. festinus* presence compared to the 'Cabernet franc' adjacent to the riparian area (Cieniewicz et al., 2019). Both clades were present in newly symptomatic CS169 vines with clade 1 isolates remaining dominant.

Our findings stress the need to carefully select planting materials derived from virus-tested vine stocks that supply rootstock cuttings and scion budwood for the production of clean grafted vines

ACKNOWLEDGEMENTS

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O3. Study of the grapevine virome in three PDO regions of Greece

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INTRODUCTION

Grapevine (*Vitis* spp.) is a host of more than 80 viral species and their presence results in great economic impact as viruses can reduce the yield and quality of fruits and alter berries composition. Although the viral status of the Greek vineyard has been studied since the seventies, the cultivation practices, the introduction of non-certified propagation material, along with the climate change, have resulted in the introduction and spread of new viral species and isolates. In this study the current viral status was assessed in three vineyards in Protected Designation of Origin (PDO) regions of Southern Greece, namely Nemea, Mantineia and Santorini, each cultivated with a different variety (cvs Agiorgitiko, Moschofilero and Assyrtiko, respectively). Grapevine samples were initially analyzed by RT-qPCR assays for the presence of seven important viruses included in the National and European Legislation. RNA-Seq analysis was performed in selected vines in order to assess their complete virome. Finally, the phylogenetic diversity of the three most prevalent viruses was studied.

MATERIALS AND METHODS

In total, 177 grapevine samples from the three PDO regions were collected during autumn 2019 – winter 2020. Total RNA was extracted with a CTAB based protocol from leaf tissues and cambial scrapping cuttings. The samples were initially analyzed by RT-qPCR assays for the presence of arabis mosaic virus (ArMV), grapevine leafroll-associated virus 1 (GLRaV-1) and 3 (GLRaV-3), grapevine fanleaf virus (GFLV), grapevine fleck virus (GFkV), grapevine virus A (GVA) and B (GVB) (Bertolini et al., 2010; Osman et al., 2007, 2008; Osman & Rowhani, 2006). Seventeen samples originating from either individual or pooled vines were selected and further analyzed by RNA-Seq using standard procedures in the Greek Genome Center (Biomedical Research Foundation of the Academy of Athens, BRFAA). De novo assembly of the obtained reads was performed with rnaSPAdes and BLASTn algorithm was used for the annotation of the obtained contigs. Finally, sequences of GLRaV-1, GLRaV-3 and GVA obtained from the HTS analyses were used for the *in silico* verification of the abovementioned RT-qPCRs' detection efficacy and for the construction of phylogenetic trees with MEGA11.

RESULTS AND DISCUSSION

The initial results obtained from the RT-qPCR analyses showed that the lowest number of viruses was observed in the self-rooted isolated vineyard of Santorini Island. Overall, the most prevalent virus in all PDO regions was GVA (83.6%). GLRaV-1 was detected in high rates in Nemea and Mantineia regions (62.7%), but it was absent in Santorini. The third most abundant virus was GLRaV-3 (56.5%) which was present in all regions. Finally, ArMV was absent from all the vineyards under investigation. The obtained HTS results revealed that the highest number of viral hits was observed in Nemea where, except for the abovementioned viruses, GFLV, GFkV, grapevine Pinot gris virus (GPGV), grapevine rupestris stem pitting associated virus (GRSPaV), grapevine satellite virus (GV-sat), grapevine virus F (GVF), GLRaV-4, GLRaV-10, grapevine rupestris vein feathering virus (GRVFV), hop stunt viroid (HSVd) and grapevine yellow speckle viroid (GYSVd) were detected. In Mantineia, grapevine samples were found also infected with GFLV, GFkV, GVB, grapevine asteroid mosaic associated virus

(GAMaV), grapevine Syrah virus-1 (GSyV-1), GRVFV, GRSPaV, GV-sat, HSVd and GYSVd. Furthermore, a new recombinant GFLV x grapevine deformation virus (GDefV) was identified. In accordance with the RT-qPCR results, the lowest number of viral sequences was recorded in Santorini region, where GLRaV-4, -5, -10, and GRVFV were also present. Finally, a viral contig resembling to the recently identified apple tombus-like virus was detected.

Due to their high prevalence and genetic variability leading in some cases to detection discrepancies, GLRaV-1, -3 and GVA were phylogenetically analyzed. As demonstrated in Fig 1a, GLRaV-1 Greek isolates were organized in three branches, with most of Nemea's isolates clustering together, whereas most of GLRaV-3 isolates of both Mantineia and Nemea clustered together in a single branch (Fig 1b). Finally, multiple GVA full length sequences were identified in most HTS analyzed vines implying a mixed infection by multiple isolates. The phylogenetic analysis showed that the Greek isolates were present in almost all GVA phylogroups (Fig 1c). Interestingly, GVA isolates of Santorini's vineyard (Fig 1c, grey shape) grouped together and in distance from the other Greek isolates.

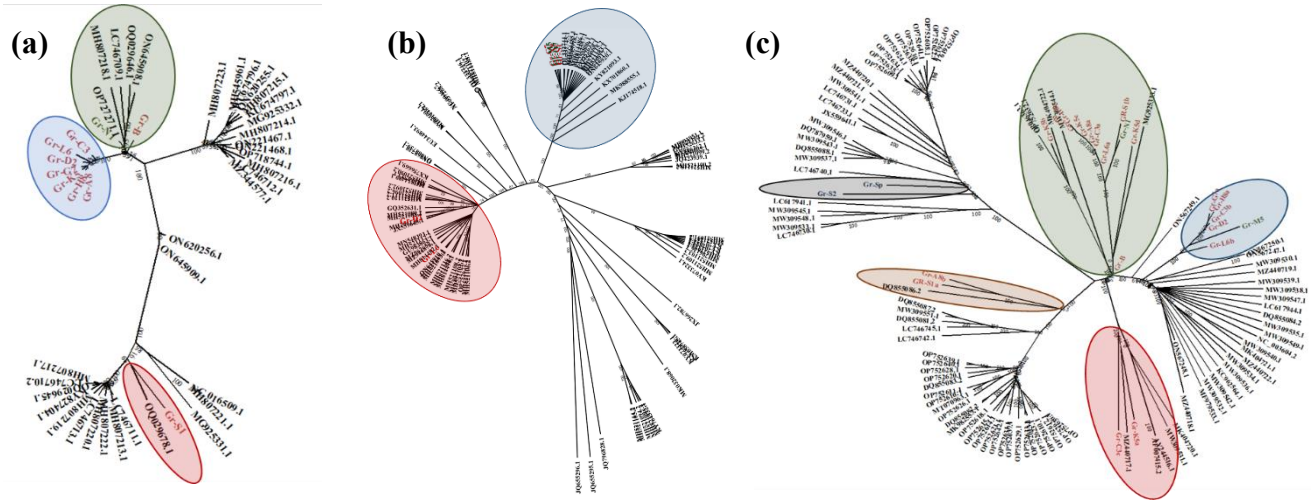

Fig 1: Maximum likelihood trees of (a) GLRaV-1 *CP*, (b) GLRaV-3 full genomes and (c) GVA full genomes. Each oval shape represents a phylogenetic clade where at least a Greek isolate is clustered. Isolates with red were identified in Nemea, with green in Mantineia and with blue in Santorini.

Overall, this study revealed the different grapevine virome complexity in each of the PDO regions studied, complexity related to their different age, size, isolation and composition, as well as the presence of a recombinant GFLV x GDefV and apple tombus-like virus not reported so far worldwide. Updating the current GLRaV-1 detection protocol was proven necessary. The need to upgrade the Greek grapevine cultivation with the establishment of certified clones is of outmost importance.

ACKNOWLEDGEMENTS

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O4. Badnaviruses infecting grapevine in Greece: A study of their epidemiological and molecular characteristics

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INTRODUCTION

Viruses belonging to the genus *Badnavirus* have been recently identified in grapevine with the help of high-throughput sequencing (HTS). These so-called pararetroviruses can infect and cause considerable losses in grapevine, in which three badnavirus species have been reported until recently, namely grapevine vein clearing virus (GVCV) (Zhang et al., 2011), grapevine Roditis leaf discoloration-associated virus (GRLDaV) (Maliogka et al., 2015) and grapevine badnavirus 1 (GBV-1) (Vončina et al., 2018). Apart from GRLDaV, which is known to occur in grapevines in Greece, GBV-1 was also detected in 2021 in a vine of the Greek variety Malagouzia in Thessaloniki. In this study, a large-scale survey was conducted to monitor the prevalence of GRLDaV and GBV-1 in major viticultural areas of the country. The genetic diversity of the two viruses was also studied through HTS. Finally, trials took place to assess the transmission potential of GRLDaV by the mealybug species *Planococous ficus* (Signoret).

MATERIALS AND METHODS

In 2016-2022, 970 grapevine samples, including leaves, petioles, or bark tissue, were randomly collected during different seasons of the year from 48 different vineyards in Greece and were analyzed for the presence of GRLDaV. Following the detection of GBV-1 in 2021, more than 200 samples were also tested to monitor its presence in Greek vineyards. Novel PCR assays targeting conserved regions located in ORF3 of the genomes of the two viruses were developed and applied herein. Five samples positive for GBV-1 and nine infected by GRLDaV were analysed through HTS, and the complete genome of both virus isolates was retrieved through Geneious prime 2023.1.2. Phylogenetic analysis was conducted in the MEGA X program.

For the transmission trials of GRLDaV, mealybugs of *P. ficus* were collected from a vineyard and kept in potato sprouts under lab conditions (24-26°C, 12h dark). A 30-year-old vine CV. Platani, infected with GRLDaV and GLRaV-3, was used as a virus source for the transmission tests. Twenty-one-rooted vine cuttings of pre-basic material of cv. Vidiano were established in pots and were used as recipient plants for virus transmission. Before the transmission trials, the vines were tested molecularly for the presence of GRLDaV and GRLaV-3. Fifteen mealybugs per plant were given a 48h AAP at the infected vine and then placed at a healthy vine for an IAP of 48h. The recipient plants were molecularly tested for the presence of the two viruses every month after the transmission from May to October 2022.

RESULTS AND DISCUSSION

Molecular detection revealed the presence of GBV-1 in 5,2% (12/231) of the samples and of GRLDaV in 17.1% (160/970). Overall, GRLDaV was detected in 32,4% (62/207) of the samples originating from self-rooted indigenous grapevine varieties, in 18,5% (96/526) of the indigenous grafted varieties, in 0.9% (2/202) of the foreign grafted varieties and 5.7% (2/35) of the rootstocks tested. GBV-1 was detected only in the indigenous grafted varieties tested at a rate of 9.6% (12/131). GRLDaV was identified in several regions of Greece with an exceptionally high incidence at the Cyclades islands and in the germplasm collections of the Agricultural University of Athens (AUA) and the National Grapevine Institute (NGI, Athens), that includes plant material of diverse geographic origins. On the

other hand, GBV-1 was detected mainly in commercial vineyards in four out of the nine geographic regions surveyed.

Whole-genome analysis of 5 GBV-1 isolates from Greece demonstrated 90-97% nucleotide (nt) identity among them and 85–88% to the Croatian virus sequence, while phylogenetic analysis placed all isolates from Greece in a divergent clade. The study of 9 complete genome sequences of GRLDaV showed 80-90% nt identity between them and the published ones. Phylogenetic analysis placed some isolates into genetically distinct clades while others clustered with previously published GRLDaV sequences (Figure 1). Interestingly, both GRLDaV and GBV-1 show common ancestry with viruses infecting fig (FBV-1 and GBV-1F, respectively), thus indicating a putative epidemiological connection between the two hosts.

Finally, in our study, GRLDaV was transmitted through mealybugs in three out of the 21 vines tested. The presence of the virus in the recipient plants was confirmed through PCR assays and Sanger sequencing of the amplicons. This significant finding advances our current knowledge of the factors affecting the epidemiology of GRLDaV.

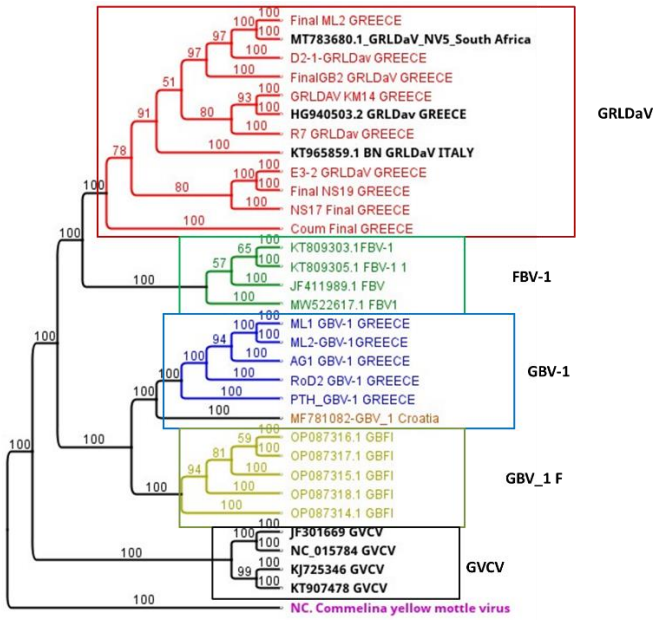

Figure 1: Maximum likelihood phylogenetic analysis of all the badnaviruses (Complete genome in nt) detected in grapevine (GRLDaV, GBV-1, GVCV). Fig badnavirus 1 and a divergent GBV-1 found in Fig (GBV-1F) are also included in the analysis. (NC001343.1 Commelina yellow mottle virus was set as an outgroup).

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O5. Preliminary survey of the main viral pathogens in the Swiss vineyards

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INTRODUCTION

Switzerland vineyards represent a total of 14,696 hectares, which is cultivated by over 2,500 winegrowers and produce 100 million liters of wine annually. The cultivation of indigenous grape varieties accounts for 36% of total cultivation and makes a significant contribution to the diversity and distinctive character of Swiss vineyards. Notable indigenous varieties include Chasselas, Gamaret, Garanoir, Arvine, Amigne and Cornalin. The wine-growing areas have been classified into six distinct wine-growing regions, each with its own unique identity, shaped by its topography, geology and climate.

As in neighboring countries, previous research in Swiss vineyards has identified grapevine leafroll and grapevine fanleaf as the two main viral diseases in terms of incidence and impact. However, no recent survey has been conducted to determine the current extent of these viral diseases and their effect on the quality and yield. This study aims to establish a protocol, employing the ELISA technique, to assess the prevalence of the most common grapevine viruses in the Valais canton, the largest wine region contributing to about 1/3rd of Switzerland's wine production. We present the results of this pilot study and compare them to data obtained over a decade ago from the Vaud region (Reynard et al., 2015).

MATERIALS AND METHODS

Ten vineyards of Pinot noir were selected from different part of the Valais canton. In addition, two adjacent blocks of Cornalin and one of Muscat were collected. During winter, 200 cane samples were collected randomly in each block and store at 4°C until the preparation. One exception is the Muscat block where only 120 cane samples could be collected. Samples were pooled in five (40 pools per block) and tested by ELISA using the standard protocol with the antibodies for the main grapevine viruses: grapevine leafroll-associated virus 1, virus 2, virus 3, and virus 4 (GLRaV-1, -2, -3, -4), Arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), grapevine Pinot gris virus (GPGV), and grapevine fleck virus (GFkV) with commercial antibodies (Bioreba AG) with a combined test for GLRaV-1 + GLRaV-3 and ArMV + GFLV.

The incidence of each block was estimated using the pooled prevalence for fixed pool size and perfect tests with a 95% interval confidence (Cowling et al., 1999).

RESULTS AND DISCUSSION

The results from the Valais region showed that the viruses responsible for the fanleaf were rare in the Pinot noir blocks tested. Over the 10 blocks, less than 1% of the plants were positive to GFLV (retest of positive for GFLV/ArMV did not detect the presence of ArMV). The relative rarity of this virus contrasted with a previous study in the canton of Vaud, where its incidence was measured at over 10%. Moreover, GPGV was also scarce in these blocks, with an overall incidence of around 2%, which also contrasts with the results observed in the neighboring countries (Bertazzon et al., 2016; Hily et al., 2020). Meanwhile, the leafroll viruses were very common, with an overall incidence amongst Pinot noir blocks of 15%. Leafroll infection was very high in three vineyards (#5, #9 and #10), with more than 37 pools (out of 40) testing positive, giving an estimated incidence of well over 40%. A retest of a subsample of the positive pools for GLRaV-1 + 3, showed that the majority were infected by GLRaV-1 (71%) and less than half by GLRaV-3 (41%) with also some double infection (12%). Very few GLRaV-2 were detected (1%) and only one pool of Pinot noir was positive for GLRaV-4 but 8 pools of the Muscat block (out of 24) suggesting an infection of the initial planting material. The overall

incidence of leafroll viruses in a survey carried out in the Vaud vineyards in 2012 was comparable, but it was mainly composed of GLRaV-2 (Reynard et al., 2015). Finally, the second most detected virus was GFkV with an overall incidence of the Pinot noir estimated at 14%.

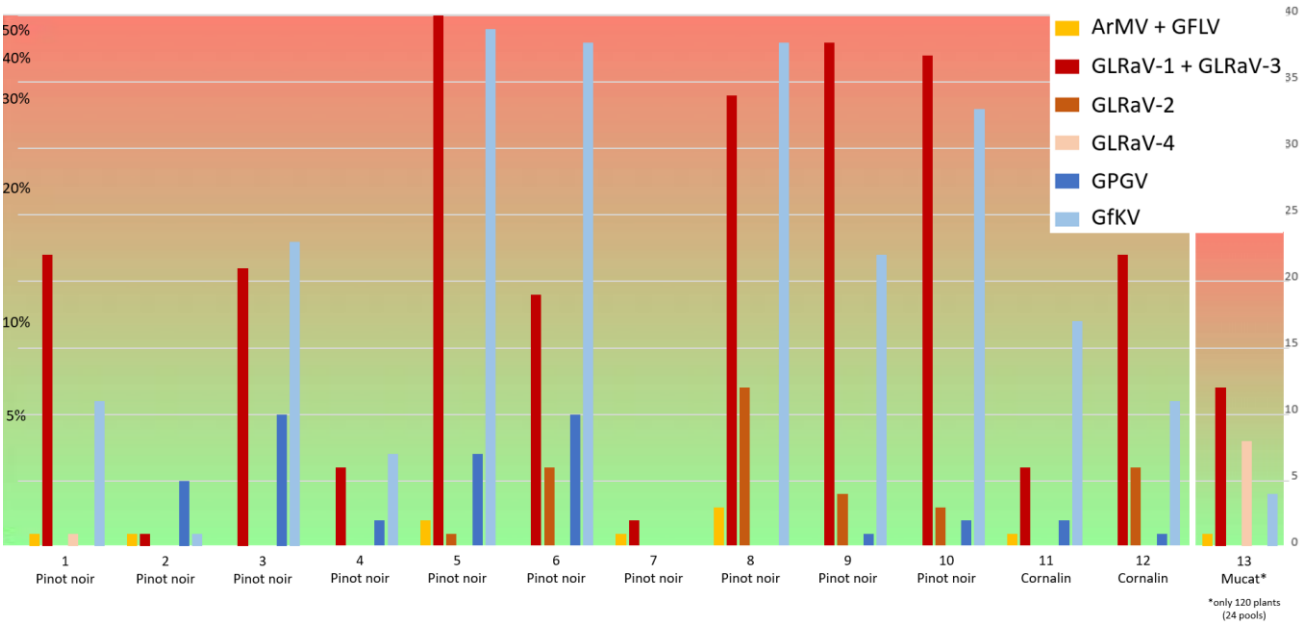

Figure 1: Virus incidence estimated from 13 vineyards located in the Valais canton. Number of pools (5 vines) tested positive by ELISA, for the fanleaf disease (ArMV or GFLV), leafroll disease (GLRaV-1/3, GLRaV-2, or GLRaV-4) GPGV or GFkV is indicated on the right ordinate. Estimated infection level marked on the left ordinate.

Although the limited size of this survey calls for caution, the results showed considerable variation between blocks and an overall high incidence of the leafroll disease. The impact of grapevine leafroll disease on yield and quality is well documented, particularly on Pinot noir including in Switzerland (Reynard et al., 2022). So far, grapevine leafroll viruses have not spread efficiently under Swiss conditions, unlike in neighboring countries (Le Maguet et al., 2012). This implies that the disease originates mainly in the propagation material and now, these plots represent a vast inoculum if an effective vector emerges in these regions.

In conclusion, this type of survey has the advantage to be cost-effective and gives a good indication of the major diseases present in a region. The winter collection assures a detection of the main pathogens and no bias toward symptomatic plants. This pilot survey will be repeated in a different Canton (Geneva) on a larger scale (50 vineyards) for the main viruses. Finally, these results will be communicated to the industry, in order to promote the usage of certified planting

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O6. The genetic origin of Syrah decline proved!

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INTRODUCTION

Because of its great potential to produce high quality wine with intense colour, Syrah was submitted to a rapid propagation all around the world. Since the 1990s, specific symptoms so-called "Syrah decline" have been described in different French vineyards. This disorder is characterized by swelling and grooving at the graft union sometimes associated to leaf-reddening during Autumn that can lead to death of vines (Renault-Spilmont *et al.*, 2007). This disorder was proved to be very present all over the world (Spilmont and Boursiquot, 2012). Numerous studies were conducted in France and an important result was obtained concerning the various "clone effect". Great differences of behaviors have been noticed between the certified clones which were then classified from "very low" to "very high" sensistiveness (Renault-Spilmont *et al.*, 2007). Into a variety, clones differ by their pathogenic and genetic status. No pathogens' involvement could be confirmed despite numerous studies (Al Rwahnih *et al.*, 2009; Beuve *et al.*, 2012; Goszczynski, 2010; Puckett *et al.*, 2018; Renault-Spilmont *et al.*, 2009; Xia *et al.*, 2022) which led us to test the genetic hypothesis.

MATERIALS AND METHODS

Obtention of genetic population

In 2007 and 2008, self-pollination was performed onto some clones previously characterized for their sensitivity towards Syrah decline. The progeny of two F1 populations were selected to study the potential trait segregation: the first was obtained from a "resistant" Syrah clone (clone 470) and the second is from a "very susceptible" Syrah clone (clone 383). All the seedlings were cultivated in a greenhouse to be grafted onto 110R. Ten grafted vines per individual were then planted in 2013 in an experimental Domain (Alaigne, Aude, South of France) to study the potential transmission and segregation of the decline trait.

Notations of symptoms of Syrah decline

Each plant was inspected for symptom development at the grafting union. The vine was considered symptomatic when characteristic swelling and cracking at the graft union were observed.
Apparition of cracks and swelling is known to be progressive during several years; precise observations were thus realized onto these populations from 2017 to 2020.

RESULTS AND DISCUSSION

Obtention of genetic population

Onto the 256 and 235 seedlings obtained for both the F1 populations, only a part of them were able to develop normally and produce canes susceptible to be grafted. At the end of the process, grafted vines produced from 93 and 45 individuals were respectively planted for the populations obtained from 470 and 383.

Notations of symptoms of Syrah decline

Many accessions appeared to be weak and significant mortality was observed in the first years.
Precise observations were done on all the well-developed vines. Typical symptoms of cracks and grooves were observed as early as the 4th year post plantation. These symptoms were **only observed**

in the population resulting from the self-pollination of the "sensitive" clone (383). Among the grafted vines well-developed, 32 % presented typical symptoms 5 years after planting (143/447 vines observed). By contrast no symptoms were observed in the population resulting from the self-fertilization of clone 470 ("resistant" clone) until the last year of notation (done in 2020).

These observations indicate that the sensitivity to Syrah decline is transmissible to the progeny which confirm the **genetic origin of this disorder**. The segregation of the sensitivity trait in the concerned population (self-pollination of "sensitive" clone) corresponds statistically (chi-Square test) to a segregation of 3/4 "sensitive" for 1/4 "non-sensitive" and leads us to propose the hypothesis of a major dominant gene at the origin of the development of specific Syrah decline symptoms.

ACKNOWLEDGEMENTS

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O7. Occurrence of *Grapevine fanleaf virus* in Turkish autochthonous grapevine varieties

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INTRODUCTION

Turkiye is located in the subtropic climatic region in the World, so inables it to produce many horticultural products such as different of fruits and grapevine varieties. Northeastern part of Anatolian peninsula located between Black Sea and Caspian Sea region is the gene source and culture area of the most important varieties of grapevine. Turkiye is one of the nations native to grapevine in the World, therefore our nation is familiar with grapevine culture for more than 6000 years and has very rich potential of both wild (*V. vinifera ssp. sylvestris*) and culture grapevine (*V. vinifera ssp. sativa)* varieties. Up today, 1437 new grapevine genotype has been determined and conserved. Turkiye has 540.000 ha of grapevine cultivation area and comes after Spain, France and Italy and is at the 4th level but in case of production is at the 6h level (TUIK, 2019).

Grapevine production has a great economic importance and consumed as fresh fruit, raisin, juice and also boiled juice (pekmez) and also vine. Dried raisins are exported.

The grapevine production is affected by several plant diseases including viruses and phytoplasma associated diseases. Recently 70 virus species has been determined to infect grapevine, among them *Grapevine fan leaf virus* (GFLV) is most destructive and causes great yield loss. The virus belongs to Picornavirales order, Secoviridae family and *Nepovirus* genus and has 30 nm circular particules and distributed vegetatively and also by nematods. It causes great leaf deformation and yield loss.

MATERIALS AND METHODS

Presence of GFLV has been investigated in Tokat (5), Corum (10) and Amasya (2) provinces which have very rich potential of autochthonous grapevine varieties. Surveys were conducted in 2017 in May and June and also for to detect the effects of pathogen in yield, in September.

DAS-ELISA and RT-PCR has been applied for to detect the virus presence. Total nucleic acid isolation has been performed by the method of Li et al. (2008) and PCR amplification by the primers of MacKenzie et al.(1997). All of the amplificatio products are subjected to sequencing and phylogenetic analysis.

RESULTS AND DISCUSSION

During the surveys performed in 2017, 418 symptomatic and 18 asymptomatic plant samples were collected from 3 provinces. Leaf distortion, chlorotic vein banding, thickness of the leaves, especially leaf lamina and petioles were detected. Widely grown local varieties were Narince, Trakya İlkeren, Çekirdeksiz beyaz, Kara, Yalova incisi, Erzincan cibini, Hasandede, Amasya beyazı, Delice, Kalecik karası.

DAS-ELISA results of the samples collected in May-June is as 260 samples were tested and 38 positives were obtained, in the surveys conducted in September 158 samples were collected and tested, among them, 46 positives were obtained, therefore total number of the infected samples were detected as 84 positives. GFLV infection rate was highest in Tokat Province and out of 161 samples 83 sample were detected as positive, in Corum 220 out of 29 and in Amasya only 11 positives were detected out of 37 samples.

7 GFLV isolate (5 from Tokat and 2 from Corum has been sequenced, replaced in NCBI genbank and phylogenetic analysis were performed according to Mega7 programme (Kumar, et al. 2016) using Clustal W parameters.

Presence of GFLV in local varieties in Northern Black Sea Region on Anatolian Peninsula was investigated by DAS-ELISA and RT-PCR. The pathogen was widely spread in Tokat province especially on Narince cultivar which is well known local grapevine variety, produced especially for the leaves rather than the berries. The virus. was distributed vegetatively and nematod vectors were not present in Anatolian peninsula.

In the phylogenetic analysis, Turkish isolates were settled in the same cluster with the Iranian isolates.

ACKNOWLEDGEMENT

The authors are gratefull to Ankara University-BAP coordination for funding this research with project number, 17L0447010

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SESSION 2

DIAGNOSTICS

Keynote speaker: Hans J. Maree

Chair: Hans Maree and Eva Varallyay

O8. Droplet digital reverse transcription-PCR for the detection of grapevine pinot gris virus infecting grapevines

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INTRODUCTION

Grapevine Pinot gris virus (GPGV), a member of the trichovirus (Betaflexiviridae) genus, was first identified in grapevines in Trentino, Italy (Malossini et al., 2015). GPGV causes leaf chlorotic mottling, puckering and deformation, stunted growth, low-quality fruit, abnormal branching, reduced yield, and vein clearing in grapevines (Saldarelli et al., 2015). Due to the genetic variability of GPGV isolates (Vu et al., 2023), precise detection of GPGV infection is symptomatic and asymptomatic vines are crucial to provide virus-free propagation material. Detection of viruses from plants with mixed infections, low-titers or phloem-limited viruses could be challenging with end-point PCR and RT-PCR methods. Droplet digital PCR (ddPCR) is a recently developed technology for absolute nucleic acid quantification (Hindson et al. 2011). Here, we tested the effectiveness of a reverse transcription ddPCR (RT-ddPCR) approach for the detection of GPGV from infected grapevine samples. One of the advantages of ddPCR is that the method can measure the absolute copy number of nucleic acid targets without the need for external standards. This study evaluated the applicability of ddPCR as a quantitative detection tool for the detection of GPGV infecting grapevines.

MATERIALS AND METHODS

GPGV infected grapevines maintained in the phytotron facility at Brock University were used as the source of viral nucleic acids for standardization of ddPCR method. Healthy grapevine samples served as the negative control in all the tests. Petiole tissue from healthy and GPGV infected was used for total RNA isolation using RNeasy Plant Mini Kit (Qiagen, Germany). Total RNA was eluted in 50uL nuclease-free water was used for all the downstream applications. GPGV forward primer GPGVMPFWD:5′GGAGCAATTGAAGCTATTAATGG3′ and the reverse primer GPGVMPREV: 5′GCCTGAAGGAGTGCCTGAGARGC3′ were designed based on multiple sequence alignment (MSA) of GPGV sequences from GenBank. The primers amplify a ~170bp region of the GPGV movement protein (MP) gene. A range of temperatures (54˚C, 56˚C, 58˚C and 60˚C) was tested in a two-step gradient RT-PCR to determine the optimum annealing conditions for the PCR amplification. The ~170bp MP gene was cloned into T/A cloning vector and transformed into DH5-α. Plasmid DNA was isolated and a series of serially diluted plasmid samples was used as the template for ddPCR to determine the detection limit and quantification. All the ddPCR assays were performed in a Bio-Rad QX200 AutoDG system droplet generator using EvaGreen assay method (Bio-Rad, Hercules, CA, USA). Each PCR reaction contained 10uL QX200 Evagreen supermix (Bio-Rad), 1uL each of forward and reverse primers (200 nM), and 2uL template in a final volume of 20uL. Healthy or water sample was negative controls. The components were mixed and briefly centrifuged for droplet generation. The PCR plates were heat sealed (PX1 PCR plate sealer, Bio-Rad) and PCR was performed in a C1000 touch thermal cycler (Bio-Rad) using the following PCR profile: 94°C for 5 min, 49 cycles of 94°C for 45s, optimal annealing temperature of 59°C for 2min, 4°C for 5 min, 90°C for 5 min, and hold at 4°C; ramp rate was 1°C/s. PCR plates were transferred into a QX200 droplet reader (Bio-Rad) and reads were analyzed using QuantaSoft software (Bio-Rad).

RESULTS AND DISCUSSION

The linear regression curve was made by plotting log10-transformed copy number concentrations measured by ddPCR against log10-transformed predicted values of serially diluted plasmid DNA. The

sensitivity (detection limit) of ddPCR in detecting the GPGV MP plasmid DNA was 10^{-7} (10ag/uL) concentrations. At this concentration, the copy number was determined to be 1.7 per reaction. The ideal concentration (optimum dilution) for ddPCR detection was between 10^{-4} and 10^{-6} respectively. Specificity of ddPCR indicated no cross-reactivity in detecting GPGV from either grapevine red blotch virus (GRBV) or grapevine leafroll associated virus-3 (GLRaV-3) infected samples. cDNA or PCR-amplified products were included to determine the specificity. The utility of the proposed RT-ddPCR assay was assessed for the detection of GPGV. The ddPCR method was more sensitive in detecting GPGV compared to the end-point PCR method.

ACKNOWLEDGEMENTS

This research was supported by Genome British Columbia and Genome Canada project [code 189GRP] entitled "CLEan plAnt extractioN SEquencing Diagnostics (CLEANSED) for Clean Grapevines in Canada" and VPR Post-doc internal grant-2022-23, Brock University.

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O9. dsRNA: a universal genetic template for cost-effective virus and viroid detection and characterization

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INTRODUCTION

Viral diseases represent a major threat to food production, worldwide. Treating virus-infected plants is impractical, unlike bacterial or fungal diseases. Therefore, reducing the impacts of viruses on crop production relies on our capacity to monitor and anticipate, outbreaks. Early detection is a critical step in defining upstream mitigation strategies to facilitate viral disease management. Because viruses lack shared conserved regions for identification at the species level, virion-associated nucleic acid (VANA) and metagenomic sequencing are commonly used instead to harness the virome. However, VANA sequencing systematically favors DNA and enveloped RNA viruses, while metagenomic sequencing is influenced by large-genome organisms and their prevalence. We have improved double-stranded RNA (dsRNA) extraction methods by optimizing the existing cellulose-based method and developing new methods employing dsRNA-binding proteins.

MATERIALS AND METHODS

Plant samples: The grapevine plants used in this study were all grown in three different vineyards located in Quebec, Canada. Mature leaf materials from grapevine plants of different varieties were collected from 2019 to 2022. Five leaves per plant (three from the bottom, one from the middle and one from the top of the canopy) were harvested and immediately frozen in liquid nitrogen and stored at -80ºC for further analysis. All plants were visually inspected for virus-like symptoms, mainly those caused by grapevine leafroll-associated viruses and GPGV.

Soil samples: Soil sample were collected in two different soil types (mineral and organic) at 0-15 cm depths. The samples were properly stored at −20 °C until proceeding with nucleic acids extraction. Each of the eight samples was homogenized and divided in the three different groups corresponding to the three extraction methods, VANA, dsRNA extraction using cellulose-column chromatography (CCC-dsRNA extraction) and dsRNA extraction method using RNeasy Power Soil ki (RPT-dsRNA).

Botrytis mycelium samples: Isolates of *B. cinerea were* collected in different crops in Quebec and fitness and pathogenicity criteria, including number of sclerotia, conidiation, radial growth, and morphotype, were evaluated. Mycelia were collected from these isolates grown in PDA and stored at −20 °C until proceeding with nucleic acids extraction.

Viral nucleic acid extraction and sequencing: For each of these aforementioned samples, different nucleic acids extractions methods were used to compare their efficiency of capturing viral diversity and characterizing viral genomes. These methods are virion-associated nucleic acid (VANA), total RNA extraction and double-stranded RNA extraction with three different methods, a CCC-RNA, a RPT-dsRNA and a double-stranded RNA binding protein-based. All these methods are described in different manuscripts (Poursalavati et al. 2023, Fall et al. 2023, Javaran et al. 2023, Clément et al. 2021, Fall et al. 2020 and Baiersdörfer et al. 2019). These methods were used to sequence dsRNA using the Illumina MiSeq and Nanopore MinION platforms.

RESULTS AND DISCUSSION

The results demonstrate that dsRNA sequencing is a powerful universal tool for the detection and genomic characterization of viruses regardless of the genome type, size or the genomic heterogeneity.

In plant leaves, dsRNA sequencing was also able to detect low-abundance viruses that total RNA sequencing failed to detect. In addition, total RNA sequencing resulted in a false-positive viroid identification due to the misannotation of a host-driven read (Javaran et al. 2023). In soil, the results indicated that the dsRNA method was more effective than the VANA approach in capturing the soil viral diversity. It is a cost-effective method for massive screening of soil viruses and a proven tool to expand RNA viruses diversity (Poursalavati et al. 2023). In fungal mycelium, mycoviruses were identified in 42 of 45 isolates. Most mycoviruses had positive-strand RNA and dsRNA genomes, but viruses with negative-strand RNA and single-stranded DNA (ssDNA) genomes were also identified. The detected ssDNA mycoviruses in the *Genomoviridae* family may have an extracellular activitiy and be used as biocontrol agent against Botrytis. Results from these different sample types show that dsRNA sequencing and the proposed data analysis workflows are suitable for consistent detection and genomic characterization of viruses and viroids, particularly in grapevines where mixed viral infections are common. Furthermore, the quality and quantity of reads obtained using dsRNA sequencing are ideal for viral genomes assembly and characterization. Compared to other viromics methods, dsRNA sequencing can give us an indication about virus that actively replicating, because dsRNA is mainly produced as an intermediate during the viral replication process, except for viruses that has dsRNA as primary genome. In the One health context, which requires the monitoring of known and unknown viruses throughout the virosphere (plants, animals, insects and soil), dsRNA sequencing provides a unique opportunity to increase our capacity for monitoring and anticipating, viral outbreaks.

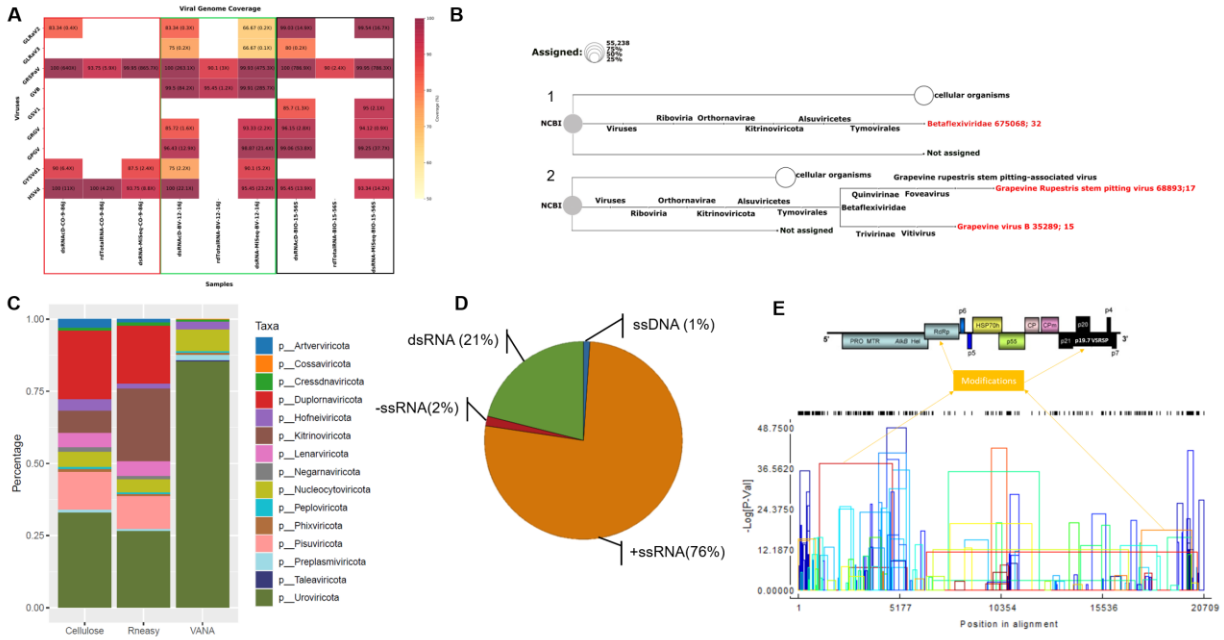

Figure. A heatmap illustrating the percentage of viral genome coverage and depth in grapevine leaves (**A**). Assignment of viral reads to taxa using different thresholds in the total RNA extraction from grapevine leaf samples (**B**); taxonomic binning was done using the default parameters in the meganization option of the MEGAN software (B-1); two thresholds Min Support Percent" and "Min Support," were turned off (=0) (B-2). Comparison of taxonomic composition of soil viral communities using three different methods (**C**). Percentage of mycoviruses belonging to different families identified in *Botrytis cinerea* isolates (**D**). Single nucleotide polymorphisms (SNPs) events in grapevine leafroll associated virus 3 (**E**).

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O10. Amplification-free CRISPR-Cas13a assay for the detection of Grapevine leafroll associated virus-3

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INTRODUCTION

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology is revolutionizing molecular diagnostics. One notable feature is collateral cleavage, where CRISPR-RNA, once bound to the target region, triggers the enzyme to cleave surrounding nucleic acids indiscriminately (1). This ability is specific to CRISPR Cas12, Cas13, and Cas14. Cas12 is most efficient at recognizing double-stranded DNA (dsDNA), while Cas14 works best with single-stranded DNA (ssDNA). Cas13a is an RNA-targeting, RNA-guided nuclease which uniquely binds to single-stranded RNA without damaging the surrounding genome (2). When developing a CRISPR-RNA system for virus diagnosis, it is crucial to consider the nature of the foreign genetic material and select the appropriate complex. Previous studies have utilized Cas12a and plasmonic gold nanoparticles as biosensors to create molecular diagnostic tests for grapevine red blotch virus (3). In this study, we have developed an amplification-free diagnostic assay for the detection of grapevine leafroll associated virus-3 from grapevine tissue using GenCRISPR™ LbuCas13a Nuclease.

MATERIALS AND METHODS

Sample Preparation: Approximately 250 mg GLRaV-3 infected grapevine petiole tissue was used for the total RNA extraction using Qiagen RNeasy Plant Mini Kit and eluted in 30µL RNase-free water. RNA extracted from healthy grapevine tissue sourced from meristem-tip tissue culture-raised grapevine was used as a negative control in all the tests. Different dilutions of these RNA samples were prepared (ranging from 10ng to 250ng). Virus infection in the samples was confirmed by RT-PCR followed by Qiaxel Advanced capillary electrophoresis.

CRISPR-RNA (crRNA) selection and synthesis: This method is for the detection of RNA viruses which targets the viral RNA without the need for the amplification of viral genes followed by detection using Cas12 complex. Instead, in this study, the Cas13a-based complex (crRNA and Cas13a enzyme) is used to carry out a collateral cleavage of the target RNA with the involvement of. Two crRNAs (acts as the gRNA) were chosen for the study. 1) that could target the partial coat protein (CP) gene and 2) target the partial HSP-70h like protein gene respectively. All the crRNAs were synthesized by Genscript, USA. The crRNA sequences that target the GLRaV-3 genes are (1) crLbu_LR3CP_1:GACCACCCCAAAAAUGAAGGGGACUAAAACUAAUGCAAUCAUAUACC CACACC targets the CP gene while (2) lcrLbu_LR3HSP70_1:GACCACCCCAAAAAUGAAGGGGACUAAAACACCAAGACCUUUUA GCGCCUCAA targets the HSP70-like protein gene. The polyU reporter sequence is 5' 6-FAM-UUUUUU-BHQ-1 3' respectively.

CRISPR-Cas13a reaction setup: Separate crRNA-Cas13a complex generation (Mix-A) was carried out with IcrLbu_LR3CP and lcrLbu_LR3HSP70 in a PCR tube with a 10uL reaction mix. Each reaction consisted of 1uL 10X Reaction buffer; 2uL (100Nm) crRNA; 3uL of Cas13a (50ng/uL); and made up to 10uL with nuclease-free water. This reaction mix was incubated at 37˚C for 10min in a thermal cycler. This reaction mixture was combined with a 40uL Mix-B consisting of 4uL of 10x assay buffer; 4uL of RNA template (100ng); 5uL of polyU reporter dye (250Nm) and 27uL of nuclease-free water. 10uL of Mix-A was added to Mix-B and the total reaction volume of 50uL was added to Corning 96 well flatbottom dark plate and the reaction was measured at ABS-490nm to EM-520 range for 90 min using the SpectraMax® M2 Systems (Molecular Devices, USA) multi detection microplate reader. This instrument is equipped with dual monochromators, dual-mode cuvette ports, and top- and bottom-

reading capability (top-reading only on the M2). Detection modalities include absorbance (UV-Vis Abs) and fluorescence intensity (FI). Endpoint, kinetic, spectrum, and area-well scanning read types and the PathCheck® Sensor allow homogeneous and heterogeneous microplate assays to be performed in one flexible system.

RESULTS AND DISCUSSION

Preliminary experimental data suggests that IcrLbu_LR3CP and lcrLbu_LR3HSP70 and Cas13a complex could be employed for the detection of GLRaV-3 in infected grapevines. The results were determined based on the data generated with the SpectraMax® M2 Systems microplate reader indicated the onset of the collateral activity of Cas13a starting 30min after the addition of Mix-A with Mix-B. The RFU values for the GLRaV-3 infected samples were recorded at least three-fold than that recorded for the healthy grapevine samples (Fig.1). The adoption of the CRISPR-Cas13a system, which avoids the need for viral genome amplification and scales viral load in the plant, promises to be an effective technique for detecting RNA viruses from plant tissues. The assay's sensitivity, robustness, and cross-reactivity will be presented in comparison to the end-point PCR and high throughput sequencing assays.

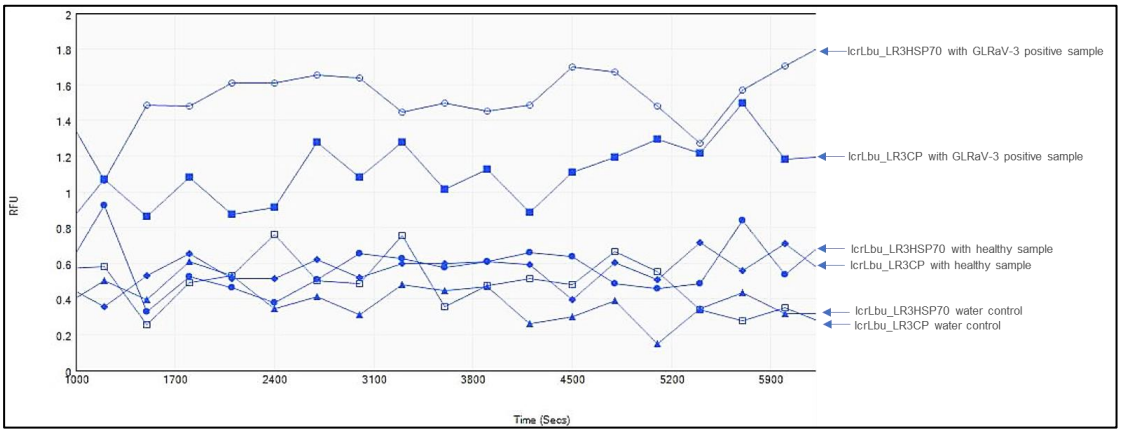

Fig 1. CRISPR CAS13a-based amplification-free detection of Grapevine leafroll associated virus 3 (GLRaV-3) using IcrLbu_LR3CP and lcrLbu_LR3HSP70. All the reactions were carried out for a period of 90min and the RFU values were recorded.

ACKNOWLEDGMENTS

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O11. Datamining, a powerful tool to unravel the worldwide dispersal and evolutionary history of grapevine viruses

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INTRODUCTION

With the dawn of high throughput sequencing (HTS), the deposit and accumulation of genetic information in digital form within dedicated databases is considerable and ever growing. Tera-bases of *in silico* data are being produced daily, for targeted clinical applications as well as for research purposes. However, only a small portion of the data is being used and analyzed, *i.e.* the part that was dedicated to answering the question for which they were produced. Still, a lot of information is yet to be evaluated and uncovered. Datamining, *i.e.,* the process of collecting, searching, extracting and discovering usable information within such large amount of data, is therefore becoming a very important and powerful tool to identify possible new pathogens, new viruses or new variants of known viruses. This was the case for example for the now well-known and infamous *Coronaviridae* family (Edgar *et al.*, 2022). These methods are not limited to the 'animal' Kingdom and can also be used for other hosts (*i.e. Vitis sp*). Our pilot project was dedicated to gain insight on a newly described *Trichovirus*, *Grapevine Pinot gris virus* (GPGV) that infects grapevine (Giampetruzzi *et al.*, 2012). The virus has now been detected in most, if not all grape-growing countries where it has been sought. Out of this 'proof of concepts' study, some advantages and pitfalls of datamining will be discussed.

MATERIALS AND METHODS

Over five hundred RNAseq datasets were used in this study, with around 120 being produced by our laboratory, dedicated to work on *grapevine fanleaf virus* (GFLV). The rest was retrieved from NCBI and were generated by many different research groups around the world. All analyses were performed using CLC Genomics Workbench 11.0 software (Aarhus, Denmark). First, reads that mapped to *Vitis vinifera* genome (http://www.plantgdb.org/XGDB/phplib/download.php?GDB=Vv), and those corresponding to grapevine transcriptome were removed. The remaining reads were then mapped on viral references for GPGV (GPGV-SK30 [KF134123], GPGV-Mer [KM491305]). using relaxed mapping stringency (0.5/0.7 corresponding to read length/similarity parameters) considering genome diversity within the virus species. Genome assembly was performed as previously described (Hily *et al.*, 2018, 2020, 2021a and 2021b).

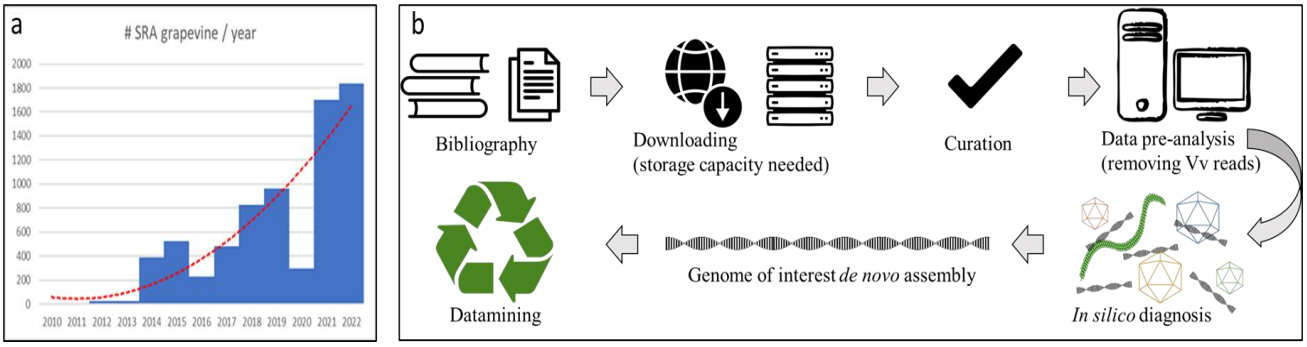

Figure 1. (a) Ever-growing HTS datasets dedicated to Vitis sp. deposited in NCBI each year. (b) Basic protocol of datamining in *Vitis sp*. for virus discovery.

RESULTS AND DISCUSSION

So far, knowledge on the genetic diversity of *Grapevine Pinot gris virus* (GPGV) was mostly limited to biased and partial genomic sequences based on PCR analyses. By performing a systematic datamining effort over 500 publicly available SRA (Sequence Read Archives) files as well as from in-house dataset, and in association with specific bio-informatic and modeling tools, we uncovered invaluable information regarding GPGV. Out of a complex set of data dedicated to serving different research topics, we identified many positive samples for the virus. From these dozens of samples, (1) we confirmed that GPGV can infect many varieties, with over 36 grape varieties found positive to the virus. (2) Those samples came from a wide range of locations, with ten countries spread out over four continents, North and South America, Europe and Asia. (3) Our work demonstrated the capacity to retrieve an important number of unbiased complete GPGV genomic sequences, with one hundred complete (to near complete) genomes being assembled, four times more than the full genome sequences available at the time (26 genomes). (4) This new set of sequences exposed a so far undescribed genetic diversity, jumping from less than 3% to 8.2% diversity. This wealth of information ultimately permitted (5) the modeling of the viral propagation at the plot level as well as the unraveling of the worldwide evolutionary history of the virus (Hily *et al.*, 2021b; Hily *et al.*, 2021a; Hily *et al.*, 2020).

A great number of viruses have been identified in the last decade, thanks to HTS. Close to 40 new viral species, displaying a great variety of genomes, have been identified to infect grapevine, with GPGV being one of them. Our work reveals another facet of HTS and its great added value. The knowledge gathered through this work is relevant at different levels and demonstrated the usefulness of datamining when dealing with grapevine viruses.

ACKNOWLEDGEMENTS

This work was supported by INRAE, by IFV and by the projects ‘VACCIVINE’ and ‘GPGV’ funded through ‘Plan National Dépérissement du vignoble’ (French Ministry of Agriculture) in 2017 and 2019, respectively. A fellowship from Moët & Chandon, CIVC, BIVB CIVA was awarded to JMH.

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O12. Grapevine leafroll disease computer vision solution in the hands of growers

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INTRODUCTION

Grapevine leafroll disease (GLD) is a widespread viral disease that negatively alters vine vigor, longevity, yield and fruit quality. In New Zealand (NZ), current management strategies rely on the identification of infected grapevines to ensure disease inoculum can be removed. For red-berry cultivars, GLD detection is currently based on visual symptom identification (VSI) for foliar symptoms (Bell et al. 2017), which is time-consuming and can be unreliable if assessors lack experience.

Technology and computer analytic advancements offer new opportunities in enhanced crop protection against diseases and whole-vineyard management with cost-effective and sustainable methods. For example, GLD detection by using standard RGB photographs and hyperspectral imaging in combination with machine learning (ML) and predictive models has been described (Bendel et al. 2020, Wang et al. 2023). Although these published methods provide potentially efficient management of disease, adoption of this technology by growers is poor and seldom reported. To ensure effective adoption of a ML solution for GLD, we established workflows and co-innovated with growers to identify how a ML solution can be implemented and integrated into day-to-day vineyard operations.

MATERIALS AND METHODS

The GLD ML detection solution used in this study was developed using RGB photographs collected over three years from *Vitis vinifera* Merlot and Pinot noir with known GLD status (Chooi et al. 2022). Over 4,000 images of leaves and whole-vine canopies of diseased and healthy grapevines were collected and processed through ML training and validation workflows. As a preliminary trial in a commercial setting, the best model generated (84% balanced accuracy for GLD detection compared with known grapevine infection status, and 98% accuracy compared with the disease status as assessed by an experienced GLD visual assessor) was applied to over 26,000 standard RGB photographs collected from commercial vineyards in the 2021 vintage across Australia, NZ, the USA. A subset of 2,704 NZ vines was screened by laboratory methods for grapevine leafroll-associated virus 2 and 3.

The described GLD ML detection model was then used in the current implementation study during the NZ 2023 vintage. With growers' involvement, nine commercial red-berry cultivar vineyard blocks were selected. These blocks included vineyards where growers currently undertake VSI for GLD management, and blocks used for VSI training. At each vineyard, grapevines suspected to have GLD were imaged, and the GPS co-ordinates collected using the Vure phone application (https://www.vurehub.com/) with positioning augmented by Trimble Catalyst GNSS receiver. The photographs were processed through the developed ML model workflow. Model results were then visualized in various forms such as static maps and tabular outputs, and interactive maps to be accessed through Vure web interface.

RESULTS AND DISCUSSION

Currently, once vines are visually assessed with disease, they are flagged with ribbon and recorded on paper, either by counting the total number of diseased vines per row or marking diseased vines on Microsoft® Excel-generated maps. In this study, disease identification was automatically logged onto

a server, and grapevine-level geospatial maps were generated for each vineyard block (total 46 ha) which chart healthy, young, missing, and GLD infected vines (Fig. 1). A total of 2,503 grapevines were observed by a human as having typical GLD foliar symptoms, of which 2,422 grapevines were also assessed for GLD by the ML model (97% agreement). Where there was disagreement, vines were probably new infections, as there was minimal foliar disease expressed. The ability to capture human and ML assessments with a mobile phone ensures all vineyard staff can become GLD VSI experts.

The digital maps provided insights into the location of new and growing disease hot-spots, and the rate of disease spread for blocks where data span multiple seasons. Additionally, key metrics are easily extracted, such as number of vines infected for a given block dimension. The demonstrated flexibility of data visualization and extraction is important, as it reassures different vineyard operators (e.g. contractors that remove vines, managing viticulturists, and accountants) that they can access the same information in formats optimal for their individual operational needs.

This study has demonstrated the use of a digital tool from the perspective of an end-user and their needs of the technology. In future, we envisage photographs for the ML solution being collected using cameras mounted on tractors or autonomous robots, to complete the automation of data capture, disease identification, and visualization, in addition to the human user interface offered by the Vure App.

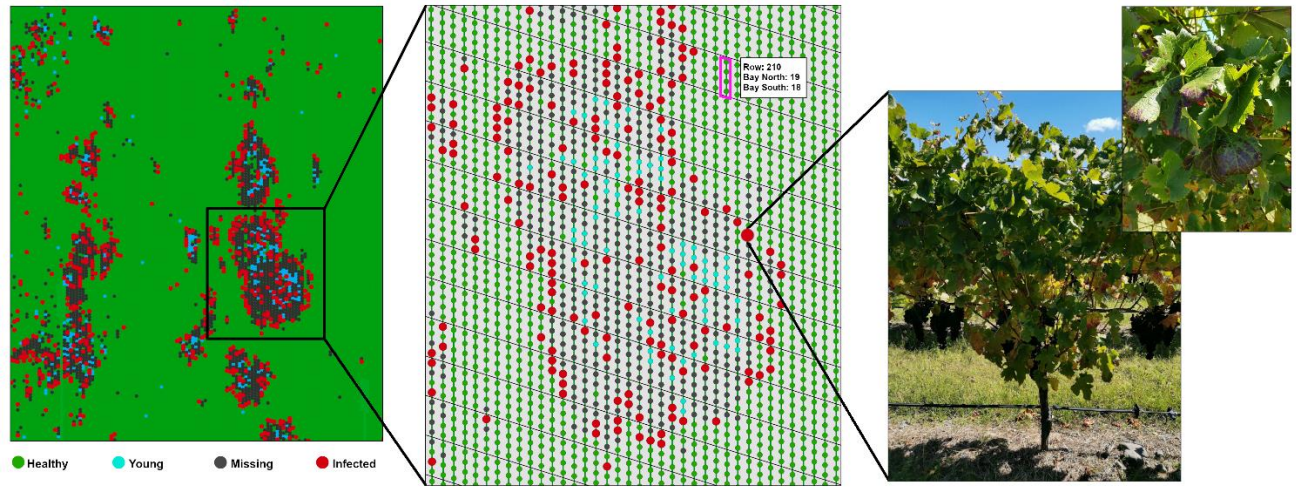

Fig. 1. Example of grapevine-level geospatial maps generated. The left panel is an overview vineyard map. The center panel is a close-up of a disease hot spot, includes rogued (because of previous virus infection) and young replant vines. Human understandable vine position can be extracted from the maps as highlighted by the pink box. Photographs demonstrate the minimal foliar disease expressed by vines where the human and ML model disagreed (right panel).

ACKNOWLEDGEMENTS

We thank all growers for their time, assistance and insights during this project.

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SESSION 3

CERTIFICATION AND OTHER MANAGEMENT APPROACHES

Keynote speaker: Maher Al Rwahnih

Chair: Maher Al Rwahnih and Giorgio Gambino

O13. In-depth studies of the virome of Slovenian grapevine clone candidates and the establishment of a protocol for virus elimination

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INTRODUCTION

Slovenia is a traditional wine-growing country with 15,075 hectares of vineyards in 2021. The area under vines is divided into three regions (Primorska, Podravje, and Posavje) with a total of nine smaller districts. The Primorska wine-growing region accounts for 40.6% of the total Slovenian vineyard area, where a successful program of clonal selection has been carried out for decades. Viruses represent one of the biggest problems for certification and clonal selection. The first objective of the presented work was to investigate the virome of Slovenian grapevine clonal candidates using small RNA sequencing technology (sRNA-seq) and to study the genetic diversity of viruses and viroids as well as co-infections. The work's second goal was to establish a successful protocol for producing virus-free plants.

MATERIALS AND METHODS

A total of 82 samples of six grapevine varieties (four white and two red) were collected from the Primorska wine-growing region. The selected samples were pooled into twelve pools. Small RNAs (sRNAs) were extracted by an enrichment procedure using a mirVana™ miRNA Isolation Kit (Ambion, Life Technologies, Waltham, MA, USA). The sRNA libraries were constructed using the Ion Total RNA-Seq Kit v2 (Ion Torrent™, Waltham, MA, USA) and barcoded using the Xpress™ RNA-Seq Barcode 1–16 Kit (Ion Torrent™, Waltham, MA, USA). Libraries were pooled at equimolar concentrations and prepared for sequencing using the Ion PI™ Hi-Q™ OT2 200 Kit and Ion PI™ Hi-Q™ Sequencing 200 Kit (Ion Torrent™, Waltham, MA, USA). Sequencing was performed on Ion PI™ chips v3 using an Ion Proton™ System (Ion Torrent™, Waltham, MA, USA). The sRNA-seq data were analyzed using the VirusDetect pipeline (Zheng et al., 2017), and validated with RT-PCR and Sanger sequencing. Twenty-eight samples were randomly selected for the virus/viroid elimination study combining in vivo thermotherapy (36–38 °C) with in vitro micrografting of meristem tips (0.1-0.2 mm) onto in vitro growing seedling rootstocks of Vialla (*Vitis labrusca* x *Vitis riparia*).

RESULTS AND DISCUSSION

sRNA-seq revealed the presence of grapevine fanleaf virus (GFLV), grapevine leafroll-associated virus 3 (GLRaV-3), grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine fleck virus (GFkV), grapevine red globe virus (GRGV), grapevine rupestris vein feathering virus (GRVFV), grapevine Syrah virus-1 (GSyV-1), grapevine Pinot gris virus (GPGV), raspberry bushy dwarf virus (RBDV), hop stunt viroid (HSVd), and grapevine yellow speckle viroid 1 (GYSVd-1). All in silico predicted viruses and viroids were validated with RT-PCR and Sanger sequencing. No vine was found to be free of viruses and viroids, while the highest number of viral entities in one plant was eight. In the second part of our study, using the described biotechnological approach to eliminate viruses and viroids, the overall regeneration rate of the plants was low (8.5%), but it is sufficient to obtain one virus-free regenerated plant per candidate that can be further micropropagated. The sanitation status

of the in vitro regenerated plants was checked with RT-PCR. All viruses were eliminated, while the elimination of viroids was less successful (39.2% for HSVd and 42.6% for GYSVd-1).

ACKNOWLEDGEMENTS

This study was funded by the Slovenian Research Agency (SRA–ARRS), grant number P4-0077; and a scholarship for nationals of the Western Balkans to postgraduate study in Slovenia (Public Scholarship, Development, Disability, and Maintenance Fund of the Republic of Slovenia, 245. Public Announcement).

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O14. Virus elimination from infected Croatian indigenous grapevine cultivars by somatic embryogenesis

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INTRODUCTION

Grapevine **(*Vitis vinifera* L.)** stands out as one of the most critical fruit species globally, offering both economic and nutritional value to numerous countries including Croatia. The majority of Croatia's cultivars are critically endangered due to lack of systematic selection and control, as well as long-term propagation of infected materials. The most important elimination technique of viruses and other pathogens is the apical meristem culture, however, the apical meristem isolation is very demanding, since the size of the explant must be below 0.5 mm for *V. vinifera* in order to reduce the risk of contact with the vascular system and the virus (Sim and Golino 2010). A low survival rate of such small explants in tissue culture further decreases the overall success of the method. An alternative method used for virus elimination is somatic embryogenesis (SE). The power of SE as a propagation tool becomes especially apparent when the goal is to generate large numbers of propagules. Successful SE, subsequent maintenance, and plantlets regeneration have been established for many species including *Vitis vinifera* L. (Gray and Meredith 1992). In this paper we report the results of a study in which SE was initiated and standardised for a set of seven indigenous Croatian cultivars. Field-grown donor plants and SE-derived plantlets were inspected for the presence of 6 typical viruses GFLV, ArMV, GLRaV-1 -2, -3 and GFkV. The results showed that viruses GFLV, GLRaV-1, -3 and GFkV present in donor plants were successfully eliminated by the SE. This is also one of only few reports published to date that reports viral detection in Croatian cultivars by RT-PCR (Vončina et al. 2017), a method that has a higher sensitivity than routinely used serological ELISA (Gambino and Gribaudo 2006).

MATERIALS AND METHODS

Plant material was collected from the National collection of indigenous grape varieties of the Republic of Croatia located in experimental field *Jazbina* (University of Zagreb, Faculty of Agriculture) during the May/June of 2014 and 2015, approximately 2-3 weeks before anthesis. Anther filaments were isolated from sterilizyed young buds (Ø 1.5 mm), and used as explants. For induction of SE, MS medium was modified in nitrogen content and supplemented with three plant growth regulators (PGRs): 6-Benzylaminopurine (BA), naphthoxyacetic acid (NOA) and 2,4-dichlorophenoxyacetic acid (2,4-D) in different combinations and ratios (Croatian patent PK20190444). Media for embryo development and regeneration were MS with activated charcoal (0.25%), without PGRs or supplemented with indole-3-acetic acid (IAA) and 1 µM gibberellic acid (GA3), respectively (López-Pérez et al. 2005). Each media was supplemented with 2 % (w/v) sucrose and 7 % (w/v) agar and pH was adjusted to 5.8 before sterilization. Media were sterilized at 121 ºC 103 kPa for 15 minutes. Reverse transcription polymerase chain reaction - RT-PCR was used to test the presence of six dominant grapevine viruses: GFLV, ArMV, GLRaV-1 -2, -3 and GFkV acording protocol described by Gambino and Gribaudo (2006).

RESULTS AND DISCUSSION

Induction of SE was succesful in all tested varieties: ’Plavac mali’, ‘Malvazija dubrovačka’, ‘Teran’, ’Babica’, ’Pošip’, ‘Babić’ and ‘Ljutun’. Proembryogenic tissue was visible as unorganized growth along the filament already 2 weeks after the explant inoculation. Four weeks later, embryogenic structures were formed (Fig. 1), while after 6-8 weeks of culturing, a sufficient amount of embryos

was reached and used for the induction of their maturation and plantlet formation. Three SE-derived plantlets of each cultivar were analysed for virus presence/absence by RT-PCR using the identical parameters as for sanitary status analysis of donor plants. Among three plantlest at least one for each cultivar was virus free. The result for ’Plavac mali’ was shown on Fig. 1.

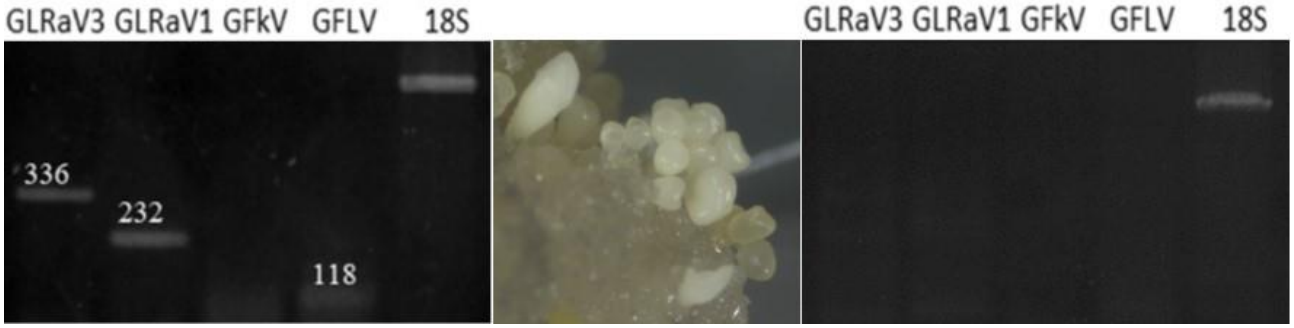

Fig. 1: Virus free (right) somatic embryos (middle) of 'Plavac mali' derived from infected explant donor (left). Among six, only viruses detected in donor plants are included.

Taken together, our protocol enabled regeneration of seven Croatian cultivars through somatic embryogenesis within 10 to 12 weeks. By using our virus elimination protocol in combination with nodal segment propagation (Malenica et al. 2019), it is possible to obtain a high number of healthy plantlets from just one healed SE-derived plant in a relatively short period of time. Therefore, our results are of great importance in respect to further virus elimination methodology development and for the establishment of a steady source of certified virus-free planting material for Croatian vineyards.

ACKNOWLEDGEMENTS

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Croatian patent: https://it-app.dziv.hr/Pretrage/hr/p/Detaljno.aspx/PK20190444

O15. Nanobody-mediated resistance to grapevine fanleaf virus and arabis mosaic virus (part I)

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INTRODUCTION

Grapevines are one of the crops with the highest number of viruses (> 85 identified), of which around 30 are of economic importance (Fuchs, 2020; Martelli, 2017). These viruses are likely to affect the survival of many vineyards worldwide. Unfortunately, to date, there is neither a cure for viruses nor a robust source of resistance in the grapevine gene pool, unlike many other cultivated plants. Consequently, virus control relies essentially on sanitary measures, such as the marketing of certified virus-free vines, removal of diseased plants and replanting after fallow periods, use of pesticides to limit vector transmission, etc... Although essential, these methods are globally insufficient to limit the progression and incidence of viral diseases and modern viticulture is facing a real technological and economical challenge.

Among the main grapevine viruses, those responsible for fanleaf disease, essentially *grapevine fanleaf virus* (GFLV) and *arabis mosaic virus* (ArMV), rank probably first in terms of worldwide distribution and economic impact. The spread of these viruses is essentially linked to human activity (grapevine trade, vegetative propagation, grafting), and to telluric nematodes vectors such as *Xiphinema index* for GFLV and *X. diversicaudatum* for ArMV (Fuchs, 2020).

Recently, we showed the exceptional potential of Nanobodies (Nbs) derived from camelid immunoglobulins to confer resistance to GFLV. Indeed, we showed that Nb23 directed against GFLV, confers strong resistance to GFLV upon stable expression in *Nicotiana benthamiana* upon mechanical inoculation or vector transmission as well as in grapevine rootstock upon *in vitro* micrografting. Resistance was effective against a broad range of GFLV isolates but not ArMV (Hemmer *et al.*, 2018). We also showed that high pressure of inoculum leads to the emergence of GFLV variants containing mutations in the coat protein that interfere with Nb23 recognition and consequently with Nb-mediated resistance (Orlov *et al.*, 2020).

In this study, we isolated Nb75 directed against GFLV that showed improved affinity and expression *in planta* compared to Nb23. We also produced bispecific Nanobodies recognizing GFLV and ArMV and showed that double resistance to GFLV and ArMV can be engineered in *N. benthamiana* and elite *Vitis* rootstock varieties.

MATERIALS AND METHODS

Source material: Nanobodies specific to GFLV were described previously (Hemmer *et al.*, 2018; Orlov *et al.*, 2020). Nanobodies from family 1 are described in Figure 1. Amino-acid differences between Nanobodies from family 1 are highlighted in cyan. Nanobodies specific to ArMV were isolated from an immune library generated against purified ArMV-S particles. Initially, 43 different Nbs belonging to 25 distinct families were isolated. From this collection, Nb1047 was purified and found by double-antibody sandwich enzyme-linked immunosorbent assay (DAS- ELISA) to recognize multiple ArMV isolates but not GFLV.

Recombinant Nanobodies were produced and purified from *E. coli* for *in vitro* studies including competitive ELISA and Surface Plasmon Resonance (SPR). *N. benthamiana* transformation was performed as reported previously (Hemmer *et al.*, 2018). All Nb used for *in planta* expression were

cloned as fusion to GFP. *Vitis* rootstock transformation was performed as described in Zekri *et al.* 2023 (see corresponding ICVG 2023 abstract). Transformed plants were genotyped using Droplet Digital PCR (ddPCR) to assess T-DNA copy number and Oxford Nanopore sequencing to precisely identify T-DNA insertions sites in *Vitis*. Nanobody expression *in planta* was assessed by fluorescence microscopy and by immunoblotting using antibodies directed against GFP.

RESULTS AND DISCUSSION

To improve Nb-resistance, we first screened our Nanobodies collection for candidates that perform better than Nb23 in terms of affinity and expression. For this, we focused on Nbs from family 1 that includes previously described Nb23 as well as related Nb101, Nb126, Nb71 and Nb75 (Fig. 1).

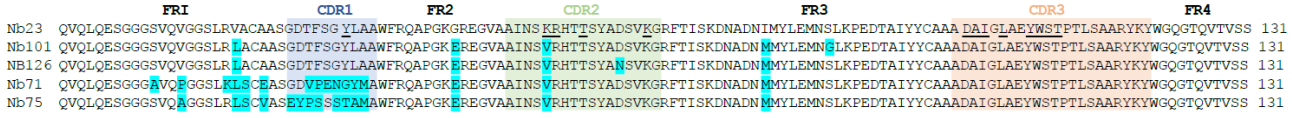

Fig. 1: Sequence alignment of family 1 members from the collection of Nanobodies directed against GFLV. The aminoacids differing from Nb23 protein sequence are highlighted in cyan. The residues involved in the interaction with the GFLV capsid protein are underlined. FR, frameworks; CDR, complementarity-determining regions.

Affinity of the various NbGFLV was assessed using competitive ELISA as well as SPR. Among the 5 candidates tested, Nb75 showed the highest affinity to GFLV and Nb23 the lowest. We also assessed the capacity of the various NbGFLV to be expressed *in planta*. Upon transient expression in *N. benthamiana*, Nb75:GFP showed the highest expression level and Nb23:GFP the lowest. Nb75:GFP was therefore selected further *Vitis* rootstock transformation. After extensive genotyping, 3 clones with single T-DNA insertion events were selected: Gravesac clone #4, 41B clone #18 and 101-14 clone #5. As shown in figure 2, high expression of the Nb75:GFP transgene was observed in all these clones including in roots, the site of entry of GFLV during natural transmission by nematodes. Similar work is in progress with the aim to confer resistance to GFLV and ArMV by combining Nb75 and Nb1047 specific to GFLV and ArMV, respectively.

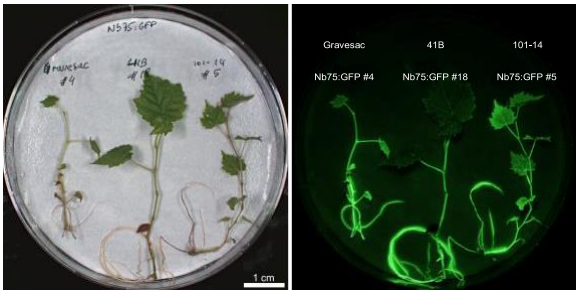

Fig. 2: Day light (left panel) and fluorescence (right panel) imaging of Gravesac clone #4, 41B clone #18 and 101-14 clone #5.

ACKNOWLEDGEMENTS

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O16. Nanobody-mediated resistance to grapevine fanleaf virus and arabis mosaic virus (part II)

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INTRODUCTION

Mercier is a leading viticultural nursery in Vendée, France, specializing in the production of vine plants. The viticulture industry in France is plagued by fanleaf disease, which has devastating effects on grapevines. This disease is caused by a complex of viruses, which severely stunt growth and reduce yields. Fanleaf is estimated to affect over 10% of French vineyards, resulting in significant economic losses. The estimated cost of fanleaf disease to the French wine industry is around €250 million per year (Lacombe *et al.,* 2013), with an additional €100 million spent on management strategies such as replanting and insecticide treatments (Poojari *et al.*, 2018). In addition to these economic impacts, fanleaf disease also has significant ecological consequences. Infected grapevines require more water and nutrients to produce a viable crop, resulting in increased environmental pressure on vineyards. The disease also makes it difficult to implement sustainable viticulture practices, such as integrated pest management and organic farming. Fortunately, biotechnologies such as genetic modification offer a promising solution to this problem. Nanobodies have emerged as a powerful tool for developing disease-resistant grape varieties (Hemmer *et al.*, 2018). This study aims to explore the potential of nanobodies against GFLV and ArMV for creating a more sustainable and resilient viticulture industry, and ultimately reducing the economic and ecological impacts of fanleaf disease.

MATERIALS AND METHODS

Vitis embryogenic calli of many varieties (15 rootstocks and 15 *Vinifera*) have been generated from flowers (ovaries, anthers) and from apical and axillary in vitro plants buds. A MS medium supplemented with plant hormones (2/4D, AIB, ANA, TDZ) sucrose and gelrite is used. After 3 to 6 months, embryogenic callus emerges from undifferentiated callus (Figure 1). It is cultured in dark and transferred every 4 weeks on a GM+ medium (Coutos-Thévenot *et al.*, 1992). Embryogenic calli of 41B, 101-14, Gravesac, 3309

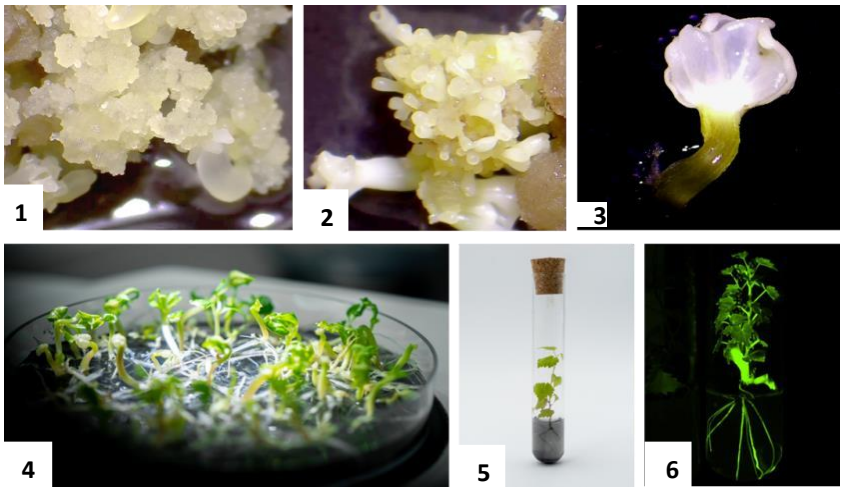

Figure 1: From embryogenic callus to whole plant. 1: Embryogenic callus. 2: Embryos differenciation., 3, 4: embryos maturation and development, 5: In vitro plant, 6: Fluorescence expression in an in vitro plant.

and Thompson Seedless were genetically transformed using an agrobacterium co-culture. Full embryo regeneration takes 1 to 6 months after co-culture. Germinated embryos were cultured on a growth chamber at 22°C under a photoperiod of 16 h light. The genotyping of all of lines are performed by the IBMP (See abstract Cousin *et al.* ICVG 2023). After the identification of the transgene insertion and the expression of Nb line by line, the final phenotyping for resistance was carried out. For this, herbaceous grafting tests were performed (Fig. 2). The first experiment consisted in grafting virus infected material onto Nb-expressing or untransformed WT rootstocks. ELISA tests were carried out 80 days after grafting on roots. In the second experiment, a double grafting was performed consisting of a GFLV-positive rootstock, a Nb75:GFP middle scion and a top GFLV-negative WT scion. The purpose of the tests was to observe the virus transmission from infected part to distal organs.

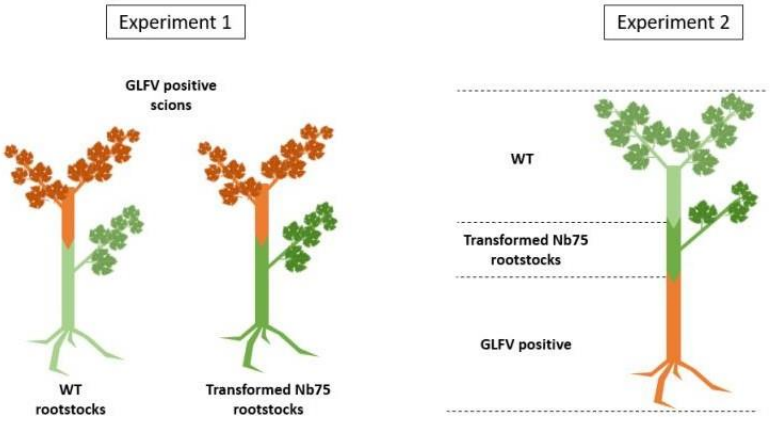

Figure 2: Herbaceous grafting. Simple grafting plant for the first experiment (1) and double grafting for the second (2).

RESULTS AND DISCUSSION

Several independent transformed lines were obtained for each rootstock varieties. Transgene localization was obtained by Nanopore sequencing (IBMP) and allowed the selection of clones with single insertions in intergenic regions. Rootstock clones were grafted with GFLV-infected scions. And roots analyzed by ELISA 80 days after grafting. Up to 100% of resistance was observed depending on the rootstock clone while control WT rootstock showed 100% of infection. The loss of resistance observed in some plants is probably caused by the strong and continuous virus source provided by the infected scion. Results of the second experiment show a migration of the virus in the susceptible WT top scion but not in the transgenic middle scion.

In conclusion, thanks to herbaceous grafting, we validated the resistance of 41B, 101-14 and Gravesac rootstock clones to GFLV. This resistance remains to be confirmed under natural transmission of the virus in controlled greenhouse conditions using contaminated soil containing viruliferous *Xiphinema index* (in progress).

ACKNOWLEDGEMENTS

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O17. Virological Assessment and Importance of Certified Propagating Material in Greek Vineyards

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INTRODUCTION

Grapevine viruses pose a significant threat to vineyards worldwide, affecting their longevity, productivity, and grape quality. This study aimed to evaluate the virological situation in Greek vineyards and emphasize the advantages of using certified propagating material. Specifically, the presence of key viruses, namely Arabis mosaic virus (ArMV), Grapevine fanleaf virus (GFLV), Grapevine fleck virus (GFkV), and Grapevine leaf-roll associated viruses 1 and 3 (GLRAV-1+3), Grapevine virus A (GVA) and Grapevine virus B (GVB) was assessed in more than 2000 samples collected from various wine-growing regions in Greece. To achieve this, Hellenifera, a research center formed through the collaboration of VNB-Bakasietas and the French Institute of Vine and Wine (IFV), played a crucial role in collecting and analyzing the samples. The center's primary objectives include the preservation and promotion of rare native grape varieties, as well as the development of certified propagating material for Greek grape varieties.

MATERIALS AND METHODS

More than 2000 samples were collected from various wine-growing zones across Greece (figure 1). The sampling process aimed to encompass a representative range of grapevine varieties and regions within the country.

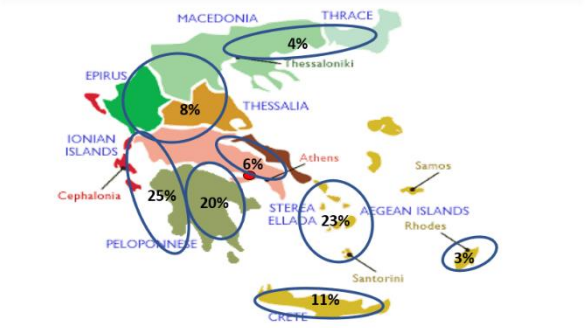

Figure 1: Sample origin and dispersion

In order to assess the presence of the aforementioned viruses, three methods were used.
- ELISA (Enzyme-Linked Immunosorbent Assay) Tests: Serological ELISA tests were conducted using a specific technique based on the reaction between antibodies (serum) and antigens (viruses).
-PCR (Polymerase Chain Reaction) Tests: PCR tests were performed using a genomic technique that amplified a DNA fragment obtained through retro-transcription. Specific primers were used to target the RNA of the virus, enabling the amplification of the desired DNA fragment (Beuve et al., 2013).
-Biological Tests (Indexing): Biological indexing was performed as a reference method for clone approval. This involved grafting a clone to be tested onto a cutting of an indicator variety capable of exhibiting characteristic symptoms of the desired viral infection. Two grafting methods were

employed: woody grafting, which involved a three-year evaluation period, and herbaceous grafting, which required six to twelve months for evaluation.

RESULTS AND DISCUSSION

Table 1 Results of virological testing of 2054 vines

	GFLV	GLRaV 1-3	GLRaV-2	GLRaV-4+	GFkV	GVA&GVB
No of positive vines	170	884	103	657	441	657
Percentage (%)	**8.3**	**43**	**5**	**32**	**21.5**	**32**

The virological analysis revealed a high incidence of viral infections in the examined vines, with GLRaV 1-3 being the most prevalent (43%). Notably, the current legislation in Greece does not include GLRaV-2, GLRaV-4+, GVA, and GVB in the list of targeted viruses. Consequently, the percentage of virus-free plants in the Greek vineyard was determined to be 37.5%. However, when considering the inclusion of these four additional viruses in the analysis, along with the results obtained from the indexing method, the proportion of virus-free plants significantly decreased to 9.5%. These findings highlight the substantial prevalence of viral infections in Greek vineyards, particularly with GLRaV 1-3. The inclusion of previously untargeted viruses underscores the urgent need for enhanced control measures to mitigate viral spread and ensure the production of healthy grapevines. Consequently, a more frequent and stricter control of mother plants of both the standard and the Certified material is imposed, since according to the recent legislation 1080/99222/08.04.2022 YA (B' 1415), there is a 0% virus tolerance in the final product.

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O18. Evaluation of Management of grapevine red blotch virus spread in vineyards by roguing infected vines

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INTRODUCTION

Grapevine red blotch virus disease (GRBD) was first recognized more than a decade ago in California (Sudarshana et al., 2015). It is now considered a major disease of wine grapes in Northern California and Oregon. A monopartite geminivirus species, *Grapevine red blotch virus* (GRBV), genus *Grablovirus*, is the causal agent of this disease (Yepes et al., 2018). Infection by GRBV causes delay in ripening, reduced total soluble solids, increases acidity, and decreases the overall quality of premium wines, and thus significantly reduces the revenue (Rumbaugh et al., 2023). While this virus is widely distributed in North America, its spread other than through planting of infected nursery stock has only been recognized in California and Oregon vineyards (Dalton et al., 2019; Flasco et al., 2023). Three cornered alfalfa hopper (TCAH), *Spissistilus festinus* Say), a membracid, has been shown to transmit the virus (Bahder et al., 2016; Flasco et al., 2023). A few other membracids have also been shown to be potential vectors of GRBV (Kahl et al., 2021; LaFond et al., 2022). Limited information on the biology, transmission characteristics and ecology of the vectors has made development of vector management options limited. Presently, roguing of infected grapevines and replanting with GRBV-free grapevines is the only option to manage GRBV. Here we report on the impact of roguing on the spread of GRBV in an experimental vineyard of Cabernet sauvignon and two commercial vineyards of Barbera and Pinot Noir in California.

MATERIALS AND METHODS

Cabernet Sauvignon grapevines on 3309C rootstock were evaluated for girdling damage by TCAH and mapped in a 1.5-acre experimental vineyard at the UC-Davis Viticulture Research Station at Oakville, planted in 2017. All vines were tested for GRBV infection by qPCR in fall of the following year, then monitored annually each fall thereafter for appearance of GRBV symptoms. Symptomatic vines were removed. Vineyard floor vegetation was mowed in early spring each year and kept free from weeds.

In Amador County, a 1-acre block of Barbera vines was planted in 2016 and maintained under standard viticultural practices for the area. When GRBV infection was first noticed in 2019, the block was mapped for symptomatic grapevines and was not subjected to roguing.

In Sonoma County, a 2-acre block of Pinot Noir/1103P.1 was planted in 2017. In fall 2019, GRBV infection in the block was recognized, and the infected vines were removed and replanted in the following spring and this was repeated in subsequent years. The vineyard was maintained with one mowing in early spring and a second one in late spring.

Incidence of GRBV was recorded at all three sites from 2019 through 2022 and confirmed by qPCR tests on a subset of samples.

RESULTS AND DISCUSSION

Our results indicate that GRBV infection was minimal in the Napa Co. experimental vineyard with a previous history of spread before being removed and replanted with GRBV-free transplants (Table 1). Despite roguing, GRBV incidence increased in the Sonoma Co. Pinot Noir block, and reached 33% after 4 years with nearly half of the vines having been infected. In the Amador Co. Barbera block,

where no roguing or vegetation management was practiced, the low incidence of GRBV in 2019 increased to 66% by 2022. These results suggest that GRBV incidence can be managed by roguing infected grapevines to reduce virus inoculum and practicing vineyard floor vegetation management to reduce the vector population.

Table 1. Incidence and prevalence of grapevine red blotch virus in three vineyards over 4 years planted with GRBV-free grapevines in 2017.

Vineyard		2019	2020	2021	2022
a) Cabernet Sauvignon	New infections	0	1	1	2
(n= 1066)	% Incidence	0	<0.1	<0.1	<0.2
	% Prevalence	0	<0.1	<0.1	<0.3
b) Pinot Noir	New infections	75	51	279	478
(n =1922)	% Incidence	3.90%	2.83%	15.9%	32.5%
	% Prevalence	3.90%	6.56%	21.1%	45.9%
c) Barbera	New infections	12	36	322	422
(n=1013)	% Incidence	1.18%	3.6%	33.4%	65.6
	% Prevalence	1.18%	4.74%	36.5%	78.2%

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O19. Roguing, an effective technique to manage Grapevine red blotch virus in British Columbia, Canada

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INTRODUCTION

Grapevine red blotch disease (GRBD) is associated with grapevine red blotch virus (GRBV), an economically important emerging pathogen with a broad distribution in North America (Rickets et al., 2017; Sudarshana et al., 2015). GRBD occurs in all grape-growing regions in Canada with highest incidence reported in Ontario (ON) vineyards (Fall et al., 2020; Poojari et al., 2017; 2020; Xiao et al., 2018). GRBV negative impacts on plant health and fruit and wine quality are severe under British Columbia (BC) growing conditions with yield losses of up to 42%. GRBV infected vines have also shown an average of 4.1 brix less than healthy vines (Bowen et al., 2020). Accordingly, management of GRBD is identified as one of the main research priorities by the BC grape and wine industry. Roguing, a technique that eliminates virus infected vines in a vineyard, has been shown to successfully manage Grapevine leafroll disease (GLRD) in different countries (Bell et al., 2017; Hesler et al. 2022). Therefore, the main objective of this study was to evaluate if roguing could be implemented in vineyards in BC to reduce the incidence of GRBD.

MATERIALS AND METHODS

Four 'Cabernet franc' ('Cf') vineyard blocks, with confirmed presence of GRBV (Poojari et al., 2017), were selected in the Okanagan Valley (BC) to determine the effectiveness of roguing to manage GRBD. Vineyard blocks were mapped each year of the study (2018-2022) by conducting two inspections per block between mid-September and end of October when GRBD symptoms are more obvious. Suspicious GRBV infected vines and their immediate within-row neighbors on each side were recorded in the map each year, flagged and tested for the presence of GRBV, *grapevine leafroll-associated virus 1* (GLRaV-1), GLRaV-2, GLRaV-3, and GLRaV-4 at the Brock University Cool Climate Oenology and Viticulture Institute, St. Catherines (ON) virus testing laboratory. Infected vines were removed in each block the following spring. GRBV incidence was determined each year as the number of confirmed infected vines divided by the total number of vines present in the block.

The effects of GRBV on fruit quality of newly infected vines was assessed in a total of 12 and seven infected vines from two different blocks, respectively. Each infected vine was paired with a healthy vine and yield and fruit quality parameters were conducted as described by Bowen et al. (2020).

Sweep net sampling was carried out in all four blocks in 2021 and 2022 with an arc over the width of the row using a standard 15" diameter cloth net with a mesh bottom. The material was placed into clear plastic 20 lb bags, tied off, and kept in a cooler with ice packs for transport. The contents were then frozen and later analyzed for the number of sharpshooters, treehoppers, froghoppers and planthoppers present in the sweeps. In 2021, sweeps were conducted once a month from June to September. In 2022 the frequency of sweeps was increased to two collection dates in May, June and September and remained at one collection date in July and August.

RESULTS AND DISCUSSION

GRBV incidence in each block at the beginning of the study (October 2018) was 11.7%, 11.2%, 9,3% and 5.2%. After roguing infected vines in the spring of 2019, GRBV incidence was reduced to 1.5%, 0.3%, 1%, and 0.2% with a total of 20, 8, 23, and 9 newly confirmed infected vines in each of the blocks. The grower could not performed roguing in the spring of 2020 and there were 1, 1, 9, and 20

newly infected vines resulting in an incidence of 1.6%, 0.3%, 1.4%, and 0.8%. Roguing conducted in the spring of 2021 and 2022 reduced the incidence of GRBV at the end of the study (October 2022) to 0.1% or less in all blocks. In total, 60% of the newly infected vines were recorded to be next to a previously rogued vine. Since all adjacent plants to an infected one were tested for GRBV each year, our results indicate that it may take one year for the virus to be detected and to show symptoms in a newly infected vine.

In 2021, vines newly infected with GRBV showed less shoots per vine, an average of 2.7 less brix, and higher TA than healthy vines. No statistical differences were observed in yield and the rest of fruit quality parameters.

Among the different insect vectors documented for secondary spread, *Spissistilus festinus*, the three-cornered alfalfa hopper, has been proven to be the most efficient candidate and responsible for GRBD spread in California (Bahder et al., 2016; Hoyle et al., 2022). Populations of potential hemipteran vectors of GRBV were very low throughout the survey in the 'Cf' blocks, and treehoppers (family *Membracidae*), including *S. festinus* as well as *Stictocephala bisonia* and *S. basalis*, other potential vector candidates found in BC (Kahl et al., 2021), were not captured on any sample date.

This study demonstrates roguing to be a successful tool to eliminate GRBD from infected vineyards in BC. GRBV detrimental effects on fruit quality can be observed in newly infected vines. Further studies need to be conducted to determine what insect vectors are responsible for the minimal GRBV spread observed in BC.

ACKNOWLEDGEMENTS

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SESSION 4

PHYTOPLASMAS

Keynote speaker: Xavier Foissac

Chair: Xavier Foissac and Martina Šeruga Musić (part I)/ Nicola Fiore and Michael Maixner (part II)

O20. The recovery on Flavescence dorée infected plants of Tocai friulano cultivar

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INTRODUCTION

Flavescence dorée (FD) is a Grapevine Yellows disease (GY) associated to FD phytoplasmas (FDp). FDp is transmitted in vineyard by the leafhopper vector *Scaphoideus titanus.* Differences in susceptibility to FD have been observed among grapevine varieties. Tocai friulano showed low susceptibility to the disease when compared to other grapevine cultivars. Recently, Casarin et al. (2023) demonstrated that FD-infected plants of Tocai friulano confined the symptoms near the area where they have appeared throughout the vegetative season. In the same paper the complete absence of the pathogen in the trunk and its disappearance in 2-year-old branches from July to November were demonstrated. Moreover, gene expression studies and metabolic analysis of secondary phloem in one-year-old canes suggested that the successful defence mechanisms activated near the symptomatic areas allowed the compartmentation of FD symptoms and phytoplasmas within the infected Tocai friulano plants (Casarin et al 2023).
Since FD symptomatic plants of Tocai friulano showed spontaneous remission of symptoms from one year to the next during field surveys, in the current work FD-infected plants of this variety were pruned for two years by removing the symptomatic parts in order to induce the state of recovery. The recovery status was then confirmed by FDp detection and metabolic analysis. Moreover, other four grapevine varieties were compared to Tocai friulano in order to explore their ability to compartmentalize FDp.

MATERIALS AND METHODS

The most part of the study was carried out in a Tocai friulano vineyard (1,5 ha) located in Friuli Venezia Giulia, Northeastern Italy. Grapevine plants were more than 25 years old and were grown with integrated pest management. The pruning system was the guyot method with two fruiting arms. All Tocai friulano plants were visually surveyed from 2019 to 2022; symptomatic plants were identified and mapped, and all symptomatic parts were labelled. During the dormancy, 4 and 61 FD-infected plants, in 2020 and 2021, respectively (treated thesis, T) were pruned by removing the symptomatic portions. Other symptomatic plants were pruned conventionally and used as a control (untreated thesis, UT).
In July and September 2022, leaf veins of surveyed plants were sampled according to the theses reported in Table 1, and analyzed for FDp detection, hydrogen peroxide (H_2O_2) titer and stilbenoid contents (piceid, piceatannol and ε-viniferin) following protocols previously published (Gambino et al. 2013; Casarin et al. 2023).
In parallel, in 2021 and 2022 GY symptom observation was carried out also in a vineyard in Piedmont, Northwestern Italy, where four varieties with variable susceptibility to FD (Moscato bianco, Arneis, Sauvignon blanc, Chardonnay) were grown. Ten plants for each variety were sampled during the dormancy at the beginning of 2023 for the FDp detection in the trunk, and processed following the protocol already reported (Casarin et al 2023). All plants showed symptoms during the vegetative season for more than one year. The same sampling and analyses were conducted on ten Tocai friulano plants.

RESULTS AND DISCUSSION

After the careful pruning in 2020 and 2021, visual surveys in vineyard showed 100% (4/4) and 77% (47/61) recovered plants in 2021 and 2022 in T plants, respectively. The experimental trial in 2022

showed a low percentage of plants that did not recover (23%). We cannot exclude new infections on these plants, as the trial was established in open field with vector presence, either we cannot exclude the failure in completely removing the inoculum during the pruning. These results suggest that the remotion of the symptomatic parts in Tocai friulano FD-infected plants is very efficient in inducing the recovery.

Leaf veins of Tocai friulano plants were tested to detect FDp in samples collected in July and September 2022 (Table 1). Results showed the presence of the FDp only in the symptomatic plants, thus, the pruning method was found to be effective in removing the FDp inoculum from the leaves.

		July		September	
Theses	**Theses ID**	**N° of samples**	**FDp titer**	**N° of samples**	**FDp titer**
Plants symptomatic since 2 years	S 2y	5	43.3±18.7	5	14. 6±13.9
Plants recovered since 2 years	R 2y	4	0.00±0.00	4	0.00±0.00
Plants symptomatic since 1 year	S 1y	/	/	5	18.9±10.9
Plants recovered since 1 year	R 1y	/	/	8	0.00±0.00
Healthy plants	H	3	0.00±0.00	8	0.00±0.00

Table 1. Relative quantification of FD phytoplasma titer (mean±st.dev.), expressed as arbitrary units, in leaf veins of Tocai friulano plants sampled in 2022. The *cytochrome c oxidase* (*COX*) gene was used for normalization.

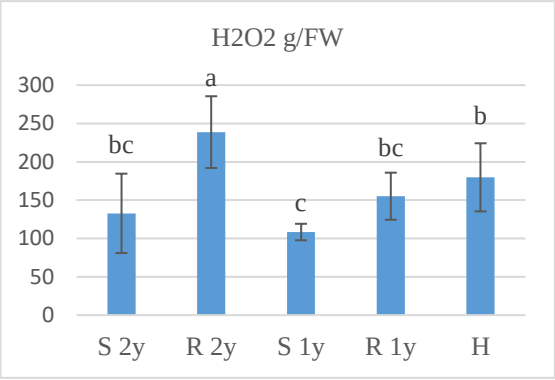

Figure 1: Hydrogen peroxide content in leaf veins sampled in September 2022 in Tocai friulano. Samples were collected from the theses listed in Table 1. Significant differences ($P \leq 0.05$) are indicated according to parametric one-way ANOVA using Student Newman Keuls test.

Metabolite contents, which were measured in leaf veins of Tocai friulano, did not show a higher accumulation of ε-viniferin or other stilbenoids analyzed in recovered plants compared to healthy or symptomatic ones. Only H_2O_2 content was higher in leaf veins of Tocai friulano plants recovered since 2 years compared to the others (Figure 1). The H_2O_2 higher accumulation confirmed the achievement of a recovery status, as previously observed in Gambino et al. (2013) and Musetti et al. (2007).

The comparison of the presence of FDp in the trunk of different grapevine varieties showing symptoms for more than one year confirmed the absence of FDp only in Tocai friulano. All the other four varieties harbored a detectable FD titer in the trunk, but at different extents. Moscato bianco and Arneis showed a very low average phytoplasma titer (0.01±0.02 and 0.05±0.06, respectively), differently from Sauvignon blanc (1.18±2.41) and Chardonnay (5.95±8.24). The results suggested that none of the observed varieties was able to confine the FDp in the one-year-old canes like Tocai friulano did, but highlighted interesting varietal features to be furtherly indagated.

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O21. Flavescence dorée phytoplasma shows different distribution and titer in woody organs of symptomatic grapevines belonging to two cultivars

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INTRODUCTION

Flavescence dorée is the most devastating grapevine diseases caused by phytoplasmas (FDp) in Europe. To prevent its spread, reliable detection is essential to identify the pathogen and to develop an effective management. Typically, FDp diagnosis in grapevine is limited to the vegetative seasonal period, from spring to autumn, when phytoplasma symptoms are evident on the leaves.
However, the ability to efficiently analyze woody canes, arms, and trunks, as recently reported, has expanded the testing timeframe (Casarin et al., 2023). Moreover, this could help to gain a better understanding of the phytoplasma-plant host interactions. The aim of this study was to investigate the presence and the titer of the FDp in roots and woody organs of Glera and Pinot gris cultivars.

MATERIALS AND METHODS

Two vineyards, cultivated with Glera or P.P. gris, were monitored in 2021 and 2022, and symptomatic plants were identified, mapped and labelled during the two years. Both vineyards were grown vertically with sylvoz pruning, with three fruiting arms. In September 2022, 12 plants, 6 for each cultivar, asymptomatic in 2021 and showing mild or severe FD symptoms in 2022, were selected, and symptomatic and asymptomatic new canes were marked. In Glera grapevines, lignified canes bore either symptomatic or asymptomatic leaves, while in P. gris lignified canes had only asymptomatic leaves.
In February 2023, secondary phloem of permanent structures (trunk, cordon), fruiting arms and 1-year-old lignified canes were sampled by longitudinally cortical scraping (10 cm in lenght), after bark removal, for a total of 523 samples. Moreover, approximately the second, eighth, twelfth and the twentieth buds were sampled in the longest 1-year-old lignified canes from symptomatic plants. After that, plants have been uprooted and root fragments were collected.
Extraction of RNA was performed using the RNeasy Plant Kit (Qiagen) for woody samples and the Spectrum Plant Total RNA Kit (Sigma) for root samples. Reverse transcription and relative quantification of FDp were performed as described in Casarin et al. (2023).

RESULTS AND DISCUSSION

Independently on the variety, the roots of 6 plants, which showed severe symptoms on the canopy, tested positive for the presence of FDp. On the opposite, among the plants with mild symptoms, only one out of the three P. gris plants tested positive, while Glera roots were always negative.
FDp was detected in all plants in at least one portion out of 24 portions of the trunk, the titer of FDp in the trunk of Glera being relatively lower than in P. gris. Interestingly, a significant difference was observed in the upper trunk, where P. gris exhibited a higher concentration of FDp compared to Glera. Concerning the cordon, FDp was detected in all the plants. Notably, Glera consistently exhibited a higher concentration of FDp in the outer part of the cordon, with an average of 7.9 arbitrary units, compared to an average of 4.8 in the inner part.
In the asymptomatic arches, FDp titre ranged from very low to zero in Glera, while P. gris exhibited higher FDp occurrence. On the opposite, in the partially or completely symptomatic arches in Glera, FDp was almost always present. In the last variety the FDp titer was higher in the outer part of the arch

(average of 9.4) compared to the inner part (average of 5.6). Moreover, desiccation was common in the three seriously infected P. gris plants, with six out of the nine arches experiencing desiccation. Desiccation was not reported in Glera.

The presence of FDp along canes was finally investigated. In Glera, 17.6% of the canes grown from asymptomatic arches tested positive for FDp, but the percentage increased at 87.5% considering canes growth from partially symptomatic arches. In P. gris, 55.5% of the canes from asymptomatic arches tested positive, while the values rose to 85.7% in partially symptomatic arches. Comparison of FDp titre along the canes, between the second and tenth buds, showed a significant difference in both cultivars. Indeed, Glera exhibited a higher concentration of FDp in the tenth bud, while P. gris had a higher concentration in the second bud. The analysis on another ten longer canes of Glera, comprising five asymptomatic canes and five lignified canes which showed symptomatic leaves, as well as five asymptomatic longer canes of P. gris, confirmed the general acropetal distribution of FDp in Glera and the basipetal distribution in P. gris (Fig. 1).

In conclusion, a strong correlation was identified between the severity of symptoms and the presence of FDp in the roots in the first year of infection, highlighting the early establishment of the pathogen in this organ. Moreover, the results demonstrated that FDp was consistently present in the trunk and cordon, albeit in varying proportions depending on the grape cultivar. These results are thus very useful for winter FDp diagnosis, when leaves are absent, and symptoms are challenging to find, as the most suitable grapevine woody organs were identified in the present work. A concentration gradient of FDp was also detected from the base to the tip of the canes, but opposite in the two cultivars. Differences in sugar and nutrient movement, related to different growth patterns of the cultivars, being Glera and P. gris with high and low vigor, respectively, can potentially explain the contrasting concentration gradients of FDp observed along the canes and other woody organs. It seems plausible that FDp migrates following the gradient of sugars and nutrients within the host plant (Prezelj et al., 2013).

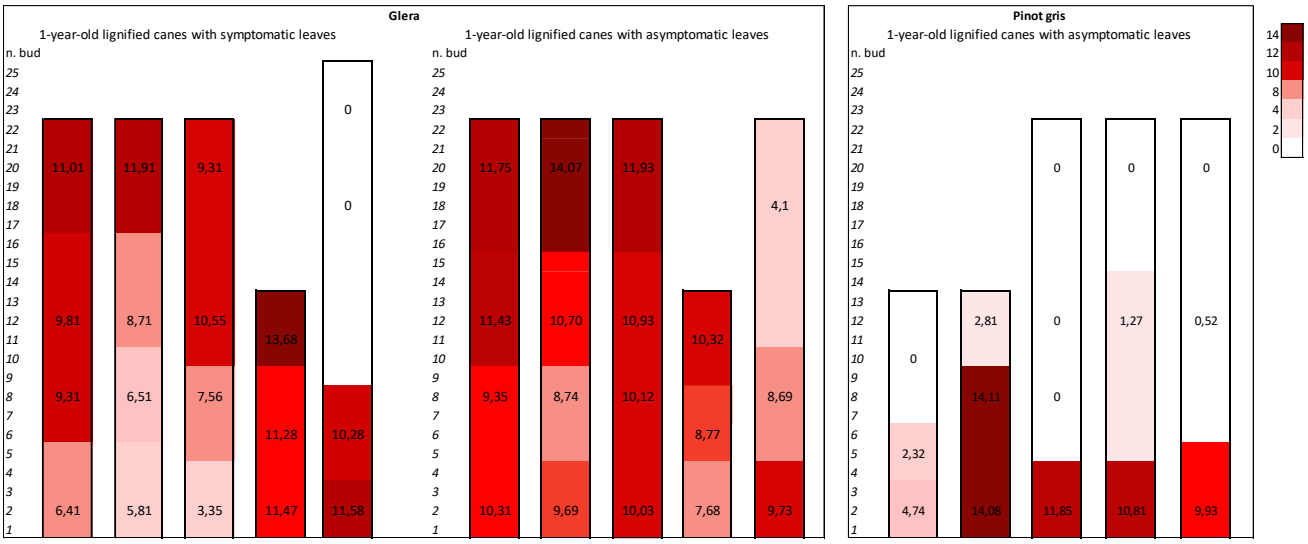

Fig. 1 Graphical representation of FDp titer measured in different points of 1-year-old canes grown from symptomatic plants of Glera and Pinot gris. A color scale was used to show the FDp titer, expressed as arbitrary units (log2).

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O22. Strain-specific response of flavonoid metabolism in grapevine var. 'Pinot gris' to *flavescence dorée* phytoplasma infection

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INTRODUCTION

Phytoplasmas (genus '*Candidatus* Phytoplasma') are a group of plant pathogenic bacteria that pose a significant threat to global agricultural productivity by causing extensive damage to crops and other plant species (Namba, 2019). They exhibit a remarkable genome plasticity resulting in various strains of certain phytoplasma species possessing distinct sets of effectors. An example of this is observed in the case of '*Ca.* P. solani' (*bois noir* phytoplasma or BNp; ribosomal group 16SrXII-A) (Šeruga Musić et al., 2019). Possible alterations in set of effectors can influence the interaction between the phytoplasma and its host plants. Indeed, previously obtained epidemiological data indicates variations in pathogenicity and prevalence among different phytoplasma strains. In the case of *flavescence dorée* phytoplasma (FDp; ribosomal group 16SrV), which is recognized as one of the causal agents of grapevine yellows disease significantly affecting viticultural production throughout Europe, a high diversity of genotypes is recorded (Plavec et al., 2019). Plants react to the phytoplasma infection by various means. One of the strategies in the case of biotic stress is production of specialized metabolites, like flavonoids, as previously reviewed (Dermastia et al., 2019). In this case, we investigated whether flavonoids potentially play a role in interactions between FDp and grapevine. Furthermore, we aimed to analyse if different FDp strainss alter the flavonoid metabolism in a different direction or severity. Our hypothesis was that more aggressive and widespread genotype M54 affects grapevine flavonoid profile more severely that less widespread M38.

MATERIALS AND METHODS

Leaves of symptomatic and asymptomatic grapevine (*Vitis vinifera* L. var. 'Pinot gris') plants were sampled throughout the development of phytoplasma infection (June-September) at three time points from a vineyard in central continental Croatia (Sveti Ivan Žabno). Using a real-time PCR assay (Pelletier et al., 2009), we confirmed the infection with the FDp in symptomatic plants. Additionally, amplification, sequencing and phylogenetic analyses of *map* gene amplicons were performed as previously described (Plavec et al., 2023). For metabolic analyses, flavonoids were extracted from leaves using a previously developed methodology (Davosir & Šola, 2023), optimized for grapevine samples. Levels of total flavonoids, as well as flavonoid subgroups were analysed using spectrophotometry-based methods, while major individual flavonoids were analysed using high-performance liquid chromatography.

RESULTS AND DISCUSSION

We detected two distinct FDp strains in sampled grapevine leaves, corresponding to the *map* genotypes M38 and M54, both belonging to the mapFD2 cluster. Therefore, we also analysed if the effect of the FDp on the flavonoid profile in grapevine is dependent on phytoplasma strain. At the beginning of symptom development, there was a significant decrease in total flavonoids (TF) in infected leaves compared to uninfected ones, with the lowest TF observed in M54-infected leaves. However, at the second and third time points, TF levels were higher in infected leaves, indicating a downregulation of flavonoid biosynthesis during the early stages of infection followed by an upregulation later on. Interestingly, the TF content showed a positive correlation with all major groups of flavonoids (flavonols, catechins, proanthocyanidins, anthocyanins), as well as individual flavonoids (quercetin and myricetin), suggesting similar patterns across the entire flavonoid pathway and suggesting a

general response of the pathway to FDp infection. Generally, M54 caused more severe downregulation of flavonoid biosynthesis at the beginning of the development of infection, and also a higher upregulation at the end of the vegetative season than M38. Considering the flavonoid compounds as notable markers of biotic stress conditions in plants, these results mark the first functional demonstration of variable impact of different FDp strains in grapevine. The findings further emphasize the importance of considering the highly adaptable phytoplasma genomes in future research, as well as their implications for the disease-causing potential of various strains.

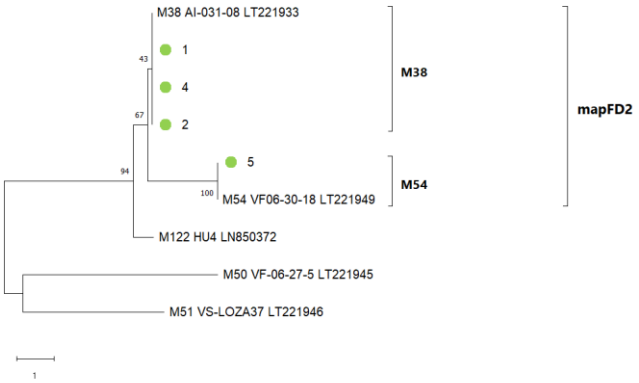

Fig. 1. Unrooted phylogenetic tree depicting the phylogenetic relationship between isolates (1-5) and reference *map* sequences, inferred by neighbour-joining analysis (number of differences) with bootstrap values obtained after 500 repeats. Affiliation to the map genotypes and clusters is shown to the right of the tree. mapFD1, mapFD2, mapFD3 = FD clusters; M38, M54 = genotypes

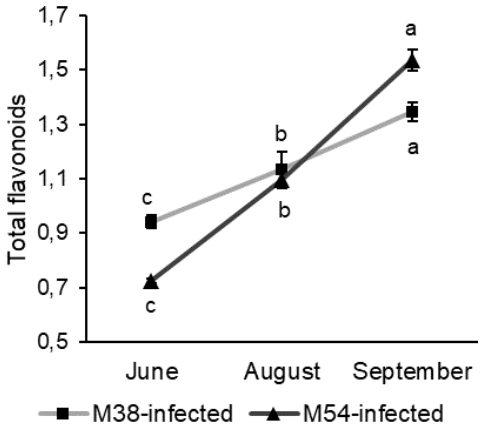

Fig.2. Content of total flavonoids of M38- and M54-infected grapevine leaves at three time points, in relation to the corresponding controls. Values represent mean ± standard deviation of three replicates. Different letters indicate a significant difference between different time points for the corresponding samples (ANOVA, Duncan test, $p \leq 0.05$).

ACKNOWLEDGEMENTS

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O23. Five leafhopper species captured in Chilean vineyards are new vectors of 16SrIII-J phytoplasmas

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INTRODUCTION

In Chile the phytoplasmas associated to grapevine yellows are '*Candidatus* Phytoplasma asteris' (ribosomal subgroups 16SrI-B and 16SrI-C), '*Ca*. P. pruni' (16SrIII-J), '*Ca*. P. ulmi' (16SrV-A), '*Ca*. P. fraxini' (16SrVII-A), and '*Ca*. P. solani' (16SrXII-A) (Gajardo *et al*., 2009; Fiore *et al*., 2015a). However, recent work showed that the phytoplasmas 16SrIII-J are the prevalent (González *et al*., 2011; Fiore *et al*., 2015b; Quiroga *et al*., 2017a, 2019a) and the "flavescence dorée" phytoplasmas and its vector *Scaphoideus titanus*, have not been found in Chile (Quiroga *et al*., 2017b). Previous studies indicate that non-grapevine plants from different botanical families present in the vineyards and in their vicinity (*Convolvulus arvensis*, *Galega officinalis*, *Polygonum aviculare*, *Malva* sp., *Brassica rapa*, *Rubus ulmifolius*, and *Rosa* sp.) are infected by 16SrIII-J phytoplasmas, and the leafhoppers *Paratanus exitiosus* and *Bergallia valdiviana* are vectors of the same pathogen. Other leafhopper species, *Bergallia* sp., *Amplicephalus ornatus*, *Amplicephalus curtulus*, *Amplicephalus pallidus*, and *Exitianus obscurinervis*, captured in a vineyard in Valparaiso region planted with cultivar Pinot noir, were infected by 16SrIII-J phytoplasmas (Zamorano *et al*. 2015; Quiroga *et al*., 2019b, 2019c, 2020). Transmission trials were performed to find out if these insect species are vectors.

MATERIALS AND METHODS

To carry out the transmission trials (TT), adult individuals were captured monthly from October 2017 to June 2021 using an entomological sweeping net. The insects were identified at the species level based on morphological characteristics. Periwinkle and grapevine cultivar Cabernet Sauvignon micropropagated phytoplasmas-free plantlets, were used in the TT. Between 4 and 6 insects of the same species were introduced to each *in vitro* plant tube. For each TT, the insects have been allowed to feed for a maximum of 7 days and then stored in 70% ethanol. At the end of the TT, the plants were treated with fungicides (captan and tebuconazole), transferred in a solid sterilized substrate composed of peat and perlite in a 2:1 ratio, and kept in a growth chamber at 25ºC under 16 h/day light. Four periwinkle and four grapevine plants, which have never been in contact with insects, were used as negative controls. Total nucleic acids were extracted with CTAB method (Angelini *et al*., 2001) from leaf midribs and insects used in the TT; the final extract, dissolved in Tris-EDTA pH 8.0 buffer, was maintained at 4°C. PCR amplification was carried out using 20 ng/µl of nucleic acid. After direct PCR with primers pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), a nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) was performed (Schaff *et al*., 1992). The amplified products (1,250 bp) were sequenced, and the identification of phytoplasmas was carried out by in *silico* RFLP using the enzymes *Hha*I, *Bst*UI and *Rsa*I (Zhao *et al*., 2013). All the plants were analyzed 3, 10, 18 and 24 months after the start of each TT.

RESULTS AND DISCUSSION

The *in vitro* TT carried out were 235, with 123 and 112 grapevine and periwinkle plants, respectively, but only 141 survived (grapevine: 76; periwinkle: 65) when transplanted from the *in vitro* to the solid substrate. All the insect species used in TT were able to transmit the 16SrIII-J phytoplasmas to grapevine and periwinkle plants: *A. ornatus* (grapevine: 3; periwinkle: 1); *A. pallidus* (grapevine: 4; periwinkle: 2); *Bergallia* sp. (grapevine: 3; periwinkle: 2); *E. obscurinervis* (grapevine 1; periwinkle: 3); *A. curtulus* (grapevine: 5; periwinkle: 3). The plants used as control were negative for phytoplasma presence. The periwinkle plants, start to show symptoms two months after the beginning of TT. The use of *in vitro* plants for TT has been successful in identifying the new insect vector species. This is due very likely to the reduced stress the insects suffer while they feed under these experimental conditions.

ACKNOWLEDGEMENTS

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O24. Management strategies for grapevine Bois noir

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INTRODUCTION

Grapevine Yellows (GY) are phytoplasma-associated complex diseases affecting the main wine-growing areas worldwide and causing important losses in berry quality and yields. Among GY, Bois noir (BN), associated with '*Candidatus* Phytoplasma solani' (CaPsol, subgroup 16SrXII-A or stolbur group), represents a serious threat in viticulture. In grapevine (*Vitis vinifera* L.), BN causes leaf yellowing, or reddening (according to white or red cultivars), down curling, flower abortion, berry shrivel, irregular ripening, and plant decline. In some cases, death can occur in 2-3 years.

The main insect vector of CaPsol is the polyphagous planthopper *Hyalesthes obsoletus* Signoret, erratically transmitting CaPsol to grapevine and living preferentially on stinging nettle (*Urtica dioica* L.) and field bindweed (*Convolvulus arvensis* L.), and also on mugworth (*Artemisia vulgaris* L.), stinking hawk's-beard (*Crepis foetida* L.), lavender (*Lavandula spica* L.), chaste tree (*Vitex agnus-castus* L.) and muscat sage (*Salvia sclarea* L.). Several studies showed the presence of many other plant hosts and insect vectors involved in the spreading of BN, unveiling a more complex and multifaceted disease epidemiology.

In this challenging scenario, disease containment resulted very difficult in vineyards and the main strategies so far adopted for BN management included: (i) eradication of infected plants, use of resistant cultivars (when possible) and phytoplasma-free propagating material, (ii) control of insect vectors and weed composition, using insecticides, herbicides, or other agronomical practices, (iii) use of antibiotics (denied by law in several Countries). Considering the negative environmental impacts related to the massive use of chemicals and the partially inadequate results so far achieved, the developing of alternative, sustainable and effective strategies aimed at limiting BN dissemination is highly needed.

This work summarizes the sustainable control strategies developed in in the last twenty years to improve BN management, analyzing their effectiveness according to specific geographical and environmental conditions.

MANAGEMENT STRATEGIES

Recent investigations have successfully obtained phytoplasma elimination from portions of CaPsol-infected plants through treatments with hot water, cryotherapy and tissue cultures (Křizan et al., 2008). A spontaneous symptom remission along with the elimination of the pathogen (recovery) can be also induced in many grapevine cultivars by agronomical practices, such as partial uprooting and pulling (Romanazzi et al., 2013), pruning or pollarding of the symptomatic portions (Riedle-Bauer et al., 2010) and grafting of recovered material onto infected rootstock (Moussa et al., 2022). Promising results were also reported in studies evaluating effects of non-pathogenic endophytic bacteria and use

of resistance inducers. In this field, the systemic introduction of *Frateuria defendens* showed a significant reduction of phytoplasma titer and symptom intensity in several plant species, including Chardonnay plantlets. A considerable decrease of symptomatic plants was also obtained using benzothiadiazole, glutathione and biostimulants (elicitors) from animal or plant-derived amino acids (Romanazzi et al., 2013; Moussa et al., 2022).

Concerning the containment of insect vector populations, use of biocontrol agents as entomopathogenic fungi (EPFs) and nematodes (EPNs) in greenhouse conditions have showed a significant reduction of *H. obsoletus* populations (adult and nymph stages). Similar results should be confirmed in field, evaluating effects on non-target organisms (Guerrero and Pardey, 2019). Further promising approaches can be provided by the understanding and the managing of microbiomes associated with target vectors to limit development and spreading of plant diseases (Microbial Resource Management, MRM) (Iasur-Kruh et al., 2018). A remarkable control strategy was proposed in a study conducted by Mori et al. (2016) in vineyards located in Northern Italy, where nettle represents one of the most important inoculum sources of CaPsol. In this study, the reduction of nettle density and death of *H. obsoletus* nymphs was carried out within an integrated approach, including selective herbicide applications with frequent mechanical cuts of nettle. Application of repellent (push) and trap (pull) plants to repulse or attract dangerous organisms in crops is an innovative and effective technology. In Israel, the "push and pull" strategy was tested reveling that *Vitex agnus-castus* was the most attractive host to the vector (Sharon et al., 2005). An appropriate weed management is important to mitigate the risk of infection by BN, reducing the inoculum in the vineyard.

CONCLUSIONS

This work summarizes the novel and sustainable strategies can be successfully adopted for BN management in vineyards. Based on natural contexts, the synergistic effects of multiple strategies adopted simultaneously and/or within the same plant growing season can represents the suitable methodology to improve the effectiveness in BN management.

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O25. "Flavescence dorée" strains in selected Italian wine-producing areas

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INTRODUCTION

The study of the genetic variability of phytoplasmas is a tool to clarify their epidemiology and to implement an effective management of their associated diseases. "Flavescence dorée" (FD), a threatening disease of grapevine associated to phytoplasmas belonging to 16SrV-C and -D subgroups (Martini *et al*., 1999) and its insect vector(s), is(are) distributed within the most important European wine-producing areas. This quarantine disease has severe effects on both vineyard productivity and landscape management and, despite the efforts to contain the pathogen dissemination, the disease is epidemic in several viticultural areas of northern Italy. During the past 30 years FD phytoplasmas belonging to both ribosomal groups were detected in several viticultural areas of Italy (Angelini *et al*., 2018), however sequencing of non-ribosomal genes, such as *secY*, *map* and *rpsC*, allowed the identification variants within the FD phytoplasma populations that seems in several cases consistent with an increased bacterium virulence (Bertaccini *et al*., 2021; Contaldo *et al*., 2021). From 2018 to 2022 focused surveys were conducted in FD infected vineyards to verify molecular variability of the FD phytoplasma strains and their relationship with outbreaks.

MATERIALS AND METHODS

Grapevine samples were collected in Treviso, Belluno and Verona provinces in Veneto, Trento and Bolzano provinces in Trentino-South Tyrol, Modena and Forli-Cesena in Emilia-Romagna and Siena in Tuscany. The differentiation of FD-D and FD-C on the 16S rRNA gene was performed as reported (Martini *et al*., 1999). MLST analysis on *secY-map*, *secY* and *rpsC* genes was carried out on symptomatic samples FD-infected of Chardonnay, Sangiovese, Trebbiano, Teroldego, Garganega and Pinot noir varieties, according to literature (Martini *et al*., 2002; Arnaud *et al*., 2007; Lee *et al*., 2004). RFLP analyses on *rpsC* gene were performed with *Tru1*I (Fermentas) according with the manufacturer instruction in polyacrylamide 6.7% gel. Sequencing of selected *secY* and *secY-map* gene amplicons was performed in both direction using the same primers employed in amplification. The sequences were assembled, aligned and used for phylogenetic analyses with MEGA 7 (Kumar *et al*., 2016).

RESULTS AND DISCUSSION

The geographic distribution of FD-C and FD-D strains was confirmed to be different. FD-C strains were prevalent in Treviso and Siena provinces (Contaldo *et al*., 2021), while FD-D strains were detected in Verona, Modena and Forlì-Cesena provinces (Bertaccini *et al*., 2021); both FD-C and FD-D were detected in Trentino-South Tyrol region (Fattorini *et al*., 2022). Multilocus molecular analyses on *map-secY* genes allowed the identification of genetic variants M54 among FD-D populations in the different regions, while among FD-C strain, variant M3 was found in Veneto and M51 in Trentino-South Tyrol (Figure 1a). Furthermore, phylogenetic analysis on *secY* gene showed the presence of variability among FD-D phytoplasmas, with 3 clusters enclosing samples from Veneto and Emilia Romagna (Figure 1b). This shows for the first time a clear presence of genetic FD-D variants in Italy for which since their first molecular identification in 1996, very little variability was detected. In particular, the sequencing of secY amplicons highlighted the presence of the same SNP in samples of cultivars Teroldego and Trebbiano collected in Verona province and in grapevines cultivar Sangiovese located in Modigliana (Forlì-Cesena province). On the other hand, among the FD-C phytoplasma

strains the highest variability was obtained on *rpsC* gene, with 5 restriction profiles identified after RFLP analyses in samples from cultivar Glera collected in Treviso and Belluno provinces (data not shown). The epidemiology of the FD disease must be further monitored, especially after the recent finding of alternative insect vectors/plant hosts species possibly responsible for the emergence of new FD variants. Therefore, a continuous and capillary monitoring of the presence and emergence of new and genetically homogeneous FD strains associated with the disease is useful for the application of the most appropriate and stringent control measures aimed to avoid epidemic spread of virulent FD strains.

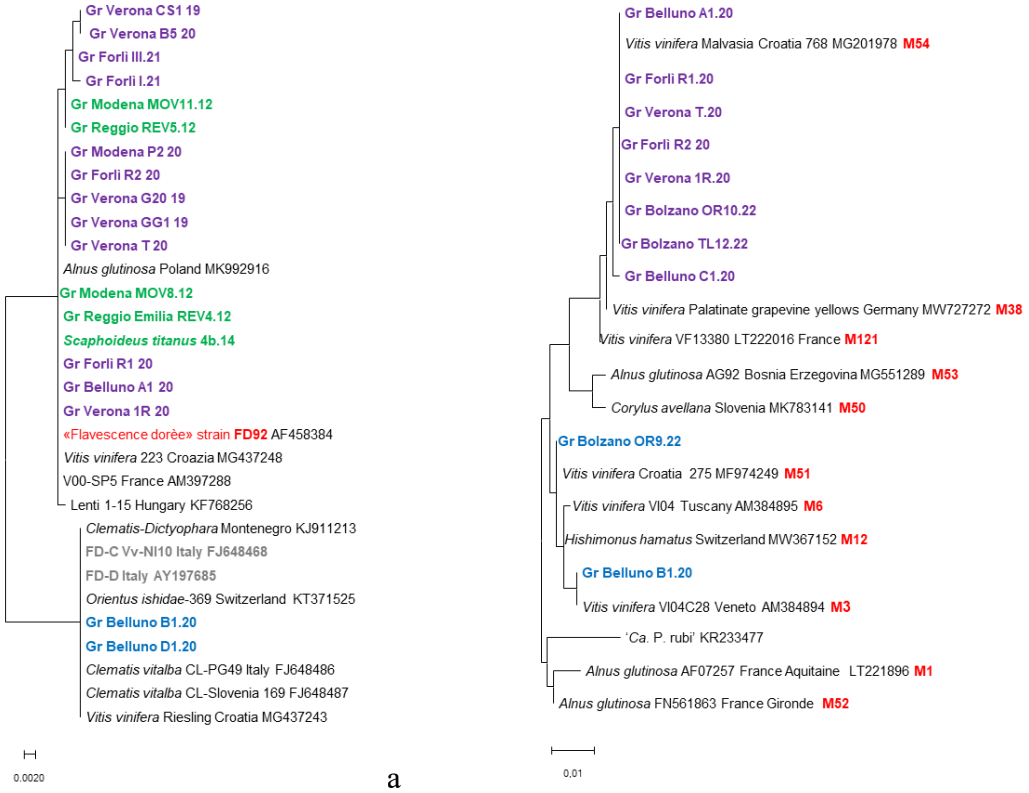

Figure 1. Phylogenetic trees of FD-D and FD-C strains from north Italy: a) *secY*, b) *secY-map* genes.

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O26. Investigations on the origin of "Flavescence dorée" isolated cases in North-East France

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INTRODUCTION

Flavescence dorée (FD) is a severe disease in European vineyards caused by phytoplasma genotypes of the 16SrV-C and D subgroups. FD phytoplasmas (FDp) also infect wild European alders (*Alnus glutinosa*) and clematis (*Clematis vitalba*), considered as the original reservoir plants. The main vector of FDp on grapevine is the North-American leafhopper *Scaphoideus titanus* (ST), introduced in Europe in the early 1900s. However, other auchenorrhynchan species, such as *Allygus* spp., *Dictyophara europaea* and *Orientus ishidae* were characterized as alternative vectors able to propagate FDp in reservoir plants from the environments and occasionally transmit them to grapevine (Filippin *et al*., 2009; Lessio *et al*., 2016; Malembic-Maher *et al*., 2020). Besides the known hosts, the recent detection of FDp in refuge plants and leafhoppers species could complexify FD epidemiological cycle (Casati *et al*., 2017). The aim of this study was to investigate the potential origin of FD isolated cases detected in vineyards of North-East France, an area that was still free of FD outbreaks, by characterizing FDp reservoir plants and potential leafhopper vector species in the vineyard landscapes.

MATERIALS AND METHODS

Isolated FD cases were detected in some vineyards of NE France between 2019 and 2021: 1 site in Alsace in Bergholtzzell (site B), and in 6 sites in Champagne in Arrentières (A), Chouilly-South (Cs), Chouilly-North (Cn), Mancy (M), Reuil (R) and Saudoy (S). In each site, ligneous trees and vines surrounding the vineyards were inventoried and wood canes were sampled from autumn 2020 to 2022. Auchenorrhynchan insects (except Thyphlocybinae subfamily) were captured on 23, 29 and 25 yellow sticky traps installed inside the vineyards and in the wild compartment in 2020, 2021 and 2022 respectively. They were renewed every two weeks, from June to September. Insects were also collected by beating the vegetation with sweeping nets and identified with a taxonomic key (Biedermann & Niedringhaus 2009). Acquisition trials of FDp on infected alders by ST were performed in confined greenhouse. Alive *O. ishidae* were captured on FDp-infected alders in Bommes (South-West France) and transferred by groups of 10 to 15 individuals on healthy alder seedlings for FDp transmission during 1 week. After 3 months incubation, 2 FDp infected alders were selected by PCR. Groups of 80 ST nymphs (L4-L5 stages) were placed twice on the infected alders during 3 to 4 days for FDp acquisition. ST individuals were transferred on 2 grapevines (Fercal) for a 4 weeks latency period. ST were then transferred by groups of 10 individuals on 12 healthy broad beans (*Vicia faba*) for FDp transmission during one week. Total DNA from plants and insects was extracted following the CTAB and TNES protocols respectively. Extracts were screened for 16SrV phytoplasmas by nested PCR on the *map* gene followed by sequencing for genotyping (Arnaud *et al*., 2007).

RESULTS AND DISCUSSION

FDp infecting isolated grapevines were M50 genotype in sites Cn, Cs and M, M50 variant (1 SNP) in sites A and S, and M38 in sites B and R. The age of the stocks, greater than 10 years, precluded a possible introduction of the phytoplasma by infected plant material and favored the hypothesis of a transfer from wild plant reservoirs. A total of 394 plant samples of 42 genera collected in the vineyard environment were tested. Alders were only present in sites B, A and R, and were highly infected by populations of 16SrV-C *map* genotypes (17/20) as already described in other European regions. The

genotypes were identified as FDp and Alder Yellow phytoplasmas (AldYp) compatible and not compatible with ST transmission respectively (Malembic-Maher *et al*., 2020). Since none of the other plant species were infected by 16SrV-C or -D phytoplasmas, alders were the only reservoir plants identified. A total of 33 921 insects belonging to 57 genera were collected and 1 665 insect samples were tested for phytoplasma infection. Nearly 25% of these samples were infected by 16SrV phytoplasmas: either by FDp M38 genotype, by AldYp genotypes, by *Candidatus* 'Phytoplasma ulmi' (16SrV-A) or by unresolved mixed infections. In Alsace, in site B, where ST is absent, the alternative FDp Deltocephalinae vectors *Allygus* spp, *O. ishidae* and *Lamprotettix nitidulus* (B. Jarausch & M. Maixner, personal communication) captured on alder and grapevine, had a high 16SrV-C infection rate ranging from 60 % to 77 % with FDp M38 genotype being predominant. They could be responsible for the single M38 isolated case detected in grapevine, suggesting a low frequency transfer from alders. Other leafhoppers individuals from the species *Euscelidius* spp., *Fieberiella* spp., *Japananus hyalinus* and *Graphocephala fennahi* were also infected by the M38 genotype. Their vectoring ability needs to be evaluated by transmission trials. In site R, where several FDp M38 scattered cases were detected in grapevine, only 3 over 48 *Allygus* spp. captured on alders were infected by M38 genotype and 25 were infected by AldYp or unresolved mixed infections. This species could be responsible for the transfer of FDp M38 from alders to the vineyards. No *O. ishidae* were captured in this site, and none of the 17 *L. nitidulus* tested were infected by M38 genotype. High ST populations were monitored in this site. One ST over 347 captured on grapevine was infected by M38 suggesting that the M38 cases in neighboring vineyards could also be the result of a low frequency transmission between grapevines by ST. As infected ST were also collected on alders (10/143 samples, 1 M38), the possible FDp acquisition by ST on alder was assessed. Acquisition trials were performed on two experimentally FDp-infected alders. At the end of the trials, 6 symptomatic broad beans and 2 asymptomatic latency grapevines were detected infected by FDp. These results indicate that ST is experimentally able to acquire FDp on alders and transmit it to grapevine and broad bean. In sites where single M50 and M50 variant genotypes were detected in grapevine, none of the tested insects were infected by these genotypes, regardless of the presence (site A) or the absence (sites Cn, Cs, S and M) of infected alders in the vicinity of the vineyards. In site A, alders and *Allygus* spp. were only infected by a mixture of AldYp genotypes. The reservoir plants and alternative insect vectors of M50 genotypes are still unknown and the origin of the isolated cases need further investigations.

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O27. Flavescence dorée phytoplasma in northern Swiss vineyards is anthropogenic

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INTRODUCTION

Flavescence dorée (FD) is a quarantine disease associated with Flavescence doréee phytoplasma (FDp), which is mainly transmitted by the leafhopper *Scaphoideus titanus* Ball (Schvester et al., 1961). In 2015, the first two outbreaks of the disease were detected in canton of Vaud (Switzerland). Since then, seven additional foci have developed within a 30 km radius, leading to the uprooting of 1,6 ha of vineyard. In order to better understand the introduction and dissemination of the phytoplasma in the region, more than 660 positive samples from all outbreaks were genetically characterized using both known and newly identified variable *loci* allowing for a better study of FD epidemiology.

MATERIALS AND METHODS

Plant material (leaves) was collected from 2015 to 2022 during annual phytosanitary inspections.
Total nucleic acids (TNA) from twelve plants representative of the different outbreaks were extracted using CTAB protocol (Anses) and sent to Macrogen for Illumina sequencing. Reads were assembled to FDp reference genome (Debonneville et al., 2022) with Bowtie2. SNPs were detected using Geneious 2023.0.4 and confirmed by Sanger sequencing.
TNA from symptomatic samples were extracted with the same CTAB protocol and presence of FDp was tested by quantitative PCR (qPCR) as previously described (Pelletier et al., 2009). Genetic characterization was done by multilocus sequence typing. *Loci* were amplified by PCR, products were sequenced forward and reverse using Sanger technology, sequences were *de novo* assembled and consensus were aligned using MUSCLE algorithm.

RESULTS AND DISCUSSION

From 2015 to 2022, samples of 2924 grapevines showing symptoms of yellowing were analyzed by qPCR. 28,8 % were found positive to FDp and 46,8 % to Bois noir phytoplasma.
A selection of representative samples from different outbreaks and years was used for multilocus genotyping. PCR and Sanger sequencing were done on 135 *map* samples, 77 *dnaK*, 648 *malG* and 43 *vmpA*. These *loci* were chosen for their proven variability of 3 to 12 SNPs per region as reported in literature (Arnaud et al., 2007; Rossi et al., 2019).
Nevertheless, the only gene showing variability in our samples was *malG*, with four SNPs. Indeed, all samples were infected by M54, dnaK1 and cluster II vmpA genotypes. Two different *malG* genotypes were found and they were spatially correlated, *i.e.* the two *malG* genotypes were geographically gathered (four outbreaks of *malG* G3/G3 and five of *malG* G1/G3). However, these markers were not considered to be sufficient to trace FDp epidemic as they could not differentiate strains from the different outbreaks within the same *malG* genotype. Therefore, we used Illumina sequencing of samples from different *foci* with the same *malG* genotype to search for hypervariable genomic regions that are likely to discriminate among genetically similar strains. Two *loci* with one SNP each were detected in genes *ftsH5* and *ftsH17* in the Illumina datasets corresponding to *malG* G1/G3. The combination of these two SNP rolled out three different genotypes inside the *malG* G1/G3 group, leading to the discrimination of samples 100 m apart within the same outbreak. Nonetheless, no SNP was detected among Illumina datasets with *malG* G3/G3 genotypes. As a result, four different genotypes of FDp were found (*Table 1*), all of them in at least two non-contiguous plots.

Table 1 Different genotypes of FDp found in canton of Vaud.

	Gene					
Genotype	***map***	***dnaK***	***VmpA***	***malG***	***ftsH5***	***ftsH17***
A	M54	dnaK1	vmpAII	G1/G3	C	C
B	M54	dnaK1	vmpAII	G1/G3	C	G
C	M54	dnaK1	vmpAII	G1/G3	A	G
D	M54	dnaK1	vmpAII	G3/G3	C	C

Data suggest that FDp was initially introduced with planting material and progressively spread at the vineyard level through *Scaphoideus titanus*, which is present in Vaud since at least 2006. Actually, M54 is known to infect only grapevines, *S. titanus* and *Orientus ishidae* (Malembic-Mahler et al., 2020; Casati et al. 2017). Indeed, the facts that M54 has never been found in other plant hosts and that *O. ishidae* (secondary vector) is extremely rare in the region (C. Linder personal communication) led us to exclude the landscape as a source of FDp in Vaud. Therefore, FDp outbreaks in Swiss vineyards in the north of the Alps are anthropogenic. This contrasts with the situation in the south of the Alps (Canton of Ticino), where landscape act as a reservoir of the disease (Oggier et al., 2023).
In conclusion, this work identified two additional hypervariable regions allowing to discriminate within G1/G3 haplotype in Vaud. It also suggests that in northern Swiss vineyards FDp was introduced *via* planting material without any influence from the environment. Thus, data highlight the importance of planting healthy vegetal material and therefore emphasize the need to use hot water treatment to provide phytoplasma-free grapevines.

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O28. Estimation of dispersal and risk factors of Flavescence dorée in Bordeaux vineyards using mechanistic and statistical models

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INTRODUCTION

Flavescence dorée (FD) is a severe disease of grapevine caused by a phytoplasma, epidemically transmitted by the leafhopper *Scaphoideus titanus* in European vineyards (Tramontini et al. 2020). Quarantine FD phytoplasma is controlled by mandatory measures: planting disease-free stocks, annual surveillance of symptoms, removal of infected plants and application of insecticides. Improving FD management strategies has much to gain from the recent advances in both statistical and mechanistic epidemiological modelling (Parnell et al., 2017). Typically, statistical spatial models can be used to identify the agronomical and environmental factors influencing plant pathogen epidemiology. Mechanistic models are able to simulate realistic patterns of epidemic spread and can be used to test the effectiveness of a range of management strategies. Here, both approaches were used to improve our knowledge on FD epidemiology and management. In a first study, we estimated the dispersal distance of FD from two snapshot maps of the FD infectious status in three fields using a mechanistic SIR model coupled with Bayesian inference framework (Adrakey et al., 2023). In a second study, we characterized field and landscape risk factors impacting FD infection in Bordeaux vineyards using spatial statistical models (Adrakey et al., 2022).

MATERIALS AND METHODS

Spatially explicit mechanistic SIR model. In 3 adjacent fields situated near Bordeaux (France) and planted with 5961 stocks of Merlot and Cabernet-Sauvignon, FD symptomatic and removed plants were precisely mapped in 2018 and 2019. A larger area of 300 m radius around the target fields was also monitored from 2014 to 2018 by recording FD-infected fields and the number of infected plants per field, without their exact location. A spatial SIR (Susceptible, Infected, Removed) model with a discrete time step of one year was used to describe the probability of infection of each plant in the focal fields. The model considered a time varying rate of primary infection, differences in cultivar susceptibility for secondary infection and a dispersal kernel representing the statistical distribution of the infected hosts after inoculum dispersal from a focal plant source. Model inference relied on data augmentation with a Bayesian approach that accounts for the missing information related to plants removed before the initial survey in order to construct the full trajectory of the epidemic.
Spatial statistical model. Between 2012 and 2016, GDON des Bordeaux organization monitored and georeferenced grapevine fields in the Bordeaux vineyard (area of 347 districts and 84000 ha), with 10 % achieved each year. A geographic information system was designed by collating the characteristics of the 34581 fields inspected over 5 years: FD-infection status, date of survey, altitude, organic or conventional practice, area, age, density of plantation and cultivar, the later extracted from the Casier Viticole Informatisé (CVI) (Fig 1). Landscape characteristics obtained from the CVI and land cover map were expressed as percentages of urban, forest, vineyard areas, vineyards with Merlot or with organic practice in radii from 50 to 5000 m around each field. The effects of these field and landscape factors on the probability that a field is infected by FD were estimated using spatial generalized linear models fitted with INLA (Integrated Nested Laplace Approximation) method.

RESULTS AND DISCUSSION

FD dispersal distance. Bayesian model inference first suggested that heavy-tailed dispersal kernels, characterized by frequent long-distance dispersal events, best fit the spread of FD. On average, 50%

(resp. 80%) of new infection occur within 10,5 m (resp. 22,2 m) of the source plant (Adrakey et al., 2023). These values are in agreement with estimates of the flying capacity of *S. titanus* using mark-capture techniques (Lessio et al. 2014). Simulations of simple removal scenarios using the fitted model predicted that the disease still spread over years despite complete removal of symptomatic plants, suggesting that cryptic infections hamper FD management. Future efforts should explore whether strategies relying on reactive removal of plants in determined buffers can improve FD management.

Effects of field and landscape factors on FD infection. Our analysis first highlighted the importance of the monitoring period with a probability of FD detection 4 times higher in September than in August. At field scale, altitude and cultivar choice were the main factors affecting FD infection (Fig 1). The odds ratio of FD infection in fields of the susceptible Cabernet Sauvignon, Cabernet Franc, or Muscadelle were twice those in fields of the less susceptible Merlot. Field infection was also affected by the field's immediate surroundings within a circle of 150 to 200 m radius corresponding to landscapes of 7 to 12 ha. In particular, the probability of FD infection increased with the proportions of forest and urban land and with the proportion of susceptible cultivars, demonstrating that the cultivar composition at landscape scale impacts FD epidemiology. The satisfactory predictive performance of the model for identifying districts with a prevalence of FD detection of >10% of the fields suggests that it could be used to target areas in which future surveys would be most valuable.

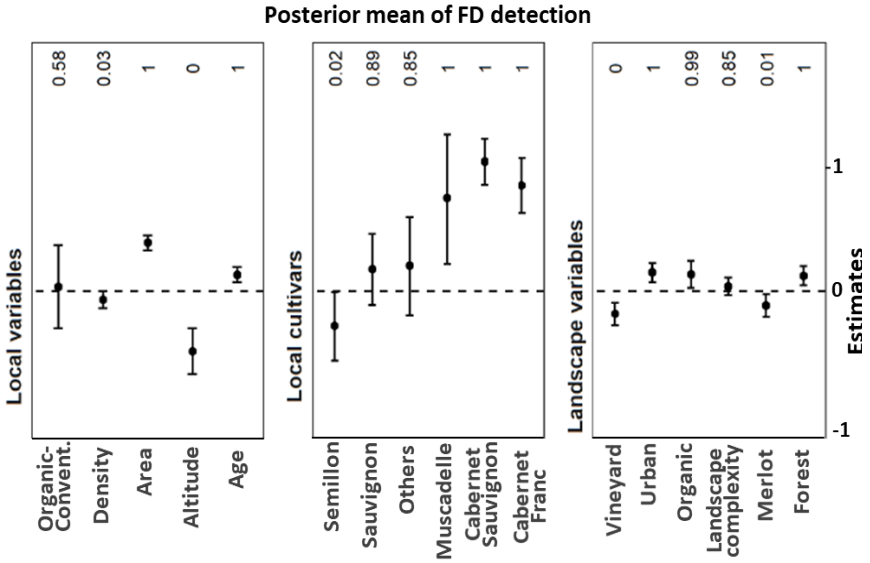

Fig 1: Effects of the local and landscape explanatory variables. For each variable, the posterior mean (dots) and 95 % credible intervals (solid lines) are displayed, together with the posterior probability of the effect being positive. The dashed lines correspond to the value 0. For estimating the local effect of cultivars, the poorly susceptible Merlot was chosen as the reference (value 0).

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O29. Bacterial microbiomes in "flavescence dorée" infected grapevines in Italy

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INTRODUCTION

When pathogens colonize a host, they are exposed to diverse complex microbial communities inhabiting the plants as endophytes therefore they compete with them for both nutrients and space. This interaction may play important roles for the success of bacterial infections and for the ability of these pathogens to be spread from plant to plant by insect vectors. "Flavescence dorée" (FD) is a threatening grapevine disease associated to phytoplasmas enclosed in 16SrV-C/D subgroups (Martini *et al*., 1999) that are quarantine organisms in Europe. The disease is distributed within the most important wine-producing areas of Europe and has severe effects on both vineyard productivity and landscape management and, despite the efforts toward its containment, it is actively spreading. The aim of this study was to investigate the bacterial composition of microbiomes mainly located in the vascular tissues of grapevine FD-infected plants to study possible relationships among bacterial genera that could led to further basic information about possibility of FD management.

MATERIALS AND METHODS

During summer 2019 and 2020 a survey for phytoplasma presence was conducted in six vineyards located in Massa Carrara (Tuscany), Belluno and Verona (Veneto) provinces in Italy. A total of 48 grapevine plants (cv Sangiovese in Tuscany and cv Glera in Veneto) showing yellows symptoms such as leaf reddening and shorter internodes were collected and total nucleic acids were extracted from leaf midribs using a phenol-chloroform method (Prince *et al*., 1993). Nested-PCR/RFLP analyses on 16S rRNA gene were carried out to identify the phytoplasmas (Zambon *et al*., 2018). Extracted DNAs were further purified and used for library preparation following the "16S Metagenomic Sequencing Library Preparation: Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System" instructions. Variants of the 799F and 1193R primers (Beckers *et al*., 2016) optimized for phytoplasma amplification based on nucleotide alignments of their ribosomal gene DNA sequences were used for amplification (Nicolaisen *et al*., 2022). All the reads were aligned to the pertinent reference sequences using USEARCH v. 10 and OTUs were then compared to the Genbank sequences using the BLAST tool.

RESULTS AND DISCUSSION

Almost all the grapevine samples (43 out of 48) resulted positive for "flavescence dorée" phytoplasmas enclosed in 16SrV-C/D subgroups and one sample from Belluno resulted with a mixed infection of 16srXII-A ('bois noir') and 16SrV phytoplasmas, after nested-PCR/RFLP analyses. After Illumina sequencing overall 431 bacterial OTUs (Organism Taxonomic Unit) were identified in the samples monitored and the bioinformatic analyses allowed the identification of the detected genera. In the infected grapevine plants the percentage of sequences related to *Acholeplasmataceae* family ranged from 95% in Tuscany and 83.4% in Veneto region (Figure 1). After BLAST analyses the prevalent OTUs identified were 100% identical to the 16S ribosomal sequences of phytoplasmas enclosed in the 16SrV group. The preliminary analysis on bacterial communities showed a slightly different composition in the two regions and grapevine varieties analyzed. While the bacteria from *Enterobacteriaceae* family were the most present in the negative samples in both regions, the composition of microbiome in infected plants varied according to geography and more likely also to

the variety. In Tuscany, the majority was represented by bacteria from *Micrococcaceae, Sphingomonadaceae* and *Beijerinckiaceae* families (Contaldo *et al*., 2023), while in Veneto together with *Micrococcaceae,* it was registered the presence of bacteria from *Methylobacteriaceae*, *Comamonadaceae* and *Pseudomonadaceae* families.

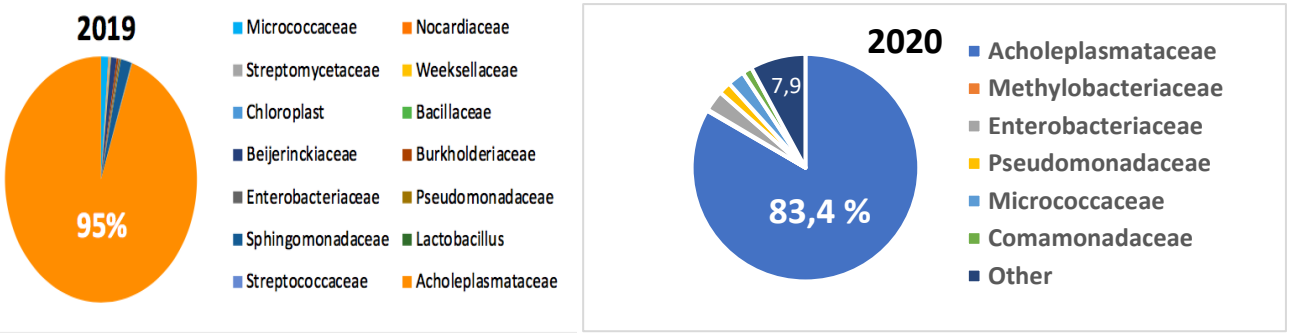

Figure 1. Bacterial family composition in grapevines from Tuscany for the cultivar Sangiovese (2019) and Veneto for the cultivar Glera (2020).

The epidemiology of the disease must be monitored, especially after the recent finding of different vectors/plant hosts species that are very likely responsible for the emergence of new FD variants. Therefore, a continuous and capillary monitoring of the presence and emergence of FD strains associated with the disease in the areas where it is present is necessary for the application of the most appropriate and stringent control measures aimed to avoid the epidemic spreading of possibly virulent FD strains. The preliminary results obtained after bacterial microbiome analyses on grapevine plants infected with "flavescence dorée" phytoplasmas could help the understanding in more details the phytoplasma infection processes and speculating the possible presence of positive and negative relationships among some of different microorganisms living in FD infected grapevines. The knowledge on microbial communities in phytoplasma infected plant host species support research on possible control strategies based also on specific bacteria application to contain phytoplasma-associated diseases reducing at the same time the dangerous application of pesticides against insect vectors.

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SESSION 5

VIRUS CHARACTERIZATION - DIVERSITY

Chair: Olufemi Alabi and Arnaud Blouin

O30. Diversity of grapevine rupestris stem pitting-associated virus in South Australian vineyards

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV; Family *Betaflexiviridae*, genus *Foveavirus*) is associated with grapevine stem pitting disease (Meng et al. 1999; Zhang et al. 1998), a disease of Rugose Wood Complex (RWC). GRSPaV is potentially one of the most widespread viruses globally (Meng and Gonsalves 2007; Meng and Rowhani 2017) and occurs with very high prevalence in Australia (Habili et al. 2006). Previous investigations have revealed that GRSPaV often consists of highly diverged virus populations within the same grapevine (Glasa et al. 2017; Lunden et al. 2010; Meng et al. 2006). The diversity of the virus in Australia has not been studied, except that two groups of symptomatic and asymptomatic variants were detected (Habili et al. 2006). Here, we report the phylogenetic analysis of the virus in various grapevine varieties in South Australia.

MATERIALS AND METHODS

Petioles or dormant canes were collected during autumn (March or May) or winter (June–August) from four grapevine growing regions in South Australia: Willunga (WIL), Langhorne Creek (LC), Barossa Valley (BV), and Urrbrae (UR). Preparation of double stranded RNA (dsRNA) and metagenomic high throughout sequencing (Meta-HTS) were done as described previously (Wu et al. 2023). The libraries were then sequenced on a NovaSeq instrument (Illumina) with a read length of 2 × 150 bp. TrimGalore (v. 0.4.2) was employed to remove Illumina adapters and discard raw reads with a quality score below 20 and length below 50 bp (Krueger 2012). The remaining reads were subjected to *de novo* assembly using SPAdes (v. 3.12.0) (Bankevich et al. 2012). To identify GRSPaV contigs, all assembled contigs were blasted against the latest release of viral sequences in the GenBank database, utilizing the "makeblastdb" function of BLAST+ (v. 2.11.0) (Altschul et al. 1997). GRSPaV contigs were then isolated from other viral contigs using the “grep” shell command. Aligning GRSPaV contigs longer than 8000 nucleotides (nts) with GenBank isolates was performed using Muscle (v. 3.8.31). The alignment was subsequently trimmed based on the shortest sequence, and phylogenetic trees were constructed using the neighbour-joining method with 1000 bootstrap replicates in MEGA (v. 7.0.26). The classification of the four phylogenetic groups was based on a previous study conducted in Slovakia (Glasa et al. 2017). Recombination events of GRSPaV contigs were carried out using the same alignment mentioned above by RDP5 (v. Beta 5.23)(Martin et al. 2021), employing the method described previously (Wu et al. 2023).

RESULTS AND DISCUSSION

A total of 55 complete or near-complete genome sequences were obtained from 15 grapevine varieties, including cv. Shiraz (WIL and LC), cv. Cabernet Sauvignon (WIL and UR), cv. Sultana (UR), cv. Grenache (UR), cv. Merlot (WIL and UR), cv. Tempranillo (UR), cv. Cabernet Franc (UR), cv. Pinot Meunier (UR), cv. Tinta Amarella (UR), cv. Viognier (UR), cv. Biancone (UR), cv. Barbera (UR), cv. Tinta Cao (UR), and cv. Carina (UR). No recombination events were detected in any of GRSPaV contigs that were used for phylogenetic analysis. Phylogenetic analysis of the complete genome sequences revealed the presence of groups 1, 2a, 3, and 4 in South Australia, while groups 2b and 2c were absent (Figure 1).

Among the 20 samples analyzed, it was found that 9/20 grapevines exhibited two distinct GRSPaV strains, 9/20 had three distinct strains, and 1/20 had four strains. Notably, a Portugese variety called Tinta Amarella (prime name: Trincadeira) had six genetically diverse GRSPaV strains (Figure 1). Out of these six strains, two were assigned to group 1, one to group 2a, and three to group 3. The majority of GRSPaV strains belonged to groups 1, 2a, and 3. Group 4 strains were only identified in two varieties, Sultana and Viognier from UR.

This study shows diverged GRSPaV populations in several grapevine varieties in South Australia. This was also reported in other regions globally (Glasa et al. 2017; Lunden et al. 2010; Meng et al. 2006). This suggests that GRSPaV may undergo significant evolutionary processes leading to the emergence of variants with distinct genetic signatures.

Understanding the genetic variability of GRSPaV within grapevines is crucial for deciphering its pathogenicity and determining its association with other viral diseases, especially in Australia where the virus is very widespread.

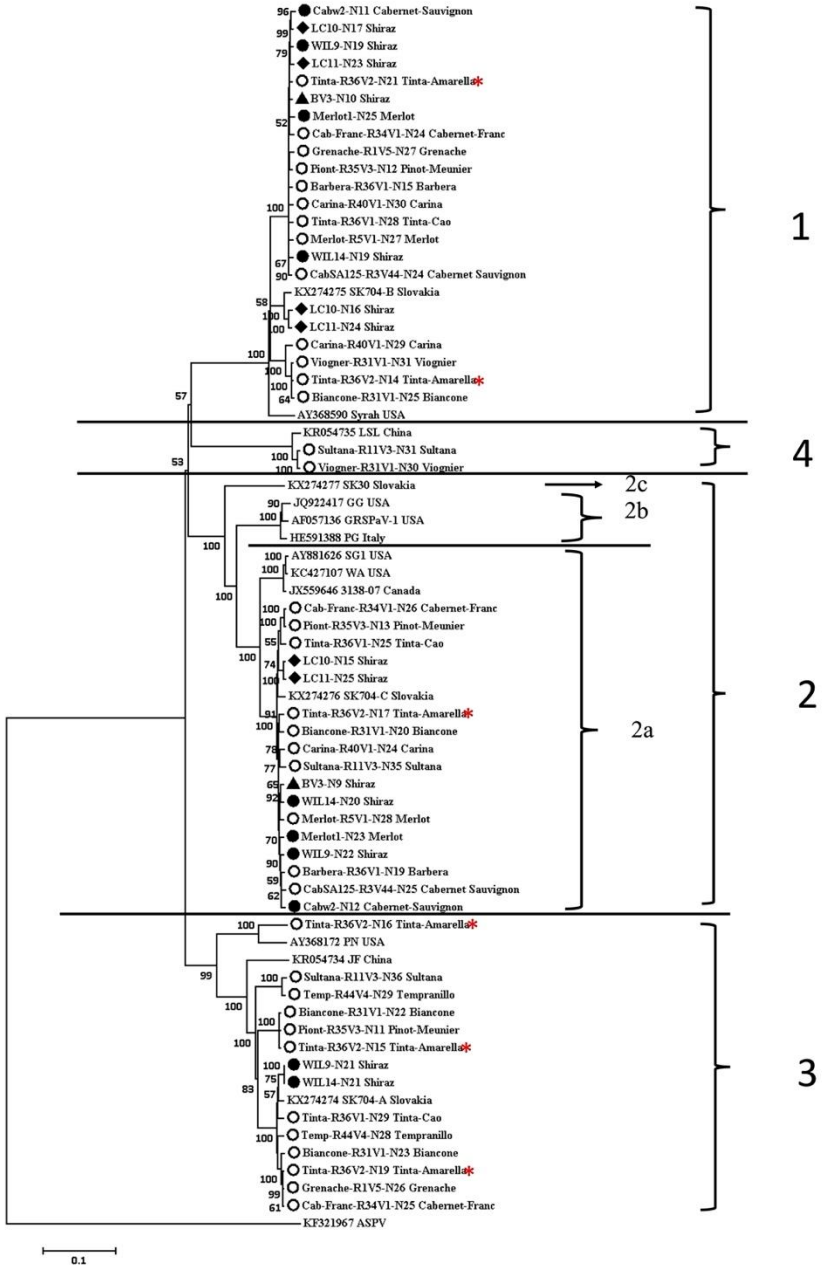

Figure 1. Neighbour-joining phylogenetic analysis of 55 complete or near complete genome sequences of grapevine rupestris stem pitting-associated virus (GRSPaV) and several representative isolates retrieved from the GenBank database. The neighbour-joining tree was constructed using a sequence alignment above 8117 nts using the MEGA (v. 7.0.26) software and the neighbour-joining method with 1000 bootstrap replicates. The hollow circles, solid circles, rhombuses, and triangles indicate the isolates from Urrbrae (UR), Willunga (WIL), Langhorne Creek (LC) and Barossa Valley (BV) of the South Australian (SA) vineyards respectively. A variety named Tinta Amarella had six distinct near full-length genomes of GRSPaV and is marked as *. The boot-strap values below 50% were not shown. Apple stem pitting virus (ASPV) (accession no. KF321967) was used as an outgroup.

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O31. Prevalence of *Grapevine Pinot gris virus* (GPGV) and grapevine leaf mottling anddeformation disease (GLMD) in France: contrasting situations in terms of virus and symptoms

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INTRODUCTION

Grapevine Pinot gris virus (GPGV) was identified by next-generation sequencing (HTS) in Italy from a Pinot gris vine expressing symptoms of chlorotic mottling and leaf deformation (Giampetruzzi *et al.*, 2012). This *Trichovirus* has now been shown to be extremely widespread over the world as reviewed recently by Saldarelli *et al.* (2017). However, this virus is frequently detected in asymptomatic vines and its involvement in GLMD (Grapevine Leaf Mottling and Deformation) disease remains unclear despite all research carried out so far (Saldarelli *et al.*, 2015; Saldarelli, 2017; Tarquini *et al.*, 2023).
A first survey realized in 2016-2017 in France have shown that GPGV was present in different regions with 65% of prevalence (Spilmont *et al.*, 2018). The protocol used (a single sample per plot, corresponding to a mix of plants) did not allow to have a precise view of the situation. A more complete study was thus conducted in 2019-2020 to precise the frequency of GPGV in different viticulture areas and try to correlate with different factors (localization, age, variety…). Specific research on GLMD symptoms was also conducted to identify the varieties expressing symptoms and assess the extent of this disease in France.

MATERIALS AND METHODS

Vineyard survey for GPGV prevalence

A network of 117 "control plots spread throughout France was identified in collaboration with our regional partners. The plots were chosen as representative of the wine-growing area (emblematic variety, localization) and with technical information available (grape variety, rootstock, year of planting…). The "symptom(s)" criterion was not used in this survey to avoid any bias for the plots' selection.
In each plot,15 vines were geolocalized and sampled for GPGV testing via Q-PCR (Bianchi *et al.*, 2015).

GLMD identification

The survey of GLMD disease symptoms was carried out in 2020 and 2021 with the help of our regional partners. First, pictures of symptomatic leaves were taken. Then, leaves were tested by Q-PCR for the presence of GPGV as well as nepoviruses (GFLV, ArMV and TBRV). Only the vines with characteristic symptoms and relevant sanitary status (GPGV positive and nepoviruses negative) were considered "GLMD confirmed" as nepoviruses produce similar symptoms. The photos were then gathered in a database shared with our partners.

RESULTS AND DISCUSSION

Vineyard survey for GPGV prevalence

The GPGV survey was carried out on a network of 117 plots, with 15 vines individually tested per plot. Amongst the 1763 vines tested, 32% were found positive for the virus. GPGV was detected in almost all the wine-growing regions. The situation varies greatly from plot to plot. Close to 70% of the plots tested displayed at least, one infected vine out of fifteen tested (Spilmont *et al.*, 2022).
The analyses focused on 40 grape varieties (dedicated to red and white wine) representing the main important French grape varieties. The infection rate was not correlated with either the scion or the rootstock variety. By contrast, statistical analyses clearly confirm a spatial effect. GPGV appears to be

less prevalent in the north-west region of France (Charentes and the west of the Loire Valley) and more present in the southeast of France (Rhône Valley and Occitanie).

GLMD identification

Identifying this disease remains complex as the symptoms can vary greatly depending on the variety. Amongst the 79 cases of GLMD under consideration, only 26 were confirmed as presenting both typical symptoms and relevant sanitary status (GPGV present and no nepoviruses). This disease was thus identified in 12 varieties from different regions: Champagne, Alsace, Burgundy, Occitanie and Bordeaux (Spilmont *et al.*, 2023).

Globally very few cases have been signaled during the survey except for Champagne, where the situation appears to be worsening (Mathieu *et al.*, 2023, this symposium). In this region, symptoms appear limited to the base of the shoots, but they concern many vines per plot. In other regions, particularly in the Mediterranean area, the symptoms pattern seems different, with a small number of isolated vines showing leaf deformations and discolorations all over the vine, sometimes associated with stunting. This can be associated with a total loss of production.

To help professionals to identify these symptoms, a photographic database of the relevant symptoms in each variety has been compiled.

No French region is totally free of GPGV, although two regions seem to be less affected (Charentes and the west of the Loire Valley). By contrast, the survey indicated a limited occurrence of GLMD symptoms in France apart from Champagne. The identification of highly contrasting situations in terms of symptoms observed in French (location, frequency, typology, expansion…) raises questions about both the cause and the possible outcome.

ACKNOWLEDGEMENTS

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O32. Molecular characterization of divergent isolates of grapevine red blotch virus from Blanc du Soleil, an interspecific hybrid white grapevine cultivar

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INTRODUCTION

The compete genomes of grapevine red blotch virus (GRBV) isolates consists of a single component of circular ssDNA of 3,205 to 3,208 nucleotides (nt) and encoded up to seven bidirectional, overlapping open reading frames (ORFs) (Cieniewicz *et al.,* 2017; Vargas-Asencio *et al.*, 2019). The GRBV virion-sense (n=4) and complementary-sense (n=3) ORFs are separated by a short intergenic region (SIR) between the V3 and C1:C2 and a long intergenic region (LIR) between the V0 and C1 cistrons. Current GRBV evolutionary data supports the existence of two distinct phylogenetic clades I and II, with up to 6.1% and 4.6% intra-clade variability exiting within them, respectively and 3.7-9.2% recombination-independent interclade variability (Cieniewicz *et al.*, 2020). Here, we report the occurrence and molecular characterization of a distinct GRBV genetic variant that was detected in a recently released interspecific hybrid white-fruited wine grapevine cultivar Blanc du Soleil (BdS).

MATERIALS AND METHODS

The diagnostic primer pair p1282-F/p1283-R (Perry *et al.*, 2016) was used to detect GRBV in several vines of BdS in a south Texas vineyard and in dormant canes of the source propagative materials from Florida A&M University (FAMU) that were received under USDA-PPQ-526 permit (P526P-22-05386). The ~3.2-kb complete GRBV genome fragment was amplified from multiple BdS samples by PCR using a pair of newly designed abutting primers GRBV-1402v/GRBV-1404c (Ouro-Djobo *et al.*, 2023) and rolling cycle amplification products as templates. Each sample-specific amplicon of ~3.2 Kb was excised, gel purified, and cloned into the pJET1.2 plasmid vector, followed by Sanger sequencing of four or more recombinant plasmid DNA samples per cloned DNA. Complete GRBV genomes were assembled per plasmid and bioinformatically analyzed (Ouro-Djobo *et al.*, 2023).

RESULTS AND DISCUSSION

Nine of the obtained BdS GRBV complete genomes (TX=7; FL=2) were 3,183-3,181 nt, indicating that they were shorter in length by 23-27 nt compared to published global GRBV sequences. The genome size reduction in the BdS GRBV isolates occurred in the LIR where two fixed deletions and a single nucleotide insertion were observed (Fig. 1). Notably, none of these indels affected the number, arrangements, and lengths of encoded virion- and complementary-sense ORFs of GRBV. Phylogenetic reconstruction revealed a monophyletic clade for the divergent (short) BdS GRBV variants basal to clade II (Fig. 2A). A phylogenetic network analysis displayed multiple reticulate edges adjacent to BdS GRBV leaf nodes (Fig. 2B), suggesting the involvement of recombination in their evolution. Tests for recombination identified three of the nine divergent BdS GRBV isolates from Florida (FLBDS92, FLBDS94) and Texas (TXBDS711) as putative recombinants with strong statistical supports (data not shown). Thus, a combination of recombination and genetic mutations likely shaped the evolution of the variant GRBV isolates obtained from BdS. This is the first report of the occurrence of GRBV in Blanc du Soleil, a white hybrid wine grapevine cultivar newly released for cultivation in regions where

Pierce's disease is a limiting factor to the propagation of *V. vinifera* cultivars. The genetic homogeneity of the variant BdS GRBV sequences obtained from both Florida and Texas, coupled with the monophyletic origin of all the nine divergent BdS variants may suggest GRBV dispersal via contaminated vines. Hence, intensive testing for GRBV may be needed for newly released *Vitis* sp. cultivars and breeding lines to be used by the industry, given its documented negative impacts (Bowen *et al.*, 2020). The potential biological consequence of the GRBV LIR indels is of interest considering that the IR of geminiviruses may contain bidirectional promoters that control the expression of the viral virion- and complementary-sense genes (Cantú-Iris *et al.*, 2019 and cited references).

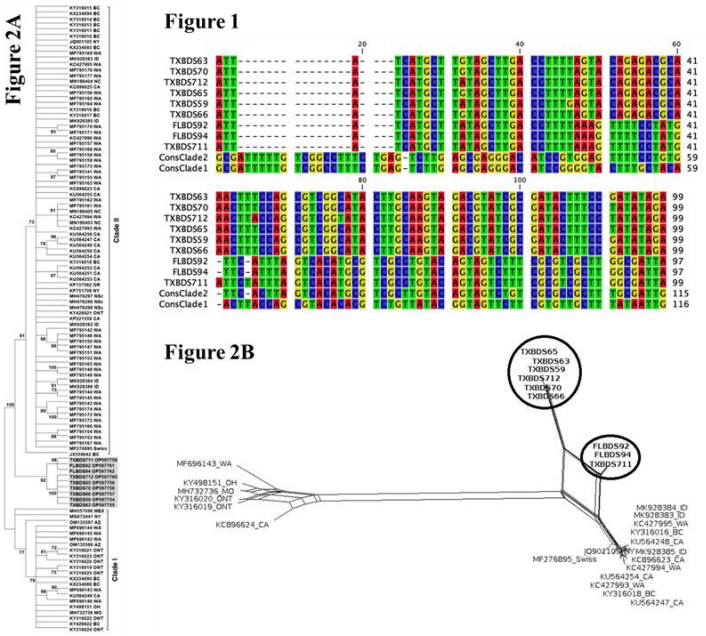

Figure 1. A compact informative view of only the "non-trivial segregating sites" in a multiple alignment of variant isolates of GRBV sampled from BdS and clade I and clade II consensus sequences.

Figure 2. Evolutionary analysis of divergent variants of GRBV derived in this study from BdS and GenBank verified sequences. **(A)** Unrooted maximum likelihood phylogenetic tree (500 bootstrap replicates); **(B)** Phylogenetic network analysis. The GRBV isolates obtained in this study (GenBank accession numbers OP597754 to OP597762) are highlighted.

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O33. Study of the etiology of grapevine leafroll disease in Greek vineyards and molecular characterization of the associated viruses

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INTRODUCTION

Grapevine leafroll disease (GLD) is one of the most important viral diseases of grapevine worldwide. Typical symptoms of the disease are the red or reddish-purple discolorations and mild yellowing or chlorotic mottling in interveinal lamina in red-berried and white-berried cultivars, respectively, in addition to downward rolling of the leaves (Naidu et al., 2014; Martelli, 2014). Several members of the family *Closteroviridae* have been associated with GLD (Martelli et al., 2012). Grapevine leafroll-associated virus 1 (GLRaV-1), GLRaV-3 and GLRaV-4 are members of the genus *Ampelovirus*, GLRaV-2 is classified in the genus *Closterovirus*, while GLRaV-7 belongs to the genus *Velarivirus* (Martelli et al., 2012). The former viruses GLRaV-5, GLRaV- 6, GLRaV-9, GLRaV-De, GLRaV-Pr, and GLRaV-Car are designated as strains of GLRaV-4, based on their common biological, epidemiological, serological, and genomic characteristics (Martelli et al., 2012). Recently, a novel putative member of the genus *Ampelovirus* was also identified in grapevines that showed typical leafroll symptoms and named GLRaV-13 (Ito and Nakaune, 2016). Even though the GLRaVs are known to occur in Greek vineyards, no extensive study has taken place previously on the etiology of GLD. Therefore, a survey was conducted on grapevines showing typical symptoms of GLD, using HTS analysis and RT-PCR methods (real-time and conventional), while phylogenetic analysis of the identified GLRaVs was also performed in order to examine the genetic variability of the Greek isolates of GLRaVs and identify any possible correlation between intensity of symptoms and virus sequences.

MATERIALS AND METHODS

In total, 175 grapevine samples were collected from 43 commercial vineyards of 9 viticultural areas of Northern Greece. Fifty-four (54) of the collected samples were showing mild GLD symptoms (mild reddening or yellowing and/or mild leafroll), 107 of them had severe GLD symptoms (severe reddening or chlorotic mottling and leafroll) while 14 samples were showing no GLD symptoms (asymptomatic). Several of the collected samples were subjected to HTS analysis; 16 samples from 8 different commercial vineyards, 1 with typical GLD symptoms and 1 asymptomatic per vineyard, and 21 samples from vines showing mainly severe GLD symptoms collected from several commercial vineyards. Sample preparation, HTS procedure and data analysis were conducted as described by Panailidou et al. (2023). Total RNA was extracted using the modified RNA-extraction protocol developed by Ruiz-García et al. (2019) and the HTS run was performed on a NovaSeq6000 platform (Illumina, Inc.) at Macrogen Inc. (Seoul, S. Korea). The 175 grapevine samples collected were further tested for the presence of GLRaV-1, GLRaV-2 and GLRaV-3 by real-time RT-PCR assays and for GLRaV-4 by conventional PCR. In the cases of GLRaV-1, -2 and -4 new methods were developed in order to accommodate the high genetic diversity of these viruses. Four phylogenetic trees were constructed based on the complete sequences of every GLRaV obtained by HTS analysis (Greek isolates) and the corresponding isolates that are deposited in GenBank (Foreign isolates). The alignment, the selection of the substitution model, and the construction of the phylogenetic tree were carried out using the MEGA X bioinformatics suite (Kumar et al., 2018). Genetic variability studies of three genes (RdRp, HSP70 and CP) of every GLRaV were also conducted, using the corresponding genome regions from Greek and foreign isolates which were compared using Geneious Prime software (https://www.geneious.com/prime/) after alignment with MAFFT.

RESULTS AND DISCUSSION

Comparing HTS results from symptomatic and asymptomatic vines revealed that GLRaV-1 and GLRaV-3 were present in most of the symptomatic samples, with GLRaV-3 being the most prevalent. GLRaV-2 was identified only in 3/8 symptomatic vines tested while GLRaV-4 was found in both symptomatic and asymptomatic vines in the surveyed vineyards. In the 21 samples with severe symptoms of GLD submitted to HTS analysis, GLRaV-3 was the most prevalent virus as it was found in 89.7% of the samples, GLRaV-4 showed also high prevalence (79.3%), while GLRaV-1 and GLRaV-2 were detected in 41.38% and 27.6% of the samples analyzed, respectively. In most cases high frequency of mixed infections with several isolates of different GLRaVs was recorded. The application of the RT-qPCR assays also revealed the presence of GLRaVs in all type of samples originating from symptomatic or asymptomatic vines. Specifically, GLRaV-1 and GLRaV-3 were detected in 24.57% (42/161 symptomatic, 1/14 asymptomatic) and 67.43% (113/161 symptomatic, 5/14 asymptomatic) of the tested samples, respectively, thus indicating a strong association of GLRaV-3 with GLD as previously mentioned by Naidu et al. (2014), Burger et al. (2017) and other researchers. On the other hand, GLRaV-2 and GLRaV-4 were also detected in a high number of tested samples. Specifically, GLRaV-2 was present in 99/161 symptomatic and in 8/14 asymptomatic vines while GLRaV-4 was detected in 68/161 symptomatic and 5/14 asymptomatic vines indicating their possible secondary role in disease symptomatology, probably affecting the intensity of the manifestation of the symptoms. The possibility of a correlation between the isolates and the intensity of symptoms was examined by the phylogenetic analysis, however no correlation existed in most cases, with the exception of the GLRaV-4 stain 10 isolates which were isolated only from asymptomatic samples and formed a divergent phylogenetic group. In addition, it seems that high genetic variability characterizes the RdRp, HSP70 and CP genes of the Greek isolates of the four viruses, with higher diversity levels appearing at the RdRp gene of GLRaV-1 and the HSP70 and CP genes of GLRaV-2 and GLRaV-4 both in nucleotide and amino acid level.

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SESSION 6

PLANT-VIRUS-VECTOR INTERACTIONS

Keynote speaker: Baozhong Meng

Chair: Baozhong Meng and Emmanuelle Vigne (part I) / Nadia Bertazzon and Gerard Demangeat (part II)

O34. The physiological significance of GLRaV-3 infection and its interaction with grapevine host over time

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3), a globally distributed *Ampelovirus* infecting Vitis plants, is still an enigma for all changes it triggers in grapevine host. It is the main causal agent of grapevine leafroll disease (GLD), provoking symptoms in grapevine hosts while disturbing sugar and anthocyanin metabolism (Song *et al.*, 2021). So far, genomic variants of GLRaV-3 are classified into eight phylogenic groups (Diaz-Lara *et al.*, 2018), with biological properties of individual variants still unknown in terms of symptom development and changes in plant metabolism, unlike those defined in other members of *Closteroviridae* family. Complex GLRaV-3 interactions with other viruses, scion-rootstock combinations and environmental factors pose as limiting factors in assessing and defining mechanisms related to the disease development (Naidu *et al.*, 2014). In this study changes in grapevine physiology and symptom development were investigated to elucidate *Vitis vinifera* response to different virus inoculums which either contained solely GLRaV-3 (LR isolates containing different GLRaV-3 genomic variants) or in coinfections with other economically important viruses (WT isolates).

MATERIALS AND METHODS

Virus free *Vitis vinifera* plants of three standard GLD indicator varieties ('Pinot Noir', 'Merlot', 'Cabernet Franc'; Martelli, 1993) and Croatian indigenous variety 'Tribidrag' were inoculated with LR and WT isolates by chip budding method. LR isolates were composed of monophyletic inoculums I, II, III, VI and VII and combinations of I/II, I/III, II/III and I/II/III, while WT isolates were composed of four GLRaV-3 mixed inoculums with other viruses, most often found in native vines of Croatian South Adriatic region. Virus transmission was confirmed by RT-PCR. In three-years long experiment, set up in the greenhouse, 36 different parameters potentially contributing to a specific virus-induced pathobiological profile were investigated. Measurements were performed 6 and 18 months post inoculation. Grapevine host response to infection was characterized through changes in nutrient status, oxidative stress parameters, primary metabolism as well as symptom expression. Data was analyzed using Discriminant analysis of principal components (DAPC; Jombart *et al.*, 2010) for each observed measurement and ANOVA test ($p<0.05$) for each individual parameter.

RESULTS AND DISCUSSION

Six months post inoculation, drastic changes had affected all varieties which in such short time already clearly discriminated control from the infected plants by DAPC method. In that period Merlot pops out as a variety with faster and more intense reaction than other varieties to virus infection, regardless of virus inoculum used (LR or WT). Clear separation amongst varieties achieved by DAPC method also proves that grapevine response to virus infection is variety-dependent. With longer infection, the differences among infected and non-infected control plants became more obvious.

Changes related to oxidative stress response had occurred in virus infected vines with greater frequency compared to non-infected ones in all varieties. Superoxide-dismutase, peroxide and total proteins were the parameters that had been most susceptible to changes caused by viral infection, regardless of the

type of inoculum, the host and the duration of the infection. For these parameters no general trend was observed, in some cases their content was significantly higher, in others lower than in non-infected controls. Concentration of malondialdehyde (MDA) increased significantly for all LR and WT inoculums in all varieties, but with longer infection.

Primary metabolism was also affected by the infection, where the concentration of carotenoids was significantly lower for LR and WT isolates 18 months post inoculation. Same trend was observed for chlorophyll a and b concentrations which were significantly reduced in LR and WT infected plants with longer infection. Nutrient status was altered as well for LR and WT infected plants, especially the concentration of N that was significantly lower for most of the LR infected plants in both observed years and Fe which was lower for both LR and WT infected plants in the first vegetation season.

No clear biological pattern was associated for different genomic variants of GLRaV-3, even though they differed in provoking changes in grapevine host. This was particularly the case for genomic variants VI and VII, that successfully provoked physiological changes in all varieties, while plants developed mild or no symptoms at all.

Symptom development varied amongst observed measurements, being more expressed in standard indicator varieties during the second growing season. 'Merlot' plants developed symptoms in the first growing season (WT infected plants) and 'Tribidrag' plants did not develop typical symptoms of GLD in both observed years even though its oxidative stress parameters were altered along with many others. This indicates that 'Tribidrag' variety is more tolerant to symptoms development than other varieties used in this experiment.

In conclusion, GLRaV-3 influence on grapevine host plants is defined through changes it causes in oxidative stress response, nutrient status, primary metabolism and symptom development. These changes are mostly variety and inoculum dependent. Changes in oxidative stress parameters could be considered as cellular markers for GLRaV-3 infection. Longer infection provokes severe symptoms in all indicator varieties and more expressed changes in host plant metabolism. Presence of different GLRaV-3 genomic variants and/or other viruses modulates disease development in grapevine.

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O35. Establishment of experimental systems to elucidate the localization and functions of unique open reading frames of *Grapevine leafroll-associated virus 3*

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is the most widespread and devastating virus impacting the global wine and grape industry (Burger et al., 2017). GLRaV-3 is the putative agent of grapevine leafroll disease (GLD). As a member of the family *Closteroviridae*, GLRaV-3 contains one of the largest genomes of the positive-sense single-stranded (+ss) RNA viruses with 12-13 open reading frames (ORF). Several of these ORFs, including *orf8*, *orf9* and *orf10*, (encoding proteins p21, p20A and p20B respectively) are unique to the virus and little is known about their function. Of the viral sub-genomic RNAs produced by GLRaV-3 during infection, those for the coat protein, and *orf8*, *orf9* and *orf10* are the most abundant in infected hosts (Jarugula et al., 2010). This suggests a consequential role in the infectious cycle of the virus. ORFs located in equivalent positions in the genomes of other viruses of the *Closteroviridae* family are known to play roles in suppressing the RNA silencing system of host defenses or systemic spread of the virus throughout the host (Donda et al., 2017). p20B has been shown to act as a viral RNA silencing suppressor (VRSS) and a potential determinant of pathogenicity for the virus (Gouveia et al., 2012). Our primary research objective is to characterize these ORFs and elucidate their role in the cycle of infection of GLRaV-3. To this end, *orf8*, *orf9* and *orf10* have been individually cloned, tagged with fluorescent proteins, and ectopically expressed within *Nicotiana benthamiana* leaves to observe their subcellular localizations.

MATERIALS AND METHODS

orf8, *orf9* and *orf10* were individually cloned from GLRaV-3 isolate 623 into pRTL-2:*EGFP* or pRTL-2:*mRFP* expression vectors and transformed into *Escherichia coli* DH5α. These cells were grown on agar containing ampicillin to select for transformed cells containing the ampicillin resistance gene *bla* encoded by pRTL-2. Colonies were cultured and lysed to isolate plasmids. Expression cassettes were excised using restriction digestion, subcloned into the binary vector pCAMBIA-0380, and transformed into *E. coli* DH5α. Cells were grown on agar containing kanamycin to select for transformed cells containing kanamycin resistance gene *nptIII* encode by pCAMBIA-0380. Colonies were cultured and miniprepped. Recombinant binary plasmids were transformed into *Agrobacterium tumefaciens* EHA105. Transformed *A. tumefaciens* cultures were then agro-infiltrated into the abaxial leaf tissue of 5-week-old *N. benthamiana* plants. 48 hours post-infiltration, cuttings from leaves were examined using the Leica Sp5 confocal laser scanning microscope (CLSM) system equipped with 488 nm Argon and 543 nm He-Ne lasers.

RESULTS AND DISCUSSION

When exogenously expressed in the leaf tissue of *N. benthamiana,* p21, p20A and p20B appear to localize to the cytosol, cortical microtubules, and the nucleus respectively. When leaf tissues expressing p21-EGFP were examined, a non-specific and diffuse pattern was observed (Fig 1A). When leaf tissues expressing p20A-mRFP were examined using CLSM, a pattern of thin cell spanning filaments was observed, characteristic of the cytoskeleton (Fig. 1B). However, when the p20A-mRFP construct was co-infiltrated with a green-fluorescent actin marker, no clear co-localization was observed. This suggests the fluorescent pattern may represent cortical microtubules, the other major cytoskeletal structure. Many other +ssRNA viruses, including those infecting grapevines, have been reported to interact with microtubules to facilitate VRC formation and viral movement (Niehl et al.,

2013). In support of the microscopy results, *in silico* structural prediction of p20A shows multivalent interfaces made up of positively charged exposed residues, a feature of microtubule-binding domains (Drechsler et al., 2019). When leaf tissues expressing p20B-EGFP were examined, a clear highlighting of the nucleus was observed. This was especially noticeable when co-infiltrated with a red fluorescent protein, which has a non-specific cytosolic distribution that passively diffuses and accumulates in the nucleus. When red and green images were merged, a significant color shift was only observed in the nucleus (Fig 1C.). The amino acid sequence of p20B was also analyzed using Plant-mPLoc and Identification Nucleus Signal Peptide software, which predicted a classical monopartite nuclear localization signal between position 109 and 120. There are several identified viral nuclear RNA silencing suppression strategies, one such example being the RNA-silencing suppressor protein p19 of tombusviruses (Hussain et al., 2021). In the nucleus, p19 sequesters siRNAs and miRNAs with high affinity, preventing them from being incorporated into Argonaute 1 (AGO1). Conversely, miR168, a miRNA that targets AGO1 mRNA, does not bind efficiently to p19, allowing it to be loaded into AGO1 and repress the translation of additional AGO1 mRNA, an essential component of viral RNA silencing (Várallyay et al., 2010). Furthering our understanding of these unique proteins could provide insight regarding GLRaV-3's paramount impact compared to other grapevine leafroll-associated viruses and lead to preventative or treatment options against GLRaV-3 infection.

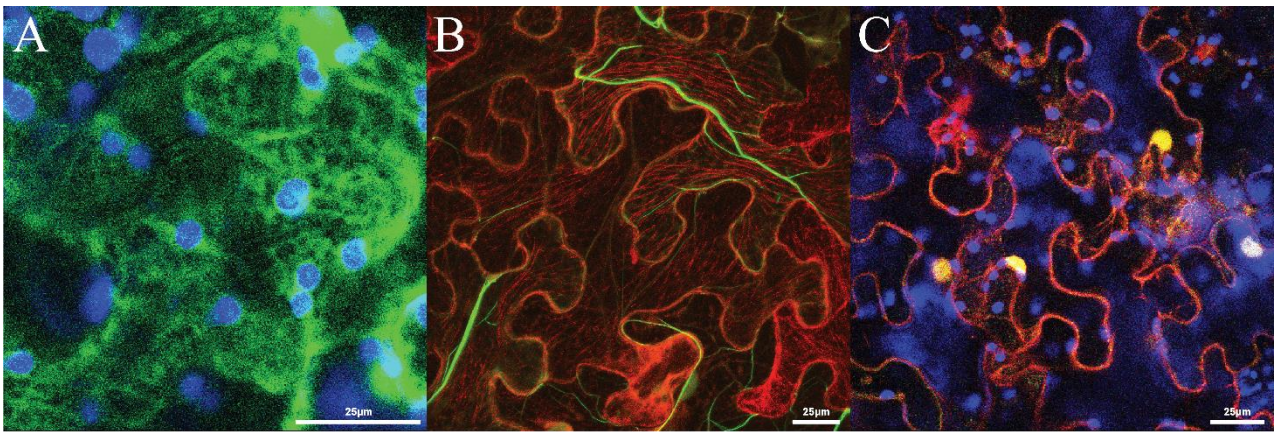

Figure 1. CLSM of *N. benthamiana* transiently expressing GLRaV-3 p21-EGFP, p20A-mRFP and p20B-EGFP. (A) *N. benthamiana* agro-infiltrated with p21-EGFP. (B) *N. benthamiana* leaf tissue agro-infiltrated with p20A-mRFP and mTalin-GFP. (C) *N. benthamiana* agro-infiltrated with p20B-EGFP and mito-mRFP. Chloroplast autofluorescence was captured and pseudo-coloured blue.

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O36. Evolution of grapevine leafroll-associated virus 3 in the alternative host *Nicotiana benthamiana*

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INTRODUCTION

Grapevine (*Vitis vinifera*) is susceptible to at least 86 viral infections, among which grapevine leafroll-associated virus 3 (GLRaV-3) is the most economically destructive worldwide (Fuchs, 2020). GLRaV-3 infection is associated with grapevine leafroll disease, causing reduced quantity and quality of yield. Despite the disease associated with GLRaV-3 in vineyards there is no cure, only costly and laborious management strategies. A preferable long-term and cost-effective solution is a mild strain of GLRaV-3, which confers mild strain cross-protection (MSCP).

As an RNA virus, GLRaV-3 is highly susceptible to mutations. To date, GLRaV-3 genotypes have been grouped into ten genetic variants (Diaz-Lara et al., 2018). Among some GLRaV-3 genetic variants, functional differences have been observed, including virus load/distribution, and symptom onset/severity, which may relate to altered virus-encoded suppression of RNA silencing (VSR) activity (Chooi et al., 2022; Chooi et al., 2016; Gouveia & Nolasco, 2012; Rohra, 2019).

Previous research has demonstrated GLRaV-3 infection in wild-type *Nicotiana benthamiana* via *Planococcus ficus* transmission (Prator et al., 2017). GLRaV-3 infection of *N. benthamiana*, an RNAi-compromised plant, creates a novel opportunity for virus evolution. Potentially, the re-specialization of GLRaV-3 to *N. benthamiana* could result in novel GLRaV-3 genotypes with attenuated virulence, with the potential to be a mild strain when returned to grapevines.

MATERIALS AND METHODS

Ten serial passages of GLRaV-3-infected *N. benthamiana* were conducted *in vitro* over 22 months. From a single donor grapevine three GLRaV-3 groups were passaged in eight lines of *N. benthamiana* plantlets (12 plantlet bioreps per line, total n=96); GLRaV-3 Group I (1 line, n=12), VI (2 lines, n=24), I+VI (2 lines, n=24), VI+X (1 line, n=12), and a VI-LSS (2 lines, low spread strain; n=24). RNAseq (Novogene, Beijing, China) of the donor grapevine, a GLRaV-3-negative *N. benthamiana* line, and each GLRaV-3-positive *N. benthamiana* line at passages 0 and 10 was undertaken using RNA libraries (n=18) containing apical sections from 3–4 plantlets. Additionally, local VSR activity of the donor and evolved GLRaV-3 p19.7 proteins were assessed by *Agrobacterium* transient GFP expression assay.

RESULTS AND DISCUSSION

For all GLRaV-3 passaged genetic variants, mutations were observed across the genomes (Figure 1). In total from RNAseq data, 79 mutations were identified, of which 39 were non-synonymous. Of the 39 non-synonymous mutations, 15 were present at host-jump (passage 0) and 21 evolved during passaging (present in passage 10 but not 0). The higher proportion of evolved mutations may indicate acclimation of GLRaV-3 to its novel host. Evolved mutations in the p19.7 gene, E62G (Group VI), and Y78H and K103M (Group X) were characterized for functional activity (Figure 2). All p19.7 proteins with amino acid mutations had less VSR activity than the donor version. The most significantly attenuated VSR activity was from p19.7 Group X K103M (~4-fold less than donor).

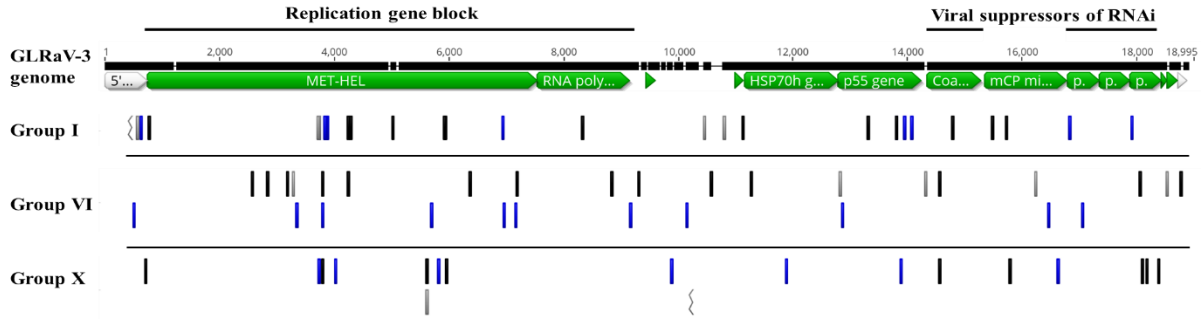

Figure 1. Grapevine leafroll-associated virus 3 (GLRaV-3) genome illustrating the location of mutations in Group I, VI, and X genetic variants during serial passaging in *Nicotiana benthamiana*. Bars in gray represent mutations unique to the passage 0 host-jump (non-retained), black represents mutations unique to passage 10 (evolved), blue represents mutations shared at passage 0 and passage 10 (retained).

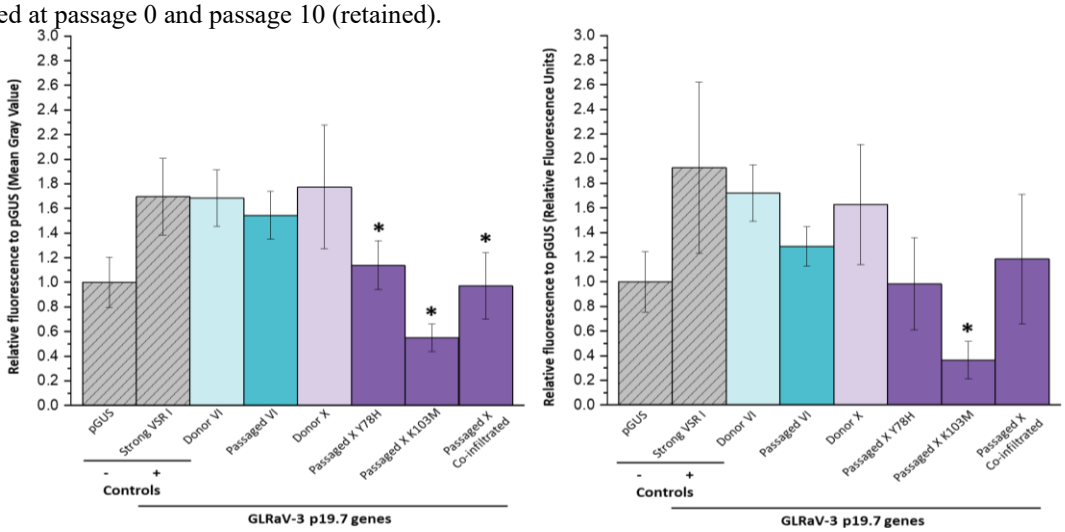

Figure 2. Local suppressor of RNAi activity of grapevine leafroll-associated virus 3 (GLRaV-3) p19.7 proteins derived from Group VI and X cloned donor and evolved genes. Relative fluorescence to pGUS assessed by (A) ImageJ (mean gray value) of infiltration images and (B) spectrophotometer (relative fluorescence units) of infiltrated leaf tissue. pGUS and GLRaV-3 Group I p19.7 proteins were used as negative and positive controls, respectively (gray striped bars). GLRaV-3 p19.7 Group VI activity (blue) and Group X evolved genotypes, Y78H and K103M (purple). Error bars represent standard deviations. Statistically significant changes between evolved and donor p19.7 proteins ($p < 0.0167$) are indicated by asterisk, inferred by median.

This is the first study to conduct and assess the genetic and functional evolution of GLRaV-3 in *N. benthamiana*. The resulting strains of GLRaV-3 may have potential application in MSCP as a long-term management strategy against GLRaV-3 for the New Zealand wine industry.

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O37. Characterization of the GRLaV-3 population among symptomatic and asymptomatic grapes

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INTRODUCTION

Grapevine leafroll disease (GLD) is a class of viral disease that affects the quality and vitality of grapevines throughout the world. One of the main viruses associated with GLD is the *Grapevine leafroll associated virus-3* (GLRaV-3), represents the genus Ampilovirus within the *Closteroviridae* family (Maree et al. 2013). GLRaV-3 considered to be the primary and most prevalent causal pathogen of GLD, though the etiology of the virus is not well characterized yet. Presently, in Israel, there are 4500-5000 hectares of vineyards planted across the country. The vegetative propagation of *Vitis vinifera*, and the ongoing search for new types and varieties have all contributed to the wide spread of the GLD throughout the years (Cabaleiro and Segura 2006). Interestingly, among vineyards that were found to be severely infected with the GLRaV-3, we occasionally observed a few virus infected but asymptomatic grapevines, which inspiring us to study and explore the viral populations within such symptomatic- and asymptomatic GLRaV-3-infected plants.

MATERIALS AND METHODS

Total RNA preparations from selected GLRaV-3-infected symptomatic and asymptomatic grapevines were extracted and used for Next Generation Sequencing (NGS) followed by bio-informatics analyses performed for virus detection. The genes encoded the RNA silencing suppressor (RSS) proteins (*p21*, *p20A* and *p20B*) (Gouveia et al. 2012) of GLRaV-3 variants from symptomatic and asymptomatic grapevine plants were cloned into pCAMBIA based expression vectors. Suppression of RNA silencing activity induced by these proteins was examined by co-expression of *p21*, *p20A* or *p20B* with a GFP tagged GVA269 minireplicon followed by confocal microscopy analysis (Brumin et al., 2009).

RESULTS AND DISCUSSION

In all tested plants, sequences of GLRaV-3 were found to be highest levels among other virus sequences. In symptomatic grapevines, various GLRaV-3 genotypes were detected, whereas, in asymptomatic grapevines, only one GLRaV-3 genotype was detected, which was found to be highly similar to the GLRaV-3 12G446 sequence reported in the GenBank data. Along with the other GLRaV-3 genotypes, this 12G446 like genotype was found to be also in symptomatic grapevines. Our research results suggest that GLRaV-3 is the primary cause of GLD, and that GLRaV-3 12G446-like variant is a mild variant. Mild virus variants considered are as highly valuable tools that can potentially utilized for cross protection and for control of plant virus diseases. Next, we investigate the activity and synergism of GLRaV-3 RNA silencing suppressor (RSS) proteins: *p21*, *p20A* and *p20B* by utilizing the GFP-tagged GVA minireplicon (GVA269) to trigger the silencing defense mechanism in *N. benthamiana* plants. Expression of GVA269 and a viral RSS protein resulting on green fluorescence that can be monitored via confocal microscopy even at 10 days post infection or infiltration (Melnyk et al. 2011). Among these 3 RSS proteins, the *p20B* was suggested to be with the highest RSS activity. Yet, co-expression of the different combinations of the three proteins induced the RSS activity, suggesting a synergism effect.

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O38. Comparative functional analysis of the movement and coat protein encoded by symptomatic and asymptomatic GPGV variants

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INTRODUCTION

During our previous project, using small RNA HTS, we have found widespread distribution of grapevine Pinot gris virus (GPGV) in Hungarian vineyards and in non-*Vitis* host plants (Demian et al., 2020; Czotter et al., 2018). This Trichovirus is putatively associated with a grapevine disease known as grapevine leaf mottling and deformation (GLMD). Yet the role of GPGV in GLMD disease is poorly understood. GPGV can be present in both symptomatic and asymptomatic grapevines, and although there are several hypotheses and observations the relationship between the presence of the virus and the appearance of symptoms is not yet fully clarified. The most significant difference in which symptomatic and asymptomatic variants of the virus differ is the C/T polymorphism in movement protein (MP). The symptomatic variant encodes a six amino acids shorter movement protein due to an early stop codon. During viral infection, RNA interference, the highly effective and specific defense reaction of the host, is activated. As a counter defence strategy of plant antiviral RNAi, viruses evolved proteins (VSR) that suppress various steps of the RNA silencing mechanism. Tarquini and coworkers (2021) revealed the ability of the GPGV coat protein (CP) encoded by ORF3 to suppress RNA silencing. In our study, we investigated and compared the potential VSR activity of the coat and movement proteins encoded by symptomatic and asymptomatic GPGV variants and looked for reasons which can explain differences in symptom development caused by these two strains.

MATERIALS AND METHODS

The VSR activity can be easily investigated in a transient gene expression system: where the potential VSR coding region of the GPGV was cloned into a binary plasmid, conjugated to *Agrobacterium tumefaciens* C58C1 strain, and then infiltrated into 3-week-old *Nicotiana benthamiana* leaves by co-infiltration. In co-infiltration experiments, GFP-expressing *Agrobacterium* and the potential VSR coding ORFs were mixed 0.4:0.6 ratio. The GFP fluorescence of infiltrated leaves (local silencing, 4dpi) and whole plants (systemic silencing, 21dpi) were examined visually by UV lamp and photographed with digital camera. The level of GFP and potential VSR protein expression was determined by Western blotting while the level of GFP mRNA expression was determined by real-time PCR.

RESULTS AND DISCUSSION

In our agroinfiltration experiments, in line with Tarquini's work, we proved that ORF3, which encodes the virus's coat protein, can suppress local RNA silencing. This suppressor effect was slightly stronger in case of the symptomatic variant. Beside local silencing, VSRs can prevent the spread of silencing signal in the plant. Investigating this feature of the ORF3 of GPGV revealed that neither symptomatic nor asymptomatic ORF3 variant have systemic silencing activity. We also tested VSR activity of the ORF2 encoded movement protein but have found neither of the two variants showing local suppressor activity. Systemic silencing activity was not observed in the case of either symptomatic or asymptomatic ORF2 variant.

However, transient expression of the ORF2 variants caused strong necrosis on the infiltrated leaf, especially in case when the symptomatic version (6 amino-acid shorter version of the MP) was used. Necrosis occurred in significantly fewer cases and much milder when the asymptomatic variant was

tested. The C/T polymorphism, which results in the symptomatic variant encoding a six amino acid shorter MP, is an important factor, but not the only one which differentiate the movement protein between the symptomatic and asymptomatic variants. There are several amino-acid changes in the MP/CP border of the virus, which role and effect are currently unknown (Fig. 1).

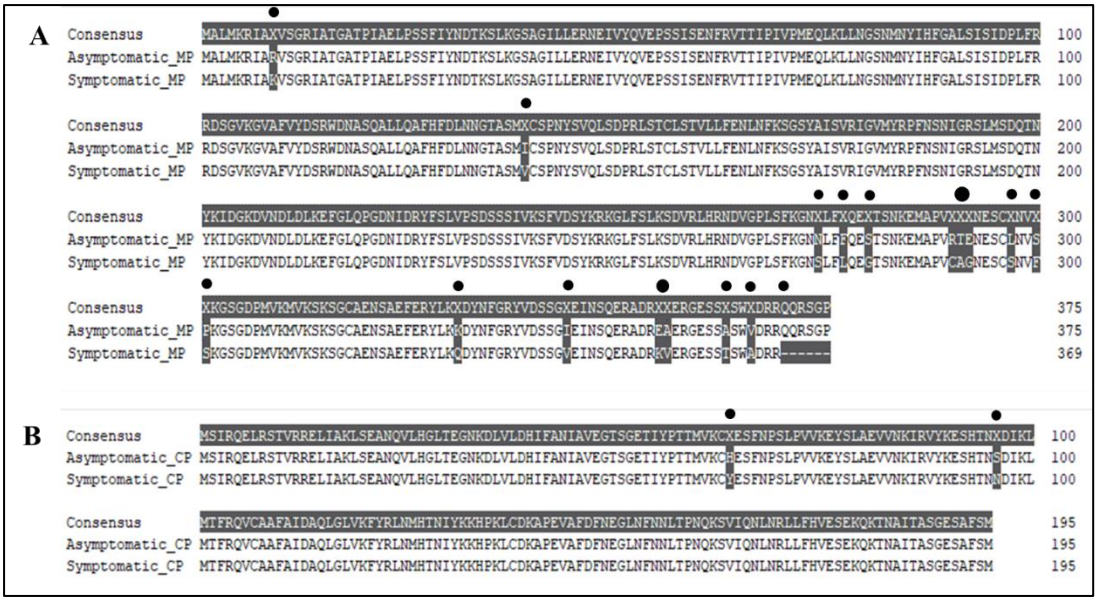

Figure 1. Alignment of the Hungarian symptomatic and asymptomatic GPGV variants, highlighting the amino acid substitutions in the MP (A) and CP (B) encoded part of the virus.

To localize the differences that could bring us closer to the cause of necrosis we performed modifications in the 3' region of the MP combining symptomatic and asymptomatic GPGV ORF2 variants features. The different mutant variants necrotized the leaf in different ways, and it seems that the polymorphisms at the 3' end of MP may play a crucial role in this process.

In conclusion, SNPs at the 3' ends of MP can influence GPGV virulence, inducing different plant response to virus infection, and could affect symptom severity in GLMD. We are currently fine mapping the differences between the two ORF2 variants to find the key factors that may play a role in the development of necrosis and, consequently, the development of symptoms.

ACKNOWLEDGEMENTS

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O39. Manipulation of host primary metabolism during grapevine Pinot gris virus infection in grapevine

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INTRODUCTION

In the last 20 years, a new grapevine pathology called grapevine leaf mottling and deformation disease (GLMD) appeared in northeastern Italy and Slovenia. Infected plants showed symptoms of leaf deformation, vein discoloration and leaf spots, as well as apex necrosis and delayed budding, in the most severe cases stunted growth and progressive loss of vigor. Grapevine Pinot gris virus (GPGV), a member of the *Trichovirus* genus in the *Betaflexiviridae* family, was identified in GLMD-symptomatic plants (Giampetruzzi et al., 2012). The role of GPGV as the causative agent of GLMD has been much debated as the virus was often detected in grapevines that did not show any symptoms. Several studies revealed that many different factors could play a role in GLMD etiology in particular, the presence of latent and virulent GPGV variants. It was hypothesized that activation/suppression of antiviral RNA silencing, induced by different populations of GPGV strains, causing alterations of some physiological pathways of the plants, leads to the manifestation of the symptoms (Tarquini et al., 2022).
The present study aims to deepen the physiological and molecular mechanisms affected by GPGV in grapevines susceptible to GLMD.

MATERIALS AND METHODS

In 2017, a vineyard cultivated with the cultivar 'Traminer aromatico' was selected for the high presence of GLMD symptomatic plants. Molecular analysis revealed the predominance of virulent GPGV variants in symptomatic plants and latent GPGV variants in asymptomatic ones. During dormancy, 1-year-old canes were collected separately from symptomatic and asymptomatic plants and grafted, as rootstocks, with healthy scions of 'Traminer aromatico', coming from Southern Italy. At least 30 grafted plants per combination were obtained, planted in pots and maintained in an insect-proof screenhouse. Plants were surveyed for two years and the presence of GLMD symptoms was recorded. In June 2020, plants were divided into five theses (T): T1) healthy, T2) plants originally infected with latent GPGV variants that never displayed GLMD symptoms, T3) plants originally infected with latent GPGV variants and showing GLMD symptoms, T4) plants originally infected with virulent GPGV variants that never displayed GLMD symptoms, T5) plants originally infected with virulent GPGV variants and showing GLMD symptoms. Leaf area index (LAI), length of shoots, length and number of internodes were determined for each vine. The following leaf eco-physiological parameters were registered using an open gas exchange apparatus LC*i T* ADC: photosynthetic rate (P_n), transpiration rate (E), stomatal conductance (g_s) and substomatal CO_2 concentration (c_i). Leaf and root samples were collected (six plants per T) and RNA was isolated using Plant/Fungi total RNA purification kit (Norgen) and Spectrum Plant Total RNA Kit (Sigma) for leaves and roots, respectively. RNAseq was carried out with Illumina Novaseq technology by Macrogen Inc. (south Korea).
The obtained sequences were filtered for quality using Trimmomatic and then assembled and quantified using Trinity -v2.13.1 pipeline. Transcripts were annotated using the annotation V1 of the 12X draft grapevine genome. For hierarchical clustering (HCL) analysis, the MeV software was used by applying the Pearson's correlation distance of log2 transformed FPKM values derived from RNAseq data. The BiNGO 3.0 plug-in tool in Cytoscape was used for GO enrichment analysis.

RESULTS AND DISCUSSION

After the second year of observations, 34% of the plants originally infected with the latent GPGV variants and 75% of those originally infected with the virulent GPGV variants showed typical GLMD symptoms. Quantification of GPGV titer, performed in leaves and roots, showed significant higher viral levels in symptomatic plants (T3 and T5, in comparison to T2 and T4). Further characterization of GPGV variants infecting grapevines revealed mixed infection of GPGV strains in all the theses, with the prevalence of the virulent variants in T3, T4 and T5. Despite the absence in T4 of typical GLMD foliar symptoms, plants belonging to T3, T4 and T5 theses shared similar eco-physiological and agronomic performances. Indeed, infection with the dominance of the virulent GPGV variants drastically reduced P_n rates and both plant vigor and growth (Fig. 1).

Hierarchical clustering of RNAseq data attested that the 9,114 differentially expressed genes (DEGs) were resolved into three major clusters, one of which enclosed 2,557 genes exclusively up-regulated in T3, T4 and T5 theses. The Gene Ontology (GO) enrichment analysis indicated that carbohydrate metabolism, energy metabolism, amino acid and protein metabolism, lipid metabolism, response to stress and secondary metabolism were the overrepresented functional categories. This transcriptomic profiling indicates a massive reprogramming of plant primary metabolism in plant cells where virus was replicating. Such a metabolic shift could explain the decrease in photosynthetic activity and in plant growth. Perturbations in primary metabolism could respond to the increased demand for energy needed to sustain viral multiplication and defense response against viruses (Llave, 2016).

Venn diagrams were used to identify genes specifically up- or down-regulated in T3 and T5, and potentially involved in the onset of foliar symptoms and in the increase in GPGV titer. Response to stress, signaling and protein degradation were the enriched functional categories. Interestingly, several genes involved in the ubiquitin-related pathway were specifically modulated in T3 and T5. Numerous indications suggested the involvement of this pathway in the plant-virus interaction, alternatively impairing and facilitating viral infection, by promoting virus replication and movement, but also by modulating the levels of RNA accumulation to ensure successful biotrophic interactions (Dubiella et al., 2021).

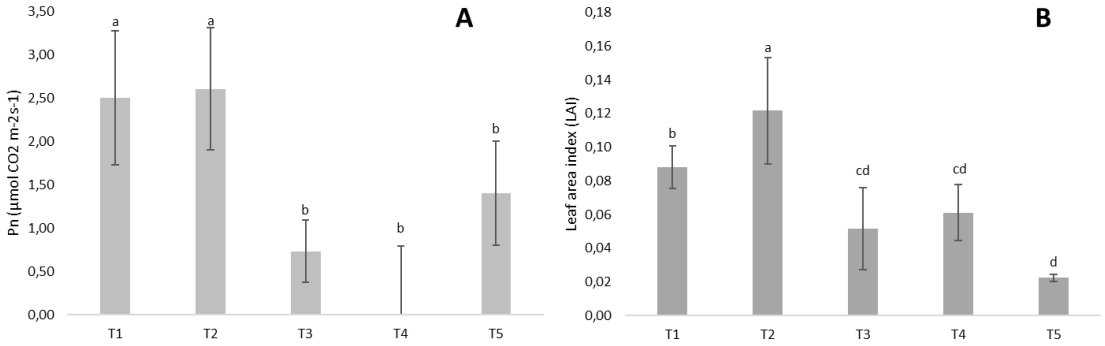

Figure 1 Changes in: A) net photosynthesis (P_n) and B) leaf area index (LAI) between different theses.

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O40. Biological interplay between viruses and powdery and downy mildews in Chardonnay (*Vitis vinifera* L.)

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INTRODUCTION

In all viticultural regions, grapevine (*Vitis vinifera* L.) is affected by several pathogens, such as fungi, oomycetes, phytoplasma and viruses, which all induce serious damages to the plants. Viruses represent a widespread class of pathogens in all grapevine species. Plant-virus interactions are generally more complex than expected (Perrone et al., 2017) and a deeper understanding of this interplay would be warranted, considering the plant as a holobiont in which micro- and macroorganisms interact with the environment as a unique organism (Dicke, 2016). If viruses are the most numerous and widespread class of pathogens in all *Vitis* spp., fungal/oomycete-associated diseases, namely powdery and downy mildews (PM and DM), undoubtedly represent the main adversities in the vineyard in terms of economic and environmental impact. The aim of this work was to study the effects of two well-known and widespread viruses, grapevine rupestris stem pitting-associated virus (GRSPaV) and grapevine fanleaf virus (GFLV), on DM and PM incidence and severity and the systemic molecular modifications induced in grapevines by these multiple interactions under controlled greenhouse conditions.

MATERIALS AND METHODS

Virus-free plantlets of *V. vinifera* cv. Chardonnay were regenerated from somatic embryos obtained from *in vitro* culture of immature anthers (Gambino et al., 2007), and a single virus-free line originated from a single somatic embryo was selected, multiplied, and used for the transmission of GFLV or GRSPaV by *in vitro* grafting (Gambino and Perrone, 2022). For each thesis, 'Chardonnay' virus-free plants, infected by GFLV, and infected by GRSPaV, were acclimated out of *in vitro* conditions. Three months after growing in greenhouse, the plants were artificially inoculated with *Plasmopara viticola* or *Erysiphe necatrix* using a suspension of $5x10^3$ sporangia/ml and $1x10^4$ conidia/ml, respectively. About 10-25 leaves/plant were visually estimated by rating the percentage of affected leaves (disease incidence, DI). The leaf area affected by the pathogen (disease severity, DS) was estimated using the severity scale 1-7 as reported by the EPPO/OEPP protocols (PP 1.31 and PP1.4). In a second experiment the inoculation by *P. viticola* was preceded and followed by treatments with fungicides (Acibenzolar-S-methyl, potassium phosphonate, essential oil of sweet orange) to analyze the multiple interactions between virus-fungi and antifungal treatments. Representative genes of the most important molecular pathways involved in the response to biotic agents in grapevine were analyzed by RT-qPCR as previously reported (Gilardi et al., 2020).

RESULTS AND DISCUSSION

Over the past decade, studies on plant-virus interactions in grapevine have gained increasing importance, suggesting that such species may be a suitable woody plant model for studies on this topic. Our analyses were focused on GFLV and GRSPaV because both are widespread viruses, well known

from the molecular point of view, and represent two extremes of the biological responses induced in grapevine (Martelli, 2017). As a rule, GFLV is quite harmful to grapevine, while GRSPaV is generally latent in *V. vinifera*. Under greenhouse conditions, grapevine-mediated interactions between the two viruses and both the DM and PM causal agents (artificially inoculated) were demonstrated. 'Chardonnay' infected with GFLV showed a higher tolerance to PM and DM with lower DS compared to virus-free plants, while GRSPaV-infected plants showed a DS not significantly different from CTR, with intermediate values between virus-free and GFLV-infected plants (Table 1).

	Plasmopara viticola		***Erysiphe necatrix***	
Virus	**Disease severity %**	**Disease incidence %**	**Disease severity %**	**Disease incidence %**
CTR	10.96 ± 1.69 c	50.34 ± 7.92 b	7.28 ± 1.3 b	58.31 ± 13.96 a
GRSPaV	6.06 ± 0.99 b	52.62 ± 4.70 b	4.87 ± 4.86 ab	58.47 ± 22.00 a
GFLV	3.45 ± 1.09 a	35.56 ± 9.12 a	2.70 ± 0.7 a	42.04 ± 16.53 a
p value	*<0.001*	*0.015*	*0.030*	*0.157*

Table 1. DS and DI of 'Chardonnay' inoculated with *P. viticola* and *E. necatrix* at the end of trial.

At molecular level, GFLV infection induces the overexpression of stilbene synthase genes and pathogenesis related proteins (*VvPR1*, *VvBgluc*) and influenced carbohydrate metabolism, thus modifying the expression dynamics of sugar transporters (*VvSTP13*, *VvSUC27*), vacuolar invertase, and sucrose synthase (*VvGIN2* and *VvSUSY4*) genes. These transcriptional changes can in turn trigger the synthesis of defense compounds, enhancing the innate plant immune responses in grapevine and increasing its resilience to biotic stresses (Gilardi et al., 2020). However, this response does not appear to be a generic reaction to all grapevine viruses. In the case of GRSPaV-infected plants, the transcriptional modulation of the candidate genes was not sufficient to confer protection against *E. necatrix* and *P. viticola*. Furthermore, to add another component to this multiple interaction and get closer to real vineyard conditions, antifungal compounds have been applied. Preliminary analysis showed a positive interaction between GRSPaV and antifungal treatments. Specifically, GRSPaV-infected plants exhibited a lower DI following treatments with antifungal compounds and artificial inoculation by *P. viticola* than virus-free and GFLV-infected plants. These analyses are still in progress and need further confirmations, but initial results suggest that GRSPaV infection primed the plant, making treatments with chemical compounds more effective in controlling *P. viticola* infection.
All these findings, at least in our experimental controlled conditions, highlight the complexity of the interactions among grapevine, viruses, fungal/oomycete pathogens, and antifungal compounds. Here, each component interacts with the others in a way that is not yet fully understood, but collectively determines the plant's responses to its environment.

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O41. Functional analysis of the silencing suppressors encoded by grapevine fanleaf virus

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INTRODUCTION

Grapevine fanleaf virus (GFLV) causes fanleaf degeneration disease, one of the most destructive viral diseases of grapevine (*Vitis* spp.) in most vineyards worldwide (Andret-Link et al., 2004; Martelli, 2019; Schmitt-Keichinger et al., 2017). Fanleaf degeneration substantially reduces yield (< 80%) and the productive lifespan of vineyards. GFLV is a member of the species *Nepovirus foliumflabelli* in the genus *Nepovirus* of the family *Secoviridae* (Fuchs et al., 2022). Recently, suppressors of RNA silencing (VSRs) were identified for GFLV (Choi et al., 2023). These include the RNA1-encoded protein 1A and the putative helicase (Hel) protein $1B^{Hel}$. The two GFLV VSRs reverse systemic RNA silencing individually or as a fused form ($1AB^{Hel}$), which is a predicted intermediary product of proteolytic processing (Choi et al., 2023). Key characteristics underlying the suppression function of GFLV VSRs are unknown. Mutations in the tryptophan-glycine (WG) or glycine-tryptophan (GW) motif of other VSRs have been shown to decrease or abolish their suppression activity and/or virus viability (Gupta & Tatineni, 2019; Karran & Sanfaçon, 2014; Pérez-Cañamás & Hernández, 2015). For example, a substitution mutation from WG to AG in the coat protein of tomato ringspot virus, another nepovirus VSR, abolished the suppression function due to the loss of binding ability to Argonaute protein (Karran & Sanfaçon, 2014). In this study, we explored the role of WG/GW in the two GFLV VSRs for RNA silencing suppression.

MATERIALS AND METHODS

In silico analyses of amino acid sequences of GFLV strain GHu (GFLV-GHu) (GenBank accession AFM91094.1) were carried out to identify a WG/GW motif using DNASTAR Lasergene v. 17. Amino acid sequences of 1A and $1B^{Hel}$ from 10 other GFLV isolates were retrieved from GenBank and included in multiple alignments using Clustal W (Sievers & Higgins, 2018). Alignments were visualized and analzyed with Jalview software 2.11.2.6. (Clamp et al., 2004).

Site-directed PCR mutagenesis was carried out using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) with specific primers generated by the NEBaseChanger® software v 2.0.0. to create W to alanine (A) substitution mutations in the WG/GW motif. The binary constructs pEarleyGate100 (pV) (Earley et al., 2006) encoding GFLV 1A, $1B^{Hel}$ and $1AB^{Hel}$ (Choi et al., 2023) were used as templates for mutagenesis experiments. The single and/or double substitution mutations were verified via Sanger sequencing at the Cornell Biotechnology Resource Center. The validated constructs were then transformed into *Agrobacterium tumefaciens* strain GV3101 for *in planta* expression. Each of the wild-type (1A, $1B^{Hel}$, or $1AB^{Hel}$) or mutant ($1A^{W293A}$, $1B^{Hel\ W538A}$, $1A^{W293A}B^{Hel}$, $1AB^{Hel\ W538A}$, or $1A^{W293A}B^{Hel\ W538A}$) GFLV VSR constructs was co-infiltrated with RNA silencing-inducing hairpin construct into transgenic *Nicotiana benthamiana* plants expressing the reporter green fluorescent protein, as previously described (Choi et al., 2023). Infiltration buffer (mock) and empty vector pV treatments were used as negative controls, while protein p24 of grapevine leafroll-associated virus 2 was used as a positive VSR control. Treated plants were monitored for systemic suppression of RNA silencing at specific wavelength light using a NIKON D850 digital camera. Next, apical leaves of treated plants were collected and used for quantification of the reporter gene expression via

fluorescence spectrometry and RT-qPCR with specific primers (Choi et al., 2023). Statistical analyses were performed using software R v. 4. 1. 3 for the ANOVA and post hoc tests.

RESULTS AND DISCUSSION

In silico analyses of GFLV-GHu 1A and $1B^{Hel}$ sequences revealed the presence of WG at amino acid positions 293-294 of protein 1A, and of GW at positions 537-538 of protein $1B^{Hel}$. Multiple amino acid sequence alignments using Clustal Omega revealed that both WG of protein 1A and GW of protein $1B^{Hel}$ are highly conserved among GFLV isolates. Substitution mutagenesis resulted in four single mutant constructs (GFLV-$1A^{W293A}$, GFLV-$1B^{Hel\ W538A}$, GFLV-$1A^{W293A}B^{Hel}$ and GFLV-$1AB^{Hel\ W538A}$) and a double mutant construct (GFLV-$1A^{W293A}B^{Hel\ W538A}$).

Transgenic *N. benthamiana* plants expressing the green fluorescent protein that were co-infiltrated with RNA silencing-inducing hairpin construct and mutant GFLV VSR constructs with W to A substitutions exhibited varying intensity of systemic suppression in apical leaves. In addition, temporal differences in the onset of systemic RNA silencing development were observed among mutant GFLV VSR constructs-treated plants. As expected, wildtype GFLV VSR constructs 1A, $1B^{Hel}$, or $1AB^{Hel}$ induced systemic suppression of RNA silencing in apical leaves, while mock- and pV-treated plants did not. Quantitative assays of the reporter gene expression confirmed visual observations. Overall, our results showed varying alterations in suppression activity of the two GFLV VSRs when mutating their WG/GW motif. This research provided preliminary insights into the functional genetic basis of GFLV VSRs, and contributed to enhancing our knlowedge of nepovirus VSRs. Moreover, this research highlighted some unique characteristics of the GFLV VSRs in comparison with previously identified plant VSRs.

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O42. Grapevine fanleaf virus elicits a hypersensitive response above and below ground in a model host

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INTRODUCTION

Grapevine fanleaf virus (GFLV, genus *Nepovirus,* family *Secoviridae*) causes fanleaf degeneration in in most vineyards worldwide (Andret-Link et al. 2004). GFLV is transmitted by the ectoparasitic dagger nematode *Xiphinema index* (Andret-Link et al. 2004) and is mechanically transmissible to herbaceous hosts (Roy and Fuchs 2022). GFLV determinants of vein clearing symptoms in *Nicotiana benthamiana* map to the amino acid in position 802 of the RNA-dependent RNA polymerase (protein $1E^{Pol}$) (Osterbaan et al. 2019). Profiling proteome and transcriptome changes during GFLV infection revealed the activation of immune responses, including a hypersensitive reaction, and the dysregulation of biochemical pathways during symptom expression (Roy et al. 2023). Although these findings contributed to our understanding of leaf symptom development, how GFLV impacts plant host root systems is unknown. With advances in root-phenotyping technologies and a library of GFLV strains we set out to explore the impact of the virus on *N. benthamiana* root system architecture (RSA) traits. We hypothesized phenotypic RSA differences upon infection with different GFLV strains.

MATERIALS AND METHODS

N. benthamiana plants were maintained in controlled growth chambers at 25 °C and 70% humidity. GFLV wildtype strains GHu and F13, as well as single amino acid mutants GFLV-GHu $1E^{Pol}_{K802G}$ and GFLV-F13 $1E^{Pol}_{G802K}$ (Osterbaan et al. 2019) were utilized to inoculate *N. benthamiana* plants. Inoculated plants were tested for the presence of GFLV by DAS-ELISA, RT-PCR, and Sanger sequencing of RT-PCR amplicons (Osterbaan et al. 2019). Infected plants were uprooted at 4-, 17-, and 26-days post inoculation (dpi). Crown root systems were washed off with distilled water, dried, and imaged on an EPSON Expression 12000XL scanner. Images were analyzed in Rhizovision Explorer (v2.0.3, Noble Research Institute, LLC). Statistical analysis of RSA traits was performed in RStudio (v4.2.2) using packages 'stats' (v4.1.3) and 'corplot' (v0.92). RNA was extracted from roots and relative GFLV titer was measured by RT-qPCR against the FBOX housekeeping gene (Osterbaan et al. 2019) using the Luna® Universal One Step kit. Quantification was done using the output Cq values of SYBR green detection. Statistical analysis was performed using log(2^-ΔΔCq) values compared to mock inoculated control plants through the Wilcoxon geometric comparison of means. 3'RNA-Seq analysis of *N. benthamiana* root tissue at 4-, 17-, and 26 dpi was performed as previously described (Roy et al. 2023). Gene set enrichment analysis contrasting 17 dpi root samples to 7 dpi apical leaf transcriptomes was performed to identify overlap of these datasets.

RESULTS AND DISCUSSION

Plants infected with wildtype GFLV-GHu exhibited vein clearing symptoms in apical tissue at 8 dpi and plants recovered by 14 dpi, as expected. No other GFLV strains elicited foliar symptoms (Fig 1A). A significantly increased root diameter and/or a significantly decreased number of root tips were observed at 17 dpi on plants infected with wildtype GFLV-GHu and mutant GFLV-F13 $1E^{Pol}_{G802K}$, both containing a lysine at protein $1E^{Pol}$ residue 802 (Fig. 1B-C). Absent (Fig. 1C) or not significantly reduced RSA traits were observed in plants infected with wildtype GFLV-F13 and mutant GFLV-GHu $1E^{Pol}_{K802}$, both containing a glycine at protein $1E^{Pol}$ residue 802. To our knowledge, this is a novel GFLV phenotype induced by protein $1E^{Pol}_{802}$ in a plant host. Other RSA traits captured by Rhizovision

Explorer were highly correlated with root tip number and average diameter, however, there was no relationship between root symptomology and relative GFLV titer. Gene set enrichment after differentially abundant transcript identification revealed overrepresentation of genes relating to hypersensitive response, abiotic stress, and protein processing/gene regulation. Further work is needed to assess the effect of modified plant host root systems on *X. index*-mediated transmission of GFLV, changes in root phenotype by distinct GFLV strains in *Vitis* spp., as well as the molecular mechanism of this root phenotype.

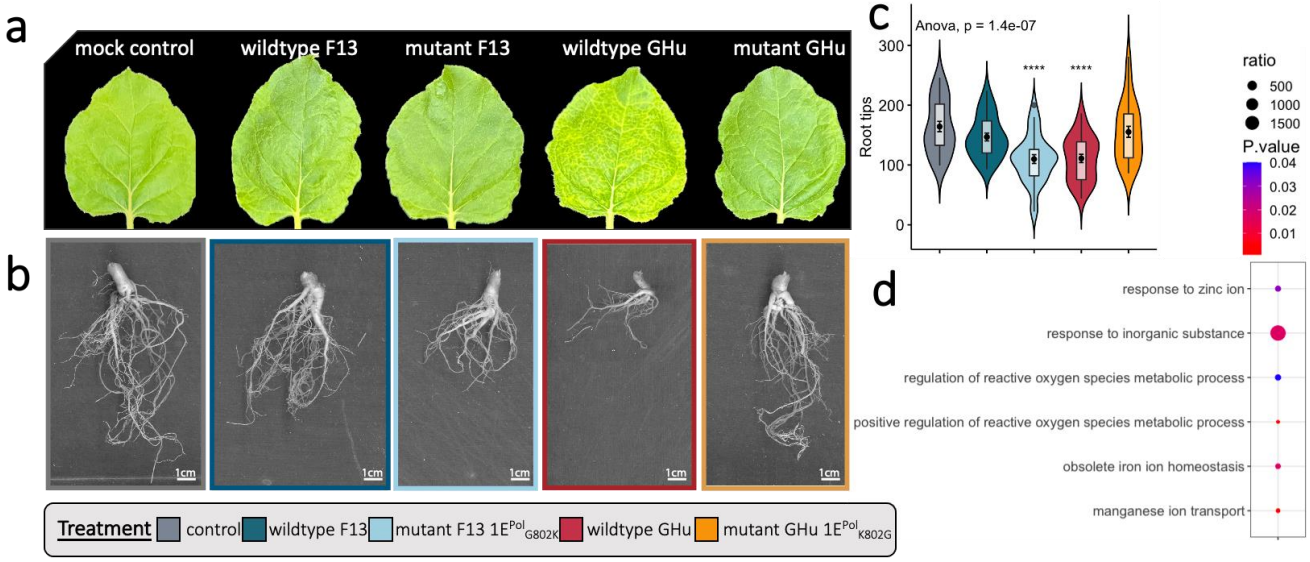

Figure 1. Grapevine fanleaf virus (GFLV) strains elicit differential symptomology in *N. benthamiana* depending on the strain. (a) At 7 days post inoculation (dpi) vein clearing symptoms are observed for wildtype GFLV-GHu infected plants while the other four treatments (mock control, wildtype GFLV-F13, mutant GFLV-F13 $1E^{Pol}_{G802K}$, and mutant GFLV-GHu $1E^{Pol}_{K802G}$) remain asymptomatic. (b) Root systems of *N. benthamiana* are altered by different GFLV strains. (c) Number of root tips as calculated by Rhizovision Explorer show statistically supported phenotypic differences among viral strains. (d) Comparative gene set enrichment analysis of wildtype GFLV-GHu vs mock control in apical leaves and root tissue reveal major common gene ontologies relating to hypersensitive response and inorganic molecules.

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O43. Searching for mild and protective grapevine fanleaf virus isolates in 'Chardonnay' vineyards in the Champagne region of France for disease management by cross-protection

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INTRODUCTION

In the global context of exploring agroecological solutions for crop production, cross-protection is an attractive biocontrol approach for managing plant viral diseases (Pechinger et al., 2019). Cross-protection consists of a deliberate infection of a plant with a mild virus isolate/strain to protect it against damage caused by severe isolates of the same virus or a related virus. For cross-protection against citrus tristeza virus, the genetic relatedness of the protective and challenge isolates is crucial, with the most effective protection obtained when the mild isolate originates from the geographical region where it is used and from the citrus cultivar to be protected (Moreno et al., 2008; Folimonova, 2013).

Grapevine fanleaf virus (GFLV) causes fanleaf disease in most vineyards worldwide with serious economic loss. This virus is transmitted from vine to vine by the ectoparasitic dagger nematode *Xiphinema index* (Andret-Link et al., 2004). Interestingly, GFLV-infected vines show a wide range of symptom types and varying degrees of severity with some plants being mild symptomatic.

No effective measures are available to winegrowers for managing fanleaf disease in infected vineyards besides the use of *X. index*-tolerant rootstocks that delays the infection of grafted wine grape cultivars (Walker et al., 1994). Cross-protection against GFLV has been studied on herbaceous model hosts with a mild strain isolated from vines introduced from Hungary (Huss et al., 1989). Subsequent work in naturally infected vineyards (in Alsace region in France) revealed that *Vitis vinifera* cv. Gewurztraminer grafted onto a rootstock infected with this mild GFLV strain exhibited a lower infection rate by vineyard isolates than control vines; its negative impact on yield made however the disease management approach with this strain impractical (Komar et al., 2008). To build on these findings we decided to further explore cross-protection against GFLV by searching for mild isolates in naturally infected vineyards in the regions where cross-protection trials will be implemented. We here report preliminary results of our work in 'Chardonnay' vineyards in the Champagne region.

MATERIALS AND METHODS

Identification and characterization of GFLV-infected vines displaying mild symptoms in highly diseased vineyards: Twenty-one 'Chardonnay' vines exhibiting either mild or severe symptoms in two commercial GFLV-infected vineyard parcels in the Champagne region of France were monitored over four years for qualitative and quantitative phenotypic traits (Kubina et al., 2022). Total RNA was extracted from leaves and used for Illumina sequencing (Vigne et al., 2018). GFLV consensus sequences were generated by *de novo* assembly, and analyzed for genetic diversity and phylogenetic relatedness (Kubina et al., 2022).

Vineyard cross-protection trial: Dormant cuttings were collected from GFLV-infected 'Chardonnay' vines exhibiting mild symptoms and propagated in the greenhouse. Following propagation and grafting, experimental vines infected by the expected GFLV isolates were obtained. These grafted GFLV-infected 'Chardonnay' vines and grafted control 'Chardonnay' vines (non GFLV-infected) were

planted in 2022 in two vineyard sites highly impacted by fanleaf degeneration disease after uprooting old vines.

RESULTS AND DISCUSSION

The 21 monitored 'Chardonnay' vines were separated in three categories based on their infectious status (presence/absence of GFLV) and on their phenotype: seven vines were asymptomatic and GFLV-free, ten vines were GFLV-infected and severely symptomatic, and four vines were GFLV-infected and mildly symptomatic. Consistent qualitative and quantitative phenotypic traits were observed across the 4-year study for the three categories of vines with significantly higher mean yields obtained from mildly (1795 g/vine) in comparison with highly (988 g/vine) symptomatic vines (Kubina et al., 2022). The complete genome of GFLV variants in the 14 infected 'Chardonnay' vines was determined by analyzing the Illumina sequences: 78% of these vines were infected with at least two genetically distinct molecules of RNA1 and/or RNA2 (Kubina et al., 2022). Knowledge of the composition of GFLV variants was used to develop diagnostic molecular tools to verify the infection status of experimental vines derived from the original mildly symptomatic vines prior to their establishment in naturally infected vineyard sites in the Champagne region. A total of 630 experimental vines were planted, including 480 (192 control and 288 GFLV-infected vines) that were assigned to eight blocks in a first naturally infected vineyard site, and 150 (60 control and 90 GFLV-infected vines) that were assigned to five blocks in a second naturally infected vineyard site. Experimental vines are currently individually monitored to assess phenotypic traits and infectious status. Results on symptom development and level of superinfection by vineyard GFLV isolates will be presented and discussed.

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O44. Molecular gut content analysis informs the landscape-level movement of *Spissistilus festinus* and epidemiological features of grapevine red blotch virus

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INTRODUCTION

Grapevine red blotch virus (GRBV) is one of the most economically important viruses in North America, with infection resulting in substantial re-plantings and management costs ranging between $2,213 to $68,548 per hectare over a 25-year lifespan of a vineyard (Ricketts et al., 2017). The three-cornered alfalfa hopper, *Spissistilus festinus* [Say, 1830] (Hemiptera: Membracidae), is a vector of GRBV in vineyards, although it is not an economically relevant pest of grape and is not considered reliant on *Vitis* spp. for feeding or reproduction (Preto et al., 2018; Hoyle et al., 2022; Flasco et al., 2023). Information on *S. festinus* phenology and its reproductive cycle is primarily gained from peanut, alfalfa, and soybean fields in southern states in the USA. Limited information is available on the behavior of *S. festinus* in vineyard ecosystems. To gain insights into *S. festinus*-GRBV-*Vitis* interactions, plant DNA from insect guts was characterized to examine the dietary history of *S. festinus* caught in vineyards. We identified yearly feeding trends, dietary profiles unique to vineyard sites, potential overwintering hosts, and an ecological connectivity between free-living vines in riparian areas and vineyard settings with regards to virus transmission. Our findings provided new insights into the landscape level movement of *S. festinus* and GRBV epidemiology that have been salient for providing growers with informed disease management strategies.

MATERIALS AND METHODS

S. festinus specimens were collected over two growing seasons in Napa Valley, California, using sweep nets and yellow sticky cards in 26 different vineyard sites and nearby riparian areas. Sticky cards were placed within vineyard rows and rotated every two weeks. Information on collection date, number, and sex of *S. festinus* was recorded. Insect were tested for GRBV presence using whole *S. festinus* specimens by multiplex PCR using primer pairs designed in the open reading frames coding for the coat protein (CP) and replication-associated proteins (RepA), as previously described (Flasco et al., 2021). Plant samples were similarly collected in and around vineyards and tested for GRBV. PCR amplicons were analyzed by agarose gel electrophoresis and visualized under UV illumination after staining with GelRed®. Negative controls included sterile water and nucleic acids isolated from GRBV-free plants and colony-maintained *S. festinus*.

Plant DNA from *S. festinus* guts were examined using an optimized version of the procedure outlined by Cooper et al. (2016). Briefly, PCR amplification of the internal transcribed spacer (ITS) DNA situated between the small-subunit and large-subunit ribosomal RNA genes, and the trnL-F intergenic spacer (trnF) of the chloroplast genome followed by sequencing of amplicons on the Pacific Biosciences platform, and sequence analyses were used to determine gut contents. Insect specimens from bean and alfalfa reared colonies were used to establish a baseline gut content. The readout of these analyses provided a guide to which plant families make up the diet of *S. festinus* and their level of interaction with various *Vitis* species.

RESULTS AND DISCUSSION

Over two growing seasons, we identified 23 and 108 unique plant genera in 2021 and 2022, respectively, in the guts of *S. festinus*. There were 40 plant genera that were consistently found over the two seasons. Among these plant genera, were primarily Asteraceae, Fabaceae, and Vitaceae family plants, followed by Solanaceae, Poaceae, and Fagaceae. Interestingly, both cultivated and free-living *Vitis* (*V. californica* and *V. californica* hybrids) species were found in the gut contents of *S. festinus*. Surprisingly, more *S. festinus* fed on free-living vines than cultivated vines during both growing seasons, with a substantial decrease in dependence on cultivated grapevines in 2022 when the weather favored the growth of plant species outside of irrigated vineyards, suggesting that *S. festinus* rely on free-living vines as feeding hosts. Among the numerous plant species tested for GRBV, only cultivated and free-living *Vitis* species were found infected with the virus, confirming previous results (Cieniewicz et al., 2019). Prior to June, the peak time for *S. festinus* presence in a vineyard in Napa Valley, gut contents pointed to potential overwintering hosts in riparian areas proximal to vineyards such as trees and shrubbery, as well as free-living vines. These studies revealed numerous feeding hosts of *S. festinus*, and a connectivity between free-living vines and the influx of GRBV in vineyards. Based on these findings, growers are discouraged from taking any action against *S. festinus* but are encouraged to carefully select their plant stocks by ascertaining the cleanliness of their rootstock and scion materials.

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Poster Session Abstracts

- **Diagnostics** **Posters P1 – P7**
- **Disease Etiology - Epidemiology** **Posters P8 – P13**
- **Certification and other management approaches** **Posters P14 – P23**
- **Phytoplasmas** **Posters P24 – P30**
- **Virus Characterization - Diversity** **Posters P31 – P38**
- **Plant-Virus-Vector Interactions** **Posters P39 – P48**

P1. A DNA-capture approach for detection and genome-wide sequencing of Flavescence dorée phytoplasma

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INTRODUCTION

Flavescence dorée phytoplasma (FDp) is a non-cultivable quarantine bacterial pest causing outbreaks in the vineyards of 10 European countries where it is transmitted by the leafhopper *Scaphoideus titanus* (Jeger et al., 2016). FDp can be detected by various real-time PCR assays and FDp strains can be genetically characterized by Multilocus sequence typing (Arnaud et al. 2007). However, the genetic characterization of FDp is often limited due to the low amount of FDp DNA in grapevine nucleic acid. Recently, a DNA capture approach was described to enrich DNA of the citrus pathogen "*Candidatus* Liberibacter asiaticus" prior to Illumina sequencing (Cai et al. 2019). We describe the design of a SureSelect (Agilent) RNA probe system to capture FDp DNA. The FDp enrichment probe consisted of RNA probes covering all coding sequences (CDS) of the FD92 FDp genome (Carle et al. 2011). RNA probes were preferred to take advantage of the high strength of RNA/DNA hybridisation expected to compensate the lower capture efficiency due to the low GC % in the FDp genome (21.7%). This approach was applied to DNA extracts of field -collected or insect-inoculated FDp-infected grapevines. We present here the outcome in terms of enrichment and sequencing coverage for pure extracts and for serial dilutions mimicking decreasing rates of infection.

MATERIALS AND METHODS

The first grapevine sample (VIS) was inoculated with FDp genotype M54 using infectious *S. titanus* under greenhouse-controlled conditions (Eveillard et al., 2016). The second grapevine sample (VIT), found to be infected with the M54 FDp genotype, was collected in Faleyras (Gironde, France). Total nucleic acids were extracted from 1.5g of petioles according to standard procedure (Maixner et al., 1995), treated with 1 µg/µl RNAse A for 30 min at 37°C and purified on Promega Wizard® SV Gel and PCR Clean-Up System columns. DNA concentration was measured with Qubit-4 fluorimeter. The number of FDp genome copies per µl of DNA extract was measured by qPCR (Eveillard et al. 2016). Serial dilutions in healthy grapevine DNA were prepared.

Probes consisted of (i) 560 CDS and (ii) 16S and 23S rRNAs of the genome of strain FD92 of genotype M54 and vmpA and B cluster II which predominates in European vineyards, (iii) *vmp*D and *vmp*E adhesin gene sequences specific to strain FD-CAM05 of genotype M50 and (iv) *vmp*A, *vmpB* and *imp* genes representative of the FDp strains diversity. A total of 48,649 RNA probes long of 120 nucleotides were covering 495,524 bp with tiling coverage of 3X were synthetized by Agilent.

Samples were processed according to "SureSelectXT HS target enrichment system for Illumina multiplexed sequencing platforms" Agilent manual version D0 (August 2020). Pair-end Illumina sequencing 2 x 150 bp was performed on Miseq at the Plateforme Génome Transcriptome de Bordeaux (PTGB). Sequences were trimmed to eliminate adaptor sequences. Sequences with length shorter than 50 bp or quality below phred20 were eliminated. Finally, sequences were mapped to the FD92 FDp genome using Bowtie 2 and mapping results analyzed with Samtool coverage under Galaxy.

RESULTS AND DISCUSSION

The selected samples were highly infected. According to qPCR, VIS and VIT grapevine DNA extract contained 0.754 % and 0,46 % of FDp DNA. After SureSelect enrichment, 90.34 % and 84.69 % of

the sequence reads corresponded to FDp for VIS and VIT pure extracts. The enrichment was of 120 for VIS and of 184 for VIS. The enrichment was even higher with all the 1/16 serial dilutions of both VIS and VIT, indicating that the capture system was saturated in the case of the pure extracts. The coverage of mapping to the FDp genome was in higher than 72 % for pure extracts, the 1/16 dilutions of VIS and VIT and for the 1/256 dilution of the VIS sample. It must be noted that the probes themselves are covering 75 % of the 647 kbp of the FD92 FDp genome. Therefore, higher coverage indicated that intergenic sequences bordering the RNA probes were partly captured. For higher dilutions of VIS and VIT, the coverage was limited to 24.3 % and 45.3 % respectively. Depth of 329 and 224 however indicate that some parts of the genome were more efficiently captured.
These results indicate the efficiency of the SureSelect RNA probe capture system to enrich grapevine DNA in FDp DNA and give sufficient data for a genome-wide genetic characterization of FDp strains.

Table 1: Statistics of Illumina sequencing after enrichment capture on FDp SureSelect probes.

samples	Total reads	reads mapped	Coverage (%)	Depth (X)	% of FDp reads after capture (A)	% of FDp DNA in initial extract (B)	Enrichment A/B
VIS pure	46,837,748	42,313,330	84.5	11,162	90,34	0.754 (1)	120
VIS dil 16	25,037,388	11,052,656	82.2 %	3,059	44,14	0.0471 (2)	937
VIS dil 256	22,617,282	1,403,579	72.2 %	436	6,21	0.00295	2103
VIS dil 4096	24,746,846	405,857	24.3 %	329	1,64	0.000184	8913
VIT pure	55,337,744	46,864,812	85.4 %	12,468	84,69	0,46	184
VIT dil 16	21,441,258	3,881,349	78.0 %	1,229	18,10	0.0286	633
VIT dil 256	19,491,652	483,904	45.3 %	224	2,48	0.0018	1379

(1) Evaluated by qPCR, (2) Calculated from the dilution factors

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P2. From Illumina-based amplicon sequencing approach to a potential homologation protocol and forthcoming regulation of GFLV cross-protected grapevines in France

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INTRODUCTION

Disease management in crop production is a critical issue especially when current solutions do not match with low environmental impact or meet technical and regulation impasses; thus, exploring innovative protection strategies to fight plant pathogens is a requirement in the agroecological transition in agriculture. Cross-protection has been studied as a biocontrol anti-viral approach in crops for some time (Pechinger et al., 2019). The ability of a mild viral isolate to confer protection to the plant it infects against other related viruses is multifactorial: fitness and genetic relatedness between viruses seem to be key but underlying mechanisms remain unresolved. Cross-protection has also been tested in grapevine against the *Grapevine fanleaf virus* (Komar et al., 2008), the causing agent of fanleaf disease, being a major threat to the viticulture industry worldwide (Schmitt-Keichinger et al., 2017), with no solution so far. Field assays, complementing laboratory experiments on both natural and herbaceous hosts, are conducted to develop an effective cross-protection in multiple French vineyards (Kubina et al., 2022). In this aim, the diagnostic of the vines primo-infected with GFLV candidate isolate for cross-protection is of the most importance. Current serological or indexing methods are not suitable nor informative enough to evaluate the sanitary status at the viral variant level but high throughput sequencing could be a solution, as shown to assess diversity (Tay et al., 2022). Early HTS study on the GFLV-population composition allowed us to synthesize a cocktail of primers recognizing a large GFLV diversity, at least in France, and specifically those dedicated to cross-protection (Kubina and Garcia, unpublished). From this, we developed molecular biology tools and complementary HTS protocols to establish the infectious status meaning: confirm the presence of the expected protecting GFLV variant while identifying all the other GFLV sequences in the vine. This poster illustrates our experimental approach, using Illumina-based amplicon sequencing, and its evaluation as a potential homologation protocol for GFLV cross-protected vines.

MATERIALS AND METHODS

Nucleic acids preparation: Three GFLV strains (F13, GHu and B844) were selected; two plasmids per strain (namely RNA1 and RNA2 GFLV molecules) derivatives of the corresponding infectious clones were amplified in *E. coli*. DNA was extracted with Nucleospin Plasmid kit (Macherey Nagel). Eight vines from a French commercial vineyard were sampled [either individually (N=2), or pooled (2x N=3)], and processed as described in (Vigne et al., 2018). Briefly, leaves were crushed in liquid nitrogen and both RNAtot from extraction (Qiagen RNeasy Plant mini kit), and decapsided viral RNA from Immunocapture assay (Bioreba AG GFLV kit) were obtained, then reverse transcribed to cDNA with specific reverse primer or oligodT using Superscript IV kit (Invitrogen).

PCR assay material: Around 120 complete CDS of GFLV-RNA1 and -RNA2 obtained in previous HTS study, were used to constitute two cocktails of degenerated primers recognizing a large diversity of GFLV variants (Kubina, unpublished). Primer cocktail #1 and #2 amplify around 500 pb product from RNA1 and RNA2 respectively. PCRs were carried out with cDNA or diluted plasmid in different combination, using Taq polymerase kit (Promega) under the following cycling parameters: initial denaturation at 95°C for 2 min, followed by 36 cycles of 30 s at 95°C, 30 s at 52°C and 45 s at 72°C,

ended with a 5 min at 72°C. As recommended for Illumina sequencing, all amplicons were purified using AMPureXP beads (Beckman Coulter) following adapted instructions (Hagege, unpublished). Plasmid and purified amplicons were quantified for DNA concentration by Qubit fluorometer (Thermo Fisher Scientific), and fragment quality was estimated by Bioanalyzer (Agilent Technologies).

HTS protocol: Equimolar preparation of RNA1 and RNA2 amplicons, indexing, and library sequencing is being performed by Genoscreen. The RNAtot of the eight plants will also be submitted to RNA-Sequencing; poly(A) library sequencing will be performed by GenoToul (INRAE).

RESULTS AND DISCUSSION

Various PCR assays were carried out to ascertain that high throughput amplicon sequencing could be used for the homologation of GFLV cross-protected vines.

PCR were performed using two primer cocktails constituted for the detection of a large diversity of both genomic molecules of GFLV and three GFLV strain plasmids as template. Template combinations were tested, either before or after the amplification step. (1) Post amplification and dilution, we composed 37 amplicon combinations by varying the three RNA1 (and RNA2 respectively) strain ratio (from 0 to 100% for each variant). Comparing expected to total number of reads being obtained for each combination will provide indication of the sequencing capacity to detect the different viral RNA proportion. (2) Then to obtain information on the primer cocktail amplification and assess a detection threshold, we mixed the plasmid templates before their amplification following 25 combinations by varying template ratio of the 3 strains of RNA1 (and RNA2 respectively). (3) To determine if our protocol and primer cocktails work on field samples (*i.e.* with unknown virome), vines were selected, which were then tested individually or pooled. Differential RNA preparation protocols were tested prior to PCR assays, resulting in 16 amplicon syntheses.

Altogether, more than 150 unique pool of amplicons will be submitted to Illumina sequencing.

While RNA-Seq is highly informative it remains too expensive for large scale study. We expect our Illumina based amplicon sequencing approach to be an effective approach and become a potential protocol to homologate GFLV cross-protected vines, combining HTS quality screening at an affordable cost.

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P3. Grapevine & the French post-entry quarantine station

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INTRODUCTION

ANSES is the French agency for Food, Environmental and Occupational Health & Safety. This agency employs about 1400 people and has 9 laboratories, spread all over France, and working in various fields. The authors of this poster are part of the ANSES **Plant Health Laboratory**. This Laboratory is present on six French geographical sites, each with its own specificity: tropical pests and pathogens (St Pierre, Reunion Island); nematology (Rennes); mycology (Nancy), entomology and botany (Montpellier); bacteriology, virology and genetically modified organisms detection (Angers); and finally, plant quarantine for the **Quarantine Unit (QU)** located in the Clermont-Ferrand site. The latter hosts the only **French post-entry quarantine station** which is an official, multi-species and public service station.

According to Regulation (EU) 2016/2031, the introduction of **plants of *Vitis*** is prohibited for phytosanitary reasons in all member States of the European Union. However, the European legislation provides for conditions under which prohibited plants may be introduced into the Community for trials or scientific purposes and for work on varietal selections. That is the reason why the French quarantine station receives every year grapevine samples from all over the world for the implementation of **a quarantine scheme** including analyses targeting **regulated quarantine pests** (Philippe Legrand, 2012; Renvoisé et al., 2019).

MATERIALS AND METHODS

Human resources: 10 persons. In addition, QU regularly welcomes fixed-term contracts employees, trainees and apprentices. **Know-how:** containment authorization notices and audits ; development of quarantine schemes ; organization of interlaboratory proficiency tests ; development and validation of plant health diagnostic tests ; management of pest conservatories ; maintenance of cultures (grafting, cuttings, *in vitro* propagation ; cultivation of woody and herbaceous plants ; biological indexing ; biological control and phytosanitary treatments) ; monitoring of plant symptoms and pests ; sanitary diagnosis by targeted analyses ; development of bioinformatics pipelines for the analysis of high-throughput sequencing data. **Approvals and quality management:** QU holds an official approval for containment and for quarantine release tests. It has an ISO/IEC 17025 accreditation, which guarantee the quality of its analyses reports. QU's accreditation notably includes the detection of all grapevine viruses by ELISA, of tobacco ringspot virus by conventional RT-PCR, and of *Xylella fastidiosa* by real-time PCR.

Nature of grapevine material received, processed or released: dormant shoots or vitroplants introduced into to the station for quarantine purposes ; potted plants released at the end of the process ; dormant shoots, leaves, veins or petioles for analyses needs ; healthy or infected materials (alive plant material, freeze-dried leaves, nucleic acid extracts, etc.) to enrich our conservatories in reference materials. **Containment facilities for plant cultivation, sampling and preparation:** an insect-proof tunnel ; a parcel opening laboratory with level 3 security ; climatic cabinets for *in vitro* culture ; confined climatic chambers with level 3 security ; a confined greenhouse with level 2 security ; a sample preparation laboratory with level 2 security. **Current laboratory analyses tests for grapevine sanitary diagnosis:** all phytoplasmas by real-time PCR ; blueberry leaf mottle virus (BLMoV) by ELISA ; grapevine flavescence doree phytoplasma by real-time PCR ; grapevine red blotch virus

(GRBV) by real-time PCR ; peach rosette mosaic virus (PRMV) by ELISA and real-time RT-PCR ; tobacco ringspot virus (TRSV) by ELISA and conventional RT-PCR ; tomato ringspot virus (ToRSV) by real-time RT-PCR ; *Xylella fastidiosa* by real-time PCR.

RESULTS AND DISCUSSION

From March 2007 to June 2023, the French post-entry quarantine station received 3251 plant samples, the majority belonging to the *Prunus*, *Malus* and *Solanum* genera. *Vitis* samples represent around 5% of all samples received over the period. With regard to grapevines, 14 **public bodies or private companies** (i.e. 9 French establishments and 3 other European establishments) have benefited from the French quarantine system to introduce **grapevine genetic resources** into European territory for testing, research or varietal selection purposes. Samples already received come from all over the world, from countries located in both the **northern and southern hemispheres** and listed below in alphabetical order: Argentina, Armenia, Australia, Canada, Chile, Georgia, Israel, Russia, South Africa, USA-California and USA-Wisconsin. Some geographical origins account for less than 1% of the incoming grapevine samples, while others represent up to 29% of them.

Grapevine samples are inspected and treated on receipt, and their **physiological and sanitary acceptability** is assessed (e.g.: search for symptoms of diseases, search for arthropods). Samples deemed acceptable are used for **cultivation**, and the resulting plants are maintained in containment facilities for 2 growing cycles, while all quarantine tests for **regulated quarantine pests** are carried out. In addition, the technical staff monitor plant health on a continual basis, looking for symptoms, vectors and pests. This way, the French post-entry quarantine station acts as a sanitary filter, and has already been responsible for the **interception** of grapevine quarantine pests such as PRMV, ToRSV or GRBV. In addition, **regulated non-quarantine or non-regulated pests** have been detected through health monitoring and additional analyses, such as: grapevine leafroll-associated viruses (GLRaV2, GLRaV3), grapevine virus A (GVA), grapevine fleck virus (GFkV) or grapevine red globe virus (GRGV).

Today, the station is seeking to diversify its analysis methods and is thinking about extending its services in aid of both the beneficiaries of the quarantine system and its main client (the French plant protection organisation). To improve its quarantine schemes, and in particular for the grapevine sector, QU is currently developing a PCR-based test for the detection of grapevine vein clearing virus (GVCV) and is deploying a **high-throughput sequencing (HTS)** approach for the detection of viruses and viroids (total RNA extraction in-house ; ribodepletion and Illumina sequencing subcontracted; data analysis in-house). This non-targeted method will be developed and validated through a thesis project. In addition, QU intends to develop a **grapevine sanitation** protocol as an alternative to the destruction of infected plants. To this end, QU already has regional and national scientific partnerships (IFV, INRAE, Université Clermont-Auvergne) and now aims to develop **international scientific partnerships**. The initial drafting of the European and Mediterranean Plant Protection Organization **(EPPO) Standard "Post-entry quarantine for Vitis"** by the unit and its participation in the EPPO panel for the final publication will contribute to achieve this objective.

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P4. Nanopore-based detection of grapevine viruses and viroids

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INTRODUCTION

Viruses and virus-like organisms limit grapevine production worldwide. They cause developmental and morphological malformations, reduce yields, change the chemical and sensory quality of grapes and wine, and shorten the life of vines. In the absence of chemical compounds for viral control, rapid and accurate detection is a critical step for sustainable viticulture. High-throughput sequencing (HTS) is a powerful technology with great applications in plant virus diagnostics and research, thanks to its ability to detect any viral entity in symptomatic and asymptomatic plants without prior knowledge (Al Rwahnih et al., 2009; Czotter et al., 2018; Nuzzo et al., 2022; Shvets et al., 2022). The aim of the presented work was to analyze the virome of the red vine 'Refošk' using the HTS platform - Nanopore sequencing technology.

MATERIALS AND METHODS

Vitis vinifera 'Refošk' was collected from the Primorska wine-growing region in Slovenia. RNA was isolated from leaves and petioles using Plant Virus RNA Kit (Geneaid) including a DNase step following the manufacturer's instructions. Quantitative and qualitative measurements of the isolated RNA were performed using a NanoVue Plus spectrophotometer (GE Healthcare Life Sciences) and Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA-PCR sequencing kit (SQK-PCS111) was used for library preparation. The library was then sequenced on a flow cell (R9.4.1) using the MinION device and MinKNOW software (v22.12.7). After sequencing, raw data were basecalled using Guppy (v6.4.6). Adapters were removed using Porechop. The overall quality control of the sequencing, error correction, mapping to the grapevine genome, and mapping to the viral database were performed with CLC Genomics Workbench (v23.0.3). De novo assembly was performed with Canu v2.2.

RESULTS AND DISCUSSION

After removing adapters, a total of 13,418,215 reads were obtained, of which 82.99% originated from the grapevine. The remaining reads were corrected and mapped to the virus reference database, of which 17.86% (average length: 1,168.92 nt) were identified as being virus- and viroid-derived. The used method revealed the presence of grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine Pinot gris virus (GPGV), grapevine red globe virus (GRGV), grapevine rupestris vein feathering virus (GRVFV), hop stunt viroid (HSVd), and grapevine yellow speckle viroid 1 (GYSVd-1). For GRSPaV, the reference sequence was 99.95% covered with an average sequencing depth of 124.84X. For GPGV, the reference sequence was 99.82% covered with an average sequencing depth of 120.16X. For the less abundant viruses GRGV and GRVFV, and the viroids HSVd and GYSVd-1, the reference coverage was 60.95%, 40.24%, 67.56%, and 64.67%, respectively, with an average sequencing depth of 4.70X, 8.41X, 1.68X, and 1.75X, respectively. Nanopore results were validated with RT-PCR.

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P5. Optimized Methods for Detecting of Grapevine Red Blotch Virus and a Rapid and Inexpensive Approach to Identifying its Candidate Insect Vectors in British Columbia

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INTRODUCTION

Tracking the occurrence and spread of Grapevine red blotch virus (GRBV) depends on robust and reliable diagnostics. Tissue sampling strategy and optimal choice of molecular test are critical for diagnostic success. This work investigated GRBV titres from several grapevine tissues and at different times during the growing season and winter dormancy in a Petit Verdot vineyard in the Okanagan Valley, British Columbia (BC), Canada. Two common nucleic acid extraction techniques were also compared.

When the spread of GRBV is identified through longitudinal observational study, it is critical to attempt to identify the process by which that spread is occurring. For geminiviruses like GRBV, transmission through the feeding of an insect vector is most likely. The ability of an insect to vector a plant virus has traditionally been determined through controlled greenhouse transmission experiments that are notoriously resource- and time-consuming and of unreliable outcome. Therefore, before committing to these protracted experiments, it is important that researchers identify biologically viable candidate vectors. This work presents an approach for identifying potential insect vectors of circulative plant viruses that significantly reduces the time-to-results while also reducing costs and required resources. This artificial transmission protocol using a buffered sucrose solution as the virus recipient in place of a living plant also eliminates plant-to-plant variation in susceptibility to infection, allowing assessment of virus-insect compatibility required for successful transmission of persistent viruses.

MATERIALS AND METHODS

This work investigated GRBV titres by droplet digital PCR (ddPCR) in roots, buds, cortical scrapings and leaves at varying distances from the cordons, and at different times during the growing season and winter dormancy. Two common nucleic acid extraction techniques were compared; a low-cost high-throughput nucleic acid extraction method (GES) and a DNA column purification method. Optimal combinations of sampling parameters were determined for summer and winter diagnostics.

To validate the artificial transmission approach, species of hemipteran insects, including leafhoppers, froghoppers, aphids, sharpshooters, and treehoppers, were subjected to an artificial feeding system to determine their ability to vector GRBV. Test insects were allowed to feed on a potted grapevine infected with GRBV for three days and then transferred to tubes containing the sucrose solution partitioned by a thinly stretched Parafilm™ membrane. After three days of feeding through the membrane, viruliferous test insects were stored for species identification and the sucrose solutions were tested by conventional polymerase chain reaction for the presence of GRBV DNA.

RESULTS AND DISCUSSION

In dormant samples, cortical scrapings yielded the highest GRBV titres and produced no false-negatives. In summer leaf samples, basal leaves yielded high GRBV titres throughout the summer months, with only one false negative on the earliest sample date. Cortical scrapings and basal leaves provide the best samples for reliable and reproducible GRBV diagnostics. The crude GES and DNA column purification methods performed comparably well for binary diagnostics, however, the GES method performed poorly in quantitative analysis (not shown).

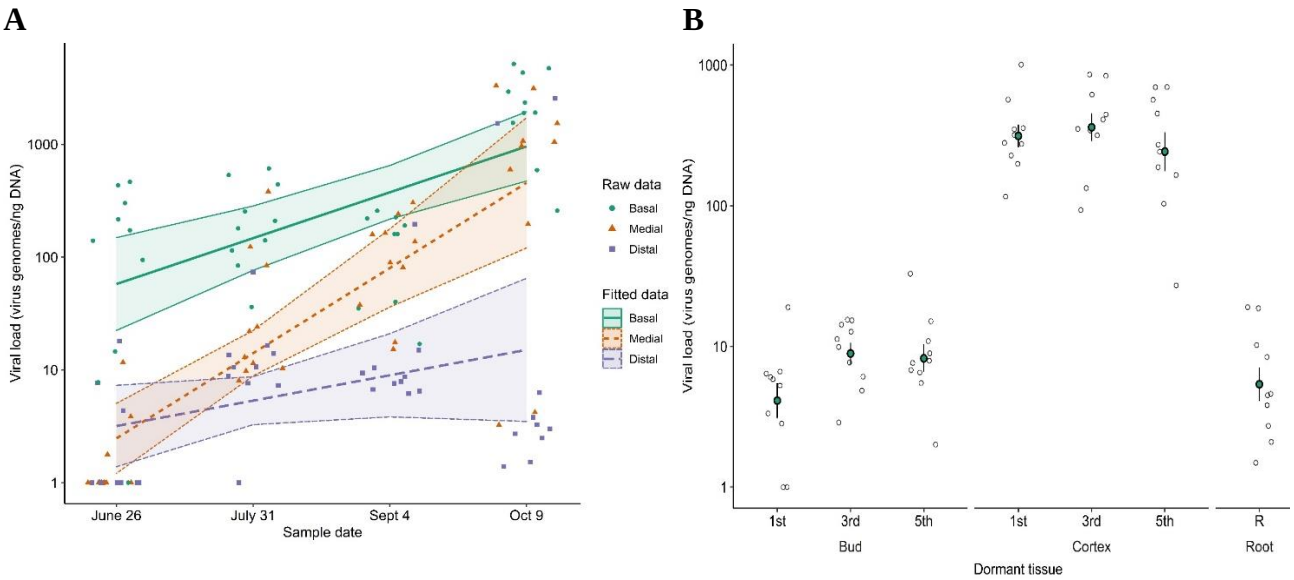

Fig 1. GRBV titres measured in infected samples collected from **a)** leaf and **b)** dormant tissues.

Out of all the insects tested in the artificial transmission system (n = 395), only nine treehoppers from two different species (*Stictocephala basali* and *S. bisonia*) successfully transmitted GRBV to the sucrose solutions, indicating a high likelihood of vector capability, to be validated by greenhouse or field experiments.

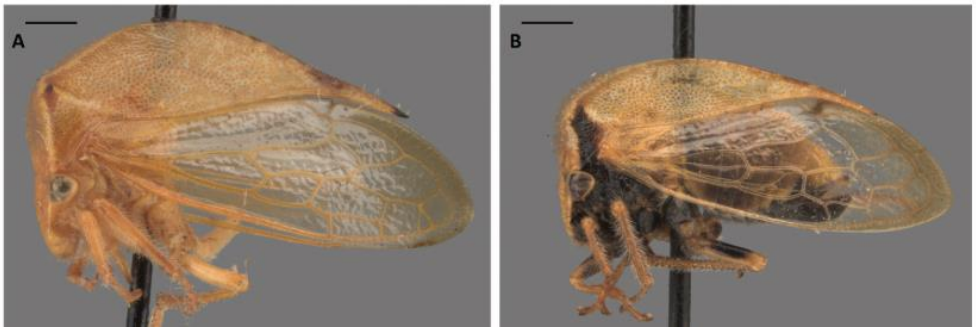

Fig 2. Two species of treehopper transmitted GRBV to solution, **a)** *S. basalis* and **b)** *S. bisonia*.

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A special thanks to Dennis Kopp for donating his personal time to taxonomically identify several treehopper specimens for us and for preparing voucher specimens (USDA National Institute of Food and Agriculture; retired). I am also grateful to the unlisted authors (see references) for their invaluable support and contributions to this work. This research was funded by the British Columbia Wine Grape Council (BCWGC), The Canadian Grapevine Certification Network (CGCN-RCCV), and Agriculture and Agri-Food Canada (AAFC) under the Canadian Agricultural Partnership (CAP) funding program.

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P6. Duplex quantitative real-time RT-PCR for detection of grapevine leafroll-associated virus 1

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INTRODUCTION

Grapevine leafroll-associated virus 1 (GLRaV-1), is an important regulated pathogen that belongs to the grapevine leafroll disease (GLD) complex, and it is transmitted by mealybugs and soft scale insects (Meng et al., 2017). The high genetic intra-species variability of GLRaV-1, which has important diagnostic implications and represents a major drawback for its control, has led to a great diversity of detection methods (Osman, et al., 2007, Alabi et al., 2011, Pacifico et al., 2011, Bruison et al., 2017, Aloisio et al., 2018). With the aim of improving GLRaV-1 diagnosis, a new and highly inclusive real-time RT-PCR method, able to detect all GLRaV-1 isolates currently known in plant material and transmission vectors, has been developed and validated.

MATERIALS AND METHODS

Plant and insect material: 65 GLRaV-1-positive samples of 16 different grapevine varieties from different geographic origins (Spain, Switzerland, Slovakia, Tunisia, Thailand, Greece and Germany) were used for validation. In addition, 241 samples from different Spanish growing areas were analyzed. *Planococcus citri* fed on GLRaV-1 infected leaves were individually analyzed after 48 h acquisition.
HTS analysis and genome recovery: Total RNA was sequenced in a NextSeq 500 platform (2x150 paired-reads) at Macrogen. HTS raw data were analyzed using CLC Genomics Workbench 10.1.1 and Geneious Prime 2022. After trimming, quality control, grapevine genome subtraction and *de novo* assembly, contigs were analyzed by BLASTN/X and GLRaV-1 related contigs extended.

Designing of specific primers and probe: GLRaV-1 specific primers and probe were designed based on the alignment of 659 full and partial genomes using Geneious algorithm.

TaqMan quantitative real-time RT-PCR method: RT-PCR was carried out in three different equipment using 1.2 µM of each of the GLRaV-1 primers, 100 nM of PEP primers, 200 nM of GLRaV-1P and 50 nM of PEP-probe. Amplification conditions consisted of 45 °C for 10 min, 95 °C for 10 min and 45 cycles of amplification (15 s at 95 °C, 1 min at 60 °C). Absolute quantitation standard curves were generated using *in vitro* transcribed RNA as previously reported (Morán et al., 2018).

In silico evaluation of GLRaV-1 detection methods specificity: The sequence mismatching of GLRaV-1 primers and probes was classified in a variant frequency score scale: <5%; 5-20%; 20-30%; >30%. Mismatch position in the sequence was also scored as critical when located less than four residues away from the 3′ end of the primer.

Validation of the designed GLRaV-1 new method: Technical sensitivity, inclusivity, exclusivity, selectivity, repeatability and reproducibility were evaluated following EPPO standards (PM 7/98).

RESULTS AND DISCUSSION

In silico analysis showed the occurrence of a high number of mismatches with variant frequency values higher than 30% as well as critical mismatches close to the 3’ end of the primers for most of the

previously reported methods, which would therefore exhibit important limitations for an inclusive GLRaV-1 detection (Figure 1).

Multiple alignment of 659 GLRaV-1 sequences, including 3 near full length sequences obtained in this study (PIN1, Spain, OQ029646; AUTH63, Greece, OQ029678; and SK809, Slovakia, OQ029645) allowed the designing of a duplex real-time RT-PCR amplifying a 186 bp fragment in GLRaV-1 CP gene and a plant internal control, the grapevine PEP gene (Morán et al., 2018), based on three specific primers and a TaqMan probe: GLRaV-1-F, 5′-GAA TGG AAA GTT GAA GCC GAA-3′; GLRaV-1-R1, 5′-TAC TGA GCT TGT CAC ATT ACT-3′ and GLRaV-1-R2, 5′-AAC CGA GCT TGT CAC ATT A-3′; and GLRaV-1P, 5′-6-FAM-TGC AGA CCW TCT TAY TCT CAR TTT AG-ZNA-4-BHQ-1-3′. In silico analysis of the designed protocol predicted a much more inclusive GLRaV-1 detection, compared to previous methods (Figure 1). The protocol has been validated according to EPPO standards.

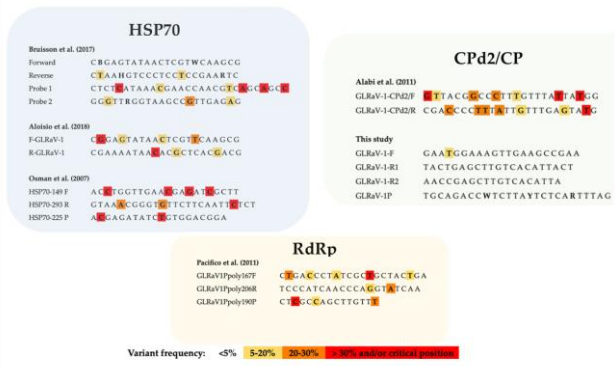

Figure 1. Frequency of primers/probes mismatches in GLRaV-1 detection methods.

Repeatability and reproducibility were evaluated by analyzing 9 technical replicates of 7 GLRaV-1 infected samples with a low relative concentration. The obtained results indicated a high level of consistency in the performance of the technique (Table 1).

The method was able to detect up to 44 copies of viral targets. The technical sensitivity in the plant material showed a detection limit of 67 viral copies.

Evaluation of analytical specificity showed that the designed technique was able to detect all the currently known GLRaV-1 diversity. In addition, the exclusivity of the method towards the presence in the matrix of common grapevine viruses, as determined by HTS, was also demonstrated.

Table 1. Evaluation of repeatability and reproducibility of the quantitative real-time RT-PCR

Samples	StepOne Plus		QuantStudio		Roche 480		All Equipments	
	Mean Ct ± SD	CV (%)	Mean Ct ± SD	CV (%)	Mean Ct ± SD	CV (%)	Mean Ct ± SD	CV (%)
91.1	32.34 ± 2.74	8.5	27.15 ± 1.79	6.6	31.37 ± 1.55	5.0	30.29 ± 2.79	9.2
91.2	31.40 ± 1.40	4.5	29.33 ± 0.95	3.3	31.54 ± 0.50	1.6	30.76 ± 1.58	5.1
91.10	33.62 ± 1.01	3.0	32.43 ± 0.58	1.8	32.43 ± 1.71	5.3	32.83 ± 0.68	2.1
91.11	33.68 ± 1.69	5.0	30.61 ± 0.73	2.4	33.84 ± 0.68	2.0	32.71 ± 1.82	5.6
98.16	34.29 ± 0.98	2.9	33.64 ± 0.84	2.5	32.65 ± 0.75	2.3	33.53 ± 0.83	2.5
102.18	24.05 ± 1.91	7.9	21.49 ± 1.53	7.1	26.48 ± 1.71	6.5	24.00 ± 2.50	10.4
102.20	25.64 ± 1.08	4.2	20.48 ± 0.39	1.9	24.50 ± 0.49	2.0	23.54 ± 2.71	11.5

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P7. Development and validation of three novel real-time RT-PCR assays for the reliable detection of grapevine virus A, grapevine virus B and grapevine virus F

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INTRODUCTION

Grapevine virus A (GVA), grapevine virus B (GVB) and grapevine virus F (GVF) belong to the genus *Vitivirus* in the family *Betaflexiviridae* (Minafra et al., 2017). GVA and GVB are associated with the Rugose Wood (RW) complex in grapevine, which causes pitting and grooving of the wood (Minafra et al., 2017), whereas the role of GVF in the RW or its impact in grapevine, if any, is yet to be determined (Mannini and Digiaro, 2017). GVA and GVB are known to occur in Greek vineyards with GVA being the most prevalent (Orfanidou et al., 2021), while GVF was first discovered in Greece in 2017 (Panailidou et al, 2019). All three viruses exhibit high genetic diversity and therefore it is essential to update the current molecular diagnostic assays for their reliable detection. In this study three novel real-time RT-PCR assays were developed. The detection range of the methods was evaluated using genetically diverse isolates from each virus. The methods were then used for testing different types of plant tissue collected during three seasons for the presence of GVA and GVF.

MATERIALS AND METHODS

For the design of primers and probes all publicly available full genome sequences of the three viruses and others obtained herein were aligned and conserved regions in their coat protein encoding genes for GVA and GVB and in their RNA binding protein encoding gene for GVF were targeted. Following optimization of the three real-time RT-PCR assays they were then subjected to further validation of their specificity and sensitivity. For this purpose, divergent isolates from each virus belonging to different phylogenetic groups, based on their full genome sequences, were used. Standard curves, using seven serial 5-fold dilution points of cDNA were constructed for the estimation of the efficiency of the three methods. For each virus, total RNA from two samples, one with a single virus isolate and one pooled sample of divergent isolates, were used. Once the efficiency of each method was established, they were applied for testing several types of tissue (leaves, petioles, phloem scrapings) collected from different grapevine shoots/canes infected with GVA or GVF from the Farm of the Aristotle University of Thessaloniki (AUTH) during three consecutive seasons, spring, summer and fall.

RESULTS AND DISCUSSION

Samples containing divergent isolates, from different phylogenetic groups, of each virus were tested with the established methods and the viruses were detected in all of them (Fig.1a, 2a, 3a). The real time RT-PCR efficiency for GVA was 96,284% (Fig.1b) for the sample that contained one isolate and 96,416% for the pooled sample that contained divergent isolates. The efficiencies for the GVB samples were 92,282% (Fig.2b) and 96,909% respectively. For the GVF samples the efficiencies were 99,793% (Fig.3b) and 108,748%. All the above results are within the acceptable range of 90-110%. Three plants from the Farm of AUTH were tested for the presence of GVA. The virus was readily detected in leaves, petioles and phloem scrapings during spring, summer and fall. Furthermore, one vine was tested for the presence of GVF, which was also detected in all samples that were collected during the three different seasons. This is the first real-time RT-PCR assay designed for the detection of GVF.

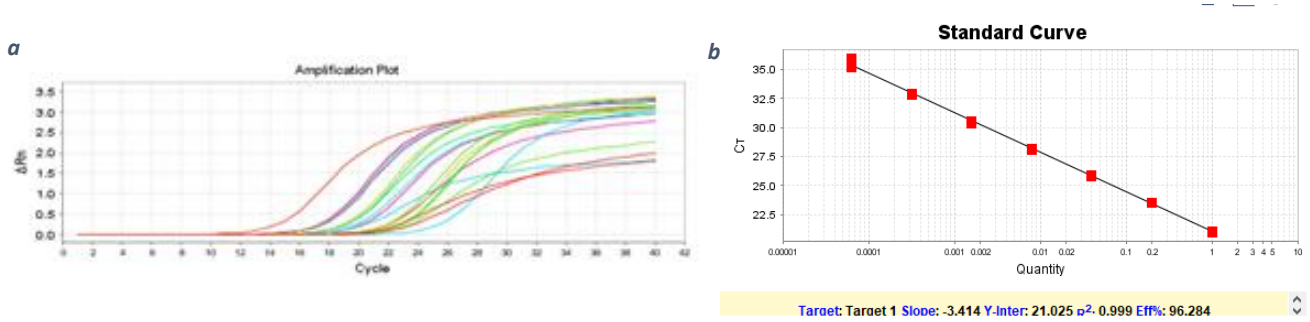

Fig.1. a. Detection of divergent isolates of GVA, ***b***. Standard curve using triplicates of 5-fold dilutions of the sample containing a single isolate of GVA.

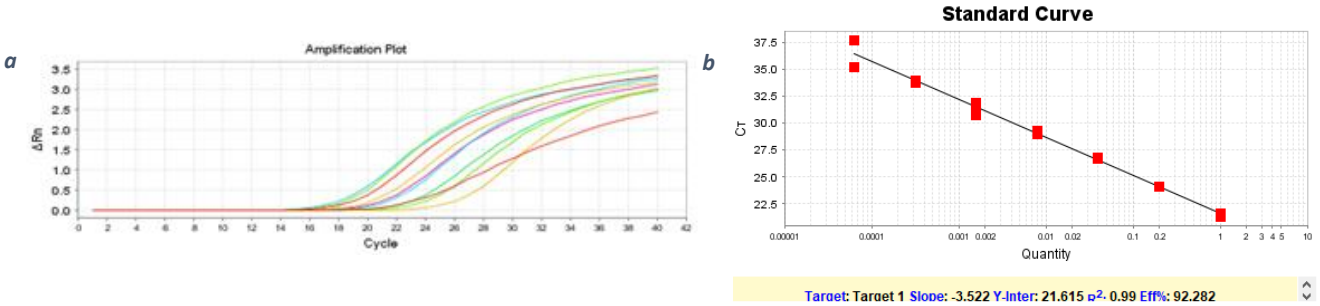

Fig.2. a. Detection of divergent isolates of GVB, ***b.*** Standard curve using triplicates of 5-fold dilutions of the sample containing a single isolate of GVB.

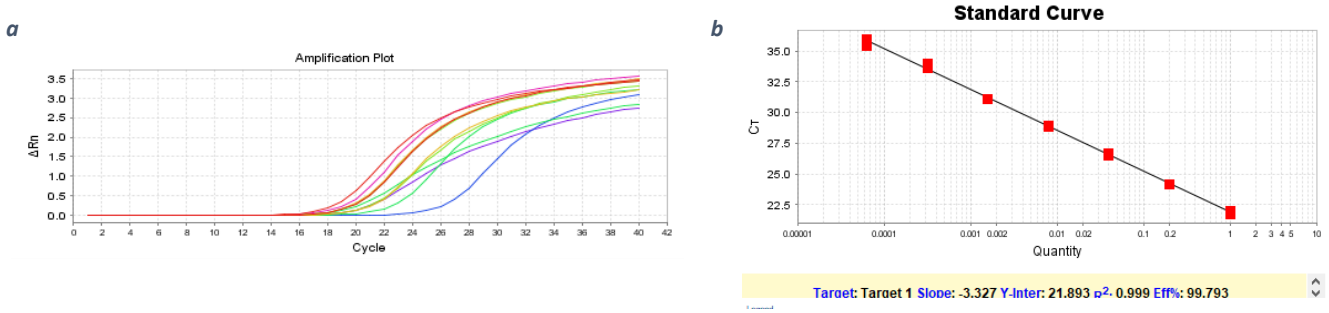

Fig.3. a. Detection of divergent isolates of GVF, ***b.*** Standard curve using triplicates of 5-fold dilutions of the sample containing a single isolate of GVF.

ACKNOWLEDGEMENTS

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P8. Occurrence of Grapevine Pinot Gris Virus in vineyards of northwestern Italy

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INTRODUCTION

Grapevine Pinot Gris Virus (GPGV) is a member of the genus *Trichovirus*, family *Betaflexiviridae*, which was characterized approximately 10 years ago in *Vitis vinifera* cv. Pinot gris in the Trentino region in Northeast Italy (Giampetruzzi et al., 2012). The virus has since been identified in most grapevine growing regions around the world and in RNA samples from grapevines collected in 2002-2005 in different European countries (Bertazzon et al., 2016). GPGV has been putatively associated with grapevine leaf mottling and deformation (GLMD) characterized by leaf mottling and deformation, short internodes, stunted shoots, and reduced yield. However, a large number of asymptomatic grapevines hosting GPGV has also been reported (Saldarelli et al. 2015; Tarquini et al. 2019). The northwestern region of Italy was considered to be few interested by the virus because GLMD symptoms were not identified in the cultivars grown in this area. However, recently some data have suggested the presence of this virus also in these vineyards (Nuzzo et al., 2022). In the present study, we assessed the occurrence of GPGV in 1,000 RNA samples collected in northwestern Italy over the period 2010-2014, and the natural spread of GPGV in commercial vineyards of the same region was investigated over two years (2022-2023).

MATERIALS AND METHODS

Ten vineyards cultivated with cv. Nebbiolo, Barbera, Chardonnay, Arneis, Freisa, Dolcetto, Moscato Bianco, and other varieties, located in Piedmont, Aosta Valley and Liguria regions, were selected to investigate the spread of the GPGV over two years (2022-2023). Leaf samples from 10 plants were collected from each cultivar in each vineyard at the end of May after visual investigations of GLMD symptoms. Total RNA was extracted following the protocol of Gambino et al. (2008) and GPGV detection was carried out by one-step real-time RT-PCR assays using SYBR Green and the primers GPgV504F and GPgV588 (Bianchi et al., 2015). To characterize the isolates of GPGV, RT-PCR amplification products obtained using the primer pairs DetF/DerR (Morelli et al., 2014), were purified and sequenced by Sanger sequencing. Furthermore, 1,000 RNA samples stored at -80°C and collected in vineyards of northwestern Italy between 2010-2014 during the routine activities of the Functional Genomics group of IPSP were analyzed for GPGV detection. Sequences of GPGV deposited in GenBank (NCBI) were aligned with the isolates characterized in this work using Multiple Sequence Alignment (MUSCLE, https://www.ebi.ac.uk/Tools/msa/ muscle/). Phylogenetic analysis based on the neighbor joining (NJ) method (with bootstrap values of 1,000 replicates) was carried out using MEGAX software version 10.1.7.

RESULTS AND DISCUSSION

Since the identification of GPGV in 2012 (Giampetruzzi et al., 2012), the virus has been considered absent or marginal for the viticultural areas of the northwestern Italy. However, its identification in some plants (Nuzzo et al., 2022) have suggested the occurrence of GPGV also in these territories. Analyzing 1,000 RNA samples extracted from plants collected between 2010-2014, GPGV was already present in 2012 in Piedmont in eight plants of 'Moscato Bianco' and in two of 'Nebbiolo' and

in two local varieties collected in the Aosta Valley in 2014. Sequence analyzes demonstrated similarities between the isolates infecting the 'Moscato Bianco' and 'Nebbiolo' with isolates collected in France in 2013 (BK011077) and 2017 (MN458444), respectively. The two samples infected by GPGV collected in the Aosta Valley showed similarities with some isolates collected in USA (MK514524) in 2017.

In 2022, 235 samples were collected from six vineyards in Piedmont and analyzed for the presence of GPGV. The typical symptomatology of GLMD has never been identified in any plants, but through molecular analyses, GPGV has been identified in 64 samples (Table 1).

Cultivar	No. of samples	No. GPGV-infected	% GPGV-infected
Nebbiolo	65	30	46.2%
Arneis	45	16	35.6%
Chardonnay	20	6	30.0%
Barbera	50	8	16.0%
Freisa	30	4	13.3%
Dolcetto	10	0	0.0%
Moscato Bianco	10	0	0.0%
Pinot gris	5	0	0.0%
Total	235	64	27.2%

Table 1. Cultivars collected in 2022 in vineyards of northwestern Italy, number/percentage of GPGV-infected plants.

The percentages of infection vary considerably according to i) the genotype, almost half of the 'Nebbiolo' samples are infected by GPGV (Table 1) and ii) the vineyard, 66% of the infected samples were collected in only two vineyards. GPGV in Piedmont showed a clear predominance for asymptomatic isolates, only three, identified in 'Nebbiolo' and 'Arneis', are close to the isolates considered symptomatic (Giampetruzzi et al., 2012; NC_015782.2), even if they appear asymptomatic in our conditions. In 2023, new vineyards were selected throughout northwestern Italy to expand the survey, and the new samples are currently being analyzed.

The results suggest that GPGV is very common in northwestern Italy even if asymptomatic, and the spread of the virus in this area started already before 2012 as reported in other viticultural regions of the world.

ACKNOWLEDGEMENTS

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P9. Dieback in vines infected with Shiraz Disease may result from double infection of grapevine virus A and a botryosephaeriaceous fungus, *Diplodia seriata*

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INTRODUCTION

Shiraz (syn. Syrah) is the principal wine grape variety in Australia. The most recent data indicated an annual yield of 425,000 tonnes in 2022 (Wine Australia). However, there have been ongoing concerns regarding the susceptibility of Shiraz to various viruses, including grapevine virus A (GVA, genus *Vitivirus*, family Betaflexiviridae), which leads to the development of Shiraz Disease (SD). GVA, especially genetic variant II (Goszczynski and Habili, 2012; Wu et al., 2023), has deleterious effects on Shiraz vines, causing significant berry loss and an inferior wine quality. Shiraz Disease was first reported by Corbett & Wiid (1985) in South Africa in Shiraz, Merlot and Gamay grape varieties. In South Africa, in addition to GVA, *Grapevine leafroll-associated virus 3* (GLRaV-3) is usually detected in SD-affected plants. Although GVA is present in other countries, it is symptomless except when infected vines are grafted on the Kober 5BB rootstock. In Australia, SD affects Shiraz, Merlot, and Malbec varieties. Many other *grapevine varieties* including rootstocks, white grapes and certain red grape varieties like Cabernet Sauvignon, Grenache and Nero D'Avola do not show the symptoms of SD. *The symptoms include late bud burst and* restricted spring growth. In autumn, red leaves curl downwards and remain on the canopy through the winter. Since 2015, we have observed dieback symptoms in GVA positive vines like those in Grapevine Trunk Disease (GTD). Here, we report preliminary results on GVA positive vines showing dieback when additionally infected with *Diplodia seriata*, a Botryosphaeriaceous fungus.

MATERIALS AND METHODS

Plant material: A total of 80 samples were collected from vineyards A and B in 2019 and 2021, from two visits each year in spring and in autumn (Table 1). Wood samples from randomly selected vines with no symptoms or symptomatic vines (with dieback) were collected for virus and for GTD testing.

Two vineyards (A and B) at Riverland, South Australia (Habili et al., 2016) and one in Yenda, NSW were studied. Vineyard A was in Glossop, SA, planted in 1994 with 7 hectares of Shiraz on Ramsey rootstock. Vineyard B was at Renmark, SA, nearly 22 km northeast of vineyard A. This 1ht vineyard was planted with Merlot on 101-14 rootstock in 1996.

Sampling The vineyards were sampled twice a year, each in spring and in autumn. Ten samples from asymptomatic vines and 10 samples from dieback ones were sampled per season from each vineyard. Wood tissue of virus-eliminated vines which had been previously generated by meristem tip culture (Malk et al., 2012), planted in 2009 at our research vineyard (Urrbrae, 290 km southwest of vineyards A and B) was used as the control. The varieties (two vines of each) included Nebbiolo, Arnies, Ramsey (*Vitis champini*), Chardonnay and Sangiovese.

Nucleic acid extraction and polymerase chain reaction: Wood from the basal canes was cut and the internal tissue surrounding the pith was stripped off. Nucleic acids were extracted from 0.1 g of scrapings by an in-house silica method described recently (Wu et al., 2023). For virus testing, scrapings from bark of phloem tissue and the nucleic acids were extracted as above. RT-PCR was carried out for the detection of viruses using primers as described (Wu et al., 2023), A segment of nucleic acid on the ITS of the nuclear rRNA gene was amplified using Botryosphaeriaceous (BOT) primers BOT100F

(5'-AAACTCCAGTCAGTRAAC-3') and BOT472R (5'-TCCGAGGTCAMCCTTGAG-3') developed by Ridgway et al. (2011), producing an amplicon size of 371-372 bp. The PCR bands were cut and sequenced on both directions using the Botryosphaeriaceous primers described above.

Fungal isolations: Five samples from symptomatic vines were sent for diagnostics from South Australia to Charles Sturt University, Plant Pathology Laboratory. Wood sample (~5 mm) were cut from the edge of the necrotic tissues and surface-sterilised following the methods described by Pitt et al. 2010. The tissues were transferred onto potato dextrose agar (PDA) and incubated at 25C in total darkness for 5-7 days and observed. All Botryosphaeriaceae-like colonies were sub-cultured onto sterile PDA and identified based on colony and spore morphology.

RESULTS

Two vineyards at Riverland, SA, were visited after the grape growers informed us of their concern. Up to 20% of vines showed dieback in 2019. In Vineyard A the symptomatic vines appeared as clusters in the rows next to a Chardonnay patch (Figure 1-1&1-2) which tested positive for GVA in a previous analysis (tested in 2015, data not shown) without showing symptoms. Randomly selected samples from vines with dieback (D) and non-dieback (ND, Table 1) were collected (40 from each vineyard). All 80 samples were tested for GVA and for BOT. The results, as summarized in Table 1, showed that a BOT species, *D. seriata*, was detected in all 80 samples irrespective of any symptoms. However, only the dieback vines tested positive for GVA (Table 1). To determine if all vines growing in the field carry *D. seriata,* we tested vines from five virus-eliminated varieties: Chardonnay, Arnies, Nebbiolo, Sangiovese and Ramsey growing at our research vineyard in Urrbrae, SA. After 11 years in the field, neither GVA nor any of the six BOT isolates were detected. In May 2022, we received two Shiraz samples showing dieback (5%) from Yenda, NSW (Australia). We detected the presence of GVA and *D. seriata* in the diseased samples confirming those at Riverland.

Table 1. The results of testing two commercial vineyards at Riverland, South Australia, for *Diplodia seriata* and for grapevine virus A (GVA).

Vineyard[1]/date of sampling	Sample number/ disease status[2]	+ve for *Diplodia*	+ve for GVA	-ve for GVA
Vineyard A- 2019 spring	5/ND + 5/D	5/ND + 5/D	5/D	5/ND
Vineyard A- 2019 autumn	5/ND + 5/D	5/ND + 5/D	5/D	5/ND
Vineyard A- 2021 spring	5/ND + 5/D	5/ND + 5/D	5/D	5/ND
Vineyard A- 2021 autumn	5/ND + 5/D	5/ND + 5/D	5/D	5/ND
Vineyard B- 2019 spring	5/ND + 5/D	5/ND + 5/D	5/D	5/ND
Vineyard B- 2019 autumn	5/ND + 5/D	5/ND + 5/D	5/D	5/ND
Vineyard B- 2021 spring	5/ND + 5/D	5/ND + 5/D	5/D	5/ND
Vineyard B- 2021 autumn	5/ND + 5/D	5/ND + 5/D	5/D	5/ND
Total samples:	80	80	40	40

[1]Vineyard A, Shiraz on Ramsey, Vineyard B, Merlot on 101-14. [2]ND, vine with no dieback; D, vine with dieback.

Fungal isolations: Botryosphaeriaceae-like isolates were recovered from all symptomatic wood samples that were plated on PDA. Of the five isolates recovered, three were morphologically identified as *D. seriata* while the other two were identified as *Spencermartinsia viticola*.

When the fungus was cultured the spore morphology showed it was most likely *Diplodia seriata.*

DISCUSSION

Shiraz vines infected with Shiraz Disease normally produce purple leaves with drooping canopies that do not enter the senescence stage (Habili et al., 2016). Here, we observed a second type of symptom, "Dieback", in Shiraz and Merlot infected with SD which expresses only in the presence of

GVA and *D. seriata*. It is not known why *D. seriata* in the absence of GVA is silent. Although the primers used here were specific for six species of Botryosphaeriaceous fungi, only *D. seriata* was detected. It is generally true that plant viruses do not directly kill their host plants. Instead, they often weaken the plants by interfering with their normal physiological processes. In Riverland, Shiraz vines which are already latently infected with the opportunistic fungus *D. seriata* (Table 1), show dieback once GVA is brought in by insect vectors. Up to date, over 133 fungal species belonging to 34 genera have been associated with GTD worldwide (Gramaje et al., 2018). In Australia, *D. seriata* accounted for almost 80% of the total number of *Botryosphaeriaceae* isolates surveyed (Pitt et al., 2010). The *D. seriata* amplicon with 372 bp was identical to the sequence of this fungus in South African vineyards (not shown). More work is needed to clarify the results obtained and to prove the application of Koch's postulates specifically for *D. seriata* in the presence and absence of GVA. The BOT primer pair may be a useful tool for nurseries to identify the most common Botryosphaeria dieback pathogen, *D. seriata*, and improve nursery hygiene. Since mechanical pruning and machine harvesting induce a lot of wounds on canopy which provide the entry points for GTD agents through two working seasons each year, these fungi have readily become widespread. An alternative management strategy should be designed as soon as possible.

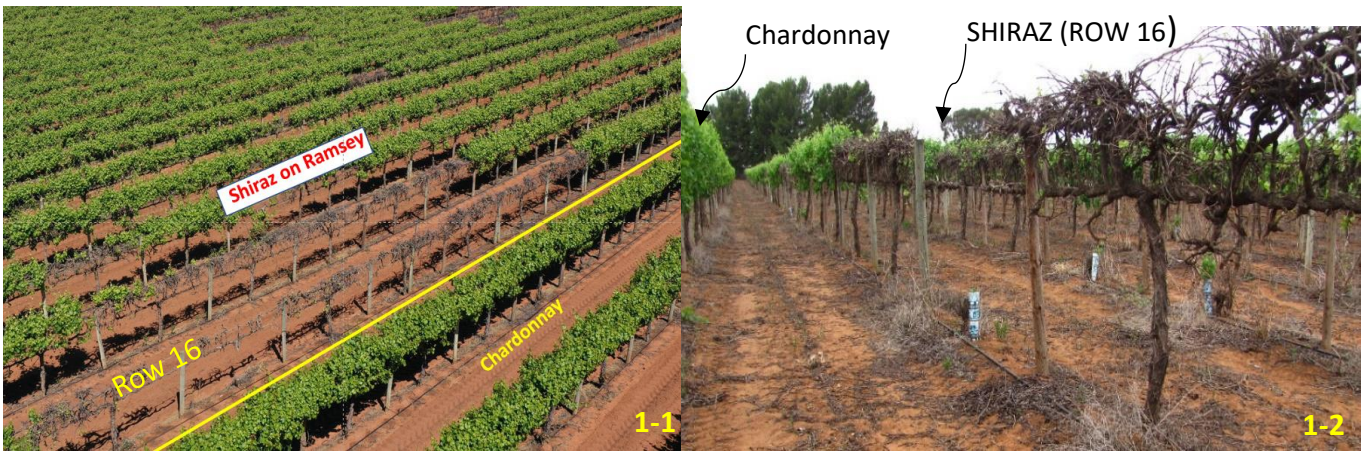

Figure 1-1 (aerial view) &1-2 (close-up view) of Vineyard A in Riverland, South Australia. The asymptomatic rows of Chardonnay are the source of grapevine virus A (GVA). All Shiraz vines tested positive for *Diplodia* while the decline vines had both *Diplodia* and GVA.

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P10. Global warming and spread of *Planococcus ficus* / GLRaV-3 under the cool climate of northwestern Spain

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INTRODUCTION

The best described epidemics of grapevine virus diseases are those caused by Grapevine leafroll associated viruses transmitted by several species of Coccids and Pseudococcids. The most widely studied are those involving mealybugs and GLRaV-3. The global warming affects the risk of arrival, survival of overwintering populations, increase of the rate of reproduction and spread of mealybugs to areas where they were almost unknown; and that could have a tremendous impact in the grapevine leafroll disease (GLD) epidemics (Cabaleiro et al., 2022). GLD affects yield and must quality and shortens the productive life of vineyards and when they are replanted winegrowers face the challenge of protecting plants from GLRaV transmission by mealybugs.

MATERIALS AND METHODS

The surveys were done in vineyards in the south of the Rías Baixas Appelation (Galicia, Spain) in three phases: 1990-1993 (36 vineyards), 2003-2004 (47) and 2014-2016 (22). Vineyards were searched for ants and mealybugs (1-3 minutes/plant) and GLRaV-3 was analyzed in randomly sampled vines by DAS or DIP-ELISA.

In a vineyard with several plots with low, medium, or high infestation by mealybugs, in pheromone-baited traps for *P. ficus* males were used to draw flight curves in 2016-2019 to know the activity period in the cool climate of the south of Galicia. The available meteorological data (2001-2022) were discharged from METEOGALICIA stations (https://www.meteogalicia.gal) to study the evolution of the number of generations that could be completed in each wine-growing area in the last two decades and the risk of mealybugs survival and spread in areas where they have not been detected so far. Subsequently, degree-days (DD) were calculated for each station, and interpolation was performed using the regression kriging tool of the SAGA extension in QGIS to generate a map depicting the distribution of degree-days in Galicia according to three models for *P. ficus* based on DD and thermal integral (Walton & Prickle, 2005; Varikou et al., 2010; González & La Rosa, 2015).

RESULTS AND DISCUSSION

In the 1990s only two vineyards with mealybugs were detected in the South of Galicia, and growers have never seen those insects in vineyards; from that <5% an increase to almost 20% was detected in the period 2003-2005 and then a clear exponential increase in prevalence happened starting after the exceptionally hot and long summer of 2006: the percentage of vineyards with mealybugs reached 85% in 2016, with some zones with 100%. *Pl. ficus* is the dominant mealybug, *Pl. citri* and *Pseudococcus viburni* were identified only in 2 two vineyards, in mix infestation with *Pl. ficus*. GLRaV-3 and clear symptoms of GLD were detected in most vineyards but if in the 1990s the incidence was an average of 20-30%, in the last survey, in vineyards with mealybugs easily observed, the incidence was over 90%. Male flight curves showed very high captures in 2017-2019 in the two plots with strong vine infestation and significantly lower in those where nymphs were difficult to observe (Fig. 1). The date of first captures was later than in Mediterranean climates, but quite irregular: August 16 in 2016; May 25 in 2017; July 30 in 2018 and June 10 in 2019; some years in December males were still caught in the traps; the curve pattern is also different (Mansour et al., 2018). Between 2001 and 2022 clear warming can be appreciated: the number of potential generations increased (Fig. 2) in the three DD

models but with clear differences among them in the predictions. The risk of survival of mealybugs exists now in all Galician grape growing areas (Fig. 2) and they are a key pest in some areas; control measurements are being implemented but even when they work to reduce populations of *Pl. ficus* as a pest, the transmission of GLRaV-3 is difficult to avoid.

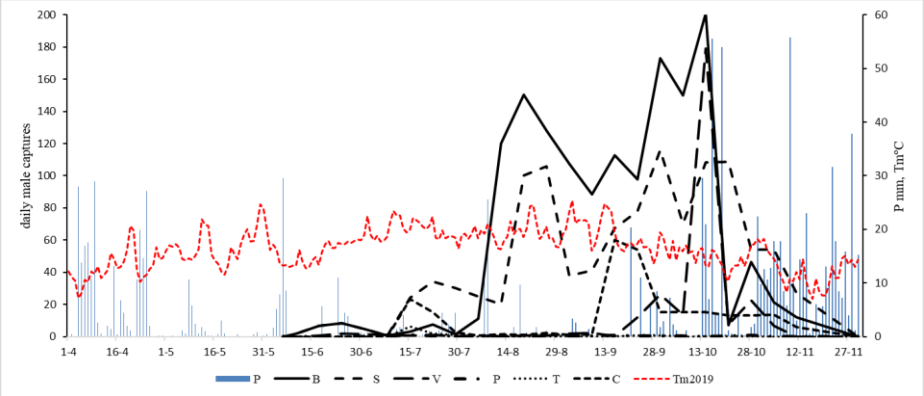

Figure 1. Counts of adult male *P. ficus* caught in pheromone-baited traps in 2019 in plots with high (B, S), medium (S) and low infestation (P, T, C). Effect of daily rainfall (mm) and average temperature on the captures.

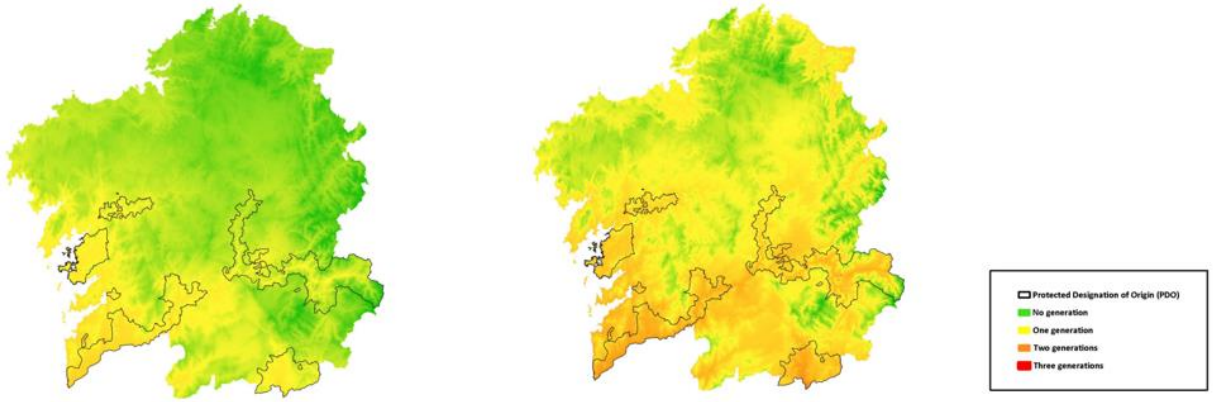

Figure 2. Maps showing the evolution of potential generations of *P. ficus* in 2002 and the average for the period 2002-2023 according to the degree-days proposed by Walton et al (2005): 16.59ºC and 235ºD.

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P11. Survey of grapevine-leafroll associated virus 3 in herbaceous plant species

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3; *Ampelovirus*, *Closteroviridae*) is one of the most widespread and economically important grapevine viruses, and the most important causal agent of grapevine leafroll disease. GLRaV-3 is graft-transmissible and is spread by infected planting material and vector transmission by mealybugs (*Homoptera: Pseudococcidae*) and/or soft scale insects (*Homoptera*: *Coccidae*) (Martelli and Boudon-Padieu, 2006). Vector transmission occurs in a semi-persistent manner, but without vector specificity with respect to mealybug transmission (Tsai et al. 2008, 2010). Although European grapevine (*Vitis vinifera* L.), American *Vitis* species and hybrids are considered the natural hosts of GLRaV-3, vector-mediated transmission to *Nicotiana benthamiana* was reported (Prator et al., 2017), and recently the virus was confirmed in peonies in Ukraine (Mishchenko et al., 2022). To investigate the host range of GLRaV-3 in weeds and herbaceous plants commonly used in plant virology experiments (e.g., *Nicotiana spp., Chenopodium spp.*), a study was conducted using vine mealybug (*Planococcus ficus*) as a vector for transmission from GLRaV-3-infected grapevine to the various herbaceous plant species.

MATERIALS AND METHODS

Grapevine accessions VVL-112 (cultivar 'Vlaška') and PMC-022 (cv. 'Plavac mali') from the Grapevine Virus Collection (University of Zagreb Faculty of Agriculture) were used as GLRaV-3 sources. Crawlers (1st and 2nd stage instars) of vine mealybug (*P. ficus*) reared on butternut squash (*Cucurbita moschata*) were used for transmission experiments. As recipient plants 16 different weed species and six different herbaceous species commonly used in plant virology experiments, together with grapevine seedlings as a positive control, were used. Transfer of vectors to recipient plants occurred at the stage of 4-6 well-developed leaves. After a 48-h acquisition access period (AAP) on the GLRaV-3 source plants (VVL-112 and PMC-022), crawlers were transferred in groups of 10 individuals with a paint brush for a 48-h inoculation access period (IAP) on the recipient plants, and then removed either mechanically or by spraying with an insecticide (imidacloprid). Transmission success was verified two (herbaceous plants) and six (grapevine) months after IAP using real-time RT-PCR with conditions, primers and probes, and 18S rRNA as an internal control as previously described (Diaz-Lara et al., 2018; Jagunić et al., 2022). Reactions were performed using the Applied Biosystems 7500 Real-Time PCR System (Thermo Fischer Scientific, USA). Extraction of RNA from plants was performed from 0.1 g of leaf tissue and petioles processed as previously described (Osman et al., 2007).

RESULTS AND DISCUSSION

None of the 417 weed plant species belonging to 16 different species from 11 different families, and none of the 140 herbaceous test plants from two families, were positive for GLRaV-3 in transmission trials with vine mealybug, as determined by real-time RT-PCR. Successful vector-mediated transmission was achieved only in grapevine to grapevine trials, with success rates ranging from 12.5 to 25% (Table 1). Further studies involving different vector species (mealybugs and soft scale insects) and different GLRaV-3 variants should be conducted to provide a comprehensive overview of the role of vegetation in vineyards in the epidemiology of GLRaV-3.

Table 1. Results of GLRaV-3 transmission to different weeds/herbaceous test plants by the vine mealybug (*P. ficus*). Experiments were conducted for four consecutive years (combined summary presented), and detection results were determined by real-time RT-PCR.

Latin name	Common name	Family	No. of GLRaV-3 positive plants / No. of plants in trial			
Weeds			**Year I**	**Year II**	**Year III**	**Year IV**
Abuthilon theophrasti Medik.	Velvetleaf	*Malvaceae*	0/10	0/36	-	0/6
Amaranthus retroflexus L.	Redroot pigweed	*Amaraanthaceae*	0/10	0/30	0/10	0/11
Ambrosia artemisifolia L.	Common ragweed	*Asteraceae*	0/2	-	0/20	0/12
Anthemis arvensis L.	Corn chamomile	*Asteraceae*	0/10	-	-	-
Asclepias siriaca	Common milkweed	*Asclepiaceae*	-	0/30	-	-
Capsela bursa-pastoris (L). Medik	Shepherd's purse	*Brassicaceae*	0/11	-	-	-
Chenopodium album L.	Lambs' quarters	*Chenopodiaceae*	0/10	0/31	0/20	-
Cirsium arvense L.	Canada thistle	*Asteraceae*	0/1	-	-	-
Datura stramonium L	Jimsonweed	*Solanaceae*	0/10	-	-	-
Daucus carota L.	Wild carrot	*Apiaceae*	0/10	-	-	-
Galinsoga parviflora Cav.	Gallant soldier	*Asteraceae*	0/10	0/31	0/10	-
Papaver rhoeas L.	Field poppy	*Papaveraceae*	-	0/24	-	0/2
Persicaria lapathifolia (L.) Delarbre	Curlytop knotweed	*Polygonaceae*	0/9	-	-	-
Portulaca oleracea L.	Common purslane	*Portulacaceae*	-	0/29	-	-
Rumex crispus L.	Curly dock	*Polygonaceae*	0/10	-	-	-
Solanum nigrum L.	Black nightshades	*Solanaceae*	0/12	-	-	-
Herbaceous test plants						
Chenopodium foetidum LAM	Stinking Goosefoot	*Chenopodiaceae*	0/9	-	-	-
Chenopodium murale L.	Nettle-leaved goosefoot	*Chenopodiaceae*	0/8	0/35	0/10	-
Chenopodium quinoa Willd.	Quinoa	*Chenopodiaceae*	0/6	-	-	-
Nicotiana benthamiana Domin.	Benth	*Solanaceae*	0/10	0/22	0/20	-
Nicotiana rustica	Aztec tobacco	*Solanaceae*	0/10	-	-	-
N. tabacum L. cv. Samsun	-	*Solanaceae*	0/10	-	-	-
Vitis vinifera	**Grapevine**	***Vitaceae***	**2/10**	**2/16**	**5/20**	**7/49**

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P12. Detection and identification of grapevine leafroll associated virus 1, 3, 4 (strain 9) isolates in south of Ukraine

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INTRODUCTION

One of the most harmful virus diseases of grapes is grapevine leafroll, which is widespread and leads to significant crop losses and deterioration of its quality. The associated viruses cause a decrease in sugar content in berries to 16%, and crop losses range from 10% to 48%. Grapevine leafroll associated viruses (GLRaVs) belong to the family *Closteroviridae* (Minafra et al., 1995, Sabanadzovic, 2001). There are five serologically distinct viruses (GLRaV 1-4, -7), of which the first and third (GLRaV-1 and GLRaV-3) are the most common in the world. The disease can be latent, but in vegetative reproduction it is the source of infection. The vectors of GLRaV-3 are mealybugs and soft scales such as *Planococcus ficus*, *Pseudococcus longispinus*. The most effective method of preventing viral diseases of grapes is obtaining a virus-free planting stock. Therefore, testing of clones for the absence of viral diseases makes it possible to determine the quality of the planting material of grapes. One of the most sensitive and specific diagnostic methods is the polymerase chain reaction with reverse transcription (MacKenzie 1997, Elbeaino, 2001). These methods allow the screening of a large number of samples in a short period of time and revealing the infection by viruses of both imported and domestic planting material.

MATERIALS AND METHODS

The purpose of our work was the development and optimization of the PCR-analysis method for the diagnosis of GLRaV-1, 3, 4 (strain 9). During the period 2019-2021, a study was carried out of 699 samples selected on clonal bush and common plantations of grapes of different varieties in order to detect latent infection of GLRaV 1, GLRaV 3, GLRaV 4 (strain 9). With the onset of colds, the isolation of the virus was carried out in woody shoots. The samples were transported to the laboratory and examined the same day or stored at - 20 ° C for several months. Samples for PCR preparation were prepared according to Rowhani et al. (2000). To set up RT-PCR in real time with hybridization-fluorescence detection, sample preparation and RNA isolation were performed as for classical RT-PCR with gel electrophoretic detection. RT-PCR in real time was performed using forward and reverse primers, fluorescently labeled DNA probes, reaction mixture, in the amount of 20 µl

To test for the presence of GLRaV 1,3 4 (strain 9), the magnesium concentration was optimized and was 1.3 mmol. The following primer pairs were used: LR1 HSP70-149 f/ LR1 HSP70-293 r/ LR1 HSP70-225 p (GLRaV-1), GLRaV-3 56 f / GLRaV-3 285 r/ GLRaV-3 181p (GLRaV-3) and LR9-F/F/ LR9-R/R (Osman et al., 2008). To the reaction mixture was added 2 µl of the prepared sample. As a positive control, infected viruses leaf twisting of the grape material. Deionized water was used as a negative control. Reverse transcription was performed in a thermostat at 52 ° C for 30 minutes. The amplification included 35 cycles (94 ° C for 30 seconds, 56 ° C for 45 seconds, 72 ° C for 60 seconds), and the elongation time in the last cycle was 7 minutes (Rowhani A., personal message). For GLRaV-1, the annealing temperature was reduced to 53 ºC during the study.

RESULTS AND DISCUSSION

As a result of the PCR analysis, data were obtained on the presence of latent infection in the experimental areas (Table 1).

Table 1: Testing grape plants for latent infection with viral diseases on different types of plantations.

Type of plantation	Total number of plants tested	Infected with GLRaV-1 (%)	Infected with GLRaV-3 (%)	Infected with GLRaV-4 strain 9 (%)
Bank clones in the greenhouse	154	-	-	-
Quartermasters in the greenhouse	244	-	-	-
Industrial vineyards	301	-	0,5	-

As a result of research carried out in different research areas of different grape varieties, it was established that Cabernet Sauvignon, Merlot, Pinot noir, Riesling are affected by GLRAV1,3 viruses. The Cabernet Sauvignon variety was also infected with the GLRaV-4 strain 9 virus (Fig. 1).

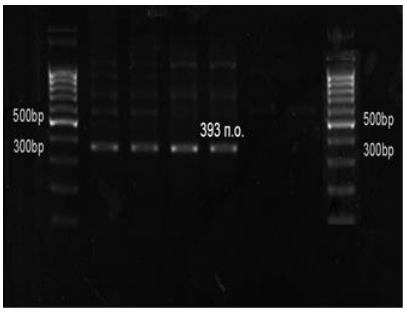

Fig. 1. Electrophorogram of the cDNA fragment of grapevine leafroll virus 4 strain 9 (GLRaV4-strain 9). Marker length of DNA fragments 100-1000 bp. (GeneRuler 100 bp DNA Ladder)

The bank of clones and breed shed in the greenhouse of grape were free of viruses GLRaV (1,3,4 strain 9). As a result of the conducted studies, it is established that GLRaV-1, GLRaV-3 are not widely spread in Ukraine's vineyards (Fig.2).

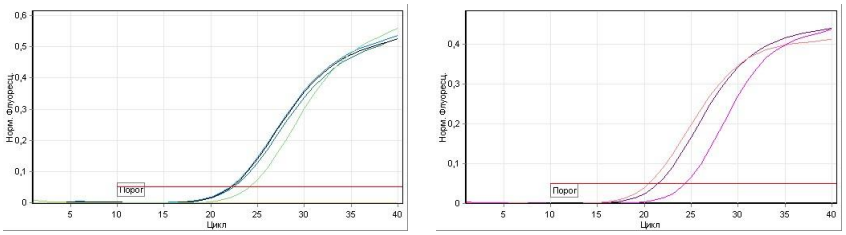

Fig. 2. Identification of grape viruses by RT-PCR. A – GLRaV1; B – GLRaV3

Single clones of clones, latently infected with viruses, uprooted. In Ukraine, the RT-PCR diagnostics method was introduced into the production system for certified planting stock of grapes for the detection of grapevine leafroll associated viruses. This method is used to test the uterine bushes of grapes at the stages of laying the bank of clones and base breed shed in specialized nurseries of Ukraine. For the first time in Ukraine, lesions of the Cabernet Sauvignon GLRaV-4 strain 9 virus were detected and identified.

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P13. Grapevine Pinot gris virus (GPGV) and Grapevine Leaf Mottling and Deformation (GLMD) disease: Champagne, a unique case in France

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INTRODUCTION

Following the identification of *Grapevine Pinot gris Virus* (GPGV) in Italy (Giampietruzzi *et al.*, 2012), some data were acquired on its possible symptomatology and agronomic impacts on a few local grape varieties (Bertazzon *et al.*, 2015). The first data obtained on the GPGV now gives us a fairly accurate picture of the presence of this virus in France. None French region appears free of the virus, although two regions (Charentes and Val de Loire) appear to be less affected for the time being (Spilmont A.-S., and *et al*., 2018 and 2022). Concerning the *Grapevine Leaf Mottling and Deformation* (GLMD) disease, supposed to be associated to GPGV, very few symptoms have been identified in France, with the notable exception of Champagne. In this region, reports are frequent, and the geographical area concerned seems to be expanding. For these reasons, agronomic impact was assessed on typical Champagne grape varieties. Boron content was also analyzed associated with symptoms expression to test the potential link between GLMD and boron (B)-deficiency symptoms (Buoso et al., 2020).

MATERIALS AND METHODS

Vineyard survey for GPGV prevalence and symptoms associated

Over the years 2016-2022, a total of 74 vineyard plots from different ages and varieties were monitored for the presence of GPGV and symptoms. For each plot, GPGV detection was performed by qRT-PCR (Bianchi *et al.*, 2015). The occurrence of the GLMD symptoms was visually surveyed. The sanitary status of the symptomatic vines was confirmed by specific qRT-PCR tests against viruses to check the presence of GPGV and the absence of three nepoviruses (GFLV, ArMV and TBRV) and the two most common Leafroll associated viruses (GLRaV-1 et -3).

Agronomic monitoring

In 2020, three vineyard plots in Champagne were studied for the agronomic impact of GLMD. Pinot noir was the variety on two of them and Meunier on the third. On each plot, thirty symptomatic vines and thirty asymptomatic vines were monitored. Each plant was controlled for the presence of GPGV and the absence of three nepoviruses and two leafroll associated viruses. The parameters monitored in this study were done on a sampling of 200 berries per modality (symptomatic vs. asymptomatic) the day of harvest: weight of 200 berries, sugars and acidity in juice, number of bunches, harvest and weight per vine.

Boron determination by petiole analysis

These analyses were performed by the Aude Chamber of Agriculture laboratory. Around flowering, a minimum of twenty-five petioles per sample were collected from groups on five vines expressing the same symptom. Sampling was performed on three different vineyards, each with six samples: three asymptomatic and three symptomatic.

RESULTS AND DISCUSSION

Vineyard survey for GPGV prevalence and associated symptoms

On the 74 vineyards analyzed since 2016, 81% had at least one vine positive to GPGV. Compared to the French national rate of 70% (Spilmont *et al.*, 2022), Champagne is one of the French regionswhere GPGV is the most present.

Even if other regions in France present a high percentage of GPGV presence, Champagne is the only one where symptoms of GLMD disease are very present. At least, thanks to the survey of Comité Champagne, 9 vineyards were identified expressing GLMD symptoms. The typology of these symptoms is always the same in this region: chlorotic mottling, decoloration and deformation of the leaves, mostly at the base of the canopy. Symptoms are stronger at the beginning of the vegetative season, while infected plants seem to recover after the veraison, with shoots and leaves growing normally after usually being symptomless. In these vineyards, a high percentage of the vines are symptomatic.

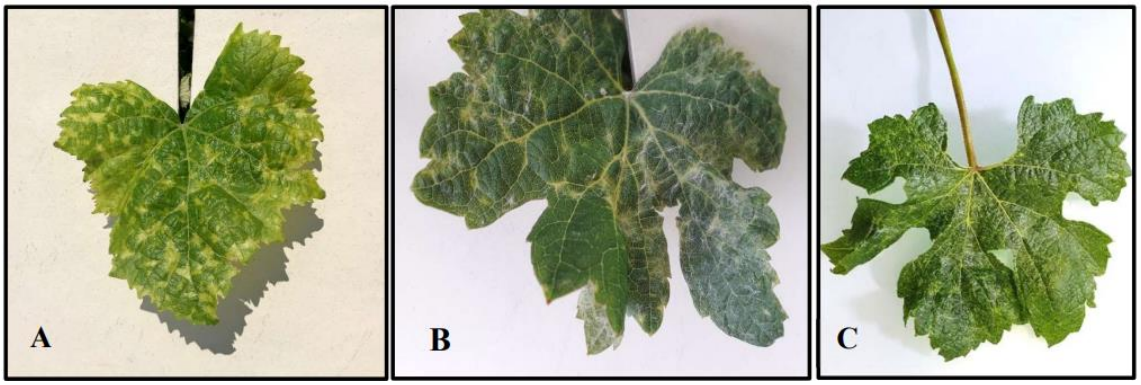

Figure 1 : Typical leaf symptoms of GLMD disease observed in Champagne on **A)** Pinot noir, **B)** Meunier and **C)** Chardonnay

Agronomic impact of GLMD and boron content

For three years, three vineyards with GLMD symptomatic and symptomless vines were monitored. Depending on the year, the average weight per bunch was down to 10 to 20 % on symptomatic vines compared to asymptomatic vines, and the yield per vine was down to 5 to 20 %. No significant differences were observed onto the other parameters between symptomatic and asymptomatic vines. In parallel no difference in petiole's boron concentration between symptomatic and asymptomatic vines was measured. Boron deficiency does not seem related to GLMD disease symptoms in this area. The survey indicated a frequent occurrence of GLMD symptoms in Champagne which make this region a unique case in France up to now. The raises questions about the cause and the possible evolution in the next years.

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P14. A simplified quantitative RT-PCR virus detection protocol used for the estimation of the sanitary status of grapevine germplasm collections in Greece

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INTRODUCTION

To obtain a comprehensive picture on the distribution and prevalence of major viruses in important grapevine germplasm collections in Greece and more importantly to identify vines free from these viruses, a screening of a total number of 255 varieties/clones was conducted from 2019 to 2021. The screening concerned not only the five viruses included in the national and EU legislation, i.e. grapevine fanleaf virus (GFLV), arabis mosaic virus (ArMV), grapevine leafroll-associated virus-1. and -3 (GLRaV-1. and -3), grapevine fleck virus (GFkV), but also grapevine virus A (GVA) and grapevine virus B (GVB), greatly undermining vine production and longevity worldwide. A simple sample preparation protocol involving the use of sandpaper (Nakaune & Nakano, 2006) to save time and effort was tested and applied on dormant vine canes, combined with RT-qPCR assays, currently the most sensitive virus detection method, based on published protocols.

MATERIALS AND METHODS

Grapevine material: In February-March 2019, 2020, and 2021, 507 grapevine samples from 96 varieties of the national germplasm collection of the Vine Department of Athens, Institute of Olive Tree, Subtropical Crops and Viticulture (IOSV), General Directorate of Agricultural Research of ELGO-DIMITRA and 263 samples from 139 varieties/clones belonging to the private Hellenifera P.C. collection were analyzed. Finally, at the end of February 2022, samples tested negative for all viruses were re-tested to confirm virus absence. Each sample consisted of 4-5 dormant canes collected along the vine cordon (bilateral Royat training system).
Sample preparation and RNA extraction: Three different sample preparation methods were assayed and compared, a. wood tissue was scraped on a sheet (5 cm×5 cm) of sandpaper (Nakaune and Masaaki, 2006) in the presence of 0.2 ml of PBS-DIECA-PVP-40 buffer and 100 mg of the resulting paste was transferred to 1ml of the same buffer; b. 0.5 g cambial scrapings were homogenized in 10 ml of the buffer mentioned above in individual plastic bags (HOMEX 5000 homogenizer, BIOREBA AG); c. liquid nitrogen was used to grind scrapings into fine powder, 50 mg of which were subjected to RNA extraction. Subsequently, two commercial RNA extraction kits PureLink™ RNA Mini Kit (Thermo Fisher) and RNeasy Plant Mini kit (Qiagen) were compared.
Quantitative RT-PCR assays: One-step RT-qPCR (Takara, One Step PrimeScript™ RT-PCR Kit) was applied with primers and TaqMan probes previously published, GFLV and GLRaV3 (Osman and Rowhani, 2006), GLRaV-1 (Osman et al., 2007), GVA and GVB (Osman et al., 2008), GFkV (Bertolini et al., 2010) and ArMV (Bertolini et al., 2003). Two final volumes of reaction cocktails were compared.

RESULTS AND DISCUSSION

Methodology: When RNA was extracted from three grapevine samples prepared each by the three different preparation methods using the PureLink^{TM} RNA Mini kit, RNA yield and purity between the classical phosphate buffer scraping homogenates and the sandpaper paste were comparable and even

better than the RNA extracted from samples treated with liquid nitrogen. The RNeasy Plant Mini kit performed worse in terms of RNA yield, particularly with the phosphate buffer scraping homogenates. To reduce the time and cost of the analyses, sample preparation with sandpaper and RNA extraction using the PureLink™ RNA Mini kit were chosen. To further reduce analysis costs five samples were analyzed for each virus using 12 and 5 µl RT-qPCR reaction volumes. No loss of sensitivity was observed (Table 1) and the volume of 5µl per RT-qPCR reaction was further used.

Table 1 Mean Cq values obtained in RT-qPCR tests for five samples/virus using two reaction volumes

Vol. (µl)	GFLV	GFKV	GLRaV-1	GLRaV-3	GVA	GVB
12	23.3 ±1	19.83 ± 1.47	23.62 ± 1	22.14 ± 1.56	19.89 ± 1.85	24.1 ± 3.35
5	22.5 ±1.4	17.86 ± 1.58	22.28 ± 0.95	21.56 ± 2.28	18.61 ± 1.88	22.84 ± 3.55

Virus screening of the germplasm collections: A total of 770 vine samples representing 255 grapevine varieties/clones, from two important grapevine germplasm collections in Greece, were tested by RT-qPCR assays. A total of 5,369 molecular analyses were performed. Results are shown in Tables 2 - 3.

Table 2 Results of virological testing of 507 vines of the IOSV germplasm collection

	GFLV	ArMV	GLRaV-1	GLRaV-3	GFkV	GVA	GVB
No of positive vines	385	0	88	468	57	481	34
Percentage (%)	**75.9**	**0**	**17.3**	**92.3**	**11.2**	**94.8**	**6.9**

Table 3 Results of virological testing of 263 vines of the Hellenifera P.C. germplasm collection

	GFLV	ArMV	GLRaV-1	GLRaV-3	GFkV	GVA	GVB
No of positive vines	57	0	88	118	35	151	2
Percentage (%)	**21.6**	**0**	**17.3**	**44.8**	**13.3**	**57.4**	**0.7**

The vines of the varieties particularly of the national IOSV germplasm collection were found heavily infected. GVA showed the highest prevalence, while ArMV was not detected. Four varieties (4.2%) of the national germplasm collection and 21 (20.7%) varieties (33 clones) of the Hellenifera collection were free from the seven viruses tested and can enter the national certification system. Although serious efforts have lately led to the release of the first certified clones in the country, the need to further reinforce programmes for grapevine sanitation and clonal selection is of primary importance. The modified protocol was proven reliable, fast, cost effective and suitable for large scale detection.

ACKNOWLEDGEMENTS

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P15. Investigation of the efficiency of chemotherapy on different multiple infected grapevine cultivars

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INTRODUCTION

Grapevine can be infected with more than 80 viruses (Fuchs, 2020), which number is constantly increasing. Having no specific methods to defend against viruses and viroids, the propagation material has to be free of them or should be eliminated. During the last decades several different virus/viroid elimination methods like meristem culture, somatic embryogenesis and plant chemotherapy, has been established and used for eliminating viruses from grapevine (Miljanić et al., 2022; Nuzzo et al., 2022; Turcsan et al., 2020). Plant chemotherapy: the usage of antiviral agents, is a fast developing technique results virus-free shoots on the treated plants (Skiada et al., 2013). Among them ribavirin, a guanosine analogue, is the most widespread antiviral chemical for grapevine virus elimination, but their efficiency depends on the cultivar and on the virus which has to be eliminated (Eichmeier et al., 2019; Komínek et al., 2016). To deal with the variable efficiency and this increasing demand expansion of the palette of useful antiviral chemicals would be necessary. Zidovudine and 2-thiouracil (2-TU) are possible alternatives, has been proven to work for eliminating viruses from several different plant species, but haven't been tried to eliminate viruses from grapevine so far. In our research we have tried ribavirin, ZDV and 2-TU alone or in combination to eliminate several different viruses from different grapevine cultivars.

MATERIALS AND METHODS

For the virus elimination studies, based on an initial RT-PCR survey, five clones, infected with different viruses were selected from our grapevine variety collection. Virome of the *in vitro* grown selected individuals were determined using small RNA HTS using our protocol (Czotter et al., 2018). Fastq files of the small RNA reads were analysed using CLC Genomic Workbench (Qiagen).
Virus elimination studies were done on *in vitro* cultures of these clones using different concentrations and combinations of the antiviral agents as detailed in Table 1.

Table 1. Experimental setup of the virus elimination studies

Antiviral chemical(s)	Applied concentration(s)	Treated genotype(s)
Ribavirin	25 mg/l	Kadarka P131 A1,Sárfehér A1, Furmint P51 ÜH2, Furmint P51 A1
Zidovudine	10, 20, 30, 40, 50, 80, 100, 120 mg/l	Kadarka P131 A1, Sárfehér A1, Kékfrankos Kt 1/2 A1
2-thiouracil	5, 10, 15, 20 mg/l	Kadarka P131 A1, Sárfehér A1, Furmint P51 ÜH2, Kékfrankos Kt 1/2 A1, Furmint P51 A1,
Ribavirin + zidovudine	25 mg/l + 50 mg/l	Furmint P51 A1, Furmint P51 ÜH2
Ribavirin + 2-thiouracil	25 mg/l + 10 mg/l	Kékfrankos Kt 1/2 A1

Plantlets regenerated from the shoot tips transferred to normal media after treatment and regenerated plants were tested for the presence of viruses and viroids by RT-PCR using virus specific primers (Gambino, 2015) to reveal the efficiency of the particular treatment.

RESULTS AND DISCUSSION

The selected mother plants were infected with different viruses, belonging to different families, as detailed in Table 2 showing the number of virus annotated contigs in the different libraries. Contigs

annotated as GFLV/ArMV/GDefV in Furmint P51 ÜH2 and GLRaV4-5-6 in Sarfeher A1 derived from a diverge GFLV and GLRaV4 variant, respectively, which molecular characterization were also carried out.

Table 2. Number of virus derived contigs in the different libraries as determined by small RNA HTS

		Nepoviruses			Grapevine leafroll viruses				Vitivirus	Tymoviruses					Viroids	
		GFLV	ArMV	GDefV	GLRaV-1	GLRaV-4	GLRaV-5	GLRaV-6	GVA	GFkV	GRVFV	GSyV-1	GRSPaV	GPGV	HSVd	GYSVd-1
1.	Kadarka P131 A1	0	0	0	113	0	0	0	0	0	0	0	9	0	4	4
2.	Sárfehér A1	0	0	0	0	9	2	6	0	0	1	0	2	10	4	7
3.	Furmint P51 ÜH2	61	19	55	0	0	0	0	0	0	0	0	4	0	1	4
4.	Kékfrankos Kt. 1/2 A1	0	0	0	0	0	0	0	0	0	32	1	0	53	4	7
5.	Furmint P51 A1	0	0	0	155	0	0	0	31	138	5	0	3	13	3	3

Our plants show very different growth rate during the *in vitro* conditions. Sarfeher was exceptional, because it shows a very good *in vitro* character. While ribavirin negatively affected the growth and rooting of the plants, zidovudin at lower concentration (10-40 mg/l) has induced it, which has been reverted at higher concentration (80-120 mg/l). 2-TU seriously blocked the growth of the plants even at low 10 mg/l concentration. 2-TU has shown particularly high phytotoxic effect as high percentage of the treated shoots have died after 1-2 months independently of the used (5, 10, 15 and 20 mg/l) 2-TU concentration. Using pre-rooted shoots for the treatment increased the survival rate and allow us to complete some tests with these antiviral agents. Unfortunately, we could not regenerate any virus-free plants during the experiments only with 2-TU or ZDV.

Ribavirin treatment of the clones proved to be very successful against GFkV, GRSPaV and GPGV as we could eliminated them from 78,3 %, 50-77,9 % and 97,1-100 % of the regenerated plants, respectively. GLRaV-1 and GVA could be eliminated from the plants with very low: 4,1 % and 6,5 % efficiency, respectively. Treatment with ribavirin and zidovudine in combination did not increase the success of elimination of either GFkV, GRSPaV or GPGV. Ribavirin combined with 2-TU was able to increase the rate of the GRSPaV-free regenerated plants to 100 %. With our experimental setup GFLV and the viroids were almost impossible to eliminate. Although our experiments are ongoing it seems very possible to conclude that chemotherapy is not able to sufficiently increase the efficiency of the elimination of all investigated viruses.

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P16. Establishment of the nuclear stock (pre-base) of the *V. vinifera* genus through phytosanitary selection in the Republic of Moldova

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INTRODUCTION

Diseases caused by phytopathogenic viruses, phytoplasmas, and bacterial cancer significantly reduce both the quantity and quality of the production, as well as influence the plants' longevity since they affect grapevines systemically, meaning that the infected tissues remain diseased throughout their lives. Adequate strategies to restrict the production and distribution of infected stocks focus on improving the cultural health (phytosanitary selection and sanitation) and certifying planting material, as combating chronic diseases through agrotechnical or chemical measures is not efficient. Phytosanitary selection is a crucial element in enhancing productivity and structural quality by eliminating diseased individuals and selecting plants that are less susceptible to diseases.

This study aims to establish a nuclear stock (pre-base) of the *V. vinifera* genus through phytosanitary selection. The research involved visual observation, biotype testing, sanitation, and *in vitro* culture to obtain grapevine clones free from viruses, phytoplasmas, and crown gall disease from 14 table grape varieties and 19 wine grape varieties. The test results revealed a widespread distribution of diseases of virotic etiology and bacterial cancer in asymptomatic form, including grapevine fleck (15-65%), *A. vitis* (26-44%), GLRaV-1 (8-26%), GLRaV-3 (7-39%).

To sanitize the selected material after testing, thermotherapy was applied at t^0 51^0C for 45 minutes. Thanks to the *in vitro* method, 7300 phytosanitary grapevine clones from local, European, and new selection varieties were multiplied, leading to the establishment of the nuclear stock. This marks the first step in the process of transferring viticulture in the Republic of Moldova based on certified planting material.

MATERIALS AND METHODS

Examinations and selection of initial clones were conducted in the old selection and industrial plantations of the Republic of Moldova during the period of 2018-2022. Testing and multiplication of the phytosanitary clones were performed at the Laboratory of Virology and Phytosanitary Control at IȘPHTA during the period of 2019-2023. The research focused on grapevine plants from 6 local varieties, 6 European varieties, 23 new selection varieties, and 6 forms. The ELISA-test diagnostic method (Clarc and Adams, 1977) was used for the following viral diseases: grapevine fanleaf virus (GFLV), grapevine fleck virus (GFkV), grapevine leafroll virus (LR) serotypes 1, 3, and grapevine virus A (GVA). Positive and negative controls were obtained from Agritest company. For testing grapevines for crown gall disease, the microbiological method was used by isolating the bacteria on the semi-selective medium of Roy & Sasser (1983). The thermotherapy method was applied in a thermostat tank LP:516, type: 1382 at a temperature of 51°C for 45 minutes. The vegetative mass was grown in a climatic chamber of type: KK 500TOP+FIT, with a thermal regime of 25°C ±1°C. The microclonal multiplication was performed using the *in vitro* culture method.

RESULTS AND DISCUSSION

The visual observation of the initial propagation material was carried out in old industrial selection plantations in the Republic of Moldova during the period from 2018 to 2022. The first stage of the selection process involved the visual observation of plants for the presence of symptoms related to viral diseases, crown goal and phytoplasma. This examination was conducted twice during the

vegetation period. After the visual examination, 118 vines with high yield and valuable grape qualities were selected and marked. These vines belonged to local, European, and newly developed grape varieties by breeders from the Republic of Moldova. At the end of the vegetation period, after the leaves had fallen, cuttings were taken from the marked vines to test for the presence of infectious agents.

The viticultural material was tested for the presence of agents that induce the main disorders of grapevines. The test results showed that visually healthy plants often act as carriers of chronic diseases in an asymptomatic form, most commonly viral ones (22.5%). Several biotypes were found to be infected with a complex of viral infections (15.3%), as well as simultaneous viral and bacterial infections (17.1%). In latent form, infections were found in both native and European grapevine varieties, as well as in newly selected ones. Thus, during the period 2019-2023, 118 plants were tested, and the most frequently diagnosed viruses were GFkV (15-65%) and agrobacteria responsible for crown gall disease (26-44%). In the territory of the Republic, GLRV viruses are widespread, being major causes of leaf curl symptoms in grapevines. Among them, GLRaV-1 (8-26%) and GLRaV-3 (7-39%) are the most widely distributed (Fig. 1). The proportion of plants tested negative for the presence of virotic and bacterial diseases ranged from 15% to 50%, indicating a high degree of prevalence of chronic diseases in asymptomatic form in grapevines.

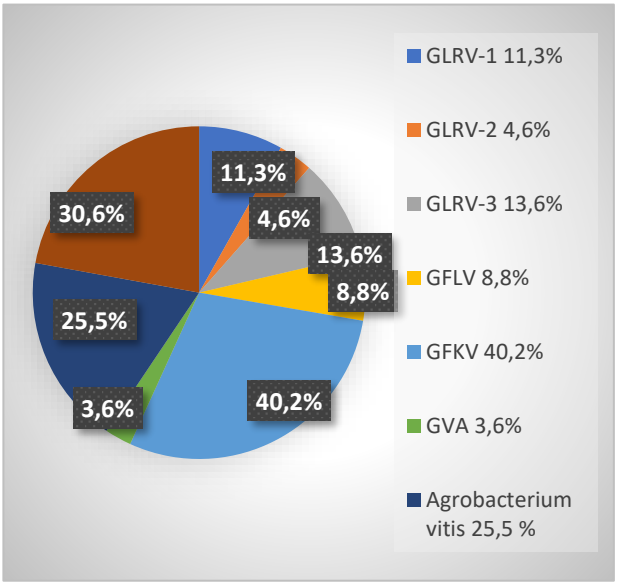

Figure 1. Average results of grapevine biotypes testing for the presence of viruses and *A. vitis* for the period 2019-2023. Whole genomes along with selected isolates from Genbank were included to define groups.

The sanitation of grapevine plants from infectious agents that cause chronic diseases is a crucial step in phytosanitary selection to ensure the production of healthy plants. Since the pathogens are not uniformly distributed within the plant (Burr *et al.*, 1983; Tarbah and Goodman, 1986; Stefani and Bazzi, 1990), and the *Bois noir* phytoplasma is widely spread throughout the country (1-100%) (Haustov *et al.*, 2020), biotypes that tested negative were subjected to thermotherapy as an additional measure. Thermotherapy has been shown to be effective in eradicating bacterial diseases such as *Agrobacterium vitis*, *Agrobacterium tumefaciens*, bacterial necrosis, and *Xylophilus ampelinus* (Hamilton R., 1997; GTNFD, 2006), eliminating phytoplasmas *Flavensce doree* and *Bois noir*, partially affecting eggs of the leafhopper *Scaphoideus titanus*, thrips, phylloxera, and being effective in treating *Xylella fastidiosa* infection (Pierce's disease) (EFSA 2015; Bloy 2016). The treatment with hot water was applied for 45 minutes at 51°C, resulting in a percentage of germinated and rooted cuttings ranging from 76% to 92% (Dubceac *et al.*, 2020).

In vitro multiplication. During the active shoot growth period, apical parts of the shoots were collected, sterilized, and then inoculated into test tubes containing agarized growth medium. When the newly formed plantlets reached a length of 5-6 internodes, they underwent accelerated multiplication using the in vitro method, which allows obtaining phytosanitary clones in a geometric progression. In this way, 7300 phytosanitary clones were multiplied from 33 grapevine varieties. Micropropagation and acclimatization of grapevine plantlets were carried out throughout the year under controlled laboratory conditions, and at the stage of growth and development, the plants were transferred to the greenhouse.

The establishment of the nuclear stock. During the period 2019-2022, the nuclear stock plantation

where 7300 self-rooted plants were planted. Among them, 14 are table grape varieties, and 19 are technical grape varieties. The purpose of this plantation is to produce superior biological category vine material of high quality and uniformity, which will serve as the foundation to ensure the quality of grapevine varieties in the wine industry. By implementing this initiative, the grapevine nursery sector in the Republic of Moldova aligns with European standards and transitions to the use of certified propagation material. Through this initiative, the Republic of Moldova strengthens its position on the global viticulture map, demonstrating its commitment to quality and sustainability in grape and wine production. This step will contribute to enhancing the reputation of Moldovan wines and foster further development and competitiveness in the international wine market.

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P17. Eradicating virus infection in grapevine by BABA (β-aminobutyric acid) induced priming of different defense pathways

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INTRODUCTION

Several pathogens including viruses, phytoplasmas, bacteria and fungi infect grapevine propagating material in latent form resulting in serious economic losses. They can cause large scale reduction in fruit quantity and quality. Currently the only viable option to fight against these pathogens is the use of certified pathogen-free propagation material which requires the establishment and maintenance of a pathogen-free prebasic collection of clones and varieties.

The most important methods for virus elimination involve in vitro meristem and shoot tip cultures. During virus elimination the most difficult and time consuming step of the tissue culture work is the fast and precise preparation of shoot tips and meristems. Viruses are usually excluded from meristems, but plant regeneration from only the meristem is very difficult. Meristems smaller than 0.2 mm are not able to develop into shoots, if its size is between 0.2 and 0.5 mm, about 10% could be regenerated and are virus free. If the shoot tip is 2mm, regeneration efficiency can reach 100% but they will still contain viruses and viroids. Therefore, our main goal is to find treatment for altering virus distribution in the shoot tips.

BABA has been reported to be capable of inducing resistance in different plant species against several pathogens (Jakab et al., 2001). BABA-induced resistance (BABA-IR) unlike to the pathogen-induced systemic acquired resistance (SAR) is not based on direct defense activation by the treatment. However, a faster and stronger activation of basal defense mechanisms was observed when the induced plant was exposed to pathogen attack or abiotic stress (Zimmerli et al., 2000; Jakab et al., 2005). Such enhanced capacity to express basal defense mechanisms is called priming (Conrath et al., 2002). By testing different defense mutants of *Arabidopsis thaliana* for their ability to express BABA-IR, it became clear that BABA-IR is not controlled by a single defense signaling pathway, both the primed expression of salicylic acid (SA)- and abscisic acid (ABA)-dependent pathway can be involved.

Besides *Arabidopsis*, BABA also successfully induced resistance against downy mildew (*Plasmopara viticola*) in grapevine. In this case, the priming of the jasmonic acid (JA) regulated defense responses proved to be important (Hamiduzzaman et al., 2005). The aim of our research is to investigate the potential protective effect of BABA in grapevine against virus infection, since successful protection of tobacco against TMV infection has been reported (Siegrist et al., 2000).

MATERIALS AND METHODS

Plant material: Grapevine (Vitis vinifera) shoots infected with different viruses were rooted and maintained in ½ MS medium under sterile conditions in growth chambers. Two Hungarian varieties, Kadarka and Furmint infected with ArMV, GFLV, GVA and GFLRaV-1 were selected for the experiments. Grapevine fanleaf associated virus-1 (GLRaV-1) is a phloem-restricted non-enveloped filamentous ssRNA(+) virus. Arabis mosaic virus (ArMV) and grapevine fanleaf virus (GFLV) are two picorna-like viruses from the genus *Nepovirus*, consisting in a bipartite RNA genome. Grapevine virus A (GVA) is a member of *Vitiviridae* and has a single ssRNA(+) genome. All of these genomic RNAs have 3′-poly A tail.

Treatments: Plants with 2-3 leaves were treated with 10fold concentrated BABA solutions as it is indicated. The BABA solution was poured onto the medium. Treated plants were allowed to grow until they were 5 leaves sized and then they were placed in a new culture vessel per leaf level on MS medium without BABA.

RNA isolation and RT-PCR: RNA isolation from grape leaves and reverse transcription were performed using oligo(dT) primer and RevertAid reverse transcriptase. The presence of the viral RNA was detected in PCR using DreamTaq and sequence specific primers designed to the different viruses.

RESULTS AND DISCUSSION

Higher concentrations of BABA inhibit plant growth: Growing control and virus infected plantlets were treated with increasing concentrations of BABA. 300 mg/L BABA solution (final concentration in MS) had lethal effect on the plants. 150 mg/L BABA still strongly inhibited plant growth, but plants treated with 50 mg/L BABA have already grown appropriately for further tests.

BABA treatment can reduce the virus titer in the growing shoot apex: To monitor the presence of the different viruses in the regenerated plantlets we used RT-PCR method. A gradually decreasing virus titer was found towards the tip of the shoot. Our results indicate that BABA treatment can help to control virus infection and movement in grapevine in a species-dependent manner. While ArMV and GLRaV-1 were regularly eliminated, GFLV and GVA resisted BABA-treatment. Based on gene expression data, this is resulted through the priming of both the jasmonic acid and the salicylic acid pathways (**Fig. 1**). In conclusion, β-aminobutyric acid can be an effective tool for elimination of virus infection in grapevine to obtain virus-free propagating material.

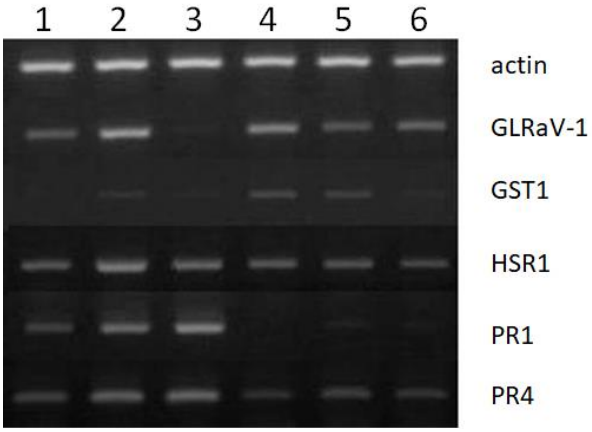

Figure 1. *Expression pattern of marker genes in the leaves of control and BABA-treated GLRaV-1 infected plants.* In contrast to control plants (lower (4), middle (5), upper (6) leaves), a gradually decreasing virus titer was found towards the tip of the shoot in the BABA-treated plants (lower (1), middle (2). Due to BABA-induced priming an increased expression of PR-1 (SA-signalling) and PR-4 (JA sgnalling) were detected in upper leaves.

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P18. From the pot to the plot: assessment of the bionematicide potential of different *Fabaceae* to delay the re-contamination of newly planted vines by grapevine fanleaf virus in vineyard

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is a widely distributed *Nepovirus* responsible for fanleaf degeneration (Meng et al. 2017). Synthetic molecules have long been used to control the populations of its soil-borne nematode vector, *Xiphinema index,* limiting the extension of fanleaf clusters and the delay between uprooting and plantation of new vines. Their proscription due to environmental impacts and healthy concerns in the early 2000s has led the vinegrowers to a technical and regulatory impasse to fight against fanleaf disease. Therefore, there is a growing interest in agroecological strategies, including plant species with antagonistic effects towards *X. index*. Hence, we evaluate the bionematicide potential of the aerial parts and roots of four *Fabaceae*: sainfoin, birdsfoot trefoil, sweet clover, and red clover, as well as that of sainfoin-based pellets in controlled conditions. Metabolomic analyzes have been performed on these plant extracts to attempt identifying bioactive molecules with nematicide properties. Meanwhile, we have designed open-field experiments highly infected by *X. index*/GFLV pathosystem. Here, we aim to determine whether we could pre-screen *Fabaceae* candidates or their dehydrated pellets in greenhouse bioassays before implementing them in vineyard experiments and eventually in schemes of integrated management of the fanleaf degeneration.

MATERIALS AND METHODS

Bioassays: *Xiphinema index rearings:* aviruliferous populations of *X. index* were reared on fig plants (*Ficus carica*) kept in 10L containers filled with an artificial substrate in a greenhouse under controlled temperature (20–25°C) and light (16 h photoperiod) conditions.

Plant materials and plant extracts: Onobrychis viciifolia cv. Perly (sainfoin), *Lotus corniculatus* cv. Altus (birdsfoot trefoil), *Trifolium pratense* cv. Formica (red clover) and *Melilotus albus* (sweet clover) seeds were provided by Jouffray-Drillaud or by Graines Baumaux, S.A.S., FR. Commercial Vitifolia® pellets were provided by Multifolia. The plant powders were mixed with a 1% phosphate buffered saline (PBS) solution.

Nematode survival bioassay in aqueous medium: adult nematodes were isolated from the rearing soil by sieving methods. Nematode viability was recorded after 24, 48 and 72 hours under binocular by scoring their mobility. Non-mobile nematodes were isolated and placed in 1% PBS for 24 hours in order to discriminate between nematostatic or lethal effect of the solutions tested.

Metabolomic analyses: Analyses were carried out using an Ultra-High Performance Liquid Chromatography system, equipped with a C18 HTec column. The chromatographic system was coupled to a High-Resolution Mass Spectrometer. The exact *m/z* and retention time of each metabolite were used for targeted metabolomic analyses using the Xcalibur software. The selected metabolites were grouped into four major chemical or functional families, including cinnamic acid and derivatives (CA), flavonoids (F), organic acids (OA), proanthocyanidins and flavanols (PF).

Vineyard experimentations: three open-field experiments have been set-up since 2015, located in Eguisheim, Bennwhir and Ribeauvillé (Alsace region, France) in highly-infected fanleaf vineyards with spreading infection. Vines and soils were analyzed randomly for the presence of GFLV and *X.*

index respectively to select the best fanleaf plots. Before spraying sainfoin pellets (3.5 T/ha) prior and during planting of the new vine, a fallow between 1 and 2 years was carried out with sainfoin or other *Fabaceae* following devitalization and uprooting. DAS-ELISA were performed each year in June to measure the kinetics of GFLV contamination between the different modalities in the grapevine leaves. These detailed experiments will be presented at the ICVG meeting.

RESULTS AND DISCUSSION

For all tested plants, either aerial or root parts, or both of them, exhibited bionematicide activities on *X. index in vitro*, sainfoin pellets being as effective as freshly harvested plant (Fig. 1).

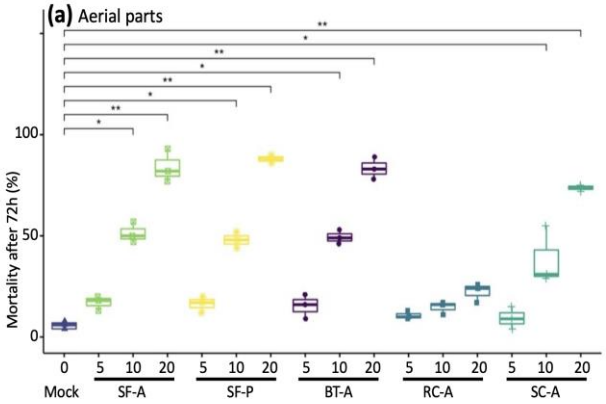

Fig. 1. *In vitro* antagonistic effect of Fabaceae aerial parts on *X. index*. Nematodes were exposed to control condition (1% PBS = Mock) or to the indicated Fabaceae extracts at 5, 10 and 20 g.L-1 for 72 h. Impact of Fabaceae extracts on *X. index* was assessed by measuring mortality rate in %. * and ** indicate significantly different mortality compared to the mock condition using a Dunn test, with p-value < 0.05 and < 0.01, respectively. SF: sainfoin, BT: birdsfoot trefoil, RC: red clover, SC: sweet clover, A: aerial parts, R: roots, P: pellets

Comparative metabolomic analyses of sainfoin did not reveal molecules or molecule families specifically associated to antagonistic properties toward *X. index*, suggesting that bionematicide effect is the result of a combination of different molecules rather than associated to a single compound (Negrel et al; 2022). However, it provides a significant and valuable study of more than 80 compounds identified in 4 different Fabaceae.

The first open-field experiments starting 8 years ago, combining a *Fabaceae* fallow, supplemented with sainfoin Vitifolia^{TM} pellets before plantation and every 2 years at a 3.5 T/ha application, shows a slower re-contamination of this modality so far compared to the controls. These preliminary results suggest that an integrated management combining complementary levers including bionematicide fallows with *Fabaceae*, their dehydrated or liquid derived compounds, innovative genetic resistances and cross-protection could eventually enable the viticulture industry to succeed in "living sustainably with the fanleaf degeneration".

ACKNOWLEDGEMENTS

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P19. *Vitis vinifera* subsp. *sylvestris*: a source of resistance towards grapevine fanleaf virus?

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INTRODUCTION

Fanleaf degeneration is one of the most detrimental viral disease of commercial grapevine. Its main causal agent is grapevine fanleaf virus (GFLV), a nepovirus specifically transmitted from grapevine to grapevine by the soil-borne ectoparasitic nematode *Xiphinema index* (Martelli et al. 2017; Andret-Link et al. 2017). So far, no effective solution exists to control fanleaf disease in a sustainable way. However, recently, a first recessive resistance factor against GFLV has been identified within *V. vinifera* subsp. *sativa* in a Riesling cultivar on chromosome 1 (Djennane et al. 2021).

The perennial wild grape *Vitis vinifera* L. subsp. *sylvestris* (*V. sylvestris*) is assumed to be the ancestor of cultivated grapevines. This wild grapevine species is recognized as a source of resistance or tolerance genes for diseases including powdery and downy mildew or grapevine trunk diseases. Although *V. sylvestris* is known to be susceptible to vitiviruses and ampeloviruses, the presence of the two nematode-transmitted nepoviruses grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV : a another nepovirus responsible for the fanleaf disease) were never reported to infect this wild species. Therefore, *V. sylvestris* may be a putative genetic resource for fanleaf resistance.

To test this hypothesis, twelve *V. sylvestris* accessions from the germplasm collection established in the Botanical Garden of the Karlsruhe Institute of Technology (KIT) were assessed for fanleaf disease resistance using adapted protocols developed with viruliferous *Xiphinema index* nematodes.

MATERIALS AND METHODS

Grapevine plant material.

Healthy or infected grapevine green cuttings were produced from woody canes cultivated on rockwool or in 10-L pots filled with sandy substrate, in a climatic chamber with a controlled temperature. After 4-5 weeks, the rooted grapevine plants were used to produce viruliferous nematodes or to be challenged with viruliferous nematodes.

Production of viruliferous nematodes. About 3000 non-viruliferous *X. index* were allowed to feed on rooted grapevine cuttings infected by the GFLV-F13 isolate in 10-L pots. Pots were cultivated in the greenhouse under controlled conditions for at least two years to allow the initial nematode population to multiply and acquire GFLV-F13.

GFLV transmission by nematodes in controlled conditions. About 300 viruliferous *X. index* were put in contact for 8 weeks with the roots of a single grapevine in 0.5-L plastic pots. After the feeding period, the grapevines were uprooted, and GFLV transmission was assessed in rootlets by detecting the virus using DAS-ELISA. The grapevines were subsequently transplanted into *X. index*-free soil and maintained in the greenhouse for at least two years. DAS-ELISA was then performed on young leaves from newly developed shoots to monitor GFLV infection and subsequent systemic virus spread.

Evaluation of nematode multiplication. Roots of grapevine cuttings were exposed to 250 viruliferous *X. index* individuals isolated from the GFLV-F13-infected grapevine rearings. The plants were cultivated in 2 L containers in the greenhouse for 10 months. After this period, all the soil was recovered to determine the total number of nematodes per plant. The ratio of the final number of *X.*

index recovered (fn) to the initial number of *X. index* (in) was used to determine the reproduction factor (RF = fn/in) for each plant. GFLV presence in the plant roots was assessed by DAS-ELISA.

GFLV transmission by nematodes under semi-natural conditions. Two-month-old plantlets from cuttings were transplanted into large containers (5 m^3) filled with soil naturally contaminated by viruliferous nematodes. The presence of GFLV was assessed by DAS-ELISA performed on leaves, each year during the spring.

RESULTS AND DISCUSSION

Our data show that one of the *V. sylvestris* accessions (Ke 3) out of 12 displays a resistant phenotype to fanleaf disease and that another accession (Ke 9) shows tolerance to GFLV systemic infection (Table1).

Accessions	Ho 1	C1-2	Ke 1	Ke 2	Ke 3	Ke 4	Ke 5	Ke 7	Ke 8	Ke 9	Ke 11	Ke12	SO_4	mock
% of GFLV infected grapevines	100	100	100	100	0	100	100	100	100	39	90	100	94	0

Table 1: Percentage of GFLV-infected grapevines determined by DAS-ELISA on leaves after 24 months following *X. index*-mediated GFLV transmission. Ho1 to Ke12 correspond to the *V sylvestris* accessions. SO_4 correspond to a GFLV sensitive rootstock. *V. sylvestris* and SO_4 accessions were challenged with viruliferous nematodes. Mock grapevines were challenged with aviruliferous nematodes.

Furthermore, the resistant accession does not slow down the multiplication of the nematode vector which demonstrates that its resistance is directed against the virus, and not against the nematode. Thus, this is the second time that a source of resistance to GFLV is discovered among *Vitis spp*.

The characterization of this resistance towards the pathosystem ArMV/*X. diversicaudatum* is currently underway as well as its durability.

The perspectives of this work are to decipher the genetic determinism underlying this resistance and to identify the genetic factor linked to this resistance. If the resistance factor carried by this *V. sylvestris* accession is different from that of the *V. vinifera* Riesling genotype (Djenanne et al., 2021), this discovery will pave the way for resistance pyramiding against a nepovirus. Finally, each identified GFLV resistance could offer a sustainable solution to control grapevine fanleaf disease in infected vineyards by introducing these genetic resistance factors in innovative rootstock genotypes combining a resistance against the vector and against the virus.

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P20. Can we transpose the results of cross-protection against *Grapevine fanleaf virus* from herbaceous hosts to grapevine?

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INTRODUCTION

Grapevine fanleaf virus (GFLV, *Nepovirus, Secoviridae*), a bipartite (+) ssRNA, is a major viral pathogen infecting grapevine across the world and causing up to 80% yield losses. While nematicides have long been used to control the populations of its soil-borne nematode vector, *Xiphinema index*, their ban in the early 2000s has led the winegrowers to a technical and regulatory impasse to fight fanleaf disease (Meng et al. 2017).

Cross-protection has proven to be a biocontrol method of choice notably to limit the devastating effects of *Citrus tristeza virus* in Brazil and those of *Pepino mosaic virus* in Europe and Morocco (Ziebell and Carr, 2010; Agüero et al. 2018). It consists of inoculating (a) primary mild variant(s) to protect a given host from the damaging effects of genetically related challenge severe variants. While this control method can indeed be effective, the still limited knowledge on the factors that impact its success, and on the mechanism(s) underlying it, has rendered the search of protective strains empirical and time-consuming (Harper et al. 2017). The search of GFLV-infected grapevines expressing no to mild symptoms in highly diseased vineyards has led us with a selection of 86 candidates. We here aimed to determine whether we could pre-screen these candidates on herbaceous plant hosts before implementing them in vineyard test plots.

MATERIALS AND METHODS

In 2015, Chardonnay - that were either GFLV-free (control vines) or infected with GFLV cross-protective candidate variants A or B - were grafted onto Kober 5BB rootstock and maintained in greenhouse conditions. In 2016, around 150 viruliferous *X. index* per plant were brought into contact with the roots of the vines thus obtained. Three different populations of nematodes were used: (i) nematodes carrying a GFLV lab-strain, (ii) and (iii) two populations of nematodes carrying GFLV variants coming from two different vineyards from the Champagne and Burgundy regions (France) (Garcia et al. 2019). Each primary GFLV-infected or control vine was challenged with the three different *X. index* populations, corresponding to a total of nine combinations (table 1), with 12 plants per combination. The control vines were followed by DAS-ELISA between 2017 and 2022 to determine the progression of the infection. Moreover, in 2022, a 450-bp amplicon per genomic viral RNA, enabling to discriminate between primary-infecting and challenge GFLV variants, was sequenced by Illumina for each plant to determine the variants being present. This allowed us to determine the percentage of superinfected plants for each combination (table 1).

During Spring 2023, the same experimental design was followed for two different GFLV herbaceous plant hosts, namely *Nicotiana benthamiana* and *Arabidopsis thaliana*. Four or seven days after mechanical inoculation of the mock and of the cross-protective candidate variants A and B, between 50 and 150 *X. index* coming from the populations described above were added into the soil of each *N. benthamiana* or *A. thaliana* plant. All the plants were tested by DAS-ELISA three weeks after primary inoculation to ensure that the (i) mock-inoculated plants were negative and that (ii) the variant A or B-inoculated plants were GFLV positive. As for the vines, the mock-inoculated plants are followed by DAS-ELISA to determine the progression of the infection.

RESULTS AND DISCUSSION

The percentage of vines for which at least one RNA of the challenge variant (*i.e.* transmitted by the nematodes) was detected by high-throughput sequencing is reported in table 1.

Primary variant	Nematode populations carrying challenge variants		
	X. index "Burgundy" population	*X. index* "Champagne" population	*X. index* lab-population
Control	90,9%	72,7%	90,9%
Variant A	16,7%	0,0%	0,0%
Variant B	27,3%	16,7%	41,7%

Table 1. Percentage of vines infected with at least one GFLV genomic segment (RNA1 or 2) of a challenge variant (*i.e.* transmitted by nematodes) in 2022.

We note that in 2022 almost all the control vines were infected while most of those that had been primary-infected by one of the two candidate variants were free of secondary infection. Nonetheless, we observed differences in superinfection rate depending on the primary variant - challenge variants combination.

The experiments on herbaceous plant hosts are still on-going and the first results will be presented during the meeting. Our goal is to compare the results obtained on vines with those obtained on *N. benthamiana* and *A. thaliana* in order to determine whether these herbaceous hosts could be suitable substitutes to test cross-protective candidates against GFLV. In other words, do we see cross-protection - and lack thereof - for the same combinations of primary and challenge variants? If this proved to be the case, this would allow us to have a rapid protocol in controlled conditions to screen the most promising cross-protective candidates before testing them in the field.

ACKNOWLEDGEMENTS

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P21. Field trials for improving grape production in vineyards heavily infected by fanleaf

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INTRODUCTION

Fanleaf is the most damaging viral disease in grapevine. It is associated to the presence of different nepoviruses, the most important and common being GFLV (Grapevine Fanleaf Virus), occurring all over the world. The virus is transmitted by nematode vectors, in particular *Xiphinema index*. Vineyards infected by fanleaf show important reduction in grape yield at the harvest. The only possible control is prevention, by planting healthy materials. The soils of several important and traditional grape growing areas in Europe, where viticulture has been carried out for centuries, are infested by infective nematodes, and only multiyear crop rotations could solve the sanitary situation. However, due to the high economic values of these soils, and to the pressure of the market, it is almost impossible that growers change crop, even only for one year.
With a practical perspective, starting from the evaluation of agronomical and physiological parameters which can be affected by GFLV, a 5-year work was performed with the aim to find some possible agronomic strategies able to reduce the losses in production in vineyards highly infected by fanleaf.

MATERIALS AND METHODS

A vineyard cultivated with the cultivar 'Corvina' was surveyed for three years, from 2018 to 2020, for the presence of various symptoms of fanleaf disease. In 2021, after having verified the presence of GFLV through serological tests, plants were classified into four categories: 1) healthy plants, 2) GFLV-infected plants that never displayed symptoms of fanleaf, 3) GFLV-infected plants showing foliar discoloration (mosaic, chlorosis, vein banding), 4) GFLV-infected plants showing foliar deformations (distortions, closer veins, toothed margins), shoot abnormalities (short internodes, fasciation) and/or growth inhibition of the plant (stunting). For each treatment, the following grape parameters and agronomic traits were analyzed on a variable number of vines (from 3 to 12): yield per vine and average cluster weight, soluble solids, pH and titratable acidity on grape, root distribution and density, leaf area development (LAI), bud fertility, bud-break and flowering phenology, some leaf eco-physiological parameters (photosynthetic rate (P_n), transpiration rate (E), stomatal conductance (g_s) and substomatal CO_2 concentration (c_i)), foliar temperature, leaf concentration of the main nutrients and root starch content.
In parallel, a total of 15 trials were established in 5 years in 12 farms, making use of fertilizers, biostimulants and plant defense stimulators. Trials were reproduced at least in two different vineyards and grapevine cultivars. The products were distributed in the canopy or in the soil, depending on the features of each product, from 1 to 5 times in each season and for at least 3 years. Visual observation on symptoms and serological ELISA test were used to detect the infected plants. Quantitative (weight of the production per plant, number of bunches) and qualitative (sugar, acidity, pH) parameters were measures at the harvest for at least 2 years in the treated and untreated symptomatic plants.
Statistical analyses were applied to identify significative differences.

RESULTS AND DISCUSSION

Agronomic and physiological parameter values evaluated in plants showing different fanleaf symptoms and compared with GFLV-infected asymptomatic and GFLV-free plants helped to identify the physiological processes most influenced by GFLV, in order to look for the best strategies to

mitigate the productive damages caused by the disease. The most interesting results were obtained comparing plants showing deformation symptoms with healthy plants.
Shoots development was significantly affected, and both canopy architecture and internode length measurements showed the lowest values in diseased plants. Interestingly, despite the reduced canopy, infected plants showed the best root development, with the highest total root density (366.3 and 185.4 roots/m^2 in symptomatic and healthy plants, respectively), good arrangement along the soil profile and optimal ratio between fine and thick roots. In agreement, normal values of foliar nutrient content and gas exchange were measured for plants infected with fanleaf, and no difference was detected for starch reserve in the roots compared to healthy plants, indicating a high-quality root system. Moreover, similar eco-physiological performance, including Pn, recorded for healthy and symptomatic plants, revealed good functionality of the canopy. Nevertheless, grape production was severely reduced in diseased plants. Despite similar values of potential fertility, the real fertility of buds was lower in symptomatic plants (0.87 and 1.21 in symptomatic and healthy plants, respectively). The flowering was significantly delayed in time, and, generally, a marked dissection of the anthers was observed, causing the abnormal development of the bunches and a drastic reduction in grape yield (66.3%), in accordance with performances reported in literature (Kubina et al., 2022).
To improve the grape production in plants showing infectious malformations, 6 different strategies were tested, but only 3 of them showed a statistically significant improvement of quality or quantity of the production, and only in cv Glera (Fig. 1). Moreover, the results were not significant in all the years. The most consistent positive results were obtained with: 1) a nutritional strategy including N, P and Ag, which was able to increase the yield of approximately 20%, maintaining at the same level all the other values; 2) a nutritional strategy including N, P, K, microelements and *Ascophyllum nodosum* extracts, which proved to increase the yield of approximately 20%, but only in the less seriously symptomatic plants; 3) a mix of *Trichoderma atroviridae* and *Glomus intraradice* strains, which in one year was associated to an increase of yield (up to 40%) and in the following year to an increase of sugar (3 brix degrees). In general, the improvement was detected on the symptoms in the bunches, but no difference was noticed on foliar symptoms. These strategies could help the grapegrowers to increase the life span of their vineyards and the economic revenues in fanleaf highly-infected areas.

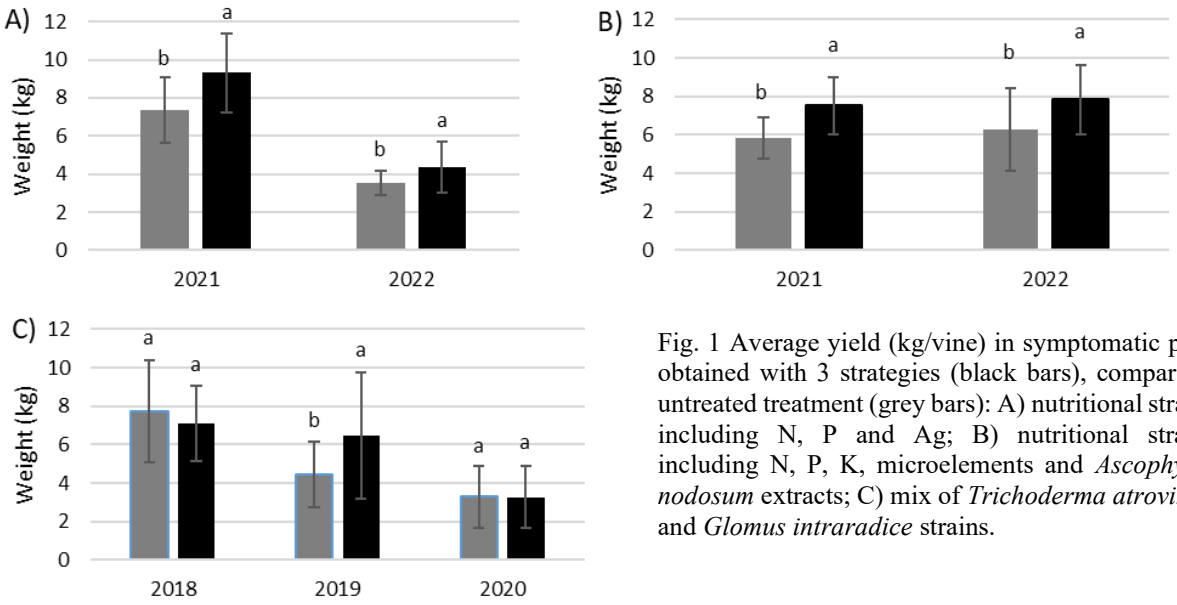

Fig. 1 Average yield (kg/vine) in symptomatic plants obtained with 3 strategies (black bars), compared to untreated treatment (grey bars): A) nutritional strategy including N, P and Ag; B) nutritional strategy including N, P, K, microelements and *Ascophyllum nodosum* extracts; C) mix of *Trichoderma atroviridae* and *Glomus intraradice* strains.

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P22. Designing cross-protection at the parcel level: the case of fanleaf degeneration

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INTRODUCTION

Cross-protection is a biocontrol method whereby the primary-infection of a plant with a mild (cross-protective) virus strain prevents it from the damage caused by the subsequent infection by another (challenge) strain of the same virus (Ziebell & Carr, 2010). It has been successfully used to protect a broad range of commercially relevant crops (including citrus and tomatoes) against the damage caused by their pathogenic viruses (Ziebell & Carr, 2010). Among the factors that appear to impact the effectiveness of cross-protection in the field, the steady mild symptoms induced by the cross-protective strains and the genetic relatedness between the cross-protective and challenge strains seem to be crucial (Ziebell & MacDiarmid, 2017). To the best of our knowledge, the complex genetic diversity of the cross-protective and that of the challenge virus strains at a local scale are rarely considered before implementation. Here, we investigate the potential of taking it into consideration while implementing a cross-protection trial against fanleaf degeneration, one of the most damaging diseases of *Vitis* spp. The main causal agent of this disease, *Grapevine fanleaf virus* (GFLV, *Nepovirus, Secoviridae*), is a bipartite virus, composed of two (+) ssRNA, specifically transmitted in a semi-persistent manner by the nematode *Xiphinema index* (Andret-Link *et al.*, 2004). Studies have revealed a high degree of polymorphism with about 0.13 nucleotide substitutions per site for both GFLV genomic RNAs (Hily *et al.*, 2021) and a genetic differentiation of GFLV population from one parcel to another (Kubina *et al.*, 2022). Based on these findings, our goal is to optimize cross-protection implementation by selecting mildly symptomatic vines infected with characterized GFLV isolates and replant them in a specific area of the same parcel where potential GFLV challengers are characterized.

MATERIALS AND METHODS

Search for GFLV cross-protection candidates in a highly infected vineyard

A vineyard parcel with 30-year-old vines in Vosne-Romanée (Burgundy region, France) was selected for this study. All the 5595 vines of the parcel consisted of non-clonal *Vitis vinifera* cv. 'Pinot noir' grafted on a rootstock of unknown-origin and cultivated in line with the organic farming standards. We first assessed GFLV prevalence by using a "W" sampling scheme, with each sample being tested by DAS-ELISA (Villate *et al.*, 2008). 36 GFLV-positive vines presenting low to no symptom were selected as cross-protection candidates in 2021. Their symptoms and fruit yield were followed for three years and compared to those of neighbouring GFLV-free vines as well as highly symptomatic vines. The presence/absence of GFLV in all monitored plants was annually assessed by DAS-ELISA. The detection of other viruses was performed using high throughput sequencing (HTS) which also allowed to reconstitute GFLV consensus sequences.

Identification of potential GFLV challengers in the future trial area

Before uprooting 200 vines in the most severely affected area of this plot, the symptoms and the yield of the vines were monitored. Moreover, all these vines were tested for GFLV by DAS-ELISA and around a quarter of them was sent for HTS to reconstitute their GFLV consensus sequences. Being present in the trial area, these GFLV variants constitute potential future challengers.

Propagating cross-protection candidates before implanting them in the future trial area
In February 2022, dormant wood cuttings were collected on the cross-protection candidates and were propagated and grafted onto 5C rootstock (*Vitis berlandieri* x *Vitis riparia*) to constitute the future experimental cohort that will be reintroduced in the trial area in 2024.

RESULTS AND DISCUSSION

The estimation of GFLV prevalence in the whole parcel revealed that over 80% of the vines could be infected with GFLV. Among the 5595 vines, 36 were considered as potential candidates for cross-protection given their low symptom expression and fruit yields that do not differ significantly from those of GFLV-free vines. Using HTS, (i) consensus sequences of GFLV RNAs found in these cross-protection candidates were obtained and (ii) the presence of other viruses was assessed. 14 candidate vines infected with grapevine leafroll associated viruses and/or grapevine pinot gris virus were no longer considered. The 59 GFLV sequences (29 RNA1 sequences and 25 RNA2 sequences) found in the 22-remaining cross-protection candidate vines were compared to those of the challenge isolates found in the 46 tested vines in the future trial area (90 RNA1 sequences and 68 RNA2 sequences). These comparisons revealed that some sequences of GFLV variants present in cross-protection candidate vines shared above 95% of nucleotide sequence identity with potential GFLV challengers making them particularly interesting for the future trial. The propagation of the 22 cross-protection candidate vines is ongoing. Knowing the phenotype of the candidate vines and the genomes of the GFLV variants infecting them as well as those of potential endemic variants will help selecting our pool of candidates to be implemented in the trial zone. The multi-year symptom monitoring and the detection of the variants present in the primary infected and control vines should eventually contribute to the identification of key features responsible for cross-protection to occur in the field.

ACKNOWLEDGEMENTS

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P23. Micrografting of shoot apices: a rapid and efficient method to sanitize grapevine infected by GVA, GVB, GRSPaV or GPGV

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INTRODUCTION

Grapevine is known to be infected with many identified viruses (Fuchs, 2020). To avoid dissemination through vegetative propagation and grafting, certification of virus-free vines has been implemented. To obtain these virus-free plants, different techniques were developed as meristem culture, somatic embryogenesis or cryotherapy (Goussard *et al.*, 1991, Maliogka *et al.*, 2015). Since several years, IFV has developed an efficient alternative method combining thermotherapy and micrografting of shoot apices shown to be efficient onto the regulated viruses (Benin *et al.*, 1984; Spilmont *et al.*, 2012). It would be interesting to evaluate its efficiency on secondary and emerging viruses.

MATERIALS AND METHODS

Sanitation process

92 accessions were submitted to sanitation by this process: 6 for GVA, 4 for GVB, 11 for GRSPaV and 71 for GPGV.

The protocol was the same as described by Spilmont *et al.*, 2012, except the number of micrografting per accession was down to 20 instead of 30. Virus detection was performed at two steps of the process. Early tests were done to evaluate the efficiency of the treatment during the process. These tests were realized by RT-PCR (or qRT-PCR depending on the virus) on mature leaves of all the plants obtained 4 to 6 months after micrografting. A second test was done at the end of the process 18 months later to check the final sanitary status. At this step only two of the sanitized clones per accession installed in the cold greenhouse were tested on mature canes.

Virus detection

Viruses studied in this article were detected by qRT-PCR. The presence of GVA and GVB were analyzed using a set of specific primers and probes internally developed (not published). As for the detection of GPGV, primers and probe reported by Bianchi *et al.*, 2015 were used. Specific primers reported by Beuve *et al.*, 2013, were used for GRSPaV.

RESULTS AND DISCUSSION

Regeneration could be very variable between grape cultivars. Regeneration rate is calculated as the number of developed plantlets obtained divided by the number of apices micrografted (a maximum of 20). In the laboratory of IFV, this rate is around 35% (Table 1). Only a very few cultivars were really recalcitrant to this technique.

Table 1: Regeneration rate obtained by year.

Year	2018	2019	2020	2021	2022
Infected accessions submitted to sanitation	63	7	6	7	52
Rate of succeeded grafting	42%	28%	40%	34%	33%

As described by Spilmont *et al.* 2012, some viruses as nepoviruses are more difficult to eliminate. With the early test, we can have a good picture of the efficiency of the sanitation for secondary viruses. Results for GVA, GVB, GRSPAV and GPGV are shown in Table 2.

Table 2: Virus detection on early and final stage of the sanitation protocol.

	Before sanitation	Number of plants confirmed negative after the sanitation process	
Viruses	**Infected accessions**	**Early test onto all the acclimated vines**	**Final test realized onto two conserved clones per accession**
GVA	6	41/41	12/12
GVB	4	29/29	7/7
GRSPaV	11	66/81	21/21
GPGV	71	193/203	134/135

As for the vitiviruses GVA and GVB, IFV sanitation protocol appears to be 100% effective. For GRSPaV and GPGV, the rate efficiency appears slightly lower as observed in early tests (81% for GRSPav and 95% for GPGV). For all these viruses, only one plant was positive for GPGV at the last stage of the protocol.

As shown with these results and those of Spilmont *et al.*, 2012, this method which combines thermotherapy and micrografting of apex is particularly efficient on all regulated and emerging grapevine viruses.

ACKNOWLEDGEMENTS

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P24. Hidden genetic diversity of '*Candidatus* Phytoplasma solani' strains in Istrian vineyards: how small can be rich

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INTRODUCTION

Grapevine Yellows (GY) are economically important diseases associated to phytoplasmas (genus '*Candidatus* Phytoplasma'), uncultivable bacteria from the class Mollicutes. In Euro-mediterranean region, GY are mainly attributed to phytoplasmas belonging to ribosomal 16SrXII-A subgroup ('*Ca.* P. solani'; *Bois Noir* phytoplasma; BNp) and 16SrV group (*flavescence dorée* phytoplasma; FDp), with the latter one being a quarantine pathogen (Plavec et al. 2015, Quaglino et al. 2013). Although symptomatology can be similar, epidemiology of FDp and BNp is very different as they are transmitted by different insect vectors and could have different alternative hosts. In Croatia, GY diseases have been continuously monitored since 1997 with FDp appearing only in 2009 (Šeruga Musić et al. 2011). While BNp is more diverse and widespread, FDp belonging to 3 *map* phylogenetic clusters are differently distributed throughout regions. Istria is heavily affected with FDp since 2014 with only one genotype (mapFD2/M54) being attributed to great losses (Plavec et al. 2019). On the contrary, information on BNp diversity is scarce with only couple of genotypes detected so far (Plavec et al, manuscript in preparation). Therefore, the aim of this case study was to assess the diversity of BNp genotypes in a small selected area of Istria, in order to draw attention to the presence and importance of BNp as GY agent in this important Croatian viticultural region.

MATERIALS AND METHODS

The samples of asymptomatic and symptomatic grapevine (cvs. 'Teran' and Chardonnay', respectively), weeds and vegetation surrounding vineyards such as *Urtica dioica L.* (nettle), *Cornus sanguinea* L. (common dogwood) and *Ulmus* sp. (elm), as well as insect vectors, were taken during late summer or early autumn in 2019 - 2022 from two selected vineyards in Vižinada area (Istria). Detection and identification of phytoplasmas was performed by triplex real-time PCR (Pelletier et al. 2009) and PCR/RFLP analyses of 16S rDNA. In order to perform multilocus sequence typing (MLST), *secY*, *stamp, tufB* and *vmp* gene fragments were amplified from BNp detected isolates. All obtained amplicons were sequenced (Genewiz-Azenta Life Sciences, Leipzig, Germany) and subjected to phylogenetic analysis (ClustalX, MEGA X).

RESULTS AND DISCUSSION

In one of the monitored vineyards with 'cv. Chardonnay' displaying strong GY symptoms, BNp infection was continously confirmed in every year of sampling, while in the other vineyard, asymptomatic cv.'Teran' vines always tested negative for phytoplasma presence. In the latter vineyard, BNp was detected in nettle as well as elm trees positioned at its's edge. More over, BNp-infected *Euscelis incisus* and *E. lineolatus* specimens were also found, which were never been reported previously to be BNp-infected in Croatia. Among detected isolates, eight were subjected to MLST so far – five from grapevine, two from elm and one originating from nettle. The results of MLST analysis have revealed the presence of 4 different *secY* genotypes (S1, S4, S6 and S39), 5 *stamp* genotypes (ST19, ST22, ST29, ST46), 2 *vmp1* genotypes (V3, V14) as well as 2 *tuf* genotypes (tuf-a, tuf-b2). Altogether, six different collective genotypes were found, with one being detected in elm and nettle samples (S1/ST29/V14/tuf-b2) close to the vineyard without BN-infected grapevine, while five different collective genotypes were detected in five 'Chardonnay' samples from the same vineyard (Fig 1., Fig. 2). Out of six detected collective genotypes under the scope of this case study, three were previosly found in Istria, while three represent newly detected genotypes for this vinegrowing region. Such a great diversity of BNp genotypes found in such a restricted and smalll area, especially in cv. 'Chardonnay' where each of the characterized isolates had a distinct collecitve genotype, point out that continous surveillance of BNp presence and genotyping should not

be neglected as a tool in our attempts of controling and preventing GY outbreaks. Moreover, presence of distinct BNp genotypes in asymptomatic vegetation in the vicinity of vineyards as well as a report of new BNp-infected insect vector species for Croatia, suggest their importance and role of these key-players in the GY epidemiology.

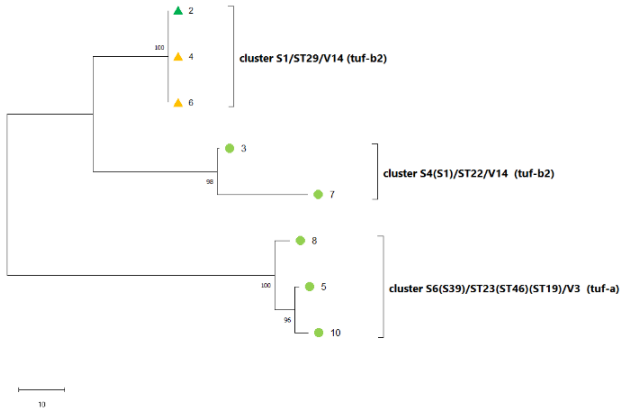

Fig 1. Unrooted phylogenetic tree depicting phylogenetic relationship of concataneted *stamp* (ST), *secY* (S) and *vmp1* (V) gene sequences, of '*Candidatus* Phytoplasma solani' isolates infecting different host plants in Istria, inferred by neighbour-joining analysis (number of differences) with bootstrap values obtained after 500 repeats. Samples originating from different plant hosts are marked as follows: green dot – grapevine, green triagle – nettle, orange triangle – elm tres.

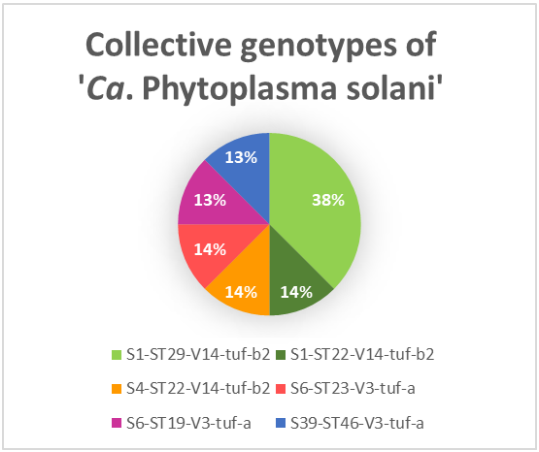

Fig 2. Distribution of collective genotypes (genes *secY, stamp, vmp1* and *tufB*) of '*Candidatus* Phytoplasma solani' isolates detected in grapevine, nettle and elm from selected vineyards in Istria.

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P25. Molecular characterization of Flavescence dorée phytoplasma isolates occurring in grapevine in Veneto (Northeastern Italy) from 2002 to 2022

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INTRODUCTION

Flavescence dorée (FD) is a quarantine disease occurring in Europe since the half of the last century. It is associated to the presence of phytoplasmas belonging to phylogenetic groups 16SrV-C and D. Several FD phytoplasma (FDp) isolates have been recorded so far, which were characterized at molecular level in different stable and variable genes. Most important are the nucleotide differences in the 16S-23S gene region, but also *rplV-rpsC, map, secY,* and other genetic traits have been used. The aim of the present work was to compare at the molecular level the FDp isolates occurring in grapevine in Veneto during the first important epidemic (1993-2002) with those present in vineyard nowadays, in order to find out if something had changed in the last 20 years.

MATERIALS AND METHODS

A total of 42 vineyards, distributed in the main grapegrowing areas of Veneto, were monitored for FD presence and symptomatic leaves from 2 to 4 plants per vineyard were sampled. DNA was extracted with the CTAB methods (Angelini et al., 2001), and a specific real time PCR was carried out to confirm the presence of grapevine phytoplasmas (Angelini et al., 2007). Samples positive to FDp were subjected to nested PCR targeting 16S-23S and *map* genetic regions (Angelini et al., 2001; Arnaud et al., 2007). Subsequently, PCR 16S-23S amplicons were digested with the restriction enzyme *Taq*I for the identification of the 16SrV-C or -D subgroup by comparing RFLP (restriction fragment length polymorphism) profiles (Angelini et al., 2001). PCR map amplicons were double-strand sequenced by automatic equipment (BMR, Padua, Italy) with the primers MAPr2 and MAPr3 (Rizzoli et al., 2021). In parallel, 14 DNA samples from FD-C and FD-D infected grapevines, collected in the 2000 years in Veneto and maintained at -20°C in the CREA collection, were amplified and sequenced with the same procedures. The raw sequence chromatograms were assembled and edited using Staden Package software (Bonfield et al., 1995).

RESULTS AND DISCUSSION

Three out of 77 samples collected in 2022 were infected by *Bois noir* (BN). All the other samples were positive to FDp with all the three PCR assays.

Molecular characterization of the 16S-23S FDp region revealed the presence of the same genetic phytoplasma groups in the two historical periods: FD-D and FD-C, as defined in Angelini et al. (2001). The geographical distribution was almost identical, with a little restriction of the area where FD-C is occurring (Figure 1).

Sequencing of the *map* gene allowed to classify all FD-C isolate as M3 *map* genotype, and all the FD-D isolates as M54 *map* genotype.

Results obtained showed that, as far as the genes indagated, no important differences were present between FDp isolates occurring 20 years ago and nowadays in the Veneto region, neither in the molecular sequence nor in the geographical distribution. FD-D/*map54* is the most common FDp isolate in vineyard in Europe, discovered in 1988 and occurring in almost all European countries where FD is present. On the oppositive, FD-C/*map3* is an “autochthonous” FDp isolate, occurring in vineyard only in the Veneto region, and thought to be originally present in clematis, and then jumped to grapevine

(Angelini et al., 2004; Filippin et al., 2009). This isolate was identified also in one *Alnus glutinosa* plant in Friuli Venezia Giulia, a region bordering Veneto (Malembic-Maher et al., 2020).
The chosen genes are quite stable and not subjected to divergent evolutionary pressure, thus deeper studies focused on other phytoplasma genetic traits, possibly more variable and associated to evolutionary changes, could reveal a different frame.

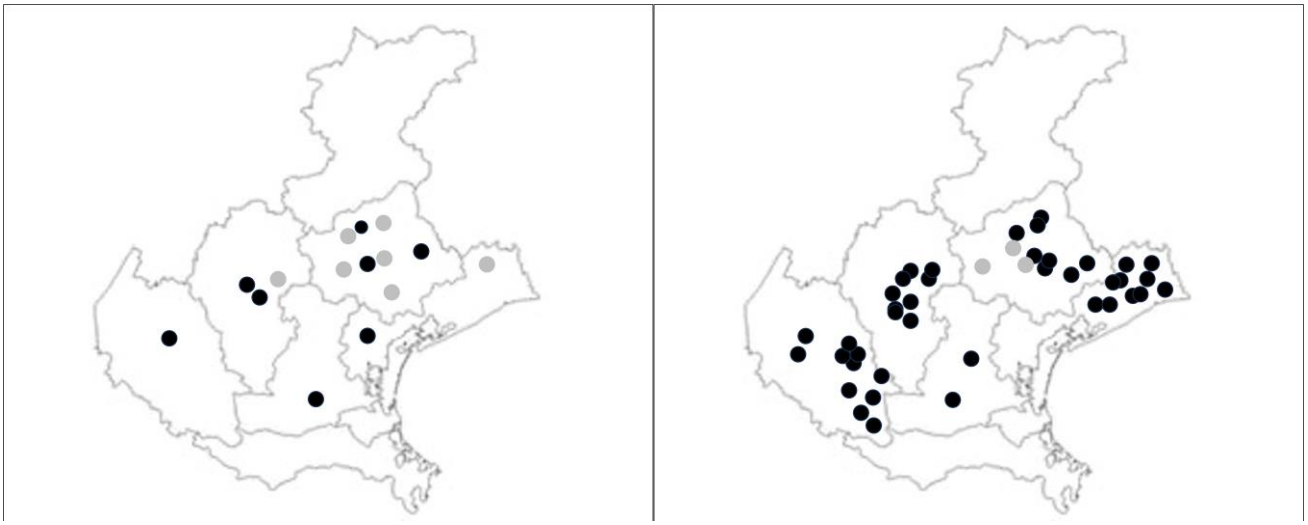

Figure 1 FD phytoplasma isolates distribution in Veneto in 2002 (left) and 2022 (right). Phytoplasma are characterized based on their *16S-23S* and *map* gene sequence. Black dots: FD-D and M54 *map* genotypes; grey dots: FD-C and M3 *map* genotypes.

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P26. Study on the occurrence of non-regulated phytoplasmas in vineyards in Greece, with emphasis on the Bois noir phytoplasma

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INTRODUCTION

Grapevine cultivation holds a prominent position in the agricultural economy of Greece and securing plant health in vineyards via robust and timely diagnostic testing for the presence of phytopathogens, is of paramount importance. Regarding prokaryotic phytopathogens, there are several bacteria and phytoplasmas that can infect grapevine, causing significant losses of crop production. Until now, the program of Official Surveys for prokaryotic pathogens in vineyards in Greece, funded by the Hellenic Ministry of Rural Development & Food as well as the EU, include only the quarantine pests: Grapevine flavescence dorée phytoplasma (FD phytoplasma) and *Xylella fastidiosa*. However, other non-regulated phytoplasmas, such as the Bois noir phytoplasma, can also have a considerable impact on this crop and thus merit a more systematic monitoring of their presence. In this study, we present data from ongoing research work conducted at the Laboratory of Bacteriology at Benaki Phytopathological Institute (BPI) in regard to the occurrence and distribution of non-regulated phytoplasmas in vineyards in Greece with emphasis on the Bois noir disease caused by '*Candidatus* Phytoplasma solani', a pathogen known to occur in Greece, affecting not only grapevine but also potato and tomato field crops (EPPO 2023).

MATERIALS AND METHODS

Historical diagnostic data regarding samples of symptomatic grapevine plants sent by growers, cooperations, agronomists or citizens for diagnostic examination at the Laboratory of Bacteriology at BPI in the period 2014 to 2023, were retrieved from the archives of BPI containing the results of laboratory examination of phytopathological samples. Those samples which had been tested positive for the presence of phytoplasmas, were selected as a source of information for phytoplasma occurrence. In parallel, the samples from asymptomatic grapevine plants sent by the Phytosanitary Inspectors in the frame of Official Surveys for FD phytoplasma in the period from June 2022 to June 2023, are being screened for the presence of other phytoplasmas by the real-time PCR method developed by Chistensen et al. (2004) for generic detection of phytoplasmas. In case of positive results, further phytoplasma identification is based on applying the specific for '*Ca*. Phytoplasma solani' real-time PCR method of Pelletier et al. (2009) and sequencing of the amplicons of the nested PCR with the primers sets P1/P7 and R16F2n/R16R2 (Jović et al., 2011). Each sample examined included shoots with leaves from forty-five (45) asymptomatic grapevine plants (composite samples) and total genomic DNA was extracted using a CTAB-based method, as described for the FD phytoplasma in the relevant EPPO diagnostic standard (EPPO, 2015).

RESULTS AND DISCUSSION

Based on the data from the diagnosis on samples of symptomatic grapevine plants which have been laboratory examined in the period from 2014 up today, nine (9) samples have been found to be infected by the Bois noir phytoplasma. These samples originated from three Regional Units: Thessaloniki, Florina and Attici. According to the sample information sheets: a) the symptoms of the plants included: leaf yellowing, decline, downwards leaf rolling, irregular ripening of wood, growth reduction (smaller than normal leaves and short internodes) and b) the disease incidence ranged from 3% to 40% in the

vineyards. About 450 composite samples from asymptomatic grapevine plants are now under testing by the real-time PCR protocol described by Chistensen et al. (2004) for generic detection of phytoplasmas and afterwards by the real-time PCR described by Pelletier et al. (2009) for '*Ca*. Phytoplasma solani' detection. In case of positive results, sequencing of nested PCR products will be performed. The final results of these tests and subsequent identification of any phytoplasma detected will be presented as a poster in the Conference.

ACKNOWLEDGEMENTS

On this occasion, it has to be acknowledged: a) the efficient collaboration with the Phytosanitry Inspectors who are sending the samples from asymptomatic grapevine plants in the frame of the Official Surveys program funded by the Ministry of Rural Development & Food and the EU, and b) the excellent assistance of the Technicians of the Laboratory of Bacteriology of BPI: Mr Ioannis Loulakidis and Mr Georgios Gagarakis in preparing plant tissue cuttings and homogenizing them to have them ready for applying the diagnostic methodology.

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P27. Survey of Flavescence dorée potential hosts plants present in the borders and surroundings of vineyards in the "Vinhos Verdes" wine area, Portugal

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INTRODUCTION

Flavescence dorée (FD) is considered a quarantine disease included in European legislation (Regulation (EU) 2019/2072) as it is one of the most serious phytosanitary problems affecting vineyards. It was detected in Portugal in 2007 (Sousa *et al*, 2007, 2010), in Portuguese cultivars, in the Cávado and Lima rivers basins, in the heart of the "Vinho Verde" wine area. Currently, FD is present in all that area, and also in "Bairrada" and "Douro" wine areas. Its spread to southern areas led the National Phytosanitary Authority (DGAV) to enforce strict measures, such as uprooting infected plants, mandatory treatments against the insect vector and mandatory hot water treatments to grapevine propagation material traded from areas affected by FD. Other secondary hosts may play a relevant role in the epidemiology of the disease, due to the possibility of being repositories of both FD and its potential vectors, and even of other vine diseases, such as Bois noir. In this work, we surveyed the surroundings of six vineyards in the "Vinhos Verdes" wine area for potencial FD plant hosts by means of detecting the phytoplasma in these plants.

MATERIALS AND METHODS

The six vineyards under study were located in old marshes, flanked by water lines. These vineyards had a previous history of FD infection and *S.titanus* presence, besides recurrent presence of a few infected plants, mainly in their borders, even though the mandatory treatment against the vector was implemented. The plants sampled for phytoplasma detection were feral *Vitis* (European and American rootstocks), as well putative host plants *Ailanthus altissima*, *Alnus glutinosa*, *Coryllus avellane, Salix atrocinera*, and *Rubus ulmifolium* located within a perimeter of about 5 meters of the vineyard (Figure 1).

Figure 1 -Example of plant sampling points and yellow traps (dots, 6 traps per farm) location in one of the studied vineyards (left). Border of one of the vineyards and FD symptomatic plant (right).

Insect yellow traps (Sousa et al 2023) were placed next to the sample plants. The FD detection in hosts plants was performed as specified in the EPPO PM 7/79 (2) -2016.

RESULTS AND DISCUSSION

In the inventory carried out, the riparian galleries were found to be colonised by invasive plants (e.g. *Ailanthus altissimo*), which are considered a high threat to the biodiversity of ecosystems. Despite

mandatory treatments against the insect vector (*S.titanus*), some grapevines with Flavescence dorée symptoms were observed in the vineyard borders, which is presumed to be due to transmission by *S. titanus* from feral *Vitis* coexisting in the riparian galleries surrounding the plots. The FD infection in symptomatic grapevines was confirmed by PCR. FD was detected in all studied plant species, except hazel (Table1).

Table 1 – Presence of 16r(V) and FD phytoplasma in plants in the sample plants as detected PCR

Plants	**16Sr(V)**	**FD phytoplasma**
Alnus glutinosa	+	+
Aillanthus altissima	+	+
Coryllus avellane	-	-
Rubus ulmifolius	+	+
Salix atrocinera	+	+
Vitis vinifera	+	+
Vitis X rootstocks	+	+

In view of these results we recommend that in an FD outbreak area and in the presence of its main vector *S. titanus* the monitoring area should be extended to secondary hosts in surrounding untreated areas of the vineyards (*Ailanthus altissima, Alnus spp, Salix spp*) and as a well as the occasional FD vectors (*Dictyophara europaea*, *Orientus ishidae*, *Phlogotettix cyclops*, *Oncopsis alni*), in order to prevent the spread and transfer of FD from those reservoirs to grapevines.

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P28. Survey of infested *Scaphoideus titanus* and other potential vectors of Flavescence dorée present in the borders and surroundings of vineyards in the Vinhos Verdes wine area, Portugal

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INTRODUCTION

Grapevine flavescence dorée phytoplasma (FD) is present in vineyards of the Vinhos Verdes wine area of Portugal since 2007 (Sousa et al. 2009) and its principal vector *Scaphoideus titanus* was first detected in Portugal in 1999 (Quartau et al. 2001). As a quarantine pest (EPPO A2 list and Regulation (EU) 2019/2072), strict measures are applied to control its spread. For instance, elimination of symptomatic grapevines and spraying against the vector in all vineyards of the area from 1 to 3 times a growing cycle, are mandatory depending on the presence of FD infected plants and the vector. In this work a survey was carried out in the borders and adjacent areas of six vineyards, for *S. titanus*, and, in particular, for potential vectors whose existence in the Vinho Verde region and/or in Portugal was unknown or little known. The survey focused on the hemipterous insects Auchenorrhyncha: *S. titanus*, *Dictyophara europaea*, *Metcalfa pruinosa*, *Oncopsis alni*, *Orientus ishidae* and *Phlogotettix cyclops*. They all feed, as nymphs and adults, on the phloem of the plants. It is through that this activity that they become infected for life and transmit the disease to healthy plants. The presence of phytoplasma was monitored in the insects.

MATERIALS AND METHODS

The six studied vineyards are located in four river basins, Cávado (Amares and Braga), Lima (Arcos-de-Valdevez), Neiva (Vila Verde) and Tâmega (Celorico de Basto e Mondim de Basto) in de "Vinho Verde" wine region in Northern Portugal. All had a previous history of FD grapevine infection and presence of *S. titanus*. The insects were captured in 6 vineyards under study (38 capture points) in yellow traps placed inside, in the border and in the surrounding area (in a perimeter of about 5 meters of the vineyard) (Sousa et al 2023). The traps were replaced weekly from May to October, during 3 years (2018, 2019 and 2020) amounting to about 2800 traps in total. All hemipterous insects Auchenorrhyncha were identified either by morphology or/ and by DNA barcoding. The detection of phytoplasma in the insects was performed as indicated in the EPPO PM 7/79 (2)- 2016.

RESULTS AND DISCUSSION

Scaphoideus titanus was captured in 31 out of 38 sampling points in all of the monitored 6 vineyards. The distribution of the captures is presented in figure 1. The following insect species were identified by morphological traits and/or confirmed by DNA barcoding: *Scaphoideus titanus* (specific FD vector), *Dictyophara europaea*, *Euscelidius* sp., *Metcalfa pruinosa*, *Orientus ishidae*, *Phlogotettix cyclops*, and *Psylla alni*, (secondary vectors). These insects can host and transmit phytoplasma of the Flavescence dorée group. We have confirmed that some of these specimens were infected with FD (table 2). Other insects were captured in the yellow traps e.g. *Macropsis fuscula* (vector of Ca. phytoplasma rubi) and *Sophonia orientalis* an invasive leafhopper which has beenfor the first recorded in mainland Portugal (Neto et al. 2021).

Figure 1- Distribution of *Scaphoideus titanus* captures over 3 years. Red - Inside the vineyard; Yellow - In vineyard borders; Grey - In the vineyard surrounding area (not treated against insects).

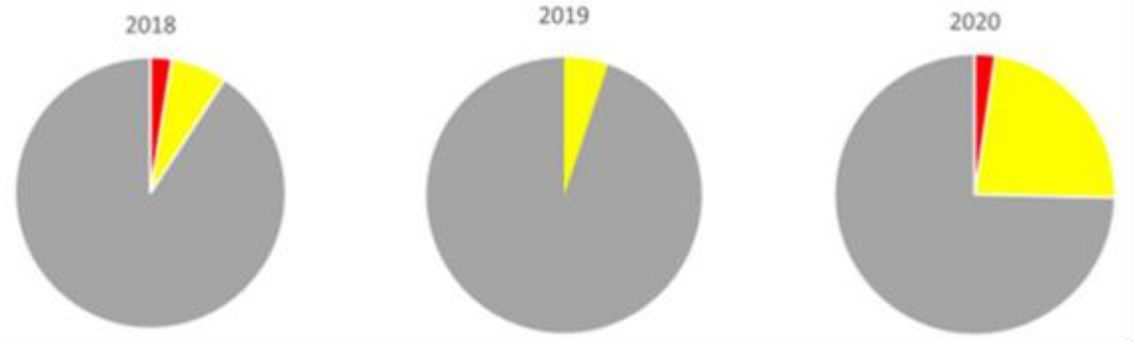

Table 1- Detection of 16Sr (V) and FD phytoplasma in capture insects

Insects	16Sr(V)	FD phytoplasma
Dictyophara europaea	+	+
Macropsis fuscula	+	+
Metcalfa pruinosa	+	+
Orientus ishidae	+	+
Psylla alni	+	+
Scaphoideus titanus	+	+

In view of these results we recommend that in an FD outbreak area and in the presence of its main vector *S. titanus* the monitoring area should be extended to occasional FD vectors (*Dictyophara europaea, Orientus ishidae, Phlogotettix cyclops, Oncopsis alni*) in the surrounding untreated areas of the vineyards in order to prevent the spread and transfer of FD to grapevines.

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P29. Molecular epidemiology and pathosystems in Croatian and Serbian vineyards: a comparative analysis of grapevine yellows phytoplasmas

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INTRODUCTION

Bacteria of the genus '*Candidatus* Phytoplasma' (class Mollicutes), commonly known as phytoplasmas, are pleomorphic intracellular pathogens responsible for numerous economically significant plant diseases worldwide. They are naturally transmitted by insect vectors that feed on plant sap, and the complex life cycles of these bacteria involve obligatory parasitism in both plant hosts and insects (Hogenhout *et al.*, 2008; Namba, 2019). *Bois noir* phytoplasma (BNp: '*Ca.* P. solani', belonging to the ribosomal16SrXII-A subgroup) and *flavescence dorée* phytoplasma (FDp: categorized under the ribosomal 16SrV group) as theprimary causal factors play an important role in the occurrence and spread of grapevine yellows (GY) diseases in Europe. (Constable 2010, Quaglino *et al.* 2013). While the symptoms may appear similar, the epidemiology of these two phytoplasmas varies due to their transmission by distinct insect vectors. BNp is transmitted by several polyphagous vectors, with *Hyalesthes obsoletus* Sign. being the predominant one, whereas FDp is exclusively transmitted to grapevine by the monophagous vector *Scaphoideus titanus* Ball.Additionally, FDp is classified as a quarantine pathogen in Europe, posing a great threat to viticulture (Schvester *et al.*, 1969; Pelletier *et al.*, 2009). The objective of this study was to examine the differences in the variability of GY phytoplasmas and their epidemiological features within the vineyards of selected viticultural regions in Croatia and Serbia.

MATERIALS AND METHODS

This study involved collecting samples from grapevines, insect vectors, and reservoir plants during late summer and autumn between 2019 and 2021, specifically in selected viticulture regions of Croatia and Serbia. Total nucleic acids were extracted from all 76 collected samples followed by PCR/RFLP analyses of 16S rDNA, as well as triplex real-time PCR in order to detect and identify the phytoplasmas. To obtain a more detailed characterization of the isolates, multilocus sequence typing (MLST) A was performed. Thisincluded amplification of *stamp* and *vmp* for BNp, and *map* and *sec* gene fragment for FDp, by using specific primers followed by sequencing (Genewiz-Azenta Life Sciences, Leipzig, Germany) and phylogenetic analysis (ClustalX, MEGA X).

RESULTS AND DISCUSSION

The MLST analysis has revealed significant variations and the existence of distinct genotypes among BNpand FDp strains found in Croatian and Serbian vineyards. In Croatia, out of 56 samples, 14 *Vitis vinifera* L., 4 *Urtica dioica* L. and 12 *H. obsoletus* samples were positive for BNp. 24 *V. vinifera* and two *S. titanus*samples were positive for FDp. Two samples of *V. vinifera* were coinfected with BNp and FDp. In Serbia,out of 20 *V. vinifera*. samples, 16 were positive for BNp and 6 samples were positive for FDp. One sample showed coinfection with BNp and FDp. Notably, the strains from Croatia displayed greater variability compared in both BNp and FDp strains from Serbia. Among the various molecular markers studied, the *stamp* gene exhibited the highest level of specificity and proved to be the most reliable marker for distinguishing between different BNp strains. Despite the geographical

proximity and similar viticultural practices between Croatia and Serbia, our findings have demonstrated distinct differences in GY pathosystems concerning the prevalent phytoplasma genotypes within the sampled vineyards. The results of the study confirmed the presence of BNp and FDp in grapevine samples from both countries, as well asin insect vectors such as *S. titanus* and *H. obsoletus*, and reservoir host plants like *U. dioica* and *Ailanthusaltissima* Mill (Fig. 1).

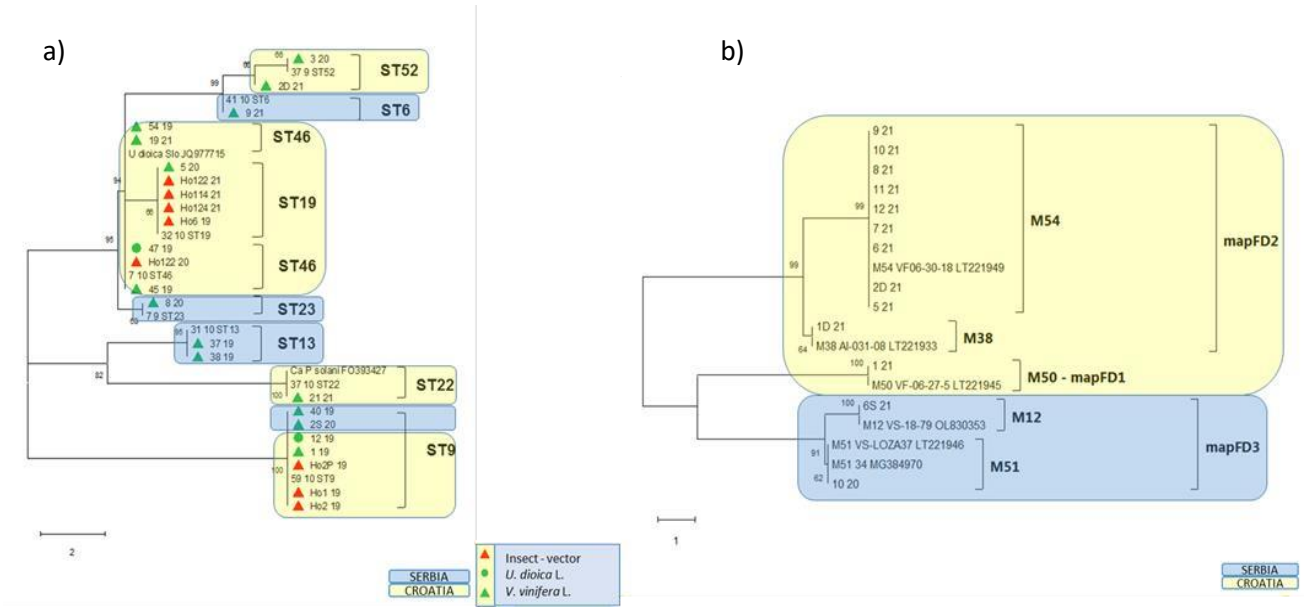

Fig 1: The unrooted phylogenetic neighbor-joining tree based on : a) *stamp* sequences of BNp isolates from Croatia and Serbia, b) *map* sequences of FDp *Vitis vinifera* L. isolates from Croatia and Serbia. Numbers on main branches correspond to bootstrap values as precentages (500 replicates).

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P30. The role of climate change in the distribution of Bois noir in the Republic of Moldova

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INTRODUCTION

A dangerous and harmful disease of phytoplasma etiology *Bois noir* (BN) has become widespread in the vineyards of Moldova. BN is naturally transmitted in the vine by the planthopper *Hyalesthes obsoletus* (Signoret, 1865) (Maixner M., 2011). The disease-causing phytoplasma BN is transmitted to the vine plant from bindweed (Sforza R. et. al., 1998) and nettle (Langer M. & Maixner M., 2004). Thus, the spread of BN disease in vineyards is determined by two factors: firstly, the presence of a plant host as a source of the disease and secondly planthopper *H. obsoletus* as a vector. The life cycle of the *H. obsoletus* is associated with weeds, and the vine plant is an occasional feeding site for the planthopper (Weintraub P.G. and Beanland L., 2006). However, if the vine plant is an accidental feeding place for this insect, it is very important to establish what is the reason for the massive spread of BN on grape plantations in the Republic of Moldova. The solution of this issue will allow to implement measures to prevent the spread of the BN disease on the grape plantations of the Republic of Moldova.

MATERIALS AND METHODS

The object of research was plantations of grapes of European and autochthonous varieties, which located in three areas of grape cultivation: Causeni region (Tenetar), Criulyan region (Slobozia-Dushka) and ATU Gagauzia (Budjak). On these plantations, experimental plots with an area of 2,5 to 6 hectares were selected. The total area of the experiment was 29 hectares. Identification of the causal agent was performed by nested PCR for phytoplasma detection. The catching of insects in the vineyard was carried out using sweep net, from July to September 2018-2019. Insect trapping was carried out from 3 vineyards, once a week. Sampling for testing of host plants *Convolvulus arvensis* L. and *Cynanchum acutum* L. was carried out from vineyards and adjacent areas. The extraction of DNA was carried out according to Angelini et. al. (2001). Molecular analysis was carried out by nested PCR, commercial kit from Qualiplant (FD / BN), primers used for the first amplification: FD9f / FD9r and STOL11f2 / STOL11r1, for the second amplification: FD9r2 / FD9f3b and STOL11f3 / STOL11r2.

RESULTS AND DISCUSSION

For three years (2017-2019), the natural distribution of BN was monitored in the experimental plots. For this purpose, each year we carried out field studies of experimental plots for the defeat of BN. At each inspection, samples of vines and herbaceous plants reservoir were taken for testing for the presence of phytoplasma, the causative agent of the disease. Tests of grapevine samples showed the presence of *Bois Noir* disease and the absence of *Flayescence doree*. PCR testing of reserve plant samples revealed a high degree of infection with BN disease. So *Convolvulus arvensis* L. is infected with phytoplasma by 62,5%, and *Cynanchum acutum* L. infected by 60%, respectively. Testing of the planthopper *H. obsoletus* found that 40,73% of the insects were infected with BN phytoplasma. During the observation of the natural distribution of BN, it was found that on some plantations there was a gradual increase in the number of bushes affected by the disease, and on others a significant

decrease. Thus, in the area of the village of Tenetari, Causeni region, the number of affected bushes in the Cabernet Sauvignon variety decreased by 6,85%, in the Pinot noir variety by 9,14% and in the Merlot variety by 36,28%, respectively.

On the site in the village Slobozia-Dushka, Criuleni region, Cabernet Sauvignon variety had an increase in the number of affected bushes by 9,14% over three years. A similar condition is on the plantations in the village of Budzhak, ATU Gagauzia. Thus, the affected of bushes of the variety Rara neagra with the BN disease increased from 14,81% to 29,50% within three years, and the affected of the variety Feteaska neagra increased from 6,91% to 11,70%.

The decrease in the number of vine bushes affected with the disease in the areas of the village of Tenetari, Causeni district is explained by the fact that starting from 2018, the soil in the vineyard was kept clean from weeds during the entire growing season. The reserve plant *Cynanchum acutum* L. was extremely rare. Removal of the plant hosts of the insect from the plantation resulted in a significant decrease in the population of the planthopper vector *H. obsoletus*, which ultimately led to a decrease in the number of infected vines.

In the areas of the village of Budzhak, ATU Gagauzia and the village of Slobozia-Dushka, Criuleni district, there are a sufficient number of factors necessary for the spread of the BN disease: vector of *H. obsoletus* and reservoirs of the disease *Convolvulus arvensis* L. and *Cynanchum acutum* L. However, if the grape is neither a host plant nor a plant of choice for *H. obsoletus*, then what forces the insect to change its habitual diet to the grape plant? In the Republic of Moldova in recent years, the average annual air temperature increased by 2.0˚C, the sum of effective temperatures increased by 446.5˚C, and the amount of precipitation decreased by 147 mm. These climatic changes have a negative impact on the biocenosis of vineyards.

Due to lack of moisture and high temperatures, herbaceous plants on vine plantations coarsen prematurely, turn yellow and dry out, which causes sucking insects to massively switch to other food sources. The vine in this period of time is in the stage of active growth and forms many lateral shoot. Viroform planthoppers, feeding on the juice of young and succulent shoots, infect them with phytoplasma, increasing the number of grapevines affected by BN on the plantation. The widespread occurrence of the BN disease on the grape plantations of the Republic of Moldova is caused both by the presence of reserve plants - the source of the disease and phytoplasma vectors, and by the recent climatic conditions that play an important role in the transition of the vector from reserve plants to grapevines.

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P31. Exploring vineyards of the Epirus region of western Greece: incidence and genetic variability of GVA, GVB, GFLV, GFkV, GLRaV-1 and GLRaV-3 in indigenous varieties

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INTRODUCTION

Grapevine fanleaf virus (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll associated virus* -1 (GLRaV-1), *Grapevine leafroll associated virus* -3 (GLRaV-3) and *Grapevine fleck virus* (GFkV) are the most severe pathogens as directed by the "*Technical Regulation for the selection and approval of clones of grapevine varieties and clones of grapevine rootstocks"* issued by the Greek Ministry of Agriculture (Issue B '1599 / 07.06.2016), to comply with the requirements for the production of certified propagating material. To investigate their incidence and genetic variability in a less studied region of western Greece, a survey was conducted after collection of indigenous vines of the Prefectures of Ioannina, Thesprotia, Arta and Preveza.

MATERIALS AND METHODS

Source material: During the period 2018-2020, woody tissue was collected from a total of 322 grapevine plants representing different autochthonous varieties such as Koutsoupia, Dichali, Korithi, Debina, Vlachiko, Kotselina, Proimo Metsovou, Goudaba, Dovrino and others. Total RNAs were extracted from cambium tissue of the selected plants using the in-house extraction protocol developed by Maliogka al. (2015).

Detection and sequencing: Samples were screened for the presence of the seven major viruses infecting grapevines. RT-PCR assay followed by a nested PCR assay were developed for the detection of each pathogen in which specific primers were designed based on conserved regions of each virus genome after the alignment of the homologous nucleotide sequences of different isolates deposited in the EMBL-EBI and NCBI databases. Selected PCR products were purified and sequenced.

Incidence and phylogenetic analysis: The results of the PCR assays reveled the incidence of each pathogen and phylogenetic trees were constructed based on partial sequences of capsid protein gene (for GVA, GVB, GLRaV-1, GLRaV-3 and GFkV) and partial sequences of the polyprotein P2 gene (for GFLV). Multiple alignments were generated using the MUSCLE algorithm. Maximum-likelihood phylogenetic trees were generated with the appropriate model after using the Bayesian Information Criterion (BIC) and 500 bootstrap replicates for assessing the confidence of the branches.

RESULTS AND DISCUSSION

The incidence of GVA, GVB, GFLV, GFkV, GLRaV-1 and GLRaV-3 was 10.2%, 1.5%, 16.5%, 12.1%, 8.4% and 10%, respectively. ArMV was not detected. Furthermore, the evolutionary relationships of collected isolates low genetic variability among isolates of each virus and close evolutionary relationship with the deposited isolates in GenBank databases, respectively. Low incidence of the above viruses highlights the importance of the findings for the production of certified propagating material from Greek indigenous vines.

ACKNOWLEDGEMENTS

The study was funded within the framework of the project "Phenotypic characterization, molecular fingerprinting and oenological evaluation of indigenous Vitis vinifera cultivars from the Epirus region of Greece".

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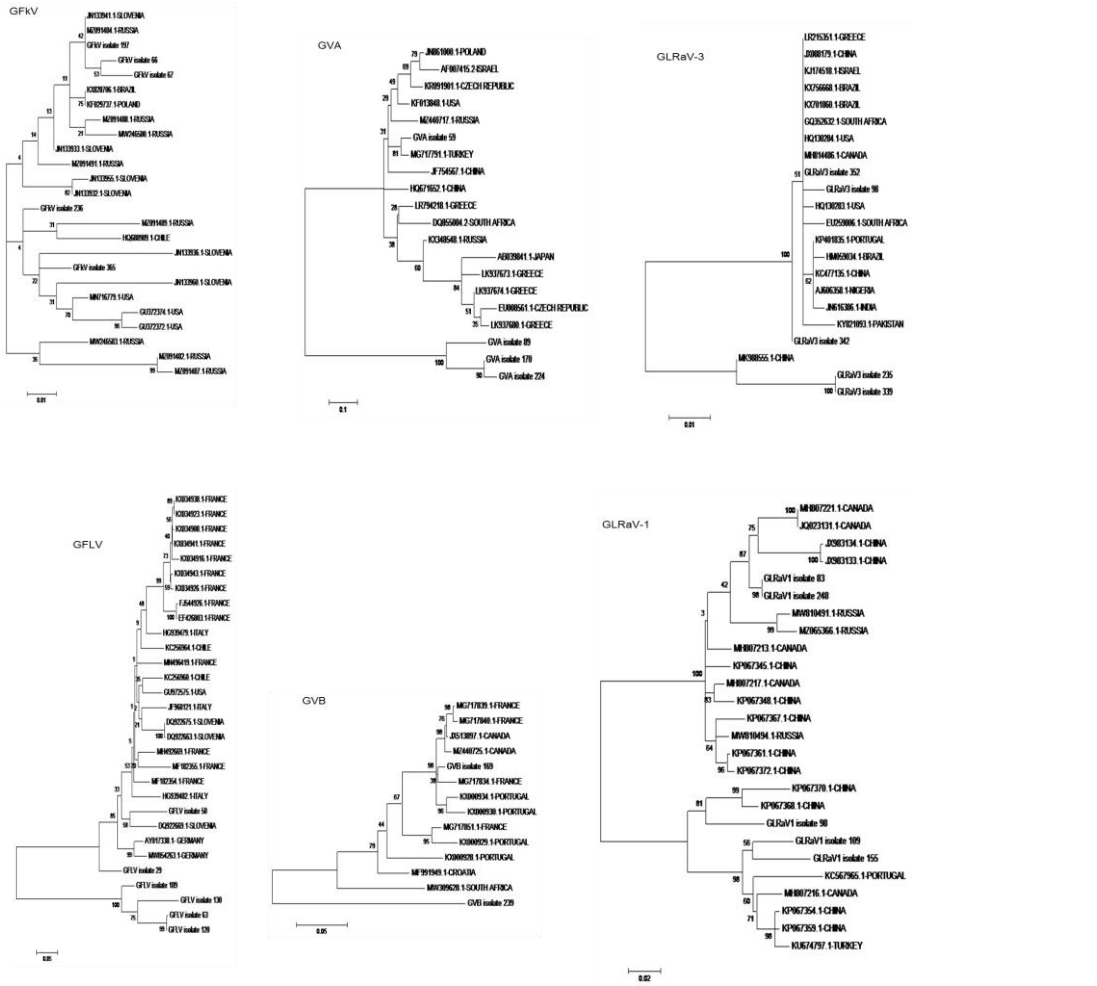

Fig.1: Phylogenetic trees constructed from GVA, GVB, GLRaV-1, GLRaV-3 and GFkV partial sequences of capsid protein gene and from GFLV partial sequences of the polyprotein P2 gene of selected isolates. ML phylogenetic trees were generated with the appropriate model after using the BIC and 500 bootstrap replicates for assessing the confidence of the branches.

P32. Rediscovering an enigmatic grapevine virus

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INTRODUCTION

Grapevine angular mosaic (GAM) was described in Greece in the late 1980s and characterized by sharp angular mosaic on leaves and malformations. This disease was specifically observed on the hybrids 'Baresana' x 'Baresana' imported from Italy, and it has been associated with grapevine angular mosaic virus (GAMV), which was classified as an ilarvirus based on the partial sequencing of the replicase (Girgis et al., 2009). In 2021, an identical sequence was identified on a novel ilarvirus infecting the annual mercury (*Mercurialis annua*) at the University of Lausanne (Switzerland). This ilarvirus was detected in mixed infection with an orthotospovirus. Herein, we investigate the pathogenicity and spread of this ilarvirus as well as its putative involvement in GAM.

MATERIALS AND METHODS

Symptomatic plants of *M. annua* were collected in the summer 2021 in Lausanne and have been maintained since then at the Agroscope institute in insect-proof cages. Samples from these plants were used for sap inoculations, electron microscopy and high-throughput sequencing (HTS) analyses as previously described (Mahillon et al. 2023). HTS was also performed on total RNA extracted from leaves of GAM-affected grapevines. Genomic and phylogenetic analyses were performed following a classic procedure (Mahillon et al. 2023).

RESULTS AND DISCUSSION

A combination of sap inoculations, transmission electron microscopy, HTS sequencing and RT-PCR identified two viruses in the diseased *M. annua* plants collected in Lausanne. The first virus was isolated on *Nicotiana benthamiana* and corresponded to an isolate of "Mercurialis orthotospovirus 1" (MerV1), which has been recently reported in Slovenia (Rivarez et al. 2023). The second virus was isolated on *Chenopodium quinoa* and corresponded to an ilarvirus (**Fig. 1A**). Pathogenicity assays indicated that the latter asymptomatically infects *M. annua*, and it was therefore named "Mercurialis latent virus" (MeLaV). Specimens of *Thrips tabaci* were visible on the collected plants and were evidenced as vectors of MerV1. This thrips species was also demonstrated to enhance the transmission of MeLaV-infected pollen.

At the genomic level, MeLaV displays the typical ilarviral, tri-segmented architecture (**Fig. 1B**), and phylogenetic analysis placed this virus within the genus *Ilarvirus* subgroup 1 (**Fig. 2C**). Strikingly, a 127-aa portion of the replicase (2a) was found to be identical to the partial sequence of GAMV. Symptomatic plants putatively infected with GAMV have been kindly given to our institute in the late 2000s (**Fig. 2C**) and were therefore analysed by HTS and RT-PCR. Results from these analyses revealed the mixed infection of grapevine leafroll-associated virus 3 and 4, grapevine virus A and grapevine rupestris stem pitting-associated virus. However, there was no trace of GAMV/MeLaV. Similar results were obtained independently by another team (R. Schoen and M. Botermans, personal communication). When grafted on Pinot noir, the symptoms of angular mosaic were not visible but the typical leafroll symptoms appeared after véraison. Several possibilities might explain the discrepancy between the original characterization of GAMV and these results, such as (i) the uneven distribution within plants, typical of ilarviruses; (ii) the initial detection through infected pollen, and (iii) the loss of the virus over time through cuttings.

Using the viral-RdRp analysis tool palmID (https://serratus.io/palmid) on Serratus (Edgar et al. 2022), fragments related to MeLaV RdRp were retrieved in other samples across Europe. Indeed, the virus was detected in multiple transcriptomes of healthy plants of *M. annua* and the related species *M. huertii* in Lausanne, supporting the idea that MeLaV infects lines used at the university. RdRp fragments slightly more divergent were detected in the RNAseq of the *Crete arum* on the Crete Island (Greece), the carnivorous plant Venus flytrap in Germany as well as the bread wheat in France. These results might indicate a wide host range for MeLaV, but could also be due to pollen contamination. In line with this idea, the virus was detected in a metaviromic study of pollinators in Belgium. Notably, no trace of MeLaV was found in any grapevine SRA. In parallel, the virus was reported in The Netherlands on *Cucurbita pepo* and *Passiflora* sp. in mixed infection with other viruses.
Altogether, these data advocate that MeLaV is widespread in Europe, but its involvement in GAM needs reconsideration.

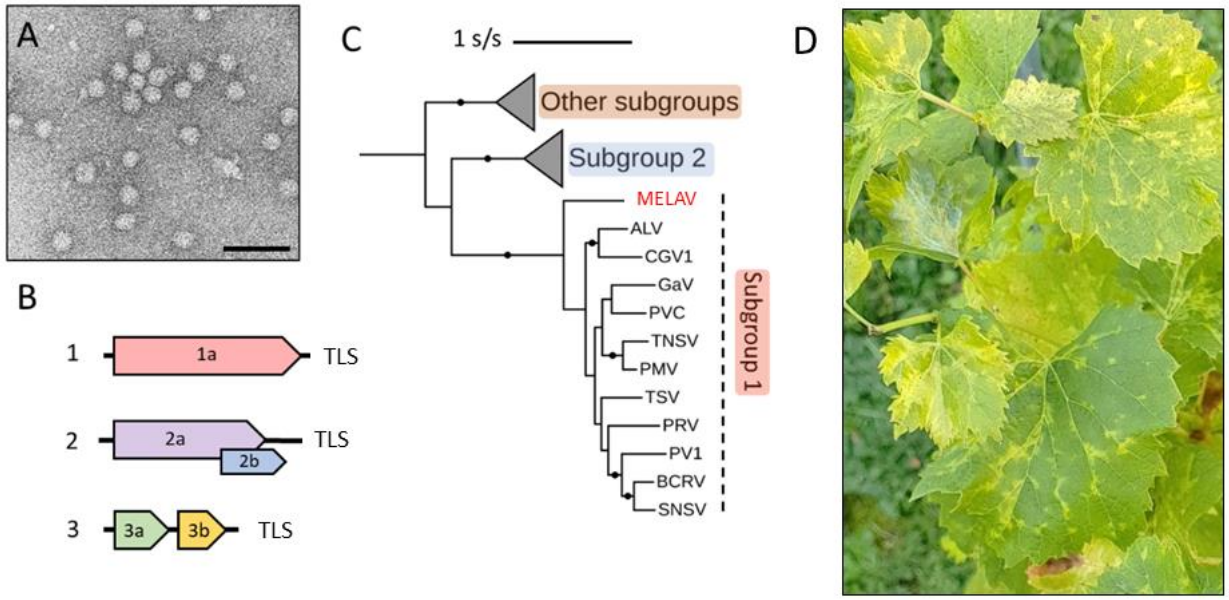

Figure 1. A: Electron micrographs of MeLaV particles. The black bar represents 100 nm. **B:** MeLaV genome. ORFs are indicated by colored arrows. 1a: replication-associated protein; 2a: RdRp; 2b: silencing suppressor; 3a: movement protein and 3b: capsid protein. TLS: tRNA-like structure. **C.** ML phylogenetic tree (model: VT+F+I+G4) for the proteins 2a of ilarviruses. The tree scale is given in substitution per site. Black circles on branch indicate bootstrap support >70%. The position of MeLaV is highlighted in red. Selected members of the genera *Cucumovirus* and *Anulavirus* were used to root the initial tree. **D:** Grapevine plant exhibiting the angular mosaic symptoms.

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We would like to thank J. Pannell and N. V. Buenrostro (University of Lausanne, Switzerland) for providing *M. annua* plants. We are also grateful to R. Schoen and M. Botermans (National Plant Protection Organization, The Netherlands) for sharing sequencing data.

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P33. Complete genome sequence of a carlavirus identified in grapevine (Vitis sp) in Greece

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INTRODUCTION

Grapevine (Vitis sp.) is host to the largest number of viruses among the cultivated plant species (Fuchs 2020). At least 86 viruses have been identified in grapevine, including 17 different Betaflexiviridae members. These include some economically important viruses such as grapevine virus A, grapevine virus B, and grapevine rupestris stem pitting associated virus. However, none of the known Betaflexiviridae members infecting grapevine belong to the genus Carlavirus. In this article, we describe the identification and characterization of a novel carlavirus identified in grapevine in Crete (Katsarou *et al.*, 2023).

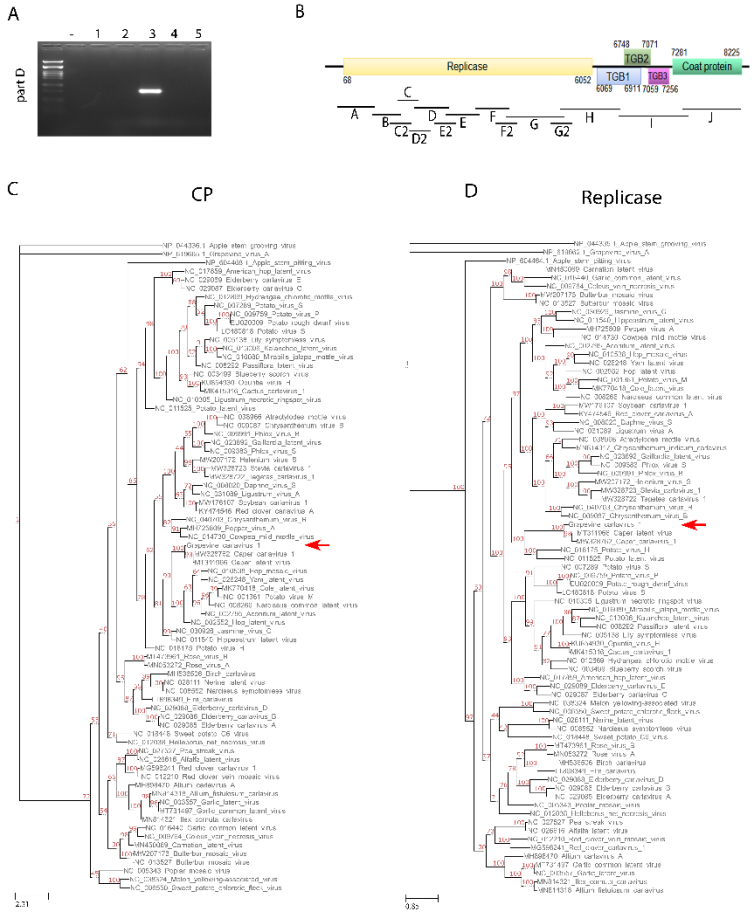
MATERIALS AND METHODS

In 2019 and 2020, ~400 grapevine samples were collected from the four prefectures (Chania, Rethymnon, Heraklion, and Lasithi) of the island of Crete, Greece. Selected samples, mostly from grapevines older than 10 years, were pooled and sequenced using high-throughput sequencing (HTS). One of these pools comprised five samples of the local grape cultivars Vilana (two plants), Kotsifali (two plants), and Mandilari (one plant), all grafted onto 140 Ruggeri rootstock, from a single location in the prefecture of Heraklion. The Vilana grapevines were 36 years old, while the Kotsifali plants were 28 years old, and the Mandilari plant was 10 years old. For sequencing, total RNA was extracted from leaves using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). Library construction was performed using a TruSeq® Stranded Total RNA with Ribo-Zero™ Plant Kit (Illumina) following the manufacturer's protocol, and 44,461,174 paired-end reads of 101 bp were generated. Bioinformatics analysis was performed using an in-house pipeline (described in Katsarou *et al.*, 2023).

RESULTS AND DISCUSSION

Following, BLAST analysis we identified common viruses found in Cretan grapes such as grapevine leafroll-associated viruses 1, 4, and 6, grapevine Pinot Gris virus, and grapevine Roditis leaf discoloration-associated virus, as well as grapevine yellow speckle viroid 1 and hop stunt viroid. However, we have also identified one contig of 8282nt that presented a 70.5% similarity to carlavirus caper latent virus (CapLV). To confirm the presence of the novel sequence in the pooled samples and to determine the full length genome sequence, a series of primers were designed and used for RT-PCR. As shown in Fig. 1A, sample number 3 from cultivar Kotsifali produced the expected PCR product while no amplification was observed when the other four samples were tested. After performing Sanger sequencing we found that the sequence of the amplified fragment was 99% identical to the HTS contig. Next, we performed RT-PCR, using overlapping regions, to obtain by Sanger sequencing the complete viral genome (Fig 1B). Finally, 5' and 3' RACE were carried out. The complete genome sequence was calculated at 8299 nt, excluding the polyA tail (GenBank no. OQ363854). Comparison of the original HTS contig and the Sanger sequence revealed they were almost identical (99.94%). Five putative ORFs were identified: ORF1 encodes a polyprotein of 228 kDa containing motifs for methyltransferase, helicase, and RNA-dependent RNA polymerase (RdRp) activity. ORFs 2, 3, and 4 encode the TGB

proteins of 31, 12, and 7.2 kDa, respectively, while ORF5 was predicted to encode a CP of 34.8 kDa. No ORF6 was identified based on this analysis (Fig. 1B)



Based on the current ICTV guidelines for carlaviruses, a member of a new virus species should possess no more than 80% amino acid (aa) sequence identity to all known viruses in its polymerase or CP coding gene, but concerns about these demarcation criteria have been raised recently (Silva *et al.*, 2022). Using the replicase aa sequence, the grapevine carlavirus showed 79% identity to caper carlavirus 1 and 78% identity to CapLV, suggesting that it is a novel member of the genus. Interestingly, the CP aa sequence identity was higher than the proposed threshold, with 87.5% aa sequence identity to caper carlavirus 1 and 86.6% identity to CapLV. Finally, the grapevine carlavirus sequence had less than 65% aa sequence identity to other members of the genus. Phylogenetic analysis was performed (ModelFinder/IQ-Tree) and The replicase and CP sequences of the grapevine carlavirus isolate clustered together with the two caper carlaviruses (Fig. 1C and D), confirming that these are the most closely related viruses in the genus. Based on the host range and replicase and CP aa sequence identity values, the virus reported in this paper is distinct from other known carlaviruses. Accordingly, we have named it "grapevine carlavirus 1"

ACKNOWLEDGEMENTS

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P34. Occurrence of grapevine leafroll virus 3 genetic variants in Blanc du Bois, aninterspecific hybrid bunch grapevine cultivar in Texas

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INTRODUCTION

Grapevine leafroll disease (GLD) is the most globally widespread disease of grapevine and grapevine leafroll-associated virus 3 (GLRaV-3) is the most prevalent and economically destructive of the complex of several GLD-associated ss(+)RNA virus species. Previous studies have documented seven distinct clades of GLRaV-3, named sequentially as phylogroups I, II, III, V, VII, IX, and X (Diaz-Lara et *et al.*, 2018). Most of the sequenced GLRaV-3 genetic variants were derived from *Vitis vinifera* cultivars and little is known about the population structure of the virus variants present in interspecific hybrid grape cultivars such as Blanc du Bois (BdB; *Vitis* spp.: 'Florida D 6-148' × 'Cardinal'). Here, we determined the occurrence of GLRaV-3 in multiple BdB vineyards and performed molecular typing of virus isolates from the different vineyards.

MATERIALS AND METHODS

Leaf tissue samples (with intact petioles) were collected from several symptomless Blanc du Bois vines (n=50/vineyard) in six locations in Austin (n=4) and Val Verde (n=2) counties. Anecdotal information from growers indicated that BdB cuttings were primarily sourced by most Texas growers from one of the six vineyards that were sampled in this study. Aliquots of total RNA extracts (Spectrum Plant Total RNA kit) of each location-specific samples were pooled into one composite RNA sample for highthroughput sequencing (HTS). HTS and bioinformatic analysis of the obtained metagenomic data were performed as described previously (Al Rwahnih *et al.*, 2018). The assembled GLRaV-3 sequences were comparatively analyzed with GLRaV-3 genomes that were retrieved from GenBank.

RESULTS AND DISCUSSION

GLRaV-3-specific HTS contigs of variable lengths were obtained from the different BdS vineyards (Table 1).

Table 1. Number and nucleotide range of GLRaV-3 sequence contigs recovered from Blanc du Bois RNA samples anlayzed by highthroughput sequencing.

Sample Code	Sample_ID	Region/County	No. Contigs	Length (nt)
277	TX-PAL_S	Gulf Coast/Austin	27	210 to 4,481
279	TX-PAL_NS	Gulf Coast/Austin	14	294 to 4,694
281	TX-COL	Gulf Coast/Austin	25	206 to 854
282	TX-WAT	Gulf Coast/Austin	33	216 to 7,913
H3012	Val Verde.H3012	Val Verde/ Val Verde	1	18,482
H3013	Val Verde.H3013	Val Verde/ Val Verde	1	18,509

Longer contiguous GLRaV-3 genomes were derived from each sample specific contigs by aligning them to the most identical exemplar GLRaV-3 isolate retrieved from GenBank. The gaps in alignment were replaced with 'N' to denote their relative positions. This analysis resulted in the reconstruction of one contiguous GLRaV-3 genome sequence per sample, except for TX-WAT, which appeared to contain two distinct contiguous GLRaV-3 genome genomes. Subsequently, the newly reconstructed

Texas Blanc du Bois GLRaV-3 genome sequences were assembled along with 40 published sequences of GLRaV-3 belonging to the previously identified phylogroups (I, II, III, V, VII, IX, and X), according to Diaz-Lara *et al.* (2018). The 47 sequences were aligned with the program MUSCLE and the alignment gaps, including those containing the 'N' nucleotide placeholders were manually trimmed in BioEdit 7.2. The alignment file was used for GLRaV-3 evolutionary analysis with the Neighbor-Joining method in MEGA X (Kumar *et al.*, 2018) and the resulting tree is shown in Figure 1. Six of the seven assembled GLRaV-3 genomes formed a monophyletic subclade in phylogroup I regardless of their vineyard source, indicating that GLRaV-3 spread across TX BdB vineyards likely occurred via contaminated vegetative cuttings. Interestingly, one other TX-WAT sequence clustered separately with a previously identified divergent isolate Vdl from Canada (Diaz-Lara *et al.*, 2018) indicating that this vineyard location has mixed populations of divergent GLRaV-3 variants. Notably, BdB vines were originally established in TX-WAT about 35 years ago and served as source of dormant cane distribution of the cultivar across locations in Texas. These preliminary findings underscore the importance of ensuring that only certified virus-free canes are used for vineyard establishment to curtail virus spread.

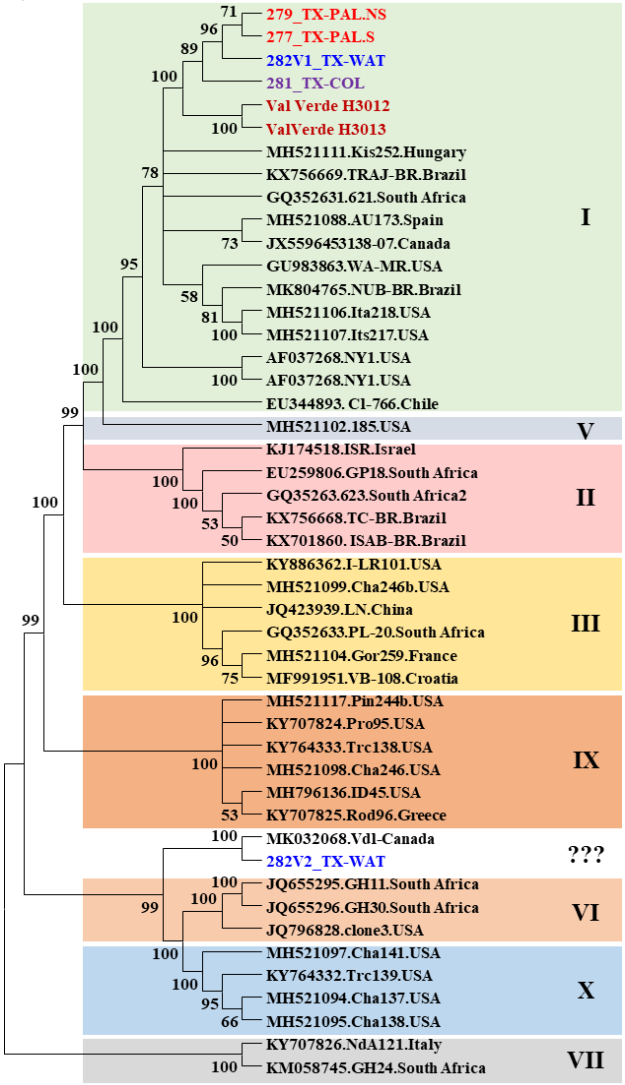

Figure 1. Unrooted Neighbor-Joining tree (1,000 bootstrap replicates) depicting the evolutionary relationships of grapevine leafroll-associated virus 3 (GLRaV-3) sequences obtained from cv. Blanc du Bois samples in Texas (n=7; colored fonts) and representative sequences belonging to the different genetic variant groups of the virus (n = 40). The shaded boxes depict different genetic variant groups according to Diaz-Lara *et al.* (2018). The analyses were conducted in MEGA X (Kumar *et al.*, 2018).

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P35. Identification, prevalence, and molecular characterization of novel vitiviruses in Greek vineyards

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INTRODUCTION

The genus *Vitivirus* of the family *Betaflexiviridae* includes several viral species that infect grapevine. Grapevine virus A (GVA), GVB, GVD, GVE, GVF, GVG, GVH, GVI, GVJ and GVL are officially members of the genus according to the International Committee on Taxonomy of Viruses (ICTV) (https://talk.ictvonline.org/taxonomy/), while GVK, GVM, GVN and GVO have been recently identified and proposed to be classified in this genus (Jo et al., 2017; Alabi et al., 2019; Debat et al., 2019; Read et al., 2022). Vitiviruses have +ssRNA genomes, 7,300 to 7,600 nucleotides (nt) long, encapsidated in non-enveloped flexuous filamentous virions and their genomes are organized into five open reading frames (ORF1-5), flanked by a 5'-end methylated cap and a 3'-end poly-A tail (Adams et al., 2012; Minafra et al., 2017). In this study, the application of high-throughput sequencing (HTS) in grapevine samples collected from different viticultural areas of Greece revealed the presence of GVE, GVF, GVG, GVH, GVI and GVL isolates, while a putative GVM-like isolate was also identified. Confirmation of the presence of each virus in the original grapevine samples was carried out by Sanger sequencing, followed by a survey on their prevalence in Greek vineyards and further characterization of their genetic diversity.

MATERIALS AND METHODS

HTS analysis was performed in 5 individual grapevine samples collected from the germplasm collections of the Viticulture Department of Athens (IOSV, ELGO-DEMETER) in Lykovrisi Attica, and the Aristotle University of Thessaloniki (A.U.TH.) and in 4 composite samples from commercial vineyards of central Macedonia and Crete. Total RNA extraction, HTS procedure and data analysis were conducted as described by Panailidou et al. (2023). Sanger sequencing was performed to verify the presence of each virus. For this purpose, RT-PCR methods were developed to amplify segments of 670, 165, 328 and 511 nucleotides (nt) of ORF1 of GVF, GVG, GVH and GVM, respectively, 644 nt of ORF3 of GVI, and 574 and 189 nt of ORF4 of GVE and GVL, respectively. Subsequently, the same RT-PCR methods were used to study their spread in Greek vineyards. A total of 809 grapevine samples collected between 2009 and 2020 from commercial vineyards of 9 geographic regions of Greece and the grapevine collections of IOSV and A.U.TH., were tested. In addition, the genetic variability and the phylogenetic relationships of Greek isolates of the identified viruses with the corresponding isolates reported from other countries were studied. For this purpose, new RT-PCR methods were developed and applied for the amplification of the coat protein gene (CP, ORF4) of each virus (except for GVM). The analysis of the CP sequences obtained by Sanger Sequencing was performed using MEGA X bioinformatics suite (Kumar et al., 2018) and a phylogenetic tree was constructed for the

complete CP nucleotide sequences of the Greek and foreign isolates of all vitiviruses, using the same software. The alignment of the CP sequences and the comparison among them were performed by Geneious Prime software (https://www.geneious.com/prime/) using MAFFT.

RESULTS AND DISCUSSION

The application of HTS revealed the presence of one isolate of GVE, GVI, GVG and GVH, 2 isolates of GVF, 5 isolates of GVL and a GVM-like isolate in the tested samples. Partial Sanger sequencing and BLAST analysis further confirmed the presence of the above-mentioned viruses in the original samples. As for their spread in Greek vineyards, GVF is the most prevalent among the six viruses, as it was detected in 24.7% (200/809) of the tested samples, followed by GVL (31/560, 5.5%), while GVG (10/352), GVI (12/554), GVH (5/245) and GVE (12/752) were present in few tested samples, showing a prevalence of less than 3%. GVE, GVI and GVG were detected mainly in samples collected from the collection of IOSV and in a small number of samples from commercial vineyards, while GVH was identified only in samples from a commercial vineyard of Central Macedonia. GVF and GVL were found in samples from grapevine collections and commercial vineyards from almost all geographic regions tested. High genetic variability was recorded in the CP gene between Greek isolates and Greek and foreign isolates of GVF, GVL and GVE. On the contrary, the Greek isolates of GVH, GVG and GVI and Greek and foreign isolates of GVG, showed low sequence divergence. Phylogenetic analysis revealed the classification of Greek isolates in more than one phylogenetic group for GVE, GVF and GVL, whereas the Greek isolates of GVI, GVG and GVH clustered together in the same phylogenetic group for each species. In the case of GVG, a novel phylogenetic group was formed, highlighting the diversity of the Greek isolates. In addition, the GVM-like isolate retrieved by HTS analysis shows high sequence similarity with other GVM isolates in the CP gene. Nevertheless, given the high sequence similarity existing between GVM and GVH in this region further analysis is needed for the classification of the identified isolate at the species level.

ACKNOWLEDGEMENTS

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P36. Update on the origin of grapevine Pinot gris virus and its molecular characterization in Germany

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INTRODUCTION

Grapevine Pinot gris virus (GPGV) is a member of the *Trichovirus* genus, with a positive single-stranded RNA genome. The virus was earlier discovered in symptomatic grapevines in northern Italy (Giampetruzzi *et al.*, 2012). Following that, the virus was reported from numerous grapevine-growing regions in many countries around the world. Since there is only one full-length sequence of the German GPGV in the NCBI GenBank (Reynard *et al.*, 2016), there is a knowledge gap regarding the virus population in Germany. In order to molecularly characterize the GPGV isolates in Germany, the MP/CP region (1517nt) of 56 isolates was used for this purpose together with 187 NCBI-retrieved GPGV sequences. Using the newly available NCBI-sequences, a time tree was generated to update the probable origin and dispersal of the virus.

MATERIALS AND METHODS

The GPGV isolates are obtained from wood samples extracted from 56 grapevines collected in vineyards of various grapevine growing regions in Germany. For phylogenetic analysis, a ML-tree was constructed based on the MP/CP region (1517nt) using the best-fit model (T92+G+I) implemented with MEGAX program (Kumar *et al.*, 2018), bootstrap value was set to 1000. The time tree was built using 175 non-recombinants NCBI-isolates, and LSD2 method using IQ-Tree2.0.3 (To *et al.*, 2016). The DnaSP v.6.12. (Librado & Rozas, 2009) was used to estimate the genetic diversity (π) and haplotype diversity (*H*d) of the German population.

RESULTS AND DISCUSSION

The ML-phylogenetic tree (Fig. 1b) based on the MP/CP sequences (1715nt) showed that the German isolates scattered all over the tree and they clustered with different isolates mainly from Europe, but also with some of worldwide origin. The German population had a high haplotype diversity for both MP and CP (*H*d>0.98), and a high nucleotide diversity for MP ($\pi = 0.023 \pm 0.00001$) and CP ($\pi = 0.024 \pm 0.00002$). The variability within the German GPGV population is particularly high and further research on this virus is necessary to obtain a clear picture regarding distribution in vineyards. The Molecular dating indicates that the basal isolates are those from Japan, probably representing the ancestral population of Chinese isolates (Hily *et al.*, 2020). The earliest node to European isolates has to be dated to 1805 CE.

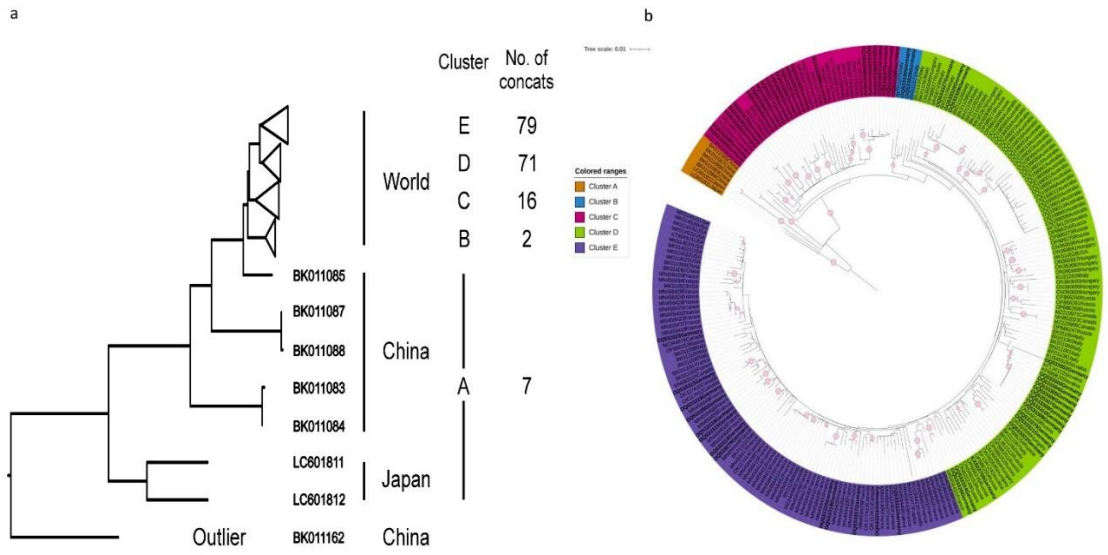

Figure 1a. A summary of an ML phylogenetic tree of 175 GPGV concats and one GINV (BK011162) concat as an outlier. **Figure 1.b**. Circular ML-tree constructed using the (T92+G+I) method, based on 243 GPGV sequences (MP/CP), in which 56 sequences are of German origin.

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P37. Genetic variability and improved detection of grapevine fanleaf virus in Greek vineyards

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INTRODUCTION

Grapevine fanleaf virus (GFLV), which belongs to the genus *Nepovirus*, family *Secoviridae*, is one of the most common grapevine viruses worldwide (Meng *et al.*, 2017). In Greece, GFLV is considered to have a limited spread. However, this could be due to the high genetic diversity that might exist in nepoviruses thus often leading to a failure of virus detection. During surveys to study the virome of Greek autochthonous grapevine varieties (*Vitis vinifera* L.) in central and northern Greece, high-throughput sequencing (HTS) analysis revealed the presence of several nepoviral sequences, most of which shared moderate to high nucleotide (nt) sequence identity with GFLV. Therefore, our aim was to study the genetic diversity of GFLV in Greek vineyards and optimize the current molecular assays so as to achieve a broad and reliable detection of its isolates. Interestingly, divergent isolates (tentatively named GDefV-like) showing high identity both with GFLV and grapevine deformation virus (GDefV) (Elbeaino *et al.*, 2012) were identified and they were further characterized.

MATERIALS AND METHODS

Eight individual and four pooled samples from indigenous varieties were collected from four different viticultural areas of Greece. Total RNAs were extracted according to the protocol developed by Ruiz-García *et al.* (2019) and were sequenced in a NovaSeq 6000 platform (Illumina, Inc.) at Macrogen Inc. (Seoul, S. Korea). Analysis of the data was performed on Geneious Prime (Dotmatics). The MAFFT algorithm pluggin in Geneious was used to construct the sequence alignments to calculate the identities between the complete genomes of the Greek nepoviruses with isolates of GFLV, GDefV and Arabis mosaic virus (ArMV), from GenBank. Additionally, the phylogeny was conducted with the use of MEGA11. Moreover, to classify the divergent isolates in a nepovirus species the amino acid (aa) sequences of the CP and Pro-Pol regions were aligned by MAFFT, the best-fit model was selected by ProtTest and their phylogeny was estimated by the PhyML online software. In order to avoid artifacts from de novo assembly, the sequence of one of the divergent isolates was confirmed by Sanger sequencing of overlapping amplicons.

Furthermore, a real-time RT-PCR detection assay developed by Bruisson *et al.* (2017), was improved by modifying the probe, to detect a wide range of known Greek GFLV isolates. Following optimization of the assay it was then subjected to further validation of its specificity and sensitivity. For this purpose, divergent isolates of GFLV belonging to different phylogenetic groups, were used. In addition, a standard curve was constructed using six 5-fold dilution points of a single isolate sample as well as of a pooled sample, which included five geographically and genetically diverse isolates.

RESULTS AND DISCUSSION

Similarity analysis of the nepovirus-related complete genomes derived from the HTS data exhibited a nt identity score of 79-85% (RNA1) and 77-91% (RNA2) with the deposited sequences of GFLV, against 79-85% and 67-70% with GDefV, respectively. Moreover, it became evident that, like other grapevine infecting viruses, more than one isolates of RNA1 and/or RNA2 coexisted in the same plant. Phylogenetically, the topology of the trees varied between RNA1 and RNA2 in respect of the species classification of the Greek isolates. The RNA2 sequences of Greek isolates were placed exclusively in the same clade with GFLV, whereas RNA1s were placed into several different clades, with the majority

grouping together with GFLV isolates. However, some divergent Greek RNA1 sequences formed a separate group with GDefV. The sequences of this group had a nt identity score between 79-81% with GFLV isolates from GenBank and 82-85% with that of GDefV. Furthermore, spatially or cultivar related groupings were not observed, as isolates originating from the same vineyard or from the same cultivar were placed in different clades indicating an active spread or multiple introductions of the virus.

The analysis performed for the species classification of the Greek grapevine infecting nepovirus-related isolates revealed that all of them belong to the *Grapevine fanleaf virus* species. More specifically, despite the lower nucleotide similarity of the divergent isolates RNA1 with GFLV than GDefV, these two seem to be closely related in aa identity in the demarcation-informative region of the Pro-Pol (RNA1) with aa identities between GFLV, GDefV and the Greek isolates being between 83-90%, which lie above the threshold of 80% according to the current species demarcation criteria for nepoviruses. The CP (RNA2) region clearly differentiated the Greek isolates as they were 91-97% identical to GFLV and only 60-64% to GDefV with a threshold of 75%.

The optimized real-time RT-PCR method developed herein was able to detect genetically diverse isolates of GFLV, from different phylogenetic groups. The efficiency of the current method was estimated 95.07%, when the target was a single isolate sample and 91.7% on a pooled sample as the target, thus detecting efficiently single and multiple isolates of GFLV in a grapevine sample.

The survey of grapevines with HTS revealed the presence of diverse isolates of GFLV in Greek vineyards, however additional sampling and HTS analysis are necessary to provide more information about the distribution and genetic variability of GFLV and possibly other nepoviruses in Greece.

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P38. Virome of Russian ampelographic collections

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INTRODUCTION

Ampelographic collections grow all over the world. Russia hosts an important portion of the total number of the world's grapevine genetic resources, which are located in various regions of the country: the Rostov Oblast, the Krasnodar Krai, the Republic of Crimea and the Republic of Dagestan (Shvets et al. 2022). Collections of grape germplasm are a place of accumulation of various pathogens, including viruses that affect the production characteristics of new varieties and hybrids. Since germplasm is used for further breeding and commercial purposes, information is needed on the phytosanitary status of these vineyards.

MATERIALS AND METHODS

Phytosanitary monitoring of the ampelographic collections was carried out in the Anapa Ampelographic Collection in the Krasnodar Krai of Russia, the Ampelographic Collection of the Dagestan Experimental Station of the N. I. Vavilov Institute of Plant Genetic Resources, the Dagestan Experimental Selection Station of Viticulture and Olericulture, the Don Ampelographic Collection named after Ya. I. Potapenko in the Rostov Oblast of Russia. In total 47 grapevine samples with symptoms of viral diseases from Anapa, 73 samples from Dagestan, 51 samples from Rostov Oblast were collected. Total RNA was isolated from a sample of shoots and leaves for each sample using the CTAB-LiCl method (Morante-Carriel, 2014). The 171 prepared libraries were sequenced on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA), producing 150 bp paired-end reads. The HTS data was processed and analyzed using the Geneious Prime (Biomatters, Auckland, New Zealand). To validate mRNA-Seq predicted viral pathogens, we performed reverse transcription PCR. To determine the 5' and 3' untranslated regions of the genome of novel viruses, the rapid amplification of cDNA ends (RACE) using the Mint RACE cDNA amplification set (Evrogen, Moscow, Russia) was performed.

RESULTS AND DISCUSSION

Using metagenomic methods, we analyzed the viromes of 171 varieties and hybrids of grapes and identified 23 viruses and 4 viroids (Fig. 1). All samples were characterized by mixed viral infection. The species compositions of viruses and viroids in all analyzed collections were 44% identical.

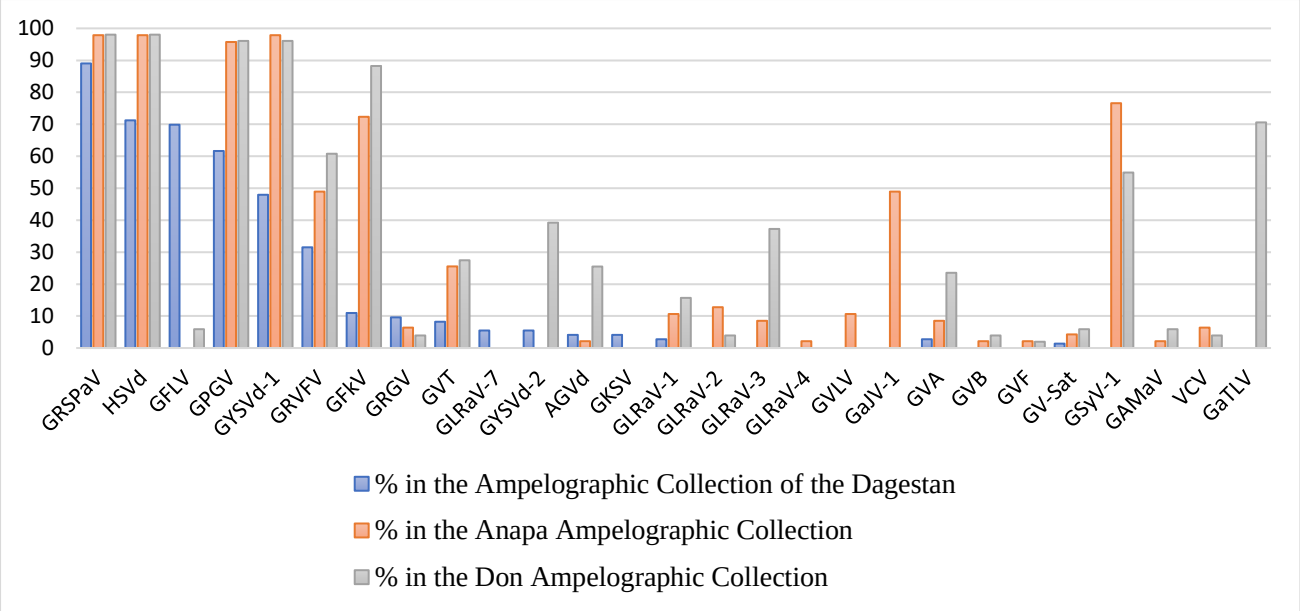

Fig. 1. Frequency of spread of grapevine viruses and virus-like organisms in analyzed ampelographic collections (percentage of the total number of collected samples).

We also report the discovery of seven novel viruses in grapes: (+) ssRNA grapevine umbra-like virus (GULV, genus Umbravirus) and three more umbra-like viruses, bisegmented dsRNA virus from family *Partitiviridae*, bisegmented (+) ssRNA virus from family *Secoviridae* and dsDNA grapevine pararetrovirus (GPRV, genus Caulimovirus).

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P39. Pol IV to the Rescue: The Enigma of Antiviral Defence in Grapevine Rootstocks

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is a major pathogen affecting grapevine cultivation worldwide, leading to significant reductions in fruit yield. In this study, we are investigating the potential role of RNA polymerase IV (Pol IV) and associated small interfering RNA (siRNA) pathways in conferring tolerance to GFLV in grapevine. Our focus is on elite rootstock varieties. Leveraging highly contiguous genome assemblies (Minio *et al.*, 2022) that include haplotype resolution of the parental lines, we will explore Pol IV contributions in antiviral defense in grapevine. In past studies, Pol IV and its partner enzyme, RNA-dependent RNA Polymerase 2 (RDR2), have been identified as factors that promote tolerance to RNA virus infection in plants (Kørner *et al.*, 2018). We hypothesize that this endogenous double-stranded RNA and siRNA production machinery could play a role in grapevine resilience to GFLV infection. By investigating the recruitment of Pol IV by proteins such as the CLASSY factors (CLSY1/2/3/4) (see review Rymen *et al.*, 2020), we aim to elucidate their involvement in grapevine defense against GFLV. To pursue these objectives, we are using genome editing to disrupt Pol IV function in grapevine, and comparing these lines to plants ectopically expressing CLSY recruitment factors (enhanced Pol IV activity). Exploring the role of Pol IV in antiviral defense presents technical challenges but could uncover novel molecular mechanisms for grapevine resilience to viral infections. By studying the role of Pol IV and its interaction with GFLV in grapevine rootstocks, this project will contribute to charting novel pathways involved in plant-virus interactions and suggest strategies for enhanced viral resistance. Long-term, these findings could thus help address the challenges posed by viral infections in viticulture and protect grapevine crops.

MATERIALS AND METHODS

Grapevine rootstock varieties corresponding to fully assembled genomes were chosen (Fig. 1).

Vectors expressing the efficient plant genome editor, zCas9io (Stuttmann *et al.*, 2021), were generated and used for grapevine callus transformations. In addition, vectors will be constructed for the ectopic/overexpression of CLSY proteins fused to epitope tags.

Edited *Vitis* rootstocks will be used in grafting experiments with GFLV-infected or non-infected *Vitis vinifera* scions, then the resulting viral titers and fanleaf symptoms will be quantified.

Immunoprecipitation assays and mass spectrometry will be performed to analyse the CLSY-Pol IV protein complexes of grapevine.

Transcriptome, proteome, epigenome, and small RNA differences will be analysed amongst wild-type, *pol IV* mutant and CLSY overexpression lines (Fig. 1).

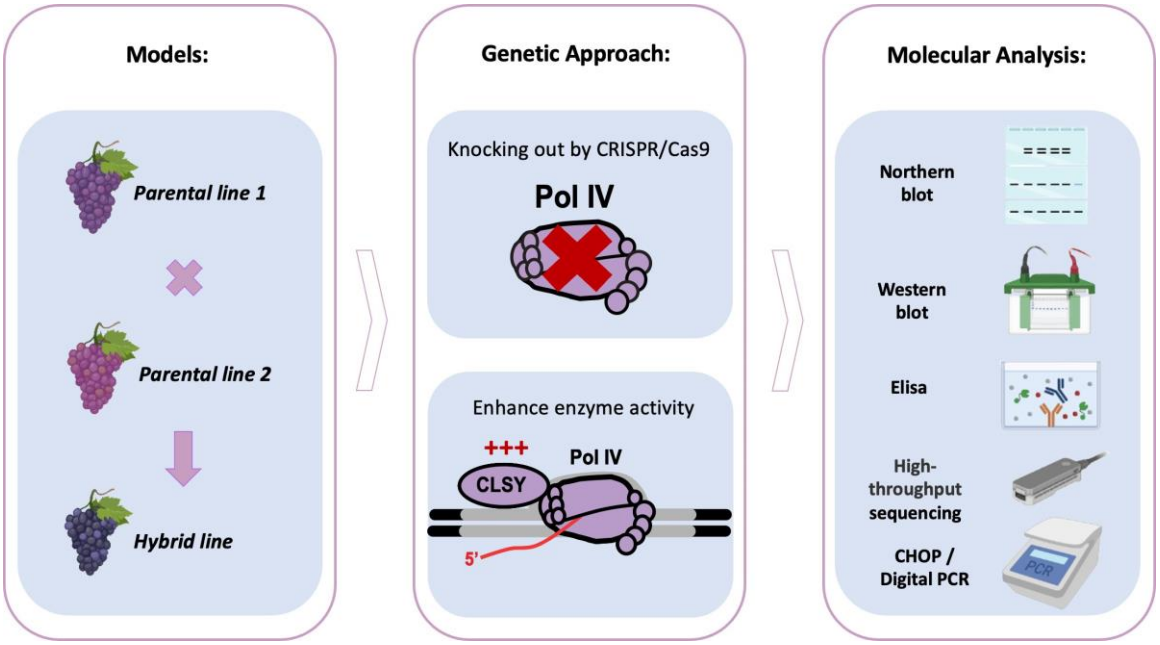

Figure 1. *Visual representation of the experimental approaches.*

RESULTS AND DISCUSSION

We are investigating the role of Pol IV and associated siRNA pathways in elite grapevine rootstocks. To this end, we have performed callus transformation with editing vectors targeting *Vitis NRPD1*, the gene encoding Pol IV's largest subunit, and will separately overexpress *Vitis* CLSY proteins fused to epitope tags. Immunoprecipitation experiments and mass spectrometry will be conducted to analyse Pol IV protein complexes and associated factors. Molecular analyses of the edited plants will include transcriptomics, proteomics, and epigenomics. We will perform grafting experiments with edited rootstocks and GFLV-infected scions. Studying the molecular profiles of *pol IV* mutants compared to wild-type rootstocks will allow us to determine the role of Pol IV in viral resistance/tolerance. This study will enhance our understanding of viral pathogenicity and plant defense in grapevine, as well as rootstock-scion interactions, and contribute to strategies for improving grapevine resilience.

ACKNOWLEDGEMENTS

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P40. Arbuscular mycorrhizal fungi may alleviate virus influence on photosynthesis related parameters in grapevine

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INTRODUCTION

Grapevine is one of the most virus-prone crops worldwide (Fuchs 2020) and viruses cause many physiological and molecular changes in its host, including perturbations in the processes of photosynthesis (Baron et al. 2012). Contrarily, grapevine roots form symbiosis with arbuscular mycorrhizal fungi (AMF) which is known to beneficially affect grapevine under stressful conditions, including biotic stress (Trouvelot et al. 2015). However, AMF influence on plants stressed by viruses is vastly underexplored, and up to now both, mycorrhiza induced resistance (MIR) and mycorrhiza induced susceptibility (MIS) are described (Pozo and Azcón-Aguilar, 2007; Miozzi et al. 2019). Multiple interactions grapevine - viral pathogens – AMF are yet to be clarified, despite their predominantly presence in agroecosystems in vineyards worldwide. Therefore, the aim of this study is to give insight into influence of AMF on photosynthetic physiology processes of virus infected grapevine. For that purpose, the ubiquitous grapevine rupestris stem-pitting associated virus (GRSPaV) is used as a less pathogenic stress inducer, and GRSPaV coinfection with grapevine leafroll-associated virus 3 (GLRaV-3) and grapevine pinot gris virus (GPGV) as a source of stronger pathogenic stress induction in the grapevine.

MATERIALS AND METHODS

The Kober 5BB rootstock (Vitis berlandieri Planch. × Vitis riparia Michx.) was grafted with Merlot (*Vitis vinifera* L.) scions and rooted in 6 L pots in the greenhouse in sterile substrate. In successfully developed plants, the presence of ten viruses was checked: GLRaV-1, -2, -3, GVA, GVB, GFkV, GFLV, ArMV, GRSPaV (Gambino 2015), and GPGV (Morelli et al. 2014). The uninfected grapevines and those which harbored only GRSPaV were further used for "chip budding" grafting with buds containing GLRaV-3, GPGV or had no viruses. Virus transmission was confirmed by qPCR (Gambino 2015) and plants with successful transmission were further treated with three mycorrhizal inoculums (only *Rhizophagus irregularis*, mixture of *R. irregularis, Funneliformis mosseae* and *Funneliformis caledonium* and autoclaved inactive AMF inoculum). Eventually, 15 treatments were set up.

Three months post inoculation, photosynthesis related parameters were measured for three leaves per plant differing in age and developmental phase: basal leaf, upper fully developed leaf and apical-not fully developed leaf. Measured parameters were: net photosynthesis rate (A_N), quantum efficiency in light (ΦPSII) and electron transport rate (ETR) and concentrations of chlorophyll and carotenoids. Open gas exchange system (Li-6400), compact porometer with pulse-amplitude modulation fluorometer (Li-600) and spectrophotometer (Lichtentaler 1987) were used.

RESULTS AND DISCUSSION

Three-way ANOVA revealed significant interaction virus × AMF × leaf type for the quantum efficiency in light (ΦPSII) and electron transport rate (ETR) ($F = 1.828$, $p = 0.035$ and $F = 1.93$, $p = 0.023$,

respectively). No interaction was found for the net photosynthesis rate but it was related to the leaf type ($F = 22.367$, $p < 0.001$) and AMF status of the treatment ($F = 63.586$, $p < 0.001$) but not to the type of virus. For ΦPSII and ETR, AMF was the factor that influenced them the most ($F = 76.78$, $p < 0.001$ and $F = 13.61$, $p < 0.001$ respectively), followed by the type of the leaf ($F = 11.93$, $p < 0.001$ and $F = 12.91$, $p < 0.001$). The lowest values of all the parameters were measured in old basal leaf, since in grapevine challenged with virus induced stress photosynthetic perturbances could occur more easily in older leaves where the accumulation of viral titer is expectantly highest (Monis and Bestwick 1996). Compared to No AMF controls, AMF alleviation influence was the strongest in basal and upper fully developed leaf. No significant differences were found between two types of AMF inoculums; both were generally represented with values higher from the non-AMF controls.

Concerning pigments' concentrations, "No AMF" treatments containing only viruses, showed no significant difference compared to the healthy control. However, addition of AMF brought significant increase above their non-AMF control for the treatments GRSPaV + GLRaV-3 and GRSPaV + GLRaV-3 + GPGV. Pigments concentrations revealed higher values when Mix AMF were in inoculum than when *R. irregularis* alone was added. Two-way ANOVA revealed significant interactions between AMF and virus compositions influencing chlorophyll *a* ($F = 2.270$, $p = 0.045$) and total chlorophyll ($F = 2.263$, $p = 0.046$). The treatments containing GLRaV-3 had the most severe depletion of chlorophyll *a* and total carotenoid concentrations, the observation that was reported in published literature and explained by heightened chlorophyllase activity (Bertamini et al, 2004).

In summary, this study gives first insight into complex and underexplored niche of AMF mediated plant response to viral induced stress. Viral influence on grapevine photosynthesis and photosynthesis related parameters is shown to be alleviated by AMF colonization, but in dependence on tissue type, since primarily effect was observed in basal and fully developed upper leaves. The beneficial role of AMF was especially seen in cases of GRSPaV coinfection with GLRaV-3 or GPGV. In virus infected grapevine mixed AMF inoculum reduced loss of leaf pigments more than *R. irregularis* alone.

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P41. Influence of arbuscular mycorrhizal fungi on distribution and relative concentration of *Grapevine rupestris stem-pitting associated virus* in Merlot

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INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) can have positive impact on plants facing biotic stress, although its impact on grapevine virus replication and spatial distribution is still ambiguous (Miozzi et al., 2019). Grapevine is perennial crop hosting plethora of viruses with significant variability of spatial and temporal distribution in the host (Setiono et al., 2018). One of the most widespread viruses in grapevine is *Grapevine rupestris stem-pitting associated virus* (GRSPaV), described by intra-plant irregular distribution with most abundant virus titer ascribing to berries (Gambino et al., 2012), phloem, and petiole tissue for in situ, and in the roots for in vitro analyzed grapevines (Hu et al., 2018). To investigate influence of AMF on GRSPaV distribution, quantification was done in three sampling points and through analysis of three tissue types. Quantification was performed for grapevine infected solely or coupled with GLRaV-3 and GPGV infection.

MATERIALS AND METHODS

The Kober 5BB rootstock was grafted with Merlot scions and rooted in 6L pots in the greenhouse. For the successfully developed plants, leaves were sampled for RNA isolation and detection of most pernicious grapevine viruses (Gambino, 2015). The grapevines harboring only GRSPaV were further infected through "chip budding" method with buds of known viral status. The buds were used as a source of GLRaV-3 + GPGV combined or had no viruses tested. To confirm the successful transmission, final detection was carried out. Two grapevine groups were formed, first group with one virus – GRSPaV and second group had GRSPaV in combination with GLRaV-3 + GPGV. Each group was subsequently treated with two types of mycorrhizal inoculums and had uninoculated control. Inoculation was carried out using one AMF species *Rhizophagus irregularis*, or mixture of *R. irregularis*, *Funneliformis mosseae* and *F. caledonium*. After successful inoculation, grapevine was sampled for virus quantification in May, June and August of one growing season. In each sampling point, three distinct tissue types (root, petiole and leaf) were sampled in order to quantify GRSPaV. From each sample, total RNA was extracted, purified and used as a template for cDNA synthesis. Relative GRSPaV quantification was carried out on real-time qRT-PCR, and normalized with actin and ubiquitin as housekeeping genes (Gambino et al., 2011).

RESULTS AND DESCUSSION

GRSPaV concentrations vary, to an extent, based on treatment and sampling period, but also on their interaction for each tissue type ($p < 0.01$, 2-way ANOVA). In all tissue types, the temporal dimension of virus concentration followed the similar trend of decrease in virus titer, with concentration of GRSPaV decreasing with each sampling point regardless of mycorrhizal inoculum, tissue type or virus combination. Since, sampling points were all positioned through late spring and first half of summer (véraison excluded), this steady decline was expected (Gambino et al., 2012). Nonetheless, differences in virus distribution throughout the plant tissues and influence of mycorrhizal inoculum was present.

The treatments without mycorrhizal fungi had generally repeatedly higher virus concentration. However, this varied with sampling time and tissue type. The least AMF influence on GRSPaV concentration was observed for the root. The most significant decrease of virus accumulation due to mycorrhizal inoculum was found in leaf tissue, especially seen in treatment where GRSPaV was in combination with GLRaV-3 and GPGV. Interestingly, uninoculated grapevine infected with virus combination had significantly greater GRSPaV concentration in leaf tissue than grapevine treated with only one virus and inoculated with three AMF species in the first sampling. This effect persisted during second sampling, where grapevine infected with 'virus mix' had significantly greater leaf GRSPaV concentration than plants infected solely with GRSPaV that received AMF inoculum – either *R. irregulars* or 'AMF mix'. Similarly, GRSPaV concentration decreased in petiole of 'virus mix' treated grapevine that received 'AMF mix' in contrast to grapevine that harbors same virus status, but was uninoculated. In summary, AMF influence on GRSPaV variability through time dimension seems to build upon general trends of GRSPaV seasonality, further lowering naturally present decrease in virus titer through summer months. Similarly, overall impact of AMF on spatial distribution of GRSPaV is present, but it is the most evident in reduction of its accumulation in leaf tissue with mixture of mycorrhizal fungi prevailing over one-species inoculum.

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P42. Protein Argonaute 1 immunopurification for the analysis of virus-derived small interfering RNA

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INTRODUCTION

Grapevine is one of the most widely grown perennial fruit crops and is the host to more than 80 viruses. Viral infections can cause severe damage to grapevine production (Fuchs, 2020). As virus enter the cells, different types of immune responses are activated. One of the defense mechanisms against viruses is RNA silencing. Viral dsRNA is cut by DCL enzymes to generate virus-derived small interfering RNA (vsiRNA). vsiRNAs are loaded into the AGO protein, guiding the RISC complex to degrade viral genome (Liu et al., 2021).
To analyze which of the vsiRNAs are actively involved in the RNA silencing process, we decided to immunoprecipitate AGO1 protein and extract small RNAs loaded in it. The extracted small RNAs can be analyzed by small RNA Sequencing.

MATERIALS AND METHODS

Firstly, we tested the protocol described by Dunker et al. (2021) on *Arabidopsis thaliana***.** For protein extraction, we used 5 grams of young leaves. A small part of the sample (crude extract, CE) was saved for Western blot analysis. Anti-AGO1 antibodies and protein A agarose were added to the rest of the sample for AGO1 immunoprecipitation. After incubation and centrifugation, a small aliquot of the supernatant (SN) was set aside for Western blot analysis. The remaining supernatant was discarded, and the pellet containing bound AGO1 was washed and resuspended. Part of a sample was saved for Western blot analysis (immunoprecipitation fraction, IP) and RNA was extracted from the rest of the sample. Western blot analysis was performed with three sample fractions (CE, SN, and IP). We performed stem-loop RT-PCR to confirm the presence of known *Arabidopsis thaliana* miRNA in the RNA extracted from the IP fraction. The PCR product was analyzed by agarose gel electrophoresis.
To test the protocol on grapevine, we first attempted to optimize the protein extraction. For protein extraction we used 5 grams of young leaves cv. Pokalca. Six different protein extraction buffers were tested. We tested three different amounts of added PVPP (1%, 5% and 10%) and two types of protease inhibitors (Protease Inhibitor Cocktail, Plant (VWR Life Science AMRESCO) and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche)). Phenylmethylsulfonyl fluoride (PMSF) was added to all extraction buffers.

RESULTS AND DISCUSSION

For *Arabidopsis thaliana* we analyzed the presence of AGO1 in three sample fractions, CE, SN and IP. Protein AGO1 signals of expected size (~ 130 kDa) were detected in IP and in CE fraction. The AGO1 signals were stronger in IP fraction then in CE. As expected, there was no AGO1 detected in SN fraction.
To assess the quality of RNA, extracted from IP fraction, we performed stem-loop RT-PCR. We successfully detected known *Arabidopsis thaliana* miRNA159.
To optimize the protein extraction from grapevine leaves, we tested six different extraction buffers. Our results demonstrate that the most efficient protein extraction was achieved using a buffer containing 5% of PVPP in combination with Protease Inhibitor Cocktail, Plant (VWR Life Science AMRESCO).
We will try to optimize the whole protocol for extraction and analysis of AGO1 bound sRNAs in grapevine.

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P43. Role of AtDRBS proteins in plant antiviral immunity

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INTRODUCTION

Fanleaf degeneration is one of the most devastating grapevine diseases, which is mainly caused by two viruses: *grapevine fanleaf virus* (GFLV) and the *arabis mosaic virus* (ArMV). Resistance strategies exist, one mediated by a transgene, the other mediated by a recessive allele (Hemmer et al., 2018; Djennane et al., 2021). Based on this observation, we were interested in discovering new factors that could mediate resistance against GFLV and even more broadly.

Viral replication requires virus- and host-encoded proteins and leads to the production of double-stranded RNA (dsRNA) replication intermediates. In the case of RNA viruses, these intermediates can act as a signal that triggers host immune responses. In animals, viral dsRNA is sensed by proteins containing dsRNA binding motifs (dRBM) such as *Staufen* (Dias et al., 2017). This sensing leads to the activation of antiviral defenses. In plants, vdsRNA are sensed by DRB4 through the RNA silencing pathway which eventually leads to the degradation or the silencing of vRNA. A recent study focused on host proteins associated to vdsRNA in *Arabidopsis thaliana (A. thaliana)*. Pull down of dsRNA and associated proteins was conducted in plants expressing GFP-tagged dsRNA-binding protein (B2:GFP) upon viral infection (Incarbone et al. 2021). In this assay, a homologous protein of AtDRB4, called AtDRB2, was identified. Interestingly, AtDRB2 relocates to viral replication complexes and its overexpression in *Nicotiana benthamiana* (*N. benthamiana*) results in a drastic reduction of GFLV replication (Incarbone et al. 2021). This reduction is also observed upon infections with other viruses. These results suggest a broad-spectrum antiviral role of AtDRB2. My research project aims to understand the role of AtDRB2 during viral infection in *A. thaliana* at the molecular level and apply this knowledge to *Vitis*.

MATERIALS AND METHODS

We decided to elucidate the AtDRB2 interaction network by Immunoprecipitation coupled with tandem mass spectrometry (IP-MS/MS) in *A. thaliana* AtDRB2-Myc complemented lines (*drb2-1/pDRB2:DRB2:Myc*) upon viral infection with GFLV. A crosslink treatment with formaldehyde was systematically performed to freeze transient interactions. GFLV infected *Arabidopsis* Col-0 plants were used as control. Three technical replicates of two independent biological replicates per genotype were performed. Statistical analysis was performed with the R package IPinquiry4 (Kuhn et al. 2023).

RESULTS AND DISCUSSION

Our study focuses on the resistance mechanism mediated by AtDRB2. As a starting point, we performed IP-MS/MS experiments using *A. thaliana* plants expressing myc-tagged AtDRB2 upon viral infection by GFLV. Interestingly, we identified proteins linked to stress granules/p-bodies assembly and RNA silencing (Figure A). Concerning viral proteins, the VPg is significantly enriched (Figure A). With the aim to globally analyze the most enriched signaling pathways of the AtDRB2 interactome in viral condition, a Gene Ontology (GO) analysis was carried out. According to the GO analysis, proteins linked to “Processing-bodies/Stress granules assembly” are the most enriched. For most of these pathways pro- or anti-viral functions have been previously described (Wu et al., 2019;

Hoffmann 2023). Moreover, the same analyses will be carried out with other viruses from different families to discriminate virus specific responses from broad-spectrum responses. In addition, we are currently performing protein-protein interaction assays to test direct interaction for a small number of candidates.

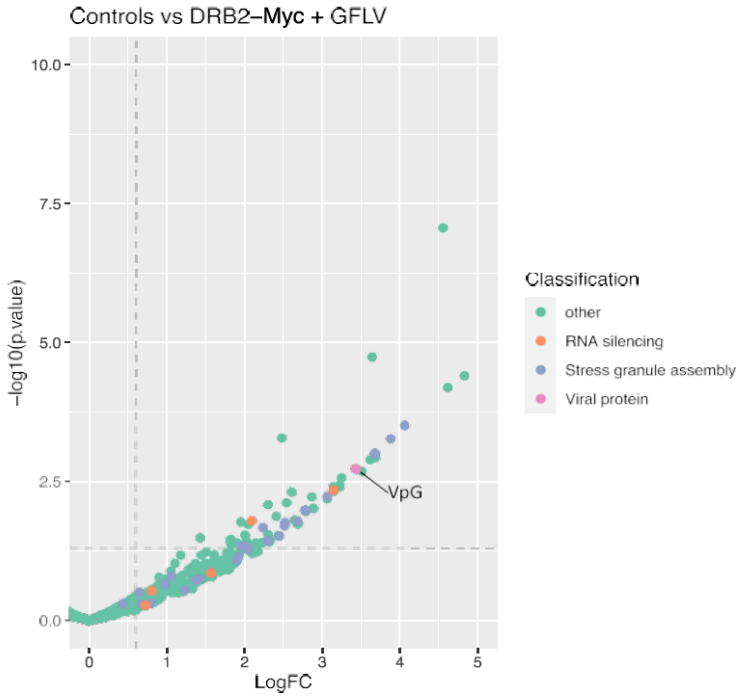

Figure: AtDRB2-Myc interactome upon GFLV infection. Volcano plot representation shows the enrichment of proteins from GFLV infected plants that co-purified with AtDRB2-Myc. Y- and X-axis display adjusted p-values and fold changes, respectively. The dashed line indicates the threshold above which proteins are significantly enriched (p. value < 0.05, Fold change >1).

In an attempt to clarify the role of AtDRB2 upon viral infection and because AtDRB2 has previously be linked to small RNA biogenesis, we also started RNA seq and sRNAseq analyses of infected *drb2-1* and *drb4*-1 single and double null mutant plants. Our overall goal is to understand the role of AtDRB2 in antiviral immunity and exploit this knowledge to design novel strategies for viral resistance.

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P44. Grapevine fanleaf virus avirulence factor $2A^{HP}$ interacts with several proteins of *Nicotiana occidentalis* involved in plant immunity

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INTRODUCTION

To ensure their growth and survival, plants have developed an arsenal of defense responses to pathogens, including hypersensitive response triggered by the perception of effectors or avirulence factors (Avr). The intracellular sensors of Avrs are predominantly proteins of the nucleotide-binding leucin rich repeat (NB-LRR) class that can recognize their targets either by directly interacting with it or by detecting a change in a host factor highjacked by the virus and thus considered as a cellular guardee. A conformational modification of the NB-LRR following sensing induces a set of defense reactions leading to programmed cell death and pathogen restriction at, or near, its entry site into the plant. This hypersensitive response (HR) is said gene-for-gene or race specific because it depends on both the pathogen and plant genotypes (Ngou *et al.*, 2022).

Grapevine fanleaf virus (GFLV) is one of the most devastating viruses in vineyards, worldwide. It belongs to the genus *Nepovirus* in the family *Secoviridae*. Its genome is composed of two single stranded positive sense RNAs each coding for a single polyprotein. Polyprotein P1 coded by RNA1 is proteolytically processed into proteins 1A to 1E and polyprotein P2 gives the homing protein $2A^{HP}$ involved in replication of RNA2, the movement protein $2B^{MP}$ and the capsid protein $2C^{CP}$ (Schmitt-Keichinger *et al.*, 2017).

In the herbaceous plant *Nicotiana occidentalis* strain F13 of GFLV induces a genuine hypersensitive response characterized by an increase in the accumulation of pathogenesis related protein (PR) 1c, phytoalexins and reactive oxygen species (ROS) resulting in localized cell death and a more or less effective restriction of the virus spread. Protein $2A^{HP}$ was identified as the Avr factor. On the contrary, strain GHu of GFLV develops an asymptomatic compatible reaction on this host (Martin *et al.*, 2018). The use of chimeric clones allowed to delineate the 50 C-terminal aminoacids of $2A^{HP}$ of strain F13 ($2A^{HP}$-F13) as the viral determinant of the HR. In order to identify plant factors involved in the recognition of the viral $2A^{HP}$ and the induction of the HR, we expressed different EGFP-tagged $2A^{HP}$ of GFLV to seek proteins preferentially associated with $2A^{HP}$-F13. Candidate proteins purified by co-immunoprecipitation and identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) are presented and discussed.

MATERIAL AND METHODS

Expression of EGFP-tagged proteins in N. occidentalis

Different EGFP-tagged proteins were transiently expressed by agroinfiltration: $2A^{HP}$ of GFLV-F13 ($2A^{HP}$-F13), $2A^{HP}$ of GFLV-GHu ($2A^{HP}$-GHu) and the chimeric $2A^{HP}$ encoding the 209 first residues of strain F13 and the 50 last residues of strain GHu ($2A^{HP}$-F209G). Free EGFP served as a control. Proteins were expressed from recombined pK7FWG2 plasmid that allows C-terminal fusions to EGFP (Karimi *et al.*, 2002). The viral suppressor of RNA silencing P19 was co-expressed with all constructs to increase their expression. Comparable levels of accumulation were obtained as checked by fluorescence intensity and western blot.

Co-immunoprecipitation and LC-MS/MS analyses

For each condition 2 agro-infiltrated leaves were collected from 3 different plants at the very first signs of necrosis in leaves expressing $2A^{HP}$-F13:EGFP. After grinding and clarification of the samples, proteins were precipitated using the µMACS GFP isolation kit (Miltenyi Biotec) following the manufacturer's instructions. Three biological replicates were performed. LC-MS/MS was performed at the Strasbourg-Esplanade proteomics facility. Proteins were reduced and treated with trypsin. Peptides generated after trypsin digestion were analyzed by nanoLC-MS/MS and identified with Mascot algorithm. The genome of *N. benthamiana* served as a reference genome (Kourelis *et al.*, 2019).

RESULTS AND DISCUSSION

HR-inducing ($2A^{HP}$-F13) and HR-non-inducing ($2A^{HP}$-GHu and $2A^{HP}$-F209G) EGFP- tagged $2A^{HP}$ proteins of GFLV were transiently overexpressed in *N. occidentalis* using agro-infiltration. The accumulation of the bait proteins was checked. At the first signs of necrosis induced by $2A^{HP}$-F13 leaf samples were collected and subjected to co-immunoprecipitation. LC-MS/MS analyses identified 1360 proteins coprecipitated with EGFP and 1900 to 2500 proteins co-precipitated with the $2A^{HP}$:EGFPs.

Among the proteins that were enriched in the HR-inducing $2A^{HP}$-F13 fraction, many are involved in the synthesis of terpenes and phenylalanine and in the regulation of the HR. In addition, four proteins were identified that are likely involved in HR induction. The sequences encoding these candidate proteins were amplified from *N. occidentalis* and cloned in expression vectors for validation. In a first step we are confirming the interactions using a GST tag for the $2A^{HP}$ proteins. Then we will use a gain-of-function assay to functionally validate the candidates for their involvement in the defense response.

This approach also identified a common interactome of proteins $2A^{HP}$ for the two GFLV strains. These 263 candidates clustered in 9 "molecular function" groups defined using Cytoscape and the ClueGO plugin, including protein synthesis and trafficking. These interactants are good candidates to find or generate recessive resistance to GFLV if (i) they are critical for the life cycle of the virus and (ii) if allelic variants or mutants losing the interaction with the virus are found.

In conclusion, the ongoing work presented here aims at identifying cellular partners of a replication accessory protein of GFLV that has been shown to induce HR in *N. occidentalis*. This will lead to the characterization of a dominant resistance to the virus and will help identifying candidate genes for designing recessive resistance.

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P45. Effects of grapevine leafroll-associated virus 3 on plant health and fruit quality in white cultivars in British Columbia, Canada

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INTRODUCTION

Grapevine leafroll disease (GLD) is the most widespread viral disease of *Vitis vinifera* and it is considered one of the main biotic constraints to grapevine health worldwide (Naidu et al., 2014). Several grapevine leafroll-associated viruses (GLRaV) are known to be associated with GLD with GLRaV-3 reported to be the most economically destructive (Rickets et al., 2015). The effects of GLD on plant health and fruit quality have been widely studied primarily resulting in decrease vine vigor and total yield, reduce plant photosynthetic capacity, and decrease in total soluble solids (brix) (Song et al., 2021). GLRaV-3 is widespread in Canada (Fall et al., 2020; Poojari et al., 2017; 2020; Xio et al., 2018) and its effects on plant health and grape and wine quality in red cultivars have been studied in British Columbia (BC) (Bowen et al., 2018). Currently, 2,275 ha (45% of the total) are planted with white cultivars in BC thus, representing a critical part of the BC grape and wine industry economy (BCWGC, 2023). Accordingly, the main objective of this study was to determine the effects that GLRaV-3 has on plant health and fruit quality on selected white cultivars in the Okanagan Valley, the main grapevine production region in BC.

MATERIALS AND METHODS

The effects of GLRaV-3 on plant health and fruit quality were evaluated in 'Sauvignon blanc' (Sb) (2019 to 2022) and in 'Chardonnay' (Ch) and 'Semillon' (Se) (2021 and 2022) vineyard blocks located in the Okanagan Valley (BC). In October of the previous year of the start of each trial, a total of 100 leaf composite samples (one composite sample equals five vines within a panel for a total of 10 panels from 10 rows) were collected and tested for GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, and grapevine red blotch virus (GRBV) at the Brock University Cool Climate Oenology and Viticulture Institute (CCOVI), St. Catherines (ON) virus testing laboratory. Based on the virus testing results, 20 GLRaV-3 infected vines, interspersed through the block, and non-infected vines flanking, in the row one vine away on each side of the infected vines, were selected for study. Each of the 20 sets of three vines was considered a replicate block for statistical analysis of GLRaV-3 effects on plant health and fruit quality and included an extra healthy vine in case one became infected with GLRaV-3 during the study. All 60 selected vines were tested for GLRaV-3 before harvest each year to verify their infection status as described above. Plant health parameters included leaf greenness measured three times during the growing season and pruning weights collected in winter. Yield components and fruit composition were collected at harvest as described by Bowen et al. (2020) and included, clusters/vine, yield(g)/vine, average berry weight(g)/vine, pH, brix, and titratable acidity (g/L). In addition, the cold hardiness of buds was measured using differential thermal analysis for low temperature exotherm (LTE) detection, following the methods of Bowen et al. (2016).

RESULTS AND DISCUSSION

Samples screened at the CCOVI virus testing facility showed infected vines from 'Sb', 'Ch' and 'Se' to host only GLRaV-3. All samples resulted negative for GLRaV-1, GLRaV-2, GLRaV-4, and GRBV. Though some healthy vines became infected with GLRaV-3 during the duration of the trials, there were enough healthy vines left to compare against infected vines and complete statistical analyses. Overall, results from this study showed no statistical differences between GLRaV-3 infected and

healthy vines on the number of clusters per vine, yield per vine, brix, pH, TA, leaf greenness, and bud hardiness. Though not statistically different, 'Sb' healthy vines had higher number of clusters. Similarly, though not statistically different, 'Ch' and 'Se' healthy vines resulted on higher brix than infected vines. Berry weight was lower in 'Sb' and 'Ch' healthy vines when compared against infected vines. In 'Se', GLRaV-3 infected vines showed a 20% yield reduction (avg. 2,135.25 g/vine) when compared against healthy vines (avg. 2,650.55 g/vine) but only in the 2022 harvest. Previous studies have reported GLRaV-3 to negatively impact white cvs. in other countries (Cabaleiro et al. 2021). Results from this study indicate GLRaV-3 to have minimal impacts on plant health and fruit quality parameters on white cultivars 'Sb', 'Ch' and 'Se' under BC growing conditions. Major effects such as the yield reduction observed on 'Se' appear to be year dependent as previously observed in different fruit quality indicators on red cvs. in BC (Bowen et al. 2018). Among many other factors, GLD detrimental impacts on plant health and fruit quality have been reported to depend on age of the vine, cultivar, scion-rootstock combination, and the duration of the infection in the plant (Naidu et al., 2014; Song et al., 2021). Though GLRaV-3 may have lower impacts on plant health and fruit quality in white than red cvs., disease management, including insect vector control and 'roguing' should still be implemented in BC to avoid the spread of GLD.

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P46. In vitro early detection of grapevine virus-induced graft incompatibility in Syrah/R110: the involvement of GRSPaV

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INTRODUCTION

More than 80% of the vineyards in the world are currently composed of *Vitis vinifera* L. scions grafted onto phylloxera-resistant American hybrids rootstocks (Ollat et al., 2016) a practice used since the end of the 19th century to control the damage caused by *Daktulosphaira vitifolia* Ficth. However, graft incompatibility can affect grape yield and plant longevity. Graft incompatibility has been classified as (i) translocated when associated with starch accumulation and phloem degeneration, (ii) localized, when characterized by weakness of the graft union, and (iii) virus-induced when due to viral infections (Mosse, 1962). In grapevines, incompatibility manifests in short-term graft failure or long-term decline of vineyards both causing economic losses to nurseries and growers. Here, we assessed the suitability of *in vitro* systems coupled with histochemistry analysis as early detection methods for grapevine incompatibility using certified homografted (i.e., a graft between the same genotype) and heterografted (i.e., a graft between two genotypes) grapevine micrografts, with known graft compatibility response when grafted onto the worldwide used rootstock 110R (*V. berlandieri x V. rupestris*). To prove the involvement of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) in Syrah/110R incompatibility, we silenced GRSPaV transcripts by ectopically applying a GRSPaV siRNA-inducing dsRNA.

MATERIALS AND METHODS

Certified plants of two clones of 'Syrah', ENTAV-INRA/FR 383 and 470 (SY383 and SY470 respectively) and 'Ritcher-110' JBP/PT (R110) were established *in vitro*. At the time of collection, the absence of the EU certification mandatory viruses ArMV, GFLV, GFkV, GLRaV-1 and -3, as well as GLRaV-2, -4, GVA, and GVB was confirmed by ELISA. The presence of GRSPaV in non-grafted *in vitro* plants was analysed by qRT-PCR and micrografts of SY383/SY383, SY470/SY470, 110R/110R, SY383/110R, and SY470/110R were performed and their graft unions processed for histochemical analysis at 28 and 49 days after grafting (DAG). Grafts were considered successful when scion growth and/or rooting of the rootstock was observed at 49DAG. Histochemical analysis was performed with five different tests: calcofluor to stain cellulose in the cell wall; acridine orange for differentiating xylem; phloroglucinol-HCl for lignin; potassium iodine (I_2KI) for starch; and aniline blue for callose (Tedesco et al., 2023). Microscope-stained images coupled with statistical analysis were used to establish differences among the graft combinations. One of the most representative GRSPaV transcript variants found in SY470 served as a DNA template to produce a dsRNA targeting the viral coat protein and was applied in SY470 homo- and heterografts before grafting. At 49DAG, graft success and GRSPaV levels were assessed by qRT-PCR (Tedesco et al., 2023).

RESULTS AND DISCUSSION

Graft success revealed that heterografts displayed a viral phenotype and that SY383/110R, known as incompatible (Renault-Spilmont et al., 2005), was more successful than SY470/110R. qRT-PCR on ungrafted plants revealed that GRSPaV levels were significantly higher in SY470 than in SY383 implying a negative correlation between GRSPaV presence and graft success. Calcofluor-stained sections pointed out a lower cellular arrangement for SY470/110R. Indeed, irregularly shaped cells were already reported in grapevine micrografts infected with GLRaV-1 and GVA (Cui et al., 2019). Acridine Orange stain revealed that while homografts' vasculature was more differentiated at 49 than at 28DAG, vascular differentiation was delayed in heterografts as reported in other species. Callose (indicating developing phloem cells) was depleted in hetero- compared to homografts at both times, and the intensity of phloroglucinol at the necrotic layer revealed that this was enhanced in hetero- compared to homografts and that heterografts' necrotic layer was even more stained at 49 than at 28DAG which seems to hinder vascular formation. I_2KI revealed that SY470/110R was enriched in starch compared to its SY470 homograft whereas the same did not happen in SY383/110R. Overall, these results revealed translocated incompatibility symptoms in Syrah/110R and show how micrografting coupled with histochemical analysis can be used to early-predict grapevine incompatibility. Finally, to prove the involvement of GRSPaV in Syrah/110R incompatibility, we silenced GRSPaV with a dsRNA before grafting and verified that graft success in SY470/110R was successfully rescued when compared to the control (100% and 67% of success, respectively). Furthermore, at 49DAG, more viral transcripts were recorded in the GRSPaV-silenced 110R rootstock compared to the control (at 88% confidence level) supporting the notion that incompatibility in grapevines is due to the rootstock hypersensitivity to scion-derived viruses. We propose the involvement of GRSPaV in Syrah/110R incompatibility and alert that grapevine incompatibility is a virus-induced phenomenon that can arise even in certified plants and manifests in the translocated incompatibility that we have shown in this work.

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P47. Protein interactome of grapevine leafroll-associated virus 3 and *Vitis* species

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INTRODUCTION

Grapevine leafroll disease (GLD) is a globally important disease that affects factors such as berry cluster size and sugar and acidity levels of grapes, leading to a reduction in grape yield and the quality of wine produced. *Grapevine leafroll-associated virus 3* (GLRaV-3), a member of the genus *Ampelovirus*, family *Closteroviridae*, is regarded as the primary causal agent (Maree et al., 2013). Although the effect of GLRaV-3 infection on the transcriptome and metabolome of infected vines has been investigated, functional studies to determine the exact mechanism by which these effects occur have not been performed. The aim of this project was to study the GLRaV-3 interactome to identify virus or host genes that play a key role in the proliferation of GLRaV-3 and the onset of GLD. This information could ultimately lead to the improvement of disease management strategies for GLD.

MATERIALS AND METHODS

A yeast-two hybrid (Y2H) assay was utilized to identify interactions among GLRaV-3-encoded proteins. To identify virus–host interactions, a *Vitis vinifera* Y2H prey library was constructed and screened against GLRaV-3 ORFs encoding proteins involved in virion assembly, intracellular movement, and suppression of host silencing. Bimolecular fluorescence complementation (BiFC) assays were conducted to demonstrate the *in planta* interaction of protein pairs identified using the Y2H system, and to identify pairwise interactions among GLRaV-3 transmembrane protein (TMP), heat shock protein 70 homolog (HSP70h), movement protein (MP), host silencing suppressor (p20B), and the major and minor coat proteins (CP and CPm, respectively). The membrane topology of the TMP was investigated *in silico*.

RESULTS AND DISCUSSION

The majority of interactions between structural proteins suggest that GLRaV-3 shares a common mechanism of assembly with members of the genus *Closterovirus*, family *Closteroviridae*. Three interacting pairs were detected using both assays. HSP70h interacted with itself and with the CP, likely facilitating its role in stabilising virions and mediating their cell-to-cell translocation (Alzhanova et al., 2001). Self-interaction of p20B was also observed and corresponds to that of closterovirus silencing suppressors (Chapman et al., 2004). BiFC screening assays revealed five additional interactions. The self-interaction of CP could enable it to partially coat viral RNA, whereas CPm likely does not self-interact in the absence of the 5′ non-translated region (NTR) of virus RNA (Alzhanova et al., 2007). The virion head structure of beet yellows virus (BYV; genus *Closterovirus*) contains the CPm, HSP70h, MP and p20, a small protein that has not been identified as a silencing suppressor, but is required for long-distance movement of the virus (Alzhanova et al., 2001; Napuli et al., 2000). The interaction of GLRaV-3 p20B with CPm, HSP70h and CP could indicate incorporation of p20B into the virion and that it serves a dual purpose, facilitating both long-distance transport and suppression of host RNA silencing. No interactions were found between CPm, HSP70h and the MP, suggesting a multi-way protein interaction or that the presence of the 5′ NTR of virus RNA is required for interaction. GLRaV-3 TMP self-interacts. However, its membrane topology was inverted compared to closterovirus TMPs. Additionally, GLRaV-3 TMP lacks a cysteine residue in the N-terminal region, which is essential for the self-interaction of BYV TMP. These differences suggest that GLRaV-3 TMP

employs a different method of dimerization, and the mechanism by which the TMP of GLRaV-3 facilitates translocation could differ from that of TMPs encoded by closteroviruses.

Five virus–host protein interactions were identified using Y2H. Two could not be demonstrated *in planta* and involved GLRaV-3 p20A, a protein of unknown function proposed to be play a role in suppression of host defence responses and long-distance transport. In yeast, p20A was found to interact with a *V. vinifera* chlorophyll a-b binding protein (CAB) and a *V. vinifera* SMAX1-LIKE 6 protein. BiFC assays further demonstrated the interaction of p20A with *V. vinifera* mitogen-activated protein kinase (MAP3KEK1) and a *V. vinifera* small HSP, as well as the interaction of GLRaV-3 CPm with *V. vinifera* 3-deozy-D-arabino-heptulosonate 7-phosphate synthase 02 (DAHPS). All five of these host proteins are connected to host defence responses against pathogens. This could indicate the action of the host defence system against GLRaV-3, or evasion of host defence mechanisms by the virus.

A working model explaining how infection by GLRaVs could lead to the symptoms of GLD was previously proposed (Song et al., 2021) and focuses on downstream physiological effects of the obstruction of sugar translocation caused by to damage to the phloem tissue by these viruses. Virus–host interactions identified in this study demonstrate that the symptoms of GLD may also be caused by interference with a variety of pathways. Furthermore, chloroplasts, DAHPS and MAP3KEK1 are all involved in salicylic acid (SA) synthesis or signalling. SA induces the expression of host RNA silencing genes and cooperates with RNA-silencing mechanisms to combat plant virus infection (Alamillo et al., 2006). Interference with these pathways could disrupt host defence mechanisms. Treatment with SA has been successful at reducing accumulation of viral RNA or virus coat proteins, or inhibiting systemic transport of viruses, in various plants (Alamillo et al., 2006) and could be considered as a tool to combat GLRaV-3 infection.

This study contributes to our knowledge on the roles of GLRaV-3-encoded proteins in its replication and spread and provides information on cellular responses by grapevine against GLRaV-3.

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P48. High grapevine Pinot gris virus titers in grapevines showing grapevine leaf mottling and deformation

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INTRODUCTION

Grapevine Pinot gris virus (GPGV) was discovered in Trentino, Italy, where it was associated with symptoms of chlorotic mottling and leaf deformation (GLMD) (Giampetruzzi et al. 2012). The virus was reported in all the main grape growing areas worldwide (Saldarelli et al. 2017), due to exchange of propagation materials and likely favored by the existence of GPGV variants not associated with disease symptoms (Saldarelli et al. 2015). Since GLMD symptoms were observed in field- and screenhouse-grown grapevines in Apulia, Italy, a further study was accomplished.

MATERIALS AND METHODS

Field-grown grapevines of the cultivar Negroamaro were in San Pietro Vernotico, Brindisi. Virus detection was carried out on total RNAs extracted from leaf and petiole tissues by a two-step real time (rt) RT-PCR protocol using SYBR green and unpublished primers sets. A RT-PCR protocol was accomplished to study the C polymorphism at the 6684-genome nucleotide position (Saldarelli et al. 2015).

Total RNAs (1µg) extracted from three symptomatic (T3, T4 and T5) and two asymptomatic grapevines (T1 and T6), were used to generate cDNAs and prepare libraries, following the Illumina protocol (Illumina, San Diego, USA). Libraries were sequenced on a Novaseq platform in paired-end 100 bp. Reads were assessed for quality by FastQC and aligned by Tophat2 tool (Kim et al. 2013) on the last improved version of grapevine genome PN40024 v4 (Velt et al. 2023). Raw reads count data from the mapping genome were determined using SeqMonk software version 1.48.1 (https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). mRNAs abundance data were analyzed by Principal component analysis (PCA) and used to identify differentially expressed genes (DEGs) between the identified clusters by DESeq2 tool (Love et al. 2014). Unmapped raw reads were *de novo* assembled by rnaSPAdes (Bankevich et al. 2012) in contigs which were searched by BLASTX against a local nr database of viral proteins. Unmapped reads were also directly mapped to a custom database of plant virus/viroid sequences to obtain genome profiles and coverages.

RESULTS AND DISCUSSION

During spring 2021 GLMD symptoms were observed in *c.* 50 years-old field-grown grapevines. rtRT-PCR analysis of 6 different plants showed that all tested positive for the presence of GPGV. Further RT-PCR investigations disclosed that they host GPGV isolates with the C polymorphism at the MP/CP genome region, therefore belonging to the symptomless clade having a 6 amino acid-longer MP. In May 2023 selected forced cuttings from these field grapevines, maintained under screenhouse conditions, displayed GLMD symptoms on three out of five plants (Table 1). A rtRT-PCR screen of these five plants showed that all were infected by grapevine leafroll-associated virus 3 and were weakly positive to grapevine virus A and B (four out of five), and negative to grapevine fanleaf virus. GPGV was detected in the three grapevines showing GLMD symptoms (Table 1).

Plant N.	GLRaV-1	GLRaV-2	GLRaV-3	GVA	GVB	GFLV	GPGV	GLMD
T1	-	-	+	+weak	-	-	-	No
T3	-	-	+	+weak	+weak	-	+	Yes
T4	-	-	+	+weak	+weak	-	+	Yes
T5	-	-	+	+weak	+weak	-	+	Yes
T6	-	-	+	+weak	+weak	-	-	No

Table 1. qRT-PCR results of the 6 screenhouse grapevines. + and - : positive and negative result. +weak : positive, Cq value > 32 on a total of 40 cycles.

RNASeq libraries from leaf and petiole tissues provided 35,662,856-38,436,117 high-quality paired-end reads, of which 98.5 to 98.7 % mapped to the grapevine genome. Libraries from symptomatic grapevines separated in a PCA analysis from those of symptomatic plants. Among the genes upregulated in symptomatic plants a F-box protein PP2-B15 and an ankyrin repeat domain-containing protein were found. F-box proteins are part of the SCF (SKp1, Cullin, F-box protein) complex, which determines the ubiquitin targeting of proteins to be degraded by the proteasome (Correa et al. 2013). Viruses exploit F-box proteins to alter cellular processes such as those controlling plant hormone levels and RNA silencing. Moreover, ankyrin-repeat proteins have been involved in plant defense response and carbohydrate metabolism causing leaf abnormalities (Wirdnam et al. 2004).

Virome analysis of the five grapevines displayed an excess of GPGV reads in plants showing GLMD symptoms, representing the 52.72-77.85% of the total viral reads, while these were the 0.08 and 0.09% of the total in the viromes of the two symptomless plants. No correlation was found between GLMD symptoms and any other viruses and viroids. In particular, grapevine Roditis leaf discoloration associated virus (GRLDaV) was present in one plant while grapevine *Rupestris* stem-pitting associated virus (GRSPaV) and the two viroids (Grapevine Yellow Speckle and Hop stunt viroid) infected all plants. As expected, reads were mapped to the GLRaV-3 genome of all plants while those from several vitiviruses were erratically present. The association of high GPGV virus titers with GLMD, initially reported by Bianchi et al. (2015) is, in this study, apparently specific as it does not involve other viruses. GPGV impact is unresolved, with contrasting reports indicating its association with GLMD, and makes the virus an interesting subject of research to study plant-virus interactions (Tarquini et al. 2023) which surely deserves further studies.

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