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PROCEEDINGS

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19th Congress of the International Council for the study of Virus and Virus-like Diseases of Grapevine

WELCOME

Dear colleagues and friends,

I am very happy to receive you in the 19th version of the ICVG meeting. It is the first time that the ICVG congress lands in South America, and specifically in Chile. In these times of economic crisis that affect many regions of the world, I know very well that for several of you it has not been easy to be present. Therefore I thank you even more for the effort you have made to attend. Your presence is the demonstration that the passion for our work is stronger than any economic crisis.
As you will appreciate, the congress will present several scientific novelties, and a good number of information coming from countries that are starting to cultivate grapevine or studying the harmful effects that viruses, phytoplasmas and viroids cause in it.
I want to thank all those who have supported us for the realization of the event, especially the sponsors and the staff of the University of Chile. Without their effort and dedication, it would not have been possible to hold this congress.
I encourage you to enjoy these days and sincerely I hope that you can establish good relationships, not only of work, but also of friendship.

Good luck,

Nicola Fiore
Chair, 19th ICVG Organizing Committee

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Dedication

These Proceedings are dedicated to the memory of Dr. René Bovey, the last of the Founding Fathers of ICVG and its first, long lasting Secretary.

Contents

Poster Session

C1: Where Grapevine Virology is Heading To

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SUMMARY

In its hundred years of history, grapevine virology has gone through stages encompassing the observation and description of field syndromes, the demonstration of their graft transmissibility -hence their putative viral nature- the isolation, morphological, physico-chemical, serological and molecular characterization of the associated viruses, their mode of spreading and taxonomic allocation. These are still ongoing exercises that, in recent times, have been facilitated by the advent of next generation sequencing (NGS), a technology that allows the identification of the "virome" of field-grown grapevines -i.e. the complex of the infectious agents (viruses and viroids) that accumulate in them over time- without going through the conventional and lengthy laboratory procedures. Whereas the metagenomics approach is having a great impact on some virological activities, e.g. the discovery of new viruses and the verification of the sanitary status of certified propagative materials, other aspects of the complex world of grapevine virology continue to deserve attention. Among these, the investigations for the advancement of the epidemiological knowledge and the fostering of biotecnological applications.

Historically, three main periods, or stages, can be recognized in the course of the virological studies of grapevines: (i) the “ancient stage” [second half of the 19th century/5th decade of the 20th century] in which the degenerative conditions of the vineyards, the deformation and odd colouring of the foliage of the vines (bright yellow, or red to deep purple leaves) attracted the attention of European scientists and their infectious (i.e. viral) origin was postulated but not ultimately proven; (ii) the “descriptive stage” [5th decade of the 20th century] when these symptomatologies were carefully described in the USA, sorted out and ascribed to different diseases, whose viral nature was substantiated by graft-transmission; (iii) the “virus identification stage” [6th decade of the 20th century to date] when a number of viruses recovered from symptomatic vines were characterized and classified taxonomically (Martelli, 2017).

The latter stage was, and still is, most productive in terms of isolation and identification of the putative agents associated with field syndromes. In fact, from the early 1960s, when *Grapevine fanleaf virus* (GFLV) was first recovered by mechanical transmission to herbaceous hosts (Cadman *et al*., 1960) and shown to be serologically related to *Arabis mosaic virus* (ArMV) (Dias and Harrison, 1963), many research groups operating in the major grapevine-growing areas of the world successfully adopted the same methodology.

That was the time when there was a steady increase in the number of newly discovered viruses reported at each ICVG Meeting. This flush of new entries had an impact of some consequence also on viral taxonomy and nomenclature. In fact, the names of the genera *Vitivirus* (family *Betaflexiviridae*) and *Ampelovirus* (family *Closteroviridae*) derive from grape-related words, whereas *Grapevine fleck virus* (GFkV) *Grapevine leafroll-associated virus 7* (GLRaV-7) and *Grapevine red blotch virus* (GRDV) are the type species of the genera *Maculavirus* (Martelli *et al.,* 2002), *Velarivirus* (Al Rwahnih *et al.,* 2102; Jelkmann *et al.,* 2012; Martelli *et al.,* 2012) and *Glabrovirus* (Varsani *et al.,* 2017), respectively.

Most of the newly discovered viruses were identified following a classical stepwise approach which, as detailed in Martelli (1993), was based on: (i) virus isolation from a symptomatic vine by manual inoculation to a more or less standard set of herbaceous host; (ii) virus transfer to a wider herbaceous host range, whose reactions were compared with those described in the literature; (iii) further transfer to susceptible plant species (usually members of the genera *Nicotiana* or *Chenopodium*) that would support active virus multiplication; (iii) virus extraction from the sap of infected host tissues, concentration and purification by differential and density gradient centrifugation; (iv) use of purified virus preparations for electron microscopy (shape and size of the particles), determination of physico-chemical properties, and raising of specific antisera; (v) serological and molecular comparison with similar, already characterized viruses. This relatively lengthy and not necessarily easy process determined whether the virus under study was already known or was a previously unreported one.

The same procedure was used for non mechanically transmissible viruses, e.g. all but one member in the family *Closteroviridae* [*Grapevine leafroll-associated virus 2* (GLRaV-2); Monette and Godkin, 1993)] and all members of the family *Tymoviridae* (Sabanadzovic *et al*., 2017), in which case the starting material for virus extraction and purification were the leaves of naturally infected vines.

Up to 2016, i.e. 56 years after the pioneristic manual transmission of GFLV, the worldwide hunt in vineyards and natural environments, where wild *Vitis* species can still be found, had led to the discovery of 68 RNA and DNA viruses in 29 genera (Martelli, 2017). A year later, these figures had grown to 84 viral species in 32 genera (Annexed Table 1). This sudden increase in the number of newly discovered viruses (16 in a couple of years) was a veritable leap forward almost exclusively due to the advent of metagenomics, i.e "the analysis of microbial communities in environmental samples through sequencing" (Roossinck *et al*., 2015). In fact, of the new additions to the list of grapevine-infecting viruses only two, *Grapevine leafroll-associated virus 13* (GLRaV-13 (Ito *et al*, 2016) and *Wild Vitis virus 1* (WVV-1) (Perry *et al.,* 2018) were discovered by conventional methods. All the others were picked up by an increasingly popular metagenomics approach known as high-throughput sequencing (HTS) or next generation sequencing (NGS). Since its first applications some eight years or so (Al Rwahnih *et al*., 2009), NGS has been used in several leading grapevine-growing countries but, as reported in this Meeting, is now expanding also to countries like Nigeria, where viticulture is a novel industry (Zangoma *et al*., 2018).

The primary purpose of NGS applications used to be the uncovering of the "virome" of vine selections, i.e. the complex of the infectious agents (viruses and viroids) that, regardless of their pathogenicity, pile up over time in hosts which, like the grapevine, are exposed to viruliferous vectors and undergo grafting for propagation. If this technology would have been available in the 1980-90s when, upon a FAO assignment I happened to survey countries of the Middle-East and of the southern rim of the Mediterraneam basin, the information of the sanitary conditions of the local viticultural industries would have been much more accurate than what it was ascertained by visual inspections and limited laboratory analyses.

As the potentialities of metagenomics were explored in more detail, it became evident that NGS technology could be used for certifying the sanitary status of grapevine propagating material (Saldarelli *et al*., 2017). Although the production of certified stocks and their availability to growers is now generally accepted as an essential measure for improving the condition of the world viticultural industry -which continues to suffer a much degraded sanitary status despite of the efforts deployed in many grapevine-growing countries- still there is much variability in the certification schemes implemented in these countries (Golino *et al*., 2017).

One of the constrains is the cost of certification which, although it may vary from country to country, it is generally high. The estimates of the Foundation Plant Services of the University of California, Davis, CA, USA for conventional virus testing are: PCR assay (37 pathogens): $ 1.200; ELISA (4 pathogens): $ 250; herbaceous host indexing: $ 100; woody host indexing: $ 350, to a total of $ 1.900, and a minimum release time of 2-3 years. By contrast, NGS testing would cost $ 350 per selection, with a release time of 1-2 months (M. Al Rwahnih, personal communication). Thus, the advantages of a NGS-driven certification are undoubtful. However, such a system may not readily be implemented in places where there is not a centralized certification structure, like that of the Foundation Plant Services of California.

From all the above, the conclusion can be drawn that NGS is a handy and advantageous technology for which a brilliant future can be foreseen. Would it be, however, a sort of a “last resort” for grapevine virology?

Most certainly not, because if the discovery of novel viruses and the ascertainment of the sanitary conditions of vine stands and certified stocks are still of paramount importance, there is still a long way to go in the investigation of other facets of grapevine virology. Epidemiology is one of these.

Since experimental evidence was obtained that GFLV, the agent of the soil-borne disease "infectious degeneration" (fanleaf), is transmittted by longidorid nematodes (Hewitt *et al*., 1958; Andret-Link *et al.,* 2017), the epidemiological studies relentlessly carried out in many countries have shown that also a number of aerial vectors are able to acquire and transmit different grapevine-infecting viruses: (i) pseudoccid mealybugs and soft scale insects are vectors of the ampeloviruses *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine leafroll-associated virus 4* (GLRaV-4, strain 4, 5, 6, and 9) (Herrbach *et al*., 2017) and of the vitiviruses *Grapevine virus A* (GVA), *Grapevine virus B* (GVB) and *Grapevine virus E* (GVE) (Minafra *et al*., 2017). In both cases, transmission is semipersistent and does not appear to be vector-specific; (ii) the mite *Colomerus vitis* transmits *Grapevine inner necrosis virus* (GINV) (Kunugi *et al.,* 2000) and *Grapevine Pinot gris virus* (GPGV) (Malagnini *et al.,* 2016); (iii) the treehopper *Spissistilus festinus* is the vector of *Grapevine red blotch virus* (GRBV) (Bahder *et al.,* 2016).

Whereas this latter finding makes it plausible that other leafhopper-borne viruses, e.g. *Summer grape latent virus* (SGLV), were able to find their way into plants of *Vitis aestivalis* (Sabanadzovic, 2009) and *V. vinifera* (Al Rwahnih *et al*., 2015), the repeatedly ascertained presence in grapevines of aphid-borne viruses among which: (i) *Cucumber mosaic virus* (CMV); (ii) *Bean common mosaic virus* (BCMV) and other unidentified potyviruses (Martelli *et al.,* 2017); (iii) *Broadbean wilt virus 1* (BBWV-1) (Castrovilli *et al*., 1985), (iv) *Alfalfa mosaic virus* (AMV) (Bercks *et al*., 1973) and (v) *Grapevine fabavirus* (GFabV) (Al Rwahnih *et al*., 2016), is puzzling, unless one admits that aphids have a role in spreading these viruses. Although the number of these

records makes aphid transmission a reasonable event, this likelihood does not seem to have attracted much the attention of grapevine virologists. Thus, it remains experimentally undetermined. The same applies to the infection by GLRaV-2 of symptomless *Vitis californica* and *V. californica* x *V. vinifera* hybrids growing in riparian areas of Napa Valley away from commercial vineyards (Klaassen *et al.,* 2011), and of *Vitis aestivalis* and *Muscadinia rotundifolia* vegetating in natural environments of south-eastern USA (Abou Ghanem-Sabanadzovic and Sabanadzovic, 2015).

GLRaV-2 belongs in the genus *Closterovirus,* which is characterized by the aphid-transmissibility of many of its members. GLRaV-2, however, does not have a recognized vector and enters *V. vinifera* stands with infected propagating material. What happens then? Does this virus spread in the vineyards? Perhaps not if, as hypothesized by Angelini *et al.* (2017), it has lost the "ability to be transmitted by an arthropd vector as a result of coadaptation with the grapevine host". But, since this contrasts with the reported infection of vines growing in natural environments, a research effort to cast light on this puzzling situation would have been desirable.

Something is finally moving in this direction, as exempified by the earnest attempt to investigate the relationship of GLRaV-2 with phylloxera (*Daktulosphaira vitifoliae*), the aphid-related hemipteran that feeds on grapevine roots. However, as the experimental transmission tests were negative (Wistrom *et al*., 2017), the epidemiology of GLRaV-2 continues to remain wrapped up in mistery.

Another unresolved problem is the epidemiology of the viruses of the "fleck complex" the most widespread of which is *Grapevine fleck virus* (GFkV), a virus that does not induce symptoms in European grapes and most of the American *Vitis* species, except for *Vitis rupestris* (Hewitt *et al*., 1972). Although circumstantial evidence from Japan (Yamakawa, 1989), South Africa (Engelbrecht and Kasdorf, 1990) and Italy (Fortusini *et al*., 1996) indicates natural field spreading of GFkV, no attemps have apparently been made to identify its possibile vector(s). Yet, the phloem-restricted nature of this virus would suggest the involvement of phloem sap-feeding insects, e.g. like those vectoring vitiviruses and ampeloviruses.

Equally desirable would be to carry out investigations for: (i) identifying the vector of *Grapevine vein clearing virus* (GVCV), a badnavirus whose natural spread is substantiated by its occurrence in cultivated *V. vinifera* and native wild *V. rupestris* (Qiu and Shoelz, 2017); (ii) elucidating the still unknown aspects of grapevine-ampelovirus interactions as discussed by Herrbach *et al.* (2107); (iii) shedding light on the epidemiology of *Grapevine rupestris stem pitting-associated virus* (GRSPaV), one of the most widespread grapevine viruses, which is neither mechanically not seed-transmissible notwhitstanding its presence in pollen grains, and has no known vector (Meng and Rowhani, 2017); (iv) further developing the integrated control strategy that has been designed and successfully applied in some relevant viticultural countries (Pietersen *et al*., 2013; 2017).

As mentioned, none of the grapevine closteroviruses, except for GLRaV-2, can be multiplied in herbaceous hosts, a condition that facilitates the study of their properties and opens to their use for biotechnological applications. Thus, the infection of *Nicotiana benthamiana* with GLRaV-3 successfully mediated by the vine mealybug *Planococcus ficus* (Prator *et al*., 2017) appears of great relevance, as it may open the way to the transfer of other non-mechanically transmissible closterovirids to herbaceus hosts.

DNA transcripts of plant viruses can be engineered as vectors for functional genomics and the expression of foreign proteins, a kind of biotechnological application for which also some grapevine viruses have been used, i.e. GVA (Haviv *et al*., 2006), GLRaV-2 (Kurth *et al*., 2012) and GRSPaV (Meng *et al*., 2013). The inherent technical difficulties and the cost of this kind of studies slows a research activity which, as discussed by Dolja and Meng (2017), has a great potentiality for practical applications.

Much easier and less costly is the transgenic approach for producing GM vines resistant to different viruses. Much work along this line was carried out at the turn of the last century in Europe (Laimer *et al*., 2009), where (France) field trials were established with vines expressing tha coat protein gene of GFLV which, as stated by Fuchs *et al*. (2000), "exhibited a promising level of resistance" to this virus. It is most unfortunate that these trials were discontinued, first in France because of the intervention of "GMO contras" who destroyed the experimental fields, then, for other reasons, in California where they had been resumed (Fuchs and Lemaire, 2017). Despite of the difficulties encountered in many countries (Europe in particular) due to the persisting strong anti GMO feeling, the transfer of "resistance genes" using either a transgenic, cisgenic, intragenic (Holme *et al*., 2013) or a genome editing [CRISP, Bortesi and Fischer (2015)] approach remains a valuable technology, whose application deserves renewed attention.

CONCLUDING REMARKS

Even from a hasty perusal of this script, the reader would realize that a great deal of the information it contains reflects the contents of the book *Grapevine Viruses: Molecular Biology, Diagnostics and Managment*, edited by B.

Meng, G.P. Martelli, D.A. Golino and M. Fuchs and published last year by Springer International Publishing. This book, a nearly 700 page volume comprising 33 chapters contributed by 74 authors from 10 countries, is an exhaustive source of up-to-date information on the multiple facets that have characterized the birth of grapevine virology and its progression from the early 20th century to date. Hence, it can righteously be regarded as the veritable highlight of the year 2017.

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O1: Ecology of *Grapevine red blotch virus* in US vineyard ecosystems.

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INTRODUCTION

Grapevine red blotch virus (GRBV) causes red blotch disease (Fuchs et al., 2015) and is a member of the genus *Grablovirus* in the family *Geminiviridae* (Varsani et al., 2017). GRBV is the first geminivirus described in *Vitis* spp (Cieniewicz et al., 2017a; Martelli, 2017). The economic impact of GRBV in US vineyards is estimated to range from $2,213/ha in eastern Washington State, when disease onset occurs at a low initial infection level and there is a low price penalty for poor fruit quality, to $68,548/ha in Napa Valley, California, when initial infection rates and quality penalties are both high (Ricketts et al., 2017). Limited information is available on the ecology of red blotch disease in vineyard ecosystems. Therefore, we investigated spread attributes of GRBV in diseased vineyards, identified potential insect vectors of epidemiological importance, and assessed the role of free-living *Vitis* species in disease epidemics.

MATERIALS AND METHODS

A 2-hectare *Vitis vinifera* cv. 'Cabernet franc' vineyard in California and a 1-hectare *V. vinifera* cv. 'Merlot' vineyard in New York were selected to monitor GRBV spread over a four-year period (2014-2017). These two vineyards were chosen based on an overall low disease incidence and confirmed presence of GRBV in some symptomatic vines by multiplex PCR (Krenz et al., 2014). The two study vineyards were planted in 2008. Individual vines were monitored for disease symptom development each year in late summer or fall, and the spatiotemporal distribution of diseased vines was mapped. Ordinary runs analysis and Spatial Analysis by Distance IndicEs (SADIE) were used for spatial analyses of diseased vines and the Monte Carlo Markov Chain method was applied to epidemic spread fitting a stochastic spatiotemporal model (Cieniewicz et al., 2017b).

Sticky card traps were placed in the diseased 'Cabernet franc' vineyard in California from March to November in 2015 and 2016 to determine the epidemiological role of the three cornered alfalfa treehopper (*Spissistilus festinus* [Say]) (Bahder et al., 2016a) and assess the diversity and distribution of vector candidates. Insects on sticky card traps were identified to species when possible by morphological characteristics and sequencing of the mitochondrial cytochrome C oxidase subunit 1 gene (Cieniewicz et al., 2017c). Subsets of insect species/taxa were removed from sticky cards and tested for the presence of GRBV by multiplex PCR (Krenz et al., 2014).

Twenty samples of free-living *Vitis* species, i.e. *Vitis riparia*, *V. aestivalis*, *V. labrusca*, *V. californica* and hybrids with *V. vinifera* or rootstocks derived thereof, were collected in seven counties in California and New York, and tested for GRBV by multiplex PCR (Krenz et al., 2014) to determine their potential epidemiological role. The genetic relatedness of GRBV variants from infected free-living *Vitis* sp. and *V. vinifera* cultivars in vineyards was compared by sequencing coding regions for the coat protein and replicase-associated protein.

RESULTS AND DISCUSSION

The incidence of diseased vines increased by 1-2% annually in the 'Cabernet franc' vineyard in California. Spatial analysis of diseased vines in each year demonstrated a strong within-row aggregation, particular at the edge of the vineyard close to a riparian area (Cieniewicz et al., 2017b). Spatiotemporal analysis of spread between consecutive years revealed a strong overall association among years, indicating that disease incidence in a given year is influenced by disease incidence the previous year. Stochastic modeling of the epidemic identified strong evidence for localized (within vineyard) spread (Cieniewicz et al., 2017b). In contrast, no evidence of spread was obtained in the 'Merlot' vineyard in New York over four years.

From insect traps placed in the 'Cabernet franc' vineyard in California, GRBV was consistently detected in *S. festinus* (Membracidae), *Colladonus reductus* (Cicadellidae), *Osbornellus borealis* (Cicadellidae) and a *Melanoliarus* species (Cixiidae) (Cieniewicz et al., 2017c). Populations of these four candidate vectors peaked from June to September with viruliferous *S. festinus* culminating from late June to early July in both years. An

assessment of co-occurrence and co-variation between GRBV-infected vines and viruliferous insects using the association function of SADIE identified a significant association between the spatial distribution of infected vines and viruliferous *S. festinus* (Cieniewicz et al., 2017c). These findings revealed the epidemiological significance of *S. festinus* as a vector of GRBV and the need for testing the transmission capability of *C. reductus, O. borealis,* and the *Melanoliarus* species.

Surveys of free-living *Vitis* species showed the presence of GRBV in many samples from grape-growing California counties such as Napa, Sonoma, Solano, Sacramento and Sutter but not in samples from Glenn and Butte counties where grapes are not grown commercially. Infection was latent is all GRBV PCR-positive free-living *Vitis* samples. Identical GRBV variants were found in free-living *Vitis* sp. and vineyards, suggesting connectivity between *Vitis* hosts in vineyards and proximal riparian areas, likely explained by the dispersal of insect vectors. None of the free-living *Vitis* samples from New York tested positive for GRBV.

Our research provided insights into the ecology of GRBV in vineyard ecosystems. These are important to devise optimal disease management strategies.

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O2*: Grapevine leafroll-associated virus 3* retention and transmission by *Pseudococcus calceolariae*

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INTRODUCTION

The citrophilus mealybug, *Pseudococcus calceolariae*, transmits *Grapevine leafroll-associated virus* 3 (GLRaV-3), an economically significant virus of grapevines. Several mealybug species are known to transmit GLRaV-3, however *P. calceolariae* is the most common of the three in New Zealand that is strongly associated with GLRaV-3 infected vineyards in New Zealand. In one study of an organic vineyard with high mealybug populations, no GLRaV-3 spread was observed (Bell 2015). This prompted the hypothesis that viruliferous mealybugs feeding on non-virus hosts such as groundcover plants may lose GLRaV-3, therefore disrupting virus transmission. To test this hypothesis, it was first imperative to determine the retention and transmission of GLRaV-3 by *P. calceolariae* after access to an alternate plant host (a non-virus host such as white clover).

MATERIALS AND METHODS

Vitis vinifera Merlot grape plants, free of GLRaV-3 as tested by ELISA were used as GLRaV-3 recipients. GLRaV-3 was detected in individual mealybugs and grapevines by reverse transcription quantitative real time PCR.

A sub-colony of *P. calceolariae* was established from eggs collected from an existing laboratory colony maintained on sprouted potatoes, *Solanum tuberosum* ('Agria'), at The New Zealand Institute for Plant and Food Research Limited. For the retention assay, <24 h old neonates were transferred to infected (GLRaV-3 positive grape leaves) or GLRaV-3-free excised grape leaves maintained with cut ends of stems in tubes of water. After a 5 day acquisition access period (AAP), nymphs were transferred from GLRaV-3-infected or GLRaV-3-free grape leaves to either 'Huia' white clover (wc) (Treatment 1: Virus/wc or Treatment 2: No virus/wc) or GLRaV-3-free grape leaves (Treatment 3: Virus/GV), or maintained without movement (Treatment 4: Virus or Treatment 5: No virus). Maintenance of mealybugs on wc provided an alternate host feeding (AHF) period. First instar *P. calceolariae* from all five treatments were collected after 1, 2, 3 and 4 days feeding. Second instar and exuviae were also collected from all treatments except Treatment 3 (Virus/GV). The GLRaV-3 retention assay was conducted twice.

To understand GLRaV-3 transmission, four treatments were used. This included an AAP period of neonates on infected grape leaves followed by first or second instar mealybugs having an AHF period on wc (Treatment 1 and 2, respectively) or no AHF (Treatment 3 and 4, respectively) and then transferred for an inoculation access period (IAP) on GLRaV-3-free receipt Merlot grapevines (Table 1). After the IAP of 7–10 days, systemic insecticide was applied to the recipient grapevines and a pathogen- and pest-free environment was maintained. Transmission of GLRaV-3 to the recipient grapevines was determined 4 to 5 months after systemic insecticide application by reverse transcription quantitative real time PCR, and the entire transmission experiment was conducted twice.

Table 1: Summary of the four treatments used to understand GLRaV-3 transmission.

	Mealybug life stage:	First instar		Second instar	
	Days:	1 - 5	5 - 10	11 - 16	16 +
Treatment 1	First instar on alternative host	GLRaV-3 infected leaf	White clover	Healthy grapevine	Healthy grapevine
Treatment 2	Second instar on alternative host	GLRaV-3 infected leaf	White clover	White clover	Healthy grapevine
Treatment 3	First instar not on alternative host	GLRaV-3 infected leaf	Healthy grapevine	Healthy grapevine	Healthy grapevine
Treatment 4	Second instar not on alternative host	GLRaV-3 infected leaf	GLRaV-3 infected leaf	GLRaV-3 infected leaf	Healthy grapevine

RESULTS AND DISCUSSION

Following a 5 day AAP, *P. calceolariae* first instars retained GLRaV-3 for 4 days when fed on either an alternate host or GLRaV-3-free grape plants. In addition, GLRaV-3 was retained in second instar mealybugs and exuviae (cast-off exoskeleton of the first instar) after feeding on GLRaV-3 positive grapevine leaves shortly before moult. Furthermore, GLRaV-3 was still transmitted efficiently (40–60%) by *P. calceolariae* nymphs after access to wc for up to 11 days post AAP; 90% transmission was achieved when no alternative host feeding was provided. The 11 day retention period, followed by transmission of GLRaV-3 to grapevines, is the longest retention period observed in mealybugs vectoring GLRaV-3 to date.

Our results suggest that groundcover plants may act as a virus sink only if mealybugs settle and feed on them for more than 11 days. Furthermore, the retention of GLRaV-3 after moult, suggests GLRaV-3 is transmitted in a circulatory manner; further experiments are required to verify this finding.

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O3:Temporal spread of Grapevine leaf mottling and deformation disease in the field

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INTRODUCTION

Grapevine leaf mottling and deformation (GLMD) is a grapevine pathology identified in Northern Italy in 2003, which is reported to be extensively spread in Northeast Italy and Slovenia (Giampetruzzi et al., 2012; Mavrič Pleško et al., 2014; Bertazzon et al., 2017). Symptoms include chlorotic mottling, mosaic and deformation of leaves, shortened internodes, stunting, and reduced yields of production. The newly identified trichovirus *Grapevine Pinot gris virus* (GPGV) has been associated with the GLMD disease, although the manifestation of symptoms has been correlated with the presence of different viral variants and/or with different titre in infected plants (Giampetruzzi et al., 2012; Saldarelli et al., 2015; Bertazzon et al., 2017). Natural transmission of the disease in vineyards could be caused by the eriophyid mite *Colomerus vitis* (Pagenstecher), a vector of the virus in controlled conditions (Malagnini et al., 2016). In the present study the natural spread of GLMD in vineyard was investigated over three years.

MATERIALS AND METHODS

Eight highly infected vineyards (with more than 3% of symptomatic plants), mainly cultivated with cv. Glera and located in the Veneto region, were selected to investigate the epidemiology of the disease over three years (from 2014 to 2016). In 2014, from each vineyard, 12 leaf samples were collected from six symptomatic and six asymptomatic plants and tested for the presence of GPGV.

Two vineyards named CIS and COL, with high occurrence of the disease and significant clustering of grapevines with symptoms, which had been verified by spatial analysis with PATCHY program according to Bertazzon et al. (2017), were chosen among those above describes for field trials. In 2014, 66 grafted plants of cv. Glera were grown in early spring in greenhouse and tested for the presence of GPGV and other grapevine viruses. In the late spring the plantlets were planted into the two vineyards: 20 plantlets were placed in the CIS vineyard inside the patch with symptomatic plants and 46 plantlets were placed in the COL vineyard, 18 of them in the area where symptomatic plants were clustered and 28 far from the patch with diseased plants. Visual surveys and molecular analysis for GPGV detection were performed on the newly planted vines for three years.

GPGV detection was carried out by means of SYBR Green real-time RT-PCR assays with primer pair CPF3/R3 followed by conventional RT-PCR and RFLP analysis for the discrimination of GPGV isolates belonging to different clades (Bertazzon et al, 2017).

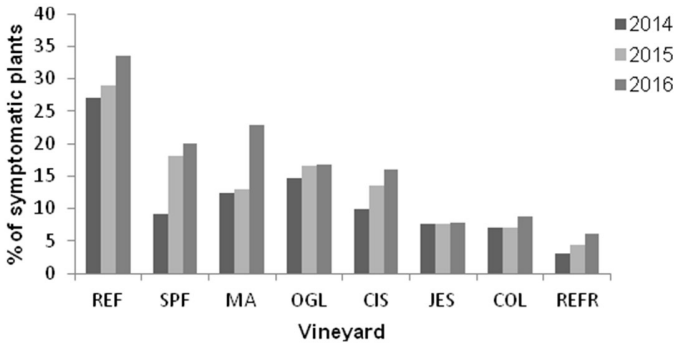
RESULTS AND DISCUSSION

The presence of GPGV was initially assessed for 96 samples collected from the eight vineyards with high incidence of symptomatic plants (12 plants per vineyard). The virus occurred in all the samples collected from symptomatic plants and in 61 out of 64 samples collected from asymptomatic plants inside and far from the patch with the diseased grapevines. The incidence of GPGV observed in this work (96.9%) confirms that elevate rates of GPGV infection are harbored in vineyards with a lot of symptomatic plants, as reported in Bertazzon et al. (2017). In the same vineyards, spatial and temporal patterns of disease symptoms were monitored from 2014 to 2016 and bidimensional maps were produced every year (Fig. 1). In general an increasing of symptomatic plants was observed in all the surveyed vineyards. Half of them showed an increase of symptomatic plants higher than 60% during three years (SPF, MA, REFR and CIS), while a lower increase as detected for other vineyards (REF, OGL, JES and COL). No correlation was found between the increment of symptomatic plants and the characteristics of the vineyards, such as year of planting or geographical disposition. However, it is interesting to note that the higher increases of symptomatic plants, recorded in the vineyards SPF (98,8% from 2014 to 2015) and MA (76% from 2015 to 2016), were associated with changes in the winter pruning strategies made by grape growers. It would be interesting to deepen the role of alterations of the sap fluxes in the manifestation of symptoms, caused by training change.

Among vineyards selected for the planting of new plantlets (CIS and COL), the higher increase of symptomatic plants was observed in the vineyard CIS (60%). Indeed, the number of symptomatic plants raised from 48 in 2014 to 77 in 2016, with all the new symptomatic plants aggregated in close proximity to formerly diseased grapevines. Conversely, an increase of only 19% of symptomatic plants was detected in the COL vineyard. Differences in the

symptom emergence rate between the two vineyards were also observed in the new plantlets. During the three years of observations, no symptoms appeared in plants collocated far from the symptomatic zone in the COL vineyard, while, at the third year symptoms were observed in six out of 17 and three out of 18 plants that had been placed inside the patch with the diseased vines in the CIS and COL vineyards, respectively.

Figure 1: Percentage of symptomatic plants observed in the eight surveyed vineyards along three years.



Together with the observation of symptoms, analyses of GPGV were performed on the newly planted vines every year. After the sprouting, 12 out of 66 grafted plants, found to be GPGV-infected with the asymptomatic viral variants (clade A), were divided between CIS and COL vineyards, and no symptom was observed in these vines during the three years of monitoring. Molecular analysis performed on the remaining originally healthy plants revealed that the average percentage of GPGV infections increased from 20% after one season, to 33% at the second year and reached 77% after the third year. In details, in CIS vineyard, during the three years of surveys, ten out of 14 healthy plants became infected with the symptomatic GPGV variants (clade B/C) and symptoms of the disease were observed in six out of eight GPGV-infected plants (two out of ten infected plants died before the third year). A similar high occurrence of GPGV was observed in COL vineyard, with 31 out of 40 plants that became infected during three years with viral variants of clade A or B/C. In particular, plants collocated inside the patch with the disease were mainly infected with variants of the clade B/C, while those planted far from the area with the aggregation of symptomatic plants showed a prevalence of GPGV-variants of clade A. In COL vineyard, among GPGV-infected plants, only three grapevines infected with variants of clade B/C and located in the patch with the disease, showed the symptoms.

Collectively, the data reported in this work indicate that, for the surveyed vineyards, both symptoms and GPGV-infection increase over time. In particular, the spreading of the virus seems very fast in field and it is followed by symptom appearance only afterwards.

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O4: Role of *Colomerus vitis* (Pagenstecher) in the epidemiology of grapevine leaf mottling and deformation in North-eastern Italy

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INTRODUCTION

Grapevine Pinot gris virus (GPGV) was discovered in Italy in 2012 (Giampietruzzi et al., 2012) and successively in several grape-growing regions worldwide infecting different varieties. Studies associating GPGV with symptoms of leaf mottling and deformation (GLMD) showed that different strains of the virus responsible for eliciting or not the symptoms exist (Saldarelli, 2015) and that *Colomerus vitis* (Pagenstecher) collected from infected grapes were able to transmit GPGV to healthy grapevines (Malagnini et al., 2016). GPGV represents a potential threat for grapevine production in Europe and elsewhere (e.g., Beber et al., 2013; Beuve et al., 2015; Raiola et al., 2013; Morelli et al., 2014; Fan et al., 2015; Al Rwahnih et al., 2016). Acquisition and transmission by an arthropod vector is central to the infection cycle of the majority of plant pathogenic viruses. Filling the gap of information of epidemiological aspects of GPGV strains/*C. vitis* interactions would help in implementing efficient strategies of control of the associated GLMD disease. The current study was aimed at identifiying the main drivers of GPGV spread and define the epidemiology of GLMD disease in North-eastern Italy vineyards. In particular, the spatio-temporal distribution of GPGV symptomatic grapes and *C. vitis* was studied in two vineyards between 2013 and 2015 growing seasons. Later, GPGV and *C. vitis* distributions were coupled assuming the existence of a potential relationship between eriophyoid mites and GPGV spread. Moreover bait grapevine plants were used in a symptomatic vineyard to verify the progress of natural infection.

MATERIAL AND METHODS

Acquisition and transmission trials were carried out under controlled conditions (22±2°C, 70 % RH±10, 16:8 L:D). In transmission GPGV infected buds and leaf erinea infested by *C. vitis* were placed onto GPGV free vines and left until tissue desiccation. After transmission trials, the presence of GPGV in single individuals of C. vitis and in grapevine plants was ascertained by RT-PCR. Total RNAs of single *C. vitis* specimens were extracted using a modified Trizol (Malagnini et al., 2016) method and GPGV detection was carried out using RT-PCR (Glasa et.al. 2014; Saldarelli et al. 2015) and real time RT-PCR (Ratti personal communication 2015). PCR products were both sequenced and compared with GenBank.
In acquisition trials eriophyid mites collected from healthy plants were put on infected grapevine leaves and resampled after 1, 4 and 8 hours for GPGV detection.
The spatial distribution of GPGV symptomatic grapes was studied in three subsequent growing seasons (2013, 2014 and 2015) in two vineyards located in the Trento Province, North-eastern Italy. In 2015 the spatial distribution of *C. vitis* was also assessed. Indices of local aggregation were interpolated by Kriging analysis and were mapped. The degree of spatial association among the different considered variables was also quantified. Each vineyards was subdivided in plots of 5 plants using a regular grid and the number of plants with symptoms of *C. vitis* and GLMD was counted. Spatial Analysis by Distance IndicEs (SADIE) were performed to map the spatial distribution pattern of plants with symptoms of *C. vitis*, GLMD and new GLMD symptomatic plants occurring during the trial. Using SADIE red – blue methods, we evaluated the local contribution to a group (cluster) of relatively high-density (patch) or to a group of zero or relatively small counts (gap) over the period of study. Tests of non-randomness based on overall index of aggregation (Ia) were performed ($\alpha = 0.05$). The similarity between the spatial patterns of *C. vitis*, GLMD and new GLMD symptomatic plants was also quantified by estimating the spatial association index and its associated probability (Px, two-tail test $\alpha = 0.05$).

To assess GPGV natural infection bait grapevine plants, previously tested free of GPGV, were placed close to symptomatic vines in an infected vineyard. Each group of bait vines consisting of ten plants were exposed for about one month and then were placed in a screen house to avoid subsequent infection. The trial started on May 2016 and stopped on October 2016. One group of vines was left in the vineyard from October 2016 until the spring 2017. The same experiment was repeated during 2017. Each bait vine was inspected for the presence of leaf erinea and eriophyid mites were collected. All bait vines and mites were tested to assess the presence of GPGV as above described.

RESULTS AND DISCUSSION

Acquisition trials showed that *C. vitis* can assume GPGV from both symptomatic and asymptomatic-infected leaves in four hours. Transmission trials confirmed that *C. vitis* can transmit GPGV to healthy grapevine plants under controlled condition as described in Malagnini et al (2016), moreover these data suggest that *C. vitis* can transmit GPGV from infected symptomatic and asymptomatic vines to healthy grapevine plants under controlled condition.

An aggregated distribution was found in both vineyards for GLDM while the distribution of plants bearing *C. vitis* galls was aggregated only in one vineyard. The distributions of GLDM symptomatic plants (= GPGV symptomatic grapevines) observed in the three growing seasons (2015, 20016 and 2017) were always associated among each other's. In one vineyard the distribution of *C. vitis* and of plants showing "new occurring" GLMD symptoms was associated. Spatial distributions of GLMD symptomatic plants over the seasons were substantially stable showing a slight increase of symptoms. However, *C. vitis* distribution was only partially associated with that of GLMD symptomatic plants and further studies are required to clarify this aspect.

GPGV was detected in bait plants placed in infected vineyards. Eriophyid mites collected from leaf erinea and buds of these vines were positive to GPGV.

These results represent an important milestone to understand the spread of GPGV in field.

However further studies are requested to improve our knowledge on relationships between vectors and virus spread.

ACKNOWLEDGEMENTS

This research has been funded by the Fondazione E. Mach in the framework of the project "Studio su una nuova malattia della vite in Trentino".

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O5: Detection of *Grapevine Pinot gris virus* in different non-*Vitis* hosts in Hungary.

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INTRODUCTION

Grapevine Pinot gris virus (GPGV), a member of *Trichovirus* (*Betaflexiviridae*), was first identified in Trentino vineyards in Italy (Giampetruzzi et al., 2012) however the symptoms of the new disease called Grapevine Leaf Mottling and Deformation (GLMD) (Martelli, 2014) were reported since 2003. Reports about GPGV reveal a widespread occurrence of the virus in many wine-producing European countries and outside of Europe, such as Korea, China, United States and Canada. Molecular characterisation and phylogeny of different isolates are indicating the existence of viral and latent GPGV variants (Saldarelli et al., 2015), (Bertazzon et al., 2017). Furthermore, GPGV has been detected in two herbaceous hosts *Silene latifolia subsp. Alba* (Mill) and *Chenopodium album* L (Gualandri et al., 2017). As the virus is transmitted by grafting (Saldarelli et al., 2015), it spreads mainly by propagating material. Moreover without regular tests of the stock cultivars and absence of symptoms on rootstocks is spread cannot be reliable controlled. As GPGV seems widespread in Slovakia (Glasa et al., 2014), Moravia (Eichmeier et al., 2016) and Hungary, it is a possibility that GPGV spread from Eastern Europe to Italy (Bertazzon et al., 2016) and from Europe to other parts of the world. A recent study reports that GPGV is present in the body of the eriophyid mite *Colomerus vitis* and is transmitted to healthy vines through mite infestation (Malagnini et al., 2016).
In our work we investigated endemic plants and weeds surrounding the vineyards to check the presence of GPGV to add new information for its epidemiology studies.

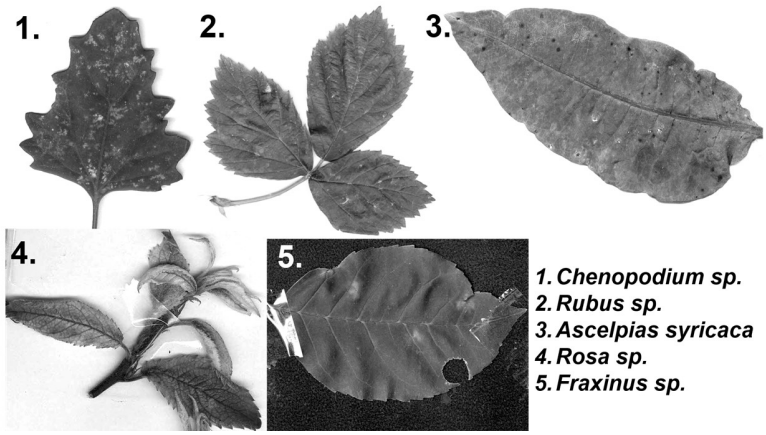
MATERIALS AND METHODS

Sampled vineyards were selected according to our preliminary survey and were positive for the presence of GPGV, but were asymptomatic. The 5 selected vineyards situate in different wine-growing regions of Hungary. Leaf samples from the grapevine and from the presenting weeds, showing symptoms were collected in May-August in 2015-2017. RNA was extracted from the grapevine and rose by an optimized CTAB based method, while RNA was extracted from the weeds by phenol-chloroform extraction method.
For RT-PCR reactions cDNA were synthetized using random (in case of grapevine samples) or GPGV specific GPGV7220R: GTTACGTGCTCCTATGAGA primer (in case of the weeds) and Thermo Scientific Revert Aid First Strand kit. Presence of GPGV was checked by a diagnostic primers (Glasa et al., 2014) amplifying a 411bp part of MP-CP. For sequence comparison a 2005 bp part of the 5'UTR (amplified by GPGV10F: CAATTGATCCCGTGTAGTGC and GPGV2015: CAGGTTCATYTTTGGATTCAAC and a 1600bp product from the 3'MP-CP coding region (amplified by GPGV5557F: ACTTATCTGATGGCTCTGATG and GPGV7220R or GPGV5578F: CAGGTACCATGGCTCTGATGAAGAGGAT and GPGV 7177R: TCTAGACTACATACTRAATGCACTCTCC) were purified by Thermo Scientific GeneJet Gel Extraction Kit then cloned into pJET1.2 vector and sequenced. To compare the sequences and generate the phylogenetic trees MEGA 7.0 program was used.
For Northern blot analyses 2-4 µg of total RNA was separated on formaldehyde-1.2% agarose gels and blotted to Nytran NX membrane (GE Healthcare), by capillary method using 20xSSC. Hybridization was carried out at 65°C in Church buffer (0.5M Phosphate buffer, pH7.2 containing 1% BSA, 1mM EDTA, 7% SDS) overnight with the appropriate radioactively labelled probe, washed for 5 minutes in 2xSSC, 0.1% SDS and for 15 minutes in 0.5xSSC, 0.1% SDS at the temperature of the hybridization and exposed to an X-ray film. Virus-specific, P^{32}-labelled, DNA probes were prepared by using the Thermo Scientific Decalabel DNA labelling kit.

RESULTS AND DISCUSSION

GPGV was detected all of the examined plantations and beside grapevine its presence were detected on 5 different non-V*itis* hosts: *Chenopodium sp., Asclepias syriaca, Rosa sp, Rubus sp.* and *Fraxinus sp.* by RT-PCR, but only in the case when cDNA was amplified by virus specific probe, indicating a lower virus level comparing to grapevine. In case of *Rosa* and *Rubus* we could support this finding by Northern blot detection of the virus. In *Chenopodium* we could only amplify a 411bp product from the MP/CP region with the diagnostic primers (Glasa et al., 2014) which were optimized for Eastern European strains of GPGV. In *Fraxinus* we could not amplify the 5' part, indicating low level of the virus or more variable 5' region in this host. The presence of GPGV in vineyard's

neighboring woody or perennial hosts suggests that it could be true that GPGV endemic in Eastern Europe and spread from here to the other part of Europe. Phylogenetical analysis of the replicase fragment and MP/CP region of the samples showed that GPGV variants were clustered according to the vineyards and not according to the hosts. The shorter MP variant accused for symptom presence was not found neither in grapevine nor in the weeds, suggesting a widespread presence of the latent variant. Host range of a virus is determined by several factors, but depends on its vector. *Colomerus vitis* is monophagous and known feeding only on grape (Malagnini et al., 2016), therefore it is possible that another polyphagous vector exists for GPGV which assists the virus transmission between the hosts, but this theory needs further investigation.



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O6: Temporal dynamics and spatial distribution of major grapevine viruses under cool climate field conditions in Ontario, Canada

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INTRODUCTION

The grape and wine industry is an essential element of the Canadian economy. The province of Ontario is the largest grape and wine producer generating an economic impact estimated at $4.36 billion (Grape Growers of Ontario, 2017; Rimerman and Eyler, 2017). One of the major concerns of grape growers and wine makers is virus diseases causing significant reduction in both the yields and quality of grapes, leading to major economic losses and shortened lifespan of commercial vineyards (Maliogka *et al*., 2015; Martelli, 2014). *Grapevine leafroll-associated virus*-2 (GLRaV-2) and GLRaV-3 are two RNA viruses from the family *Closteroviridae* which are involved in grapevine leafroll disease (GLRD), the most widespread and destructive disease of grapevine in the world. *Grapevine red blotch-associated virus* (GRBaV) is a circular, single-stranded DNA virus from the family *Geminiviridae* which causes red blotch disease (GRBD), a recently recognized important disease in North America (Krenz et al., 2012; Al Rwahnih*et al*., 2013; Martelli, 2014). Outbreaks of GLRD and GRBD were recently reported in Canada and caused serious concerns to the grape and wine industries. Although the importance and impact of these viruses on the grape and wine industries has been clearly recognized in other major grape-producing countries of the world, there is a lack of information on the seasonal dynamics of these viruses in grapevine, especially in cool climate viticulture regions such as Ontario, Canada. This poses major limitations not only to our understanding of the biology of these diverse viruses with vastly different genome structures and expression strategies, but also to the effectiveness and reliability of their diagnostics. The aim of this research was to elucidate the seasonal dynamics and spatial distribution of these three viruses in infected *Vitis vinifera* cultivars under field conditions in the major grape production region of Ontario, Canada.

MATERIALS AND METHODS

Based on results from an earlier preliminary survey, positive samples from different commercial grapevine varieties including three vines each from two white cultivars (Chardonnay and Riesling) and three red cultivars (Cabernet Franc, Merlot and Syrah) with GLRD and GRBD were chosen and labelled for this assay. Different tissues (leaves, petioles, berries and canes) were collected monthly from the vineyards from May through October for each year for two years (2015 - 2016). Total nucleic acids were isolated from grapevine samples and cDNAs were prepared with random primers using M-MLV reverse transcriptase. Subsequently, the resulting cDNAs were used as templates for PCR and assayed with virus-specific primers designed based on consensus sequence from the coat protein gene of the genomes available in GenBank combined with SYBR Green qPCR assay to compare virus titer across different months and also different tissues collected at the same time point. In addition to nucleic acid-based assays, Western blots also were employed to study the seasonal dynamics of the coat protein levels of GLRaV-2 and GLRaV-3 under field condition.

RESULTS AND DISCUSSION

The titers of the viruses assayed vary considerably among different tissue types. In this study, different tissue types (leaf, petiole and fruit) collected at three time points (May, June and September) were tested using qPCR (for GRBaV) or RT-qPCR (for GLRaV-2 and GLRaV-3). The results have shown that leaves collected in September have the highest titer for all three viruses whereas in June the highest titer was detected in berries. The finding that young fruits contain the highest amount of all three viruses was unexpected; however, similar results were reported in Italy for another virus, *Grapevine rupestris stem pitting-associated virus* by Gambino *et al* (2012). In sharp contrast, seeds collected in September contained the lowest titer of these viruses and after that petioles or fruits had lower titer of these viruses comparing with the two other tissues, leaves and cambium.

The titer of these viruses exhibited seasonal variation**.** According to the results of RT-qPCR and qPCR at different time points, all three viruses had the lowest titer early in the season (May & June). But later in the season they had different patterns of concentration, with the highest level of virus being detected in either September or October depending on the virus **(Figure 1)**.

Based on results from Western blotting, the pattern of virus titer in different months has shown different seasonal dynamics for these viruses. GLRaV-3 and GLRaV-2 had lower viral titer at the beginning (May) and toward the end (September & October) of the season. During the growing season they had different patterns of concentration and the highest level of virus was either in June, July or August depending on the virus **(Figure 2)**. Our results differ from those reported by others (Fiore et al. 2009; Tsai et al. 2012). For example, Fiore et al. (2009) has shown that GLRaV-3 detection rate in infected plants remained constant when an ELISA-based method was used, but it decreased by the end of season when RT-PCR method was used. These discrepancies could have been due to several factors, including the assay methods used, the grapevine cultivars chosen, as well as the different climate conditions under which these studies were conducted.

In conclusion, our results suggest that the best tissue for testing in early season is young fruit and the best tissue in late season is leaf. Also the best time for testing is either in late season for PCR-based method or mid season for serological methods. This is the first report on the seasonal dynamics and spatial distribution of the most destructive grapevine viruses in commercial vineyards in Canada. Our findings provide guidelines for the reliable and effective detection of these viruses using serology or nucleic acid-based diagnostic methods.

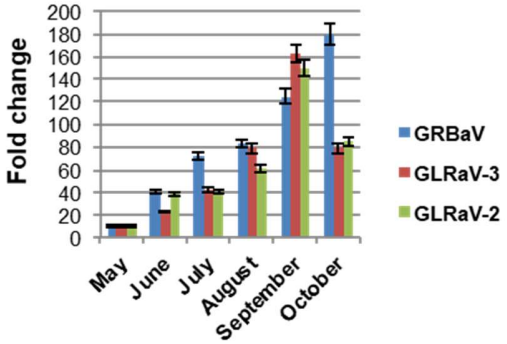

Fig. 1. Seasonal dynamic changes of three viruses in leaves as judged by qPCR or RT-qPCR.

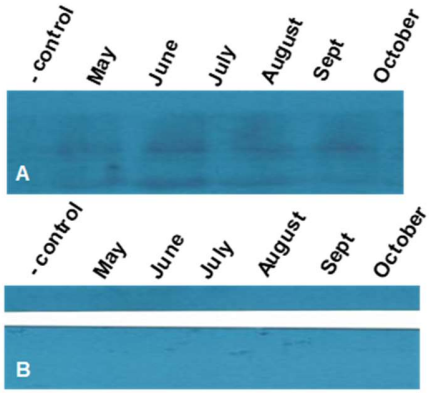

Fig. 2. Western blot showing seasonal dynamics of GLRaV-3 **(A)** & GLRaV-2 **(B)** from May to October.

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O7: Poor detection of grapevine leafroll disease in the rootstock Richter 99 (*Vitis berlandieri* X *Vitis rupestris)*

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INTRODUCTION

Grapevine leafroll associated virus 3 (GLRaV-3) does not produce symptoms and is poorly detected, for reasons that are unknown, by ELISA and PCR in the *Vitis* rootstocks commonly utilised in South Africa. This may be due to a number of reasons; 1) the rootstocks may be tolerant or immune to GLRaV-3 supporting little or no replication of the virus, 2) the rootstocks may select for strains of GLRaV-3 which are poorly detected by current methods, 3) the virus may be erratically distributed in the rootstock, 4) rootstocks may effect temporal changes in the virus titer, or 5) rootstocks may contain substances inhibitory to the PCR or ELISA detection systems. During the current study we compared the virus status of the rootstock and scion within individual vines for grapevine leafroll associated virus (GLRaV-3) strains in order to understand the poor GLRaV-3 detection. We also determined the presence in this material of other leafroll associated viruses and viruses of the Viti- and Foveavirus genera.

MATERIALS AND METHODS

Vines were collected from commercial and experimental vineyards of red and white-cultivar wine grapes from Wellington and Stellenbosch, SA, during autumn of 2014, 2015, and 2016. Samples were of scions displaying clear leafroll symptoms having lignified, relatively large canes arising from the rootstock. Cane tissue was collected separately from both the scion and the rootstock, and each assigned a separate, but linked, accession number. Total RNA was extracted from 200mg phloem scrapings from each sample using a modified cetyltrimethylammonium bromide (CTAB) method White et al., 2008.

Rootstock and scion samples collected were tested in RT-PCR for leafroll associated viruses and rugose wood associated viruses using the published primers of; Osman and Rowhani, 2006 (GLRaV-1); Bertazzon and Angelini, 2004 (GLRaV-2), Goszczynski, 2013 (GLRaV-3), Abou Ghanem-Sabanadzovic et al., 2012 (GLRaV-4 like); Al Rwahnih et al., 2012 (GLRaV-7), Dovas and Katis, 2003 (Viti and Foveaviruses), de Meyer, 2000 (GVA), and systems developed during this study for GVB, GVD and GVE

Amplicons for Illumine Miseq sequencing were selected from vine samples where both the scion and rootstock were positive in GLRaV-3 PCR, or for a number of samples yielding amplicons in the Viti-/foveaviruses PCR. CLC Genomics Workbench 6 (Aarhus) was used to carry out all trimming and analyses of the Illumina MiSeq data sets. For GLRaV-3 variant analysis the Illumina reads of each sample were reference mapped to the cognate region of Hel2F/Hel2R (Goszczynski, 2013) sequences of GLRaV-3 variant representatives.

RESULTS AND DISCUSSION

Rootstock suckers are generally pruned away in commercial viticulture and we were only able to find sufficient specimens of various scion combinations with Richter 99 rootstock (R99, *V. berlandieri* X *V. rupestris*). We confirmed the poor detection of GLRaV-3 with a broad spectrum GLRaV-3 PCR in R99 in 76 individual vines tested (Figure 1). We could find no clear evidence of selection of specific GLRaV-3 variants in those rootstocks which were infected by this virus. The possibility that unreported, heterogeneous, and hence poorly detected strains of GLRaV-3 may account for the poor detection of this virus in this tissue was therefore discounted. The possibility that the limited numbers of GLRaV-3 sources detected in R99 are resistance-breaking sources requires future investigation. Members of the Viti-Foveavirus genera were readily detected in 46 of 75 R99 tested, but were present in a further 24 scions but not the corresponding R99 rootstocks. The high yields of amplicon of Viti-Foveaviruses by PCR allowed us to discount the possibility that R99 contained inhibitors that may affect PCR. We

were able to show that GLRaV-1 and GLRaV-2, *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus E* (GVE) and *Grapevine rupestris stem pitting associated virus* (GRSPaV) were all capable of infecting R99.

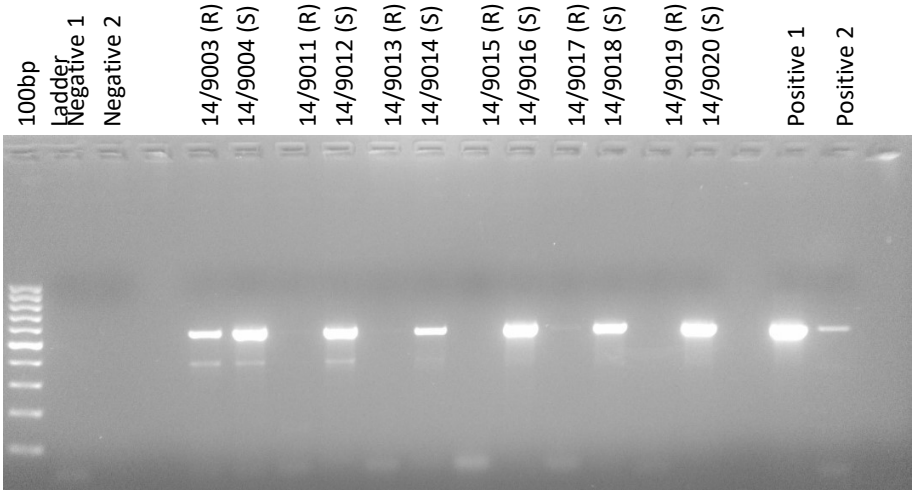

Figure 1: Representative gel of a subset of scion/rootstock samples from single vine to illustrate differences in amplification obtained when testing the rootstocks (R) and the scion (S) for GLRaV-3. The expected size of the product is about 560bp. Negative controls 1 and 2 represent "no-template" controls while Positive 1 is a control containing RNA from a known GLRaV-3 infected scion and Positive 2 is a cognate GLRaV-3 amplicon template.

ACKNOWLEDGEMENTS

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O8: Improvement of double-stranded immunocapture for grapevine virus enrichment

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INTRODUCTION

High throughput sequencing is a powerful tool for virus discovery but its usage seems disproportionate to the tiny virus genome size. In order to take full advantage of this technology, a viral enrichment method is used to increase the viral to plant sequence ratio. Double-stranded RNA (dsRNA) is the enrichment method we selected, using antibodies as a bait and magnetic beads as a support. The method has proven very effective for the detection of highly replicating viruses, mostly in herbaceous plants (Blouin et al. 2016), however when applied to grapevines it lacked sensitivity, with the majority of the sequence obtained being of plant origin, in particular matching the ribosomal RNA (rRNA). The rRNA is highly structured and forms large numbers of potential targets for the dsRNA antibodies used in the dsRNA capture experiment. We assessed the effect of different buffers and clarification processes, as well as the addition of ribonucleases on the capability to detect viruses and rRNA from grapevine tissue.

MATERIALS AND METHODS

Leaf tissue was selected from potted plants with known infection from *Grapevine leafroll-associated virus* 3 (GLRaV-3) group I. Leaf samples were harvested (central part of the leaves and about 1 cm of petiole), chopped into small pieces (< 5 mm), then mixed and divided equally into 21 bags before being dehydrated. The wet weight of each sample was 2 g fresh tissue. This experiment was divided into seven treatments, each made of three replicates. The dsRNA capture protocol was constant across treatments, with 50 µg of protein L magnetic beads (Thermo Scientific PierceTM) coated with 200 µL of hybridoma supernatant (2G4 or 3G1) per sample as previously described (Blouin 2016). The buffers tested were Tris-buffered saline with tween (TBSt : 25mM Tris, 150 mM NaCl and 0.05% tween) or CTAB buffer, as described by White et al. (2008) (2 % CTAB; 2 % PVP K-40; 25 mM EDTA; 100 mM Tris-HCl (pH 8.0); 2 M NaCl; 0.5 g/L spermidine). The buffer, antibody and protocol used in each of the seven treatments are described in Table 1. The recovered beads were resuspended in water and a reverse transcriptase was performed using the Tetro Reverse Transcriptase (Bioline). The virus and rRNA concentration was measured by hydrolysis probes in a duplex reaction using PerfeCTa Multiplex qPCR ToughMix (Quanta bio) on an EcoStudy (Illumina).

Table 1: Different protocols used for the seven treatment. All treatments were made of three replicates of 2 g of grapevine leaf tissue infected with *Grapevine leafroll-associated virus* 3 (GLRaV-3).

Treatment	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Extraction buffer	TBSt		TBSt + 2% PVP + 50 mM Na_2SO_3	CTAB + 50 mM Na_2SO_3			
Incubation	X		+ 1mL 20% SDS per 20 mL extract → 20 min at 65ºC + 5mL 5M KAc per 20 mL extract → 20 min on ice				
Clarification	8,500 g for 15 min at 4ºC and filtered						
Nucleic acids precipitation	X		+ 0.8V isopropanol followed → centrifugation at 20,000 g for 20 min at 4ºC. Pellet washed with 70% EtOH and resuspended in 10 mL TBS				
RNAse treatment	X		X		125 U RNAseT1	550 U RNAseT1	500 U RNAseT1
					→ 37ºC 30 min		
Antibodies	2G4	3G1	2G4				
Incubation	1 hour @ 37ºC						

RESULTS AND DISCUSSION

The results showed no real difference between the two mAb lines (treatment A and B) but a drastic effect of the buffers and process was observed (Figure 1). The addition of 2% PVP improved significantly the detection of viral dsRNA, with a detection of GLRaV-3 about 15 cycles earlier in treatment C versus A or B. The difference between buffers (treatment C: TBST or treatment D: CTAB) was marginal when the antioxidant and PVP were added (PVP is included in CTAB buffer), but the extract clarification and the precipitation of the nucleic acids, as described in Tzanetakis and Martin (2008), helped to enhance the viral dsRNA capture. These modifications increased the consistency of the bead recovery. Overall, the CTAB buffer was the preferred buffer for its reliability, and because it yielded smaller and cleaner pellets after the isopropanol precipitation, providing the possibility to resuspend in smaller volumes and work in a microfuge tube from that step. The pellet can even be resuspended in 1 mL without loss simplifying even more the ensuing steps (data not shown). The level of rRNA recovered was also increased by the modified treatment (C and D), but the addition of RNAse T1 (ssRNA specific) helped reduce the rRNA by more than 10 cycles. The increased concentration of RNase T1 (F and G) did not further decrease the rRNA concentration, suggesting that the remaining rRNA was highly structured.

The total extraction, from homogenisation to the recovery of the beads, was completed in less than 6 hours and did not require the use of solvents. The method was developed for virus discovery and large ecological surveys of grapevines by high throughput sequencing (HTS). Preliminary results show a much improved enrichment for viral reads (up to 73% of the total reads).

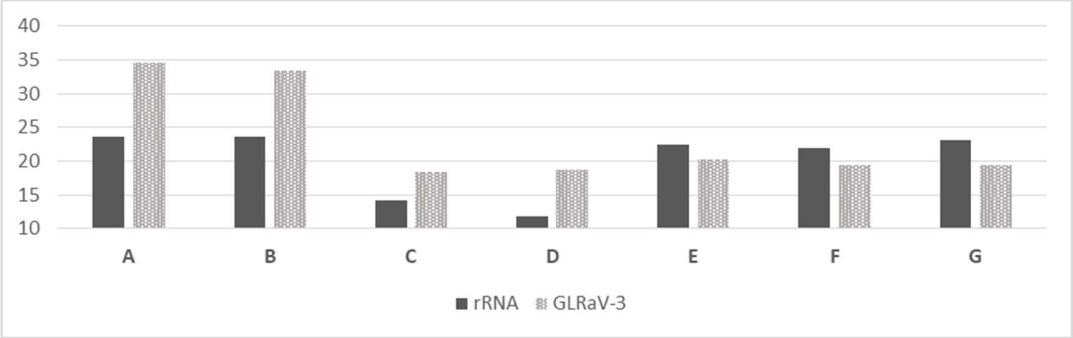

Figure 1: Effect of the different protocols on the yield of ribosomal RNA (rRNA; black) and *Grapevine leafroll-associated virus* 3 (GLRaV-3; grey) measured by Taqman assay and expressed in Ct values from leaf samples of *Vitis vinifera*. Each treatment comprises of 3 x 2g replicates and two technical replicates. Treatments are described in table 1.

ACKNOWLEDGEMENTS

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O9: Nanobody-based reagents as diagnostic tools for Grapevine viruses

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INTRODUCTION

With more than 70 different viruses species identified so far, grapevine (*Vitis spp*) is the crop with the highest number of infecting viruses (Martelli, 2014). Although the pathogenicity of all of these viruses has not been established, a number of them are considered as severe grapevine pathogens such as the emerging *Grapevine red blotch virus* or *Grapevine Pinot gris virus* in addition to the well described viruses responsible for rugose wood, leafroll- and fanleaf degeneration-diseases (Basso et al., 2017; Maliogka et al., 2015). These viruses cause substantial crop losses, reduce berry quality and shorten the longevity of grapevines, hampering for the soil-borne nepoviruses infesting high-value vineyards, the cultivation of grapevines. No germplasm resistance to these viruses has been reported so far in *Vitis* species. However, while many efforts are being made, no effective and economically acceptable solution to eradicate or efficiently limit the disease is yet established. One of the most efficient approaches to limit the spread of the virus is the release by the nurseries of virus-free grapevine material through systematic and reliable certification schemes.

The certification of propagative material is mainly based on double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using immunochemical reagents derived from polyclonal or monoclonal antibodies. Their production can be expensive and time-consuming, requiring specific structures and skills, and the availability and quality of antibodies produced can be prone to variations in performance. These classical ELISA reagents could favorably be replaced by Nanobodies (Nbs). Nbs are small peptides derived from heavy-chain-only antibodies found in camelids (Fig.1) (Muyldermans, 2013). They are the smallest naturally occurring intact antigen-binding domains known to date. They are monovalent, stable, soluble, and recognize cryptic epitopes inaccessible to common antibodies. They can be easily tailored and produced to almost unlimited amounts in bacteria such as *E. coli*. We recently also established that Nb possx ess antiviral activity by showing that constitutive expression of Nanobodies directed against *Grapevine fanleaf virus* (GFLV) conferred resistance to the cognate virus in *N. benthamiana* and grapevine rootstocks (Hemmer et al., 2017).

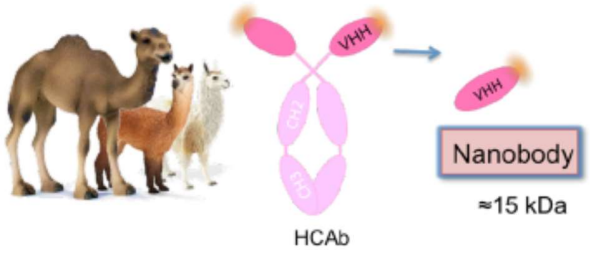


Fig.1 Nanobodies derived from heavy-chain-only antibodies.

MATERIALS AND METHODS

Virus isolate and virus purification: *Strawberry latent ringspot virus* (SLRSV) -T29 was originally isolated from naturally infected grapevines and maintained by mechanical inoculation on *Chenopodium quinoa* or *N. benthamiana*. The viral particles were purified from infected *C. quinoa* and purified using standard nepovirus purification procedure consisting of clarification and sucrose gradients.

Nanobodies production: *Camelidae* were immunized with purified virus particles at weekly intervals for 6 weeks. SLRSV specific single domain antibodies (Nbs) or (VHH) were generated according to Ghassabeh *et al.,* 2010. The resulting VHH libraries were screened by phage display for virus-specific binders against SLRSV purified virions. Nbs were tailored with appropriate tags (ie His6 tag, alkaline phosphatase or fluorescent proteins) using standard molecular biology protocols. Large-scale production of Nbs was performed by expression in *E. coli* and soluble Nbs further purified by affinity and size exclusion chromatography.

DAS-ELISA assessment of Nbs reactivity: Virus detection was performed from SLRSV infected grapevine extracts by DAS-ELISA using anti-SLRSV IgG as capture/trapping antibodies and the tagged Nbs as detection antibodies. For the coating step, tailored Nbs were used as capture/trapping antibodies and anti-SLRSV IgG or tailored Nbs as detection antibodies. Negative control consisted of healthy plants.

RESULTS AND DISCUSSION

Two Nanobodies (Nb 1.1 and Nb 1.8) belonging to two different families, able to recognize SLRSV-T29, were identified from the screening step. To evaluate their ability to detect SLRSV in plant crude extracts, the two anti-SLRSV Nbs were tagged either with fluorescent protein or alkaline phosphatase. The tailored Nbs were successfully produced in *E. coli* and semi-purified (Fig. 2)

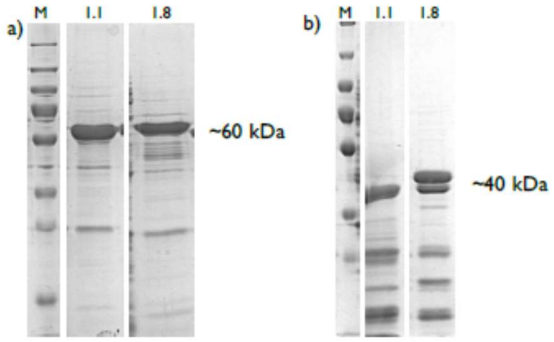
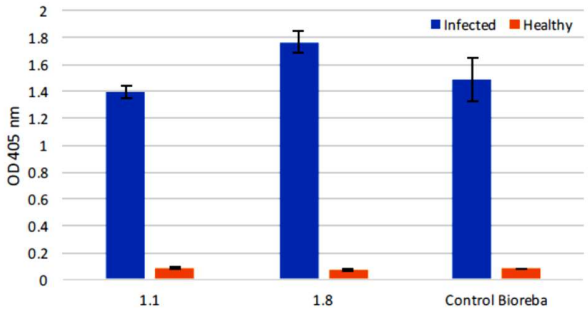


Figure. 2 SDS-PAGE analysis of the semi-purified Nanobodies tagged with alkaline phosphatase (a) and fluorescent protein (b). 1 µg from each tailored Nb after the purification process was separated by SDS-PAGE and stained with Coomassie blue. M: Ladder

The SLRSV Nanobody-based reagents were able to detect the virus from a solution containing purified particles and from infected *Ch. quinoa* and blackberry crude extracts (Fig. 3). They performed similarly to the commercial antibodies or even slightly better.

Figure 3: DAS-ELISA on SLRSV infected blackberry.



Further competition experiments were performed in order to see if the two Nbs recognize different epitopes. Even though the two Nbs have two completely different CDR3, our experimental data show that they partially share a common epitope. Finally, the anti-SLRSV Nbs were tested on the SLRSV-infected plant collections of the INRA Colmar, Agroscope and BIOREBA.

A similar approach was initiated to develop Nanobody-based reagents for the detection of *Raspberry ringspot virus* (RpRSV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll-associated virus* 1 and 3 (GLRaV-1 and GLRaV-3), and *Grapevine Pinot gris virus* (GPGV), which are associated to major grapevine virus diseases. The performance of DAS-ELISA tests using Nanobody-based reagents for other major grapevine viruses detection from leaves and woody grapevine material will be presented.

Table 1: Recognition spectrum of Nb 1.1 and Nb 1.8 mixed together and Nb 1.1 and Nb 1.8.individually in comparison to anti-SLRSV conventional antibodies. Green and red colors correspond to positive and negative SLRSV detection, respectively. "+" relates to detection levels. Note that Nb 1.1 and Nb 1.8 do not recognize the peach isolates. Nb 1.8 performs better than Nb 1.1.

Plant	Grapevine			Ch. quinoa		Blackberry	Peach
Isolate	34198+34199	38004	T35 Sylvaner	1005 (peach)	997 (blackberry)	11092	27862
ELISA	++	+	+	++	++	++	+++
Nb 1.1	+	-	-	-	+++	++	-
Nb 1.8	++	+	++	-	++++	++++	-
Nb Mix	+	+	+	-	+++	++	-

ACKNOWLEDGMENTS

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O10: Rapid and non-destructive detection of Shiraz disease and grapevine leafroll disease on asymptomatic grapevines in Australian vineyards

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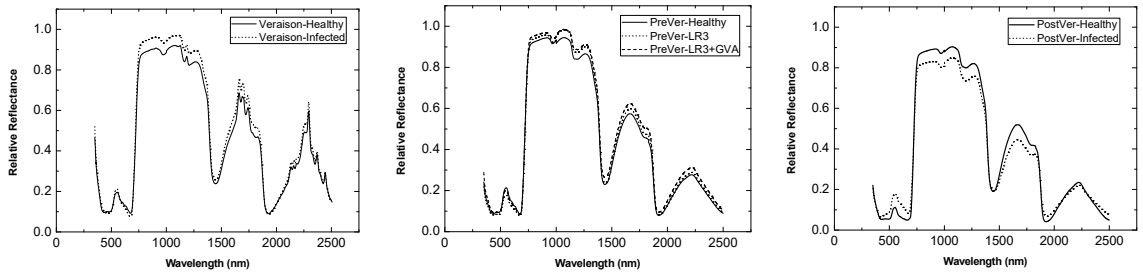
INTRODUCTION

*Grapevine leafroll-associated virus*es 1 and 3 (GLRaV-1, -3) that are associated with Grapevine Leafroll Disease (GLD) as well as *Grapevine virus A* (GVA) associated with Shiraz Disease (SD) are amongst the most widespread grapevine viruses in Australian vineyards (Habili *et al.* 1995; Habili 2013). GLD is one of the most important diseases affecting grapevine cultivars and rootstocks worldwide (Maree *et al.* 2013; Naidu *et al.* 2015) and has been reported to result in yield losses of up to 40% in Australian vineyards (Habili and Nutter 1997) costing growers between $300 and $2,400 per hectare (Nimmo-Bell 2006; Freeborough and Burger 2008; Atallah *et al.* 2012). Current approaches to detect GLD and SD include visual (symptom-based), and serological (enzyme-linked immunosorbent assay; ELISA; for GLD only) and molecular (reverse-transcription polymerase chain reaction; RT-PCR) assays. With the advent of infrared imaging spectrometers (hyperspectral sensors) that measure hundreds up to thousands of narrow band reflectance wavelengths from leaves simultaneously, it is now feasible to rapidly and non-destructively detect grapevine viruses such as GLRaV-3 (Naidu *et al.* 2009), including from airborne platforms for virus surveillance of vineyards (MacDonald *et al.* 2016). Consistent differences between the spectra of virus-infected compared to healthy leaves of grapevines allows this technique to be utilised for non-destructive detection of viruses and other pathogens. Here, we report on the use of this emerging technique for the detection of GLD and GVA in field-grown Pinot Noir and Shiraz grapevines in South Australia. One white-berried cultivar, Riesling, infected with GVA was also tested in this study.

MATERIALS AND METHODS

Three vineyard blocks of *Vitis vinifera* L. cvs. Pinot noir, Shiraz (syn. Syrah), and Riesling infected with GLRaV-3 (Pinot noir), GVA (Riesling), and a co-infection of GLRaV-3 and GVA (Shiraz) were used for this study in the 2015-16 and 2016-17 growing seasons. The presence of GLRaV-3 and GVA were detected using ELISA and PCR tests, respectively; the two red-berried vines also showed visible signs of the diseases following véraison. Both pre-veraison and post-veraison, asymptomatic (i.e. green) leaves from the same (infected) vines, as well as several vines that were confirmed healthy, were measured with a portable field spectrometer (FieldSpec 3, ASD, Boulder, CO, USA) following calibration to obtain hyperspectral reflectance measurements of the adaxial surface. Each measurement produced a reflectance spectrum ranging from 350 nm to 2500 nm. 10 leaves per vine and a minimum of 20 healthy or infected vines were measured. Data was analysed using a classification methodology based on a partial least squares discriminant analysis (PLS-DA) model using PLS_Toolbox chemometrics software (Eigenvector Research Inc., Manson, WA, USA). A subset of 20 healthy and infected leaves per cultivar whose data was excluded from the PLS-DA calibration model were used to test the model for accuracy.

RESULTS AND DISCUSSION



(a) **(b)** **(c)**

Figure 1: Hyperspectral reflectance spectra of healthy (solid lines) and infected (dotted lines) leaves. **(a)** Pinot noir with GLD; **(b)** Shiraz with GLD (middle line), and GVA co-infected with GLD (top line); and, **(c)** Riesling with GVA.

The calibration model developed using PLS-DA captured 96%, 91% and 88% of the variance in the data using between 2-6 reflectance wavelengths of a total of 2150 wavelengths. Fig. 1 shows distinct differences between the reflectance spectra of healthy versus infected leaves in Pinot noir infected with GLD (Fig. 1a), Shiraz co-infected with GVA and GLD (Fig. 1b), and Riesling infected with GVA (Fig. 1c). The spectral differences are conserved across all the leaves measured in the study (within a cultivar), and all of the spectra have some notable features, in particular the sharp absorbance peaks around 700 nm, 1450 nm, and 1950 nm, which correspond to leaf water absorbance. For GLD-infected Pinot noir, the spectral differences between infected and healthy are notably in the wavelengths between 800-1200 nm, around 1600 nm, and around 2250 nm; for Shiraz infected with GVA and GLD, the wavelengths around 1100-1300 nm, 1600 nm, and 2250 nm; and for Riesling infected with GVA, the spectral regions between 800-1300 nm and 1600-1750 nm. For the purposes of classification, several of these wavelengths were specifically chosen for the PLS-DA model to yield the highest accuracies or lowest root mean square error of calibration. For Pinot noir, the model had a prediction error of 22%, while for Shiraz and Riesling, the errors were 10% and 36%, respectively.

Our work represents the first study of non-destructive detection of GVA and GLD, including on co-infected vines, and using only asymptomatic leaves. We have also demonstrated that near- and mid-infrared spectroscopy can be a powerful tool to detect virus infections in white-berried grape cultivars that are usually asymptomatic. With additional calibration of the model, greater prediction accuracies can be achieved. Our findings open opportunities for conducting large spatial-scale virus surveillance of vineyards using manned or unmanned aerial vehicles equipped with hyperspectral cameras. This technology will allow for infected vines to be removed at the pre-symptomatic stages of growth, as well as in grapevine nurseries and vine improvement organisations to screen material prior to propagation.

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O11: Molecular characterization of newly detected viruses in Chilean vineyards.

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INTRODUCTION

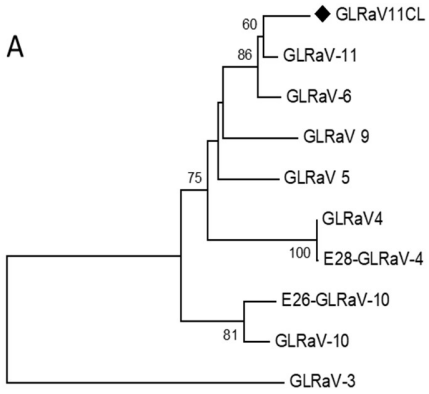
Grapevine viruses are responsible of different diseases in grapevine that are related great losses around the world. The dissemination of theses pathogens is mainly associated to the use of infected plant material for propagation, therefore, the constant surveillance of the vineyards is the main strategy for controlling viral diseases. During the last ten years, several studies have been carried out in Chile in order to know which are the more prevalent viruses affecting grapevine (Fiore et al., 2008; Fiore et al, 2011). Those studies considered the most widespread viruses but did not consider the recently detected viruses, such as *Grapevine Pinot gris virus* (GPGV), *Grapevine red blotch virus* (GRBV), *Grapevine rupestris vein feathering virus* (GRVFV), *Temperate fruit decay-associated virus* (TFDaV) and *Grapevine leafroll-associated virus-4* (GLRaV-4) variants. Thus, the main goal of this study was to detect these viruses in Chilean grapevines.

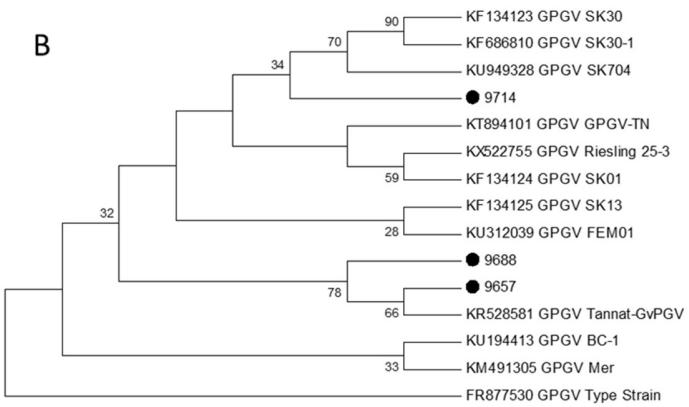
MATERIALS AND METHODS

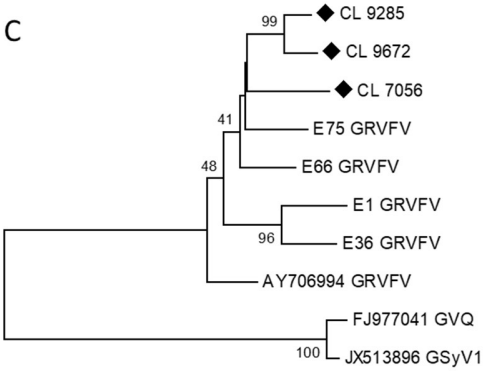
500 samples of different cultivars of grapevine (table grape and wine production), were sampled using mature lignified canes and total nucleic acids were extracted using silica capture method (Mackenzie et al., 1997, Malinowski 1997). Detection of GPGV, GRBV, GRVFV, TFDaV and GLRaV-4-variants was performed using previously described primers (Angelini et al., 2016; Al Rwahnih et al., 2013; Al Rwahnih et al., 2009; Basso et al., 2015; Fiore et al., 2016). Positive samples were cloned and three colonies were sequenced in MacrogenUSA Corp (Rockville, MD). Obtained sequences were assembled and aligned using Bioedit (Hall 1999) and phylogenetic trees were constructed using neighbor joining algorithm (bootstrap of 500 rep) in MEGA v 7.0 (Kumar et al., 2016)

RESULTS AND DISCUSSION

As expected, the rates of detection of the analyzed viruses were low. Particularly TFDaV and GLRaV-7 were not detected in any samples. GRVFV was the more prevalent virus in this survey, being detected in 35 samples, from different cultivars for wine production. There was no association of symptoms with the presence of this virus. One sample of Cabernet sauvignon that was affected by a severe reddening and leafrolling was found to be positive to a GLRaV-4 variant. BLAST nucleotide comparison gave an 87% of identity with a GLRaV-11 isolate from Greece (AM494935). GPGV was detected in 10 samples, all of them of the same clone, Grenache 136. Phylogenetic reconstruction for each virus is shown in figure 1A (GLRaV-4v), 1B (GPGV) and 1C (GRVFV)







A clear association of GLRaV-4-variant De (Acc. number AM494935) with GLRaV-11CL is observed in Figure 1A. This clustering, together with the nucleotide identity indicates the presence of a new variant of GLRaV-4 detected in Chile. Regarding GRVFV, there is a close association of Chilean isolates with those isolates detected in Spain (Fiore et al., 2016). GPGV isolates were clustered together with asymptomatic isolates according to Saldarelli et al., 2015, but according to our registry, plant infected with isolate 9688 showed leaf deformation and yellow mottles in young leaves, but we cannot discard the association of these symptoms with other pathogen affecting the plant. Regarding the importance of this finding, Agriculture and Livestock Service of the Chilean Government (Servicio agrícola y Ganadero, SAG, Gobierno de Chile) are working in the control and elimination of the plants that are infected or can be infected by this virus, considering that the hosts are restricted to the mentioned clone.
This work represents the first record in Chile of *Grapevine Pinot gris virus* and *Grapevine rupestris vein feathering virus* and is the fourth report of a *Grapevine leafroll-associated virus-4* variant (Escobar et al., 2008; Engel et al., 2008; Engel et al., 2010). Further studies are in progress in order to determine with a higher support, the phylogeny of Chilean isolates of the newly reported viruses.

ACKNOWLEDGEMENTS
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O12: Validation and harmonization of diagnostic methods for the detection of *Grapevine Pinot gris virus* (GPGV)

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INTRODUCTION

Grapevine Pinot gris virus (GPGV) has been originally found in Northern Italy associated to symptoms of stunting, chlorotic mottling and leaf deformations in grapevine plants. Successively, its presence has been reported in many countries even on asymptomatic plants (Saldarelli *et al.,* 2017). The study of this virus with regard to its biological and molecular characterization, its etiological role and spread by Eryophid mites vectors *Colomerus vitis*, the symptoms on different grapevine varieties cannot disregard the use of reliable, robust and validated diagnostic methods to implement possible prevention strategies. To this aim, a test performance study (TPS) was carried out to evaluate, harmonize and validate four diagnostic protocols used in the detection of GPGV in symptomatic and asymptomatic grapevine material. Specifically, two end point RT-PCR (Glasa *et.al.* 2014 - protocol 1; Saldarelli *et al*. 2015 - protocol 2) and two real time RT-PCR (Bianchi *et al*. 2015 - protocol 3; Ratti personal communication 2015- protocol 4) protocols have been compared. Obtained performance data from the four molecular protocols are reported and evaluated in the present Abstract.

MATERIALS AND METHODS

A total of 44 vine samples, comprising *V. vinifera* and rootstock species, of which 25 target (positive to GPGV) and 19 non target (13 virus free and 6 infected by GFLV, ArMV, GLRaV 1, 2 and 3, GVA, GVB and GFkV in single and/or mixed infection) were used for the TPS, preliminary tests and to define analytical sensitivity and specificity. Based on preliminary analyses 6 samples, showing a relatively low virus concentration were chosen.
Total RNAs were extracted from woody plant material using different grinding system (Nitrogen or mechanic) and commercial extraction kits (Qiagen and Sigma). All the isolates had been previously tested by real time RT-PCR, RT-PCR and biological tests to assess their sanitary status.
Tests were performed in 16 laboratories using the same samples (analyzed in blind conditions) and reagents; the same threshold calcolation method (Mhele *et al*., 2013) for real time RT-PCR and by analyzing the electrophoretic gels for the RT-PCR was used in each laboratory to analyze data.
The tests were performed in 16 laboratories using the same samples (analyzed in blind conditions) and reagents; in each laboratory the results have been obtained by a threshold calculation method, for the real time RT-PCR, (Mhele et al., 2013) and by analyzing the electrophoresis gels for the end point RT-PCR.
Analytical sensibility was evaluated either by analysis of 4 dilutions of the total RNAs extract and samples containing 1000, 500, 250, 50 e 12,5 copies of a plasmid DNA recombinant for the DNA amplicon targeted in each protocol. Repeatability was evaluated by three time test repetition for all samples in the same condition.
The processing of the obtained results (about 24,000 data points) has led to the definition of the validation parameters according to UNI/EN/ISO 16140 and 17025 and EPPO standards PM7/76 and PM7/98

RESULTS AND DISCUSSION

As reported in Table 1, both diagnostic methods, (end point and real time RT-PCR) were proven to be highly effective, although analytical sensitivity was more efficient for real time protocols, as expected. No statistically significant differences were observed for the other validation parameters. Moreover, all protocols gave satisfactory results even if six samples with low virus concentration were selected to stress the tests. Despite the large number of laboratories that performed the TPS and the different instruments and extraction methods (Nitrogen or different mechanic grinding system), no statistically significant differences were observed in the analytical specificity using the non target samples infected by other viruses than GPGV. All protocols showed good accuracy and robustness as proven using a large number of samples in a high number of laboratories.
In conclusion, harmonized and validated reference protocols for the diagnosis of GPGV are, for the first time, available.
Detailed protocols are available on line in CREA web site at the following link:
(http://sito.entecra.it/portale/cra_manuali_dettaglio.php?id_manuale=23504&lingua=IT)

Diagnostic method	*end point RT-PCR Protocol 1*	*end point RT-PCR Protocol 2*	*realtime RT-PCR Protocol 3*	*realtime RT-PCR Protocol 4*
Analytical sensitivity	10^{-3} (250 copies)	10^{-2} (250 copies)	10^{-4} (12,5 copies)	10^{-4} (12,5 copies)
Analytical Specificity	no cross reaction to 8 untarget viruses	no cross reaction to 8 untarget viruses	no cross reaction to 8 untarget viruses	no cross reaction to 8 untarget viruses
Sensitivity	77%	75%	85%	85%
Specificity	100%	93%	99%	99%
Accuracy	89%	84%	92%	92%
Repeatability	97%	92%	97%	100%
Reproducibility	96%	94%	99%	99%

Table 1. Summary of validation parameters obtained by the end point RT-PCR and the real time RT-PCR protocols in the GPGV TPS.

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O13: Simultaneous detection of the 13 viruses and 5 viroids affecting grapevine by molecular hybridization using a unique probe or 'polyprobe'

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INTRODUCTION

Grapevine is the most economically important fruit crop in the world, which is affected by various diseases of viral and/or viroidal etiology, which may affect the production of grapes with losses of up to 15% (Martelli, 1993). Traditionally, viral infection assays in grapevine have been based on the bioassay or on the ELISA serological technique. However, both techniques have distinct disadvantages derived from the space/time required, the inability to identify the pathogen (bioassays), the absence of antibodies against important pathogens or the inability to detect viroidal agents (e.g., ELISA). In recent years, the incorporation of detection techniques based on molecular components of pathogens (RT-PCR, real time PCR –TaqMan-, etc.) has significantly increased the detection limit but also the cost of the analysis. For this reason, trends in detection techniques have been focused on reducing the costs/time of the analysis by performing the simultaneous detection of several pathogens, allowing the analysis of up to 13 (low density array, TaqMan RT-PCR, Osman et al., 2008) or 44 (Microarrays; Engel et al., 2010) grapevine viral pathogens. However, the cost resulting from these methods is incompatible with large-scale surveys, one aspect to consider in cultures with many years of planting. In this sense, the technology based on the nonradioactive molecular hybridization is a fast, simple and reliable methodology for routine diagnosis of viruses and viroids. In our laboratories, we have developed a molecular nonradioactive hybridization for polyvalent detection of different viruses/viroids by using a single probe or 'polyprobe' containing, fused in tandem, the different viral/viroidal sequences (Herranz et al., 2005; Janet Zamora-Macorra et al., 2015). This methodology permits the simultaneous detection of different viruses/viroids in one test with limit detection similar to the highest obtained by ELISA (in the case of viruses). This technology has proved to be an efficient and cheap methodology for the detection of the main viruses and/or viroids affecting stone fruits (Herranz et al., 2005; Peiró et al., 2012), tomato (Aparicio et al., 2009) and citrus (Cohen et al., 2006). In the present work, we have developed a polyprobe with the capacity to detect 13 viruses and 5 viroids affecting grapevine plants.

MATERIALS AND METHODS

Infected plants with the different virus and viroids were subjected to total nucleic acids extraction (TNA) by the silica capture method (MacKenzie et al., 1997; Malinovski, 1997). RT-PCR reactions were performed using the TNA and the specific primers containing the 5′ and 3′ *Xho*I and *Sal*I restriction sites respectively. The following viruses and viroids were detected: *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 1*, *2*, *3, 4* (GLRaV-1, 2, 3, 4), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine fleck virus* (GFkV),*Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine rupestris vein feathering virus* (GRVFV), *Arabis mosaic virus* (ArMV), *Citrus exocortis viroid* (CEVd), *Grapevine yellow speckle viroid 1* (GYSVd-1), *Grapevine yellow speckle viroid 2* (GYSVd-2), *Hop stunt viroid* (HSVd), and *Australian grapevine viroid* (AGVd). In the case of GLRaV-4, were detected the variants 4, 5 and 6. The incorporation of the PCR fragments in the pKS + plasmid and the subsequent fusion in tandem, was performed by using the restriction sites *Xho*I-*Sal*I as described previously (Peiró et al., 2012).

RESULTS AND DISCUSSION

The use of riboprobes carrying partial sequences of different plant viruses and viroids fused in tandem, has permitted the simultaneous detection of up to ten different pathogens (twelve viruses and four viroids) using a non-radioactive molecular hybridization procedure (Peiró et al., 2012). In the present work we have generated three different polyprobes for the detection of the main viruses (13, Poly15), viroids (5, poly5) or both (poly18) affecting grapevine crops. The individual and the three polyprobes were able to detect up to 5-1 pg/µl of viral/viroidal RNA, comparable to other described probes (Sanchez-Navarro et al., 1999). The analysis of 142

grapevine samples revealed that all positives samples detected by using individual probes were also detected by using the corresponding polyprobe. The infection percentages were: GLRaV-1 (9.1%), GLRaV-2 (39.4%), GLRaV-3 (19.1%), GLRaV-4 variant 5 (8.4%) GLRaV-4 variant 6 (7.7%), GFLV (23.9%), GFkV (36.6%), ArMV (2.8%), GVA (12.7%), GVB (3.5%), GRSPaV (18.3%), GRVFV (92.2%), HSVd (100%), GYSVd-1/-2 (89.4%) y AGVd (0.7%). When the 142 samples were analyzed by the ELISA assay to detect GLRaV-1, GLRaV-3, GFLV, GFkV or ArMV, the infection percentages were similar (GLRaV-1: 9.1%; GLRaV-3: 19.1%), higher (ArMV: 4.2%) or lower (GFLV: 23.2%; GFkV: 31.7%) to that obtained by using the molecular hybridization technique. To our knowledge, this is the first polyprobe described with the capacity to detect eighteen different pathogens.

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O14: Genetic diversity and identification of putative recombination events in *Grapevine rupestris stem pitting-associated virus*

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) is a ubiquitous virus commonly detected in cultivated vines and reportedly associated with Rupestris stem pitting disorder (Maliogka et al., 2015). Currently, six distinct molecular variant groups (I, IIa, IIb, IIc, III and IV) of GRSPaV are recognised (Hu et al., 2015), each associated with variable symptoms expressed in different cultivars (Meng and Rowhani, 2017). To investigate the impact of recombination and the use of different genomic regions on variant classification, three surveys were conducted, in which a phylogenetic approach was used to classify and compare the genetic diversity of GRSPaV on a global and local level.

MATERIALS AND METHODS

Source material: To represent global diversity, a survey (NV) was performed on 59 accessions from various countries collected prior to phytosanitary regulation. A second survey (MB) was carried out on 176 plants in local mother blocks that previously conformed to certification requirements for South Africa. The final survey (OV) comprised 117 individual plants in South African vineyards established prior to the implementation of current sanitary protocols. Two cetyltrimethylammonium bromide methods (Carra et al., 2007; White et al., 2008) were used to isolate RNA from collected material.

Detection, cloning and sequencing: Samples were screened for the presence of GRSPaV. A 928 bp region encompassing the coat protein gene (*CPreg)*, and a 1668 bp area within the replicase gene (*pREP*) were amplified from positive samples. PCR products were purified, cloned, and a minimum of three positive clones for each region per sample were sequenced.

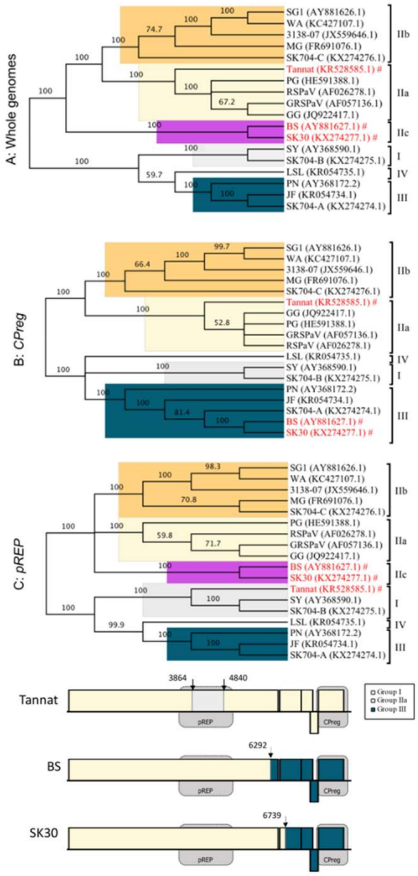
Recombination and phylogenetic analyses: GRSPaV whole genome sequences obtained from GenBank were used as references for phylogenetic groups (Glasa et al., 2017; Hu et al., 2015). Recombination and phylogenetic analyses were conducted as described previously (Glasa et al., 2017) on a whole genome alignment and two additional alignments containing only the regions of reference sequences corresponding to *CPreg* and *pREP*. Recombination analyses of survey samples were conducted in RDP4 V8.20 (Martin et al., 2015), recombinant sequences were removed and phylogenetic analyses were performed using RAxML Black Box V9.2.10 (Stamatakis, 2014).

RESULTS AND DISCUSSION

GRSPaV was detected in 72%, 29% and 6% of the samples from the NV, OV and MB surveys, respectively. GRSPaV was more prevalent in vineyards established prior to the implementation of current sanitary measures. The increased prevalence of GRSPaV in the NV survey compared to that in OV may be attributed to the diverse origins of NV source material. Furthermore, plants from the NV survey are maintained within the cultivar repository regardless of their sanitary status, whereas old vines had to remain economically viable throughout their existence. This indirect form of selection for healthy vines possibly contributed to the lower incidence of GRSPaV in older vineyards.Recombinant whole genome reference sequences correlated with inconsistencies between topologies generated by the two genome regions (Fig 1, 2). The two reference sequences for group IIc were both recombinants, belonging to group IIc based on their whole genomes and *pREP* area, but clustering within group III in *CPreg*-based trees. Similarly, isolates from survey samples of which the *pREP*-sequences belonged to group IIc, clustered within group III based on the sequence of their coat protein (Fig 3).

Recombinant sequences were detected in survey samples and removed prior to phylogenetic analyses. Between the two trees, all previously defined groups were distinguishable. Mixed infections were found to occur within single vines and the majority of isolates belonged to groups known to elicit little or no symptoms in *Vitis vinifera*. Furthermore, a distinct subgroup of lineage II, not represented by any of the reference sequences available, was observed and labelled subgroup IId. Increased knowledge of the recombination events within the GRSPaV genome could promote the development of a standardised method for variant classification and the clarification of the etiological role of the virus.

1



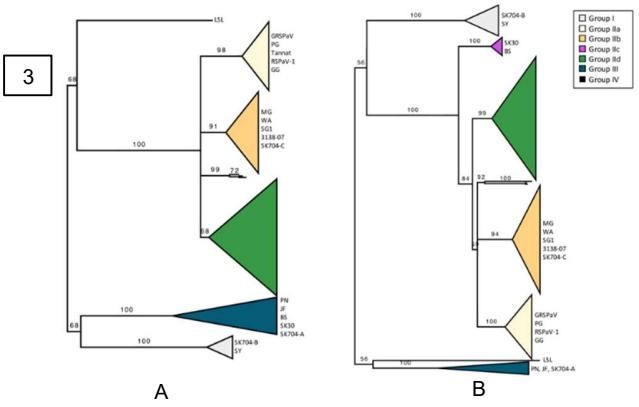
2

Fig 1: Phylogenetic trees constructed from GRSPaV (A) whole genome, (B) *CPreg*-trimmed and (C) *pREP*-trimmed reference sequences. Analyses were conducted as described previously (Glasa et al., 2017). Bootstrap values are displayed above nodes. Lineages of reference sequences are on the right of each figure. Recombinant sequences are indicated with a hash symbol (#).

Fig 2: Recombination detection results of whole genome reference sequences obtained from RDP4. Event numbers are given in brackets next to each recombinant sequence. Breakpoint positions are indicated by the black arrows.

Fig 3: Phylogenetic analysis of GRSPaV diversity based on (A) *pREP* and (B) *CPreg* alignments. In most cases, although no proof exists that fragments from the same sample originated from the same viral isolate and recombinant sequences were not taken into consideration, the classification of a collection of *CPreg* and *pREP* clones from a single sample, was consistent. Reference sequences of respective lineages within each group are indicated next to clusters, and lineage names are given at the right of each topology followed by the number of clones within the (NV, OV and MB) surveys that clustered within specific groups.

3



ACKNOWLEDGEMENTS

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O15: Occurrence of *Grapevine Roditis leaf discoloration-associated virus* in Mediterranean Commercial Vineyards in Turkey

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INTRODUCTION

Grapevine (*Vitis* spp.) is a major fruit crop with high socio-economic importance worldwide. Due to propagation vegetatively, its perennial life cycle, susceptibility to plant virus diseases and frequent exchanges of propagative material among countries contribute to spread these pathogens. Furthermore, vegetative propagation of grapevines and its perennial life cycle accelerate the mixing and introduction of several viral agents into a single plant providing the bear of complex diseases. Recently, newly emerged viruses having DNA genome has been identified in grapevine. *Grapevine Roditis leaf discoloration associated virus* (GRLDaV) was one of the virus newly emerged from Greece related to Roditis leaf discoloration disease in grapevine (Maliogka *et al.,* 2015). This DNA virus only reported from Greece, Italy and Turkey in the world (Chiumenti *et al*., 2015; Ulubaş Serçe *et al*., 2017). Since the presence in limited countries of the virus, we performed a research on the occurrence of GRLDaV in the Mediterranean Region commercial vineyards of Turkey, which the virus has been detected before.

MATERIALS AND METHODS

A total of 166 symptomatic grapevine plant samples collected from Adana, Antalya, Burdur, Isparta, Kahramanmaraş and Mersin provinces of the Mediterranean Region of Turkey, during in June to July and September of 2015-2016 (Figure1). Total RNAs were extracted using ZR RNA MiniPrep™ (Zymo Research, USA) and cDNA were synthesized by using EasyScript Plus™ cDNA Synthesis kit (Abm, Canada). PCR assays were carried out using the primer pairs of BadnUp-6262 5'- GAA AGA CGA ACC CTT CAT CAT GAA G -3' and BadnDo-6757 5'- CCC CAT CGA CAG CTC ACA AG -3' amplifying a 495 bp region of GRLDaV (Maliogka *et al.,* 2015). The primer pairs of BadnUp-6524/BadnDo-6932 (409 bp amplicon), cBadnUp-6733/cBadnDo-256 (512 bp amplicon) (Maliogka *et al*., 2015) and 11for-G/13rev-G (410 bp amplicon) (Chiumenti *et al*., 2015) were also used for cDNA amplification of GRLDaV infected samples. Several samples were also inoculated to *Nicotiana tabacum* cv. Xanthi and *Chenopodium quinoa* test plants using a phosphate buffer including 1% polyvinyl pyrolidone-40 and 1% sodium sulphite. The available sequences of GRLDaV at the GeneBank were used for the construction of a phylogenetic tree using neighbor joining method of MEGA7 software with the (Kumar et al., 2016).

RESULTS AND DISCUSSION

Out of nine among the all analyzed grapevine samples were detected as infected with GRLDaV and the infection rate was found 5.2%. The all infected grapevines were from Adana province from five different vineyards and included a local variety which was *Vitis vinifera* L. cv. Yalova Incisi. The observed symptoms on these infected grapevines were main and/or lateral vein clearing, yellowing as well as leaf deformations (Figure 1). GRLDaV infected grapevine leaves were inoculated to test plants and the symptoms were observed after 7 days of the inoculation. While the faint symptoms as vein swelling and thickening were observed on *N. tabacum* cv. Xanthi, the symptoms on *C. quinoa* were chlorotic mottling of the coming leaves and downward leaf curling (Figure 1). The test plants (both tobacco and quinoa plants) were also analyzed by PCR and GRLDaV specific 495 bp amplicons were observed on the gel, indicating the transmission of the virus to the test plants. The PCR amplifications of primer pairs of BadnUp-6524/BadnDo-6932, cBadnUp-6733/cBadnDo-256 and 11for-G/13rev-G were failed in repetitive attempts.

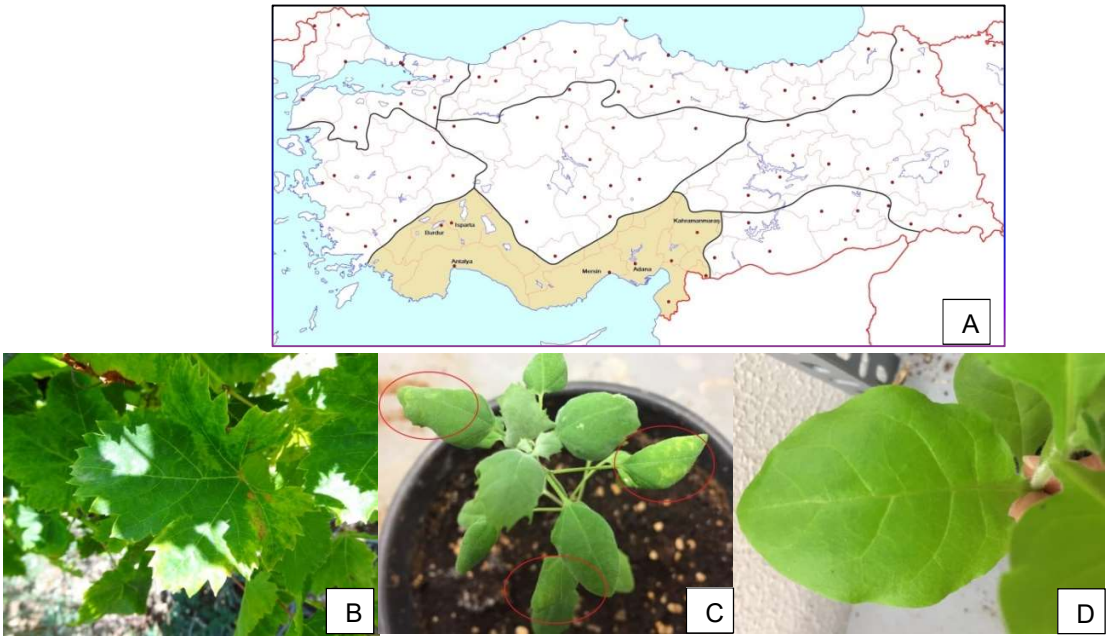

Figure 1. The Mediterranean Region and provinces in Turkey which grapevine samples were collected for the investigation of the occurrence of *Grapevine Roditis leaf discoloration associated virus* (A) and the virus detected symptomatic grapevine leaves (B). The chlorotic spot symptoms of GRLDaV infected grapevine inoculated *Chenopodium quinoa* (marked with circles) (C), and the swelling of veins on *Nicotiana benthamina* cv. Xanthi (D).

The phylogenetic tree of GRLDaV sequences revealed that the isolates from Turkey were more identical to each other than the Italian and Greece isolates according to hypothetical protein gene region of the genome (Figure 2).

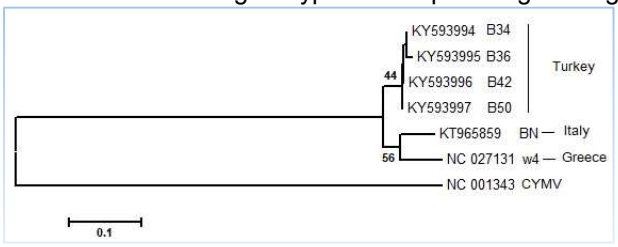

Figure 2. The neighbor joining tree (bootstrap 1000) of GRLDaV hypothetical protein gene sequences deposited in GeneBank. CYMV (*Commelina yellow mottle virus*) was used as an outgroup.

To our knowledge, the most of the GRLDaV detected vineyards located in Adana province have replaced with other fruit crops. So, even the ignorance of economic importance of the Roditis leaf discoloration disease, the growers preferred to destroy the vineyards because of the diseases. To understand the prevalence and importance of the virus the more research has been necessary.

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O16: Molecular characterization of divergent *Grapevine leafroll-associated virus* 3 isolates in California, USA

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INTRODUCTION

On a worldwide basis, *Grapevine leafroll-associated virus* 3 (GLRaV-3) is the most prevalent and economically destructive of the complex of several ssRNA virus species that are associated with grapevine leafroll disease (GLD). Early studies to determine GLRaV-3 genetic variation identified five subgroups based on phylogenetic analyses of several different genomic regions (reviewed by Burger et al. 2017). GLRaV-3 isolates that are more diverse and distantly related to variant groups I-V have been recently identified, leading to the formation of Groups VI and VII (Burger et al. 2017). Maree et al. (2015) identified eight monophyletic groups and four supergroups using GLRaV-3 sequence information available on Genbank (>602 nt). The presence of genetically diverse isolates has been well studied in the California North Coast region, especially Napa Valley, where members of Groups I-VI have been identified (Sharma et al. 2011). However, a survey of GLD symptomatic vines in other regions of the state identified grape vines that were negative for all known viruses associated with GLD, including GLRaV-3 (Sharma et al. 2015). One explanation for this result is the presence of genetic variants that cannot be amplified by the primers sets used in their RT-PCR assay. Our original GLRaV-3 RT-qPCR assay was designed to amplify all group I-V variants (Osman et al. 2007). We added an assay specific to GLRaV-3e (Sharma et al. 2011) after we determined that our original assay would not detect this group VI variant (unpublished). In this study, we tested GLD symptomatic vines from a Santa Barbara County vineyard using RT-qPCR and high throughput sequencing (HTS) to determine if additional GLRaV-3 genetic variants were present that could not be detected by our two current GLRaV-3 RT-qPCR assays.

MATERIALS AND METHODS

Three vineyard blocks (Chardonnay 4 or 5 scion; Freedom or Salt Creek rootstock) established in 1995-1996 in Santa Barbara County were selected for sampling. Five groups of four individually sampled vines were selected randomly along a “W” formation within each block. One individual sample consisted of four petioles, two from each cordon, collected near the fruiting zone (Arnold et al., 2017, Madden et al., 2007). Total RNA was extracted from petioles and tested by RT-qPCR as described in Al Rwahnih et al. (2016) with primers and probes specific to GLRaV-3 genetic variants: GLRaV-3 groups I-V (Osman et al. 2007); GLRaV-3e (Klaassen, unpublished). For HTS analysis, total nucleic acid (TNA) was prepared from leaf petioles or bark as described by Al Rwahnih et al. (2012). Aliquots of TNA samples from source trees were subjected to ribosomal RNA depletion and complementary DNA library construction using a TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina, San Diego, CA). Sequencing was performed on the Illumina NextSeq 500 platform. Samples were titrated to obtain a minimum of 25 million reads per sample and sequencing was performed using a single end 75bp regime. SPADES was used for deNovo assembly. For phylogenetic analysis, we focused on an interval containing the coat protein that was present in all sequences. Sequences were aligned using MUSCLE and a phylogenetic tree was constructed using PhyML. Groups were labeled I-VII as they appear in Maree et al. (2015). The ‘ungrouped’ accession, 43-15, appears as GLRaV-3 group ‘f’ in Sharma et al. (2011).

RESULTS AND DISCUSSION

Forty-seven of the 56 composite samples had Cq values of less than 30.0 using our GLRaV-3 (Group I-V) assay, indicating that they were GLRaV-3 positive. However, 32 samples had Cq values > 30.0 using our GLRaV-3e assay. While higher Cq values typically indicate a lower titer infection or contamination, mismatches in primer/probe binding sights can lead to a similar result. HTS analyses of five vines with higher Cq values identified the presence of a GLRaV-3 isolate in four samples that was 99% identical to NZ2, a highly divergent GLRaV-3 group VI isolate first identified in New Zealand. This is the first report of NZ2-like GLRaV-3 in the US.

In addition, a second divergent GLRaV-3 sequence that was not detected by our GLRaV-3 assays was identified in one of the four samples. It has high identity to the partial GLRaV-3f sequence first identified in Napa Valley (Sharma et al. 2011). GLRaV-3f sequences are only 75-80% similar to isolates in existing GLRaV-3 variant groups (Fig. 1).

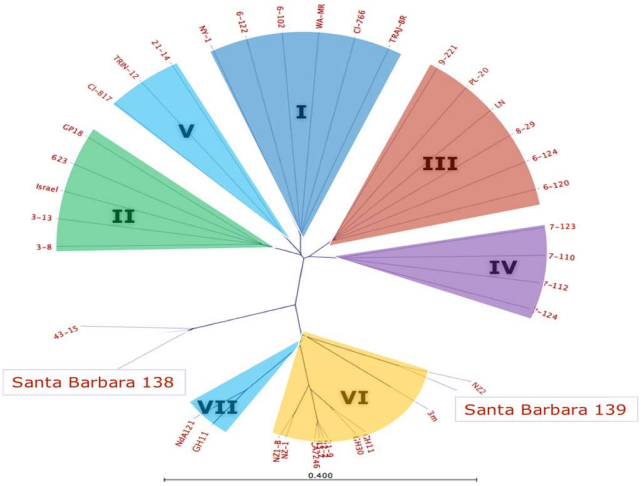

Fig. 1. Phylogenetic tree including the isolates reported here, labeled following the grouping convention of Maree et al. (2015). Whole genomes along with selected isolates from Genbank were included to define groups.

Santa Barbara 139 clusters with the NZ2 isolate that has been previously identified as related to group VI in Maree et al. 2015. Santa Barbara 138 clusters with the partial (428 nt) 43-15 isolate sequence (classified as GLRaV-3f) first identified in Napa Valley in Sharma et al. (2011).

Santa Barbara 138 and 43-15 are divergent enough that they would appear to define a new monophyletic group had they been included in the analysis of Maree et al. (2015). The results presented here support a conclusion of Sharma et al. (2011), that a single vineyard may contain highly diverse isolates of GLRaV-3. In addition, they highlight the need for research on genetic diversity and methods that improve our virus detection abilities. The HTS sequencing results are deposited in GenBank (KY764332, KY764333). Of particular importance is the nearly full-length isolate (Santa Barbara 138) that extends considerably what is known about the divergent GLRaV-3f variant.

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O17: Absence of *Grapevine red blotch virus* in Swiss vineyards

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INTRODUCTION

Grapevine red blotch is a recently identified viral disease that was first recognized as a disease in 2008, when foliar symptoms similar to leafroll were observed in the Napa Valley (California) on vines tested negative for known *Grapevine leafroll-associated virus*es. In 2012, *Grapevine red blotch virus* (GRBV) was discovered independently in California and New York and was later demonstrated to be the causal agent of what is now named red blotch disease (Fuchs et al., 2015).

After the discovery of the virus, surveys demonstrated a high prevalence of GRBV in all major grape-growing regions across the USA. Consequently, GRBV is presently recognized as an important economic threat to the US wine industry (Ricketts et al., 2017). Furthermore, GRBV is observed on native and wild *Vitis* spp. collected around infected vineyards, indicating that this virus is not only spread by viticultural practices (*i.e.*, vegetative propagation) but also naturally. Although significant advances have been made in understanding GRBV since its discovery in 2012, the epidemiology is poorly unveiled. Questions also remain about the origins of this emergent pathogen and its distribution outside North America. Here, we report the results from a large-scale survey that indicates that GRBV is not present in three main Swiss vine-growing regions.

MATERIALS AND METHODS

GRBV reference samples were collected from the Agroscope grapevine virus collection (Switzerland) (Gugerli et al., 2009). Samples for the surveys consisted of two petioles from basal leaves, collected from two separate canes of a vine. From 2014 to 2016 individual plants were sampled in commercial Swiss vineyards. Most of the sampled vines presented symptoms of reddening or yellowing, but asymptomatic vines were also randomly assessed. Additionally, 653 accessions from the Agroscope grapevine virus collection were tested in 2015.

DNA was extracted using a BioSprint semi-automated platform (Qiagen, Germany) according to the manufacturer's protocols. Purified DNA was screened for GRBV by polymerase chain reaction (PCR), using the primers Repfor and Reprev as published (Krenz et al., 2014). In addition to those primers for virus detection, primers NS7/NS8 specific for detecting a plant internal control (18S rDNA) were added to the multiplex reaction. Amplified DNA was separated on 1.5 % agarose gels and stained with ethidium bromide.

RESULTS AND DISCUSSION

In total, 3062 vines were tested from the three main grape-growing regions of Switzerland. The surveyed regions represented ca. 60 % (9,000 hectares) of vineyard area in Switzerland. All these samples tested negative for GRBV and internal control fragment was amplified in every reaction.

We monitored the presence of GRBV in the Agroscope grapevine virus collection.

We tested 653 accessions, dominantly infected by leafroll viruses collected in Swiss vineyards (447) but overall, originated from 19 different grape-growing countries (153 accessions from European origin and 53 accessions from overseas). Of these 653 accessions, only six, were infected by GRBV. All were of US origin introduced in our collection between 1985 and 2009. These introductions highlight that GBRV was already present in North America in the 1980's, although only recently identified. Therefore, GRBV has been overlooked for decades because of the lack of a specific detection method and by the symptom similarity to that of GLRaVs.

Importantly, the large-scale survey of this work demonstrated the absence of detectable GRBV in the three main Swiss grapevine-growing regions. Together with the survey in the Agroscope virus collection, these results suggest that the virus is not present in Swiss vineyards. Absence of spread of GRBV in the Agroscope grapevine virus collection for more than 30 years also points out the lack of vector or contact transmission. Thus, the epidemiological situation in Switzerland sharply contrasts with that in North America in which the virus is actively

spreading (Krenz et al., 2014), presumably transmitted through insect vectors. The cornered alfalfa treehopper *Spissistilus festinus* was recently shown to transmit the virus under experimental conditions (Bahder et al., 2016). However, *S. festinus* is a Nearctic species, thus probably absent from Europe. Because GRBV has not been reported yet in any European vineyards, the virus is on the alert list of the European and Mediterranean Plant Protection Organization (www.eppo.int/). In the absence of reports of GRBV infection in the Old World, one can speculate that GRBV moved from an indigenous wild host plant into grapevine sometime after the introduction in the 18th century of *Vitis vinifera* to North America.

In conclusion, GRBV was not detected in Swiss vineyards, and therefore, GRBV should be considered as a quarantine pathogen for Switzerland. As a precautionary measure, all accessions infected with GRBV were eliminated from our collection. To the best of our knowledge, this study is the first large-scale GRBV survey in vineyards outside North America. Further large-scale studies in other major wine-growing countries, particularly from the Old World, are necessary to evaluate more precisely the global distribution and provide further clues about the origins of this intriguing virus.

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O18: High-throughput sequencing in grapevine certification program in Spain: towards establishing a standard protocol

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INTRODUCTION

The presence of viruses in grapevine propagation material is subjected to intense regulation within the European Union (EU), aiming in minimizing the impact of the diseases produced by these viruses. At present, certification schemes for grapevine in the EU and in particular in Spain relies on biological indexing supported by other diagnostic tools such as ELISA and RT-qPCR. This protocol is generally effective and provides reliable diagnostics for the five viruses required in Spanish official certification: GLRaV-1, GLRaV-3, ArMV, GFLV and GFkV (this last only in rootstocks). The protocol, however, has some important drawbacks: requires a long-term symptom inspection (up to three years), a well-trained staff on symptom identification, costly expenses on a diagnostic field for indexing, trained people in grafting and plant management, and often results are dubious when plants show virus symptoms but there is no detection by ELISA or RT-qPCR. For example, leafroll symptoms can be induced by a leafroll-associated virus different from GLRaV-1 or -3, the only sanctioned in EU regulations. In the last years, we have been using high-throughput sequencing (HTS) to detect viruses (and viroids) present in the grapevine material submitted for certification in Spain. It allows us to compare conventional diagnostics and HTS. A cost-effectiveness analysis of HTS versus the combination of the standard methodologies for grapevine certification shows that they are in the same range. Additionally, HTS provides more valuable information on disease etiology and timely results. Finally, we claim for the development of a standard protocol for HTS in grapevine certification.

MATERIALS AND METHODS

A set of 20 samples was analyzed for virus presence by four methodologies: ELISA, RT-qPCR and Indexing performed at IMIDA (the official center for grapevine certification in Spain) following standard protocols and, in addition, HTS. Samples were dormant canes from grapevine varieties or clones provided by nurseries and public centers from all over Spain in order to be certified. Indexing consisted in bud grafting five plants each of Cabernet Sauvignon and Rupestris du Lot indicators. Visual symptoms were evaluated in a rating scale 0-5 during a three-year period. DAS-ELISA was performed using Bioreba and/or Agritest kits. For RT-qPCR detection, RNAs were extracted from phloem scrapings of dormant canes with the Spectrum RNA kit (Sigma), and specific primers described by Osman et al. (2008) were used in combination with the One-step RT-qPCR kit (Ambion). MicroRNAs for HTS were obtained from the same plant tissues using the miRCURY kit (Exiqon). Library preparation and Illumina sequencing was performed at CRG, Barcelona, Spain. Bioinformatics was performed at the online server of SCBI (Málaga, Spain).

RESULTS AND DISCUSSION

Biological indexing was able to display leafroll and fleck-like symptoms, but did not allow discriminating among leafroll viruses. Some discrepancies were observed in the results provided by indexing, DAS-ELISA and RT-qPCR. For example, DAS-ELISA was unable to detect GLRaV-2 in clone 4LS22 of variety Beba from Extremadura (Eastern Spain). However, it showed leafroll symptoms at indexing and revealed the presence of this virus by both RT-qPCR and HTS. On the other hand, clone 4LS23 (Beba, Extremadura) exhibited a very representative occurrence: strong leafroll symptoms were displayed by indexing but neither DAS-ELISA nor RT-qPCR were able to detect GLRaV-1, GLRaV-2 or GLRaV-3. However, HTS revealed the presence of GLRaV-6 discarding any other leafroll virus, later on confirmed by RT-qPCR and DAS-ELISA (Bioreba GLRaV-4-9 kit). In our knowledge, this is the first report of leafroll symptoms specifically associated to GLRaV-6 in the literature. Given that between leafroll-associated viruses only GLRaV-1 and GLRaV-3 are considered in current Spanish official certification, some materials would be suitable for certification as healthy in spite of the presence of viruses able to induce leafroll symptoms.

It worth mentioning that HTS allowed detecting GRSPaV in all the samples studied, revealing the high incidence of this virus in Spanish vines, in addition to the common presence of the viroids HSVd and GYSVd-1. Moreover,

HTS allowed the determination of *Grapevine Pinot gris virus* in clone RJ-24-2015 of variety Tempranillo Blanco from La Rioja (Northern Spain).

Based on our recent investigations, it seems clear that combined routine methodologies and HTS are comparable in terms of reliability and both approaches should be considered feasible for current EU regulations. We have compared the cost per plant of HTS versus the combination of our routine protocol (biological indexing, ELISA, RT-qPCR). Per sample and five viruses (GLRaV-1, -2, -3, GFLV, GFkV) we estimate a cost of 75 € for ELISA, whilst 200 € for RT-qPCR. We need to point out that we do not include ArMV in the routine analysis because it is considered virtually absent in Spanish vines, although it should be taken into account in a more rigorous protocol. For indexing we estimate 150 € per sample that include the cost of indicator plants (5 Cabernet Sauvignon and 5 Rupestris du Lot), soil preparation and sanitation, grafting, plants maintenance and visits for symptom inspection along three years. Thus, jointly this makes an amount of 425 € per plant for certification. In addition, we need to consider the requirement for trained personnel in serology, molecular biology, plant management and symptom inspection. Moreover, the cost of all these operations appears to be stable and there is no expectation to a decrease in the next future. In the case of HTS, cost includes RNA extraction, library preparation and sequencing. Per sample, RNA extraction and library preparation makes 300 €. Illumina sequencing of smallRNAs performed in a pool of samples up to 2 million reads/sample costs 150 €. Then, a total of 450 € resulted per sample. Although both methodologies show a similar cost, in the case of HTS it will probably drop in the coming years. In comparison with standard methodology, HTS requires trained personnel in molecular biology and bioinformatics but not in plant management or symptom inspection which needs a long training and experience. Turnaround-time is also to be considered in this comparison, given that we perform symptom recording along three years before producing the final report. However, for HTS analysis, we reached an average of six months from sample reception to results availability. Another evident advantage of HTS is the capability of detecting additional viruses and viroids in the samples.

Since the publication of Al Rwahnih et al. (2009), HTS for the detection and characterization of viruses and viroids in grapevine is widely being used in many labs, including Spain (Velasco et al., 2015, Cretazzo & Velasco, 2017). Comparison between biological indexing and HTS in grapevine virus detection has been achieved elsewhere (Al Rwahnih et al., 2015). In addition, a model has been recently proposed for HTS certification in grapevine (Saldarelli et al., 2017). Thus, the use of HTS in official grapevine certification is an opportunity to take into account in future EU regulations. The agents involved: nurseries, scientists, technicians and regulators must reach a consensus in order to develop and establish standard protocols in the application of HTS technologies in grapevine certification. The standard methodology for HTS certification should include: type of tissue for the analysis (phloem scrapings, petioles, etc.), protocol for total RNA or smallRNAs extractions, library preparation, minimum number of reads per sample and standard bioinformatics analysis. In parallel, minimum quality standards for RNA extraction, libraries and sequencing must be guaranteed.

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O19: Occurrence of *Grapevine Pinot gris virus (GPGV)* and Grapevine Leaf Mottling and Deformation (GLMD) syndrome in France: genetic diversity and field monitoring in diverse viticulture areas

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INTRODUCTION

A new virus, called *Grapevine Pinot gris virus* (GPGV) has been identified by next-generation sequencing (NGS) in Italy in a Pinot gris vine expressing symptoms of chlorotic mottling and leaf deformation (Giampetruzzi et al., 2012). This new *Trichovirus* has now been shown to be present in numerous countries over the world as reviewed recently by Saldarelli et al. (2017). The mite *Colomerus vitis* has been demonstrated to be able to transmit GPGV. This virus was also recently detected in two herbaceous species. Nevertheless, the role of GPGV as the causal agent of GLMD (Grapevine Leaf Mottling and Deformation) syndrome remains unclear as it is very frequently identified in asymptomatic vines (Bianchi et al, 2015). Different studies suggest the existence of genetically distinct variants differing in their ability to induce GLMD (Saldarelli et al., 2015; Bertazzon et al., 2016). In addition, a correlation between GPGV concentration and the expression of GLMD has also been proposed (Bertazzon et al, 2016). In France, the first identification of GPGV was done by NGS in a Merlot vine co-infected by a *Nepovirus* in Bordeaux vineyards (Beuve et al, 2015). In 2016, a first survey was carried out in different French vineyards, indicating that this virus is widespread (Spilmont et al, 2017). A larger scale survey was performed in 2017, covering the major French vineyards. Efforts were also done to identify vines potentially affected by GLMD. Symptomatic vines were identified in Champagne and in some vineyards located in south of France. Phylogenetic analyses were performed on partial sequences obtained from selected samples (either symptomatic or asymptomatic). A precise description of the kinetics of symptom expression was also done on six varieties.

MATERIALS AND METHODS

Vineyard survey for GPGV prevalence. Over the years 2016-2017, a total of 243 vineyard plots from different ages were chosen in the different French viticulture areas. Plots were selected locally to represent the diversity of the local varieties, independently of potential symptoms' presence. For each plot, one sample constituted of leaves taken from 20 vines was collected between June 2016 and July 2017. A grapevine collection was also screened for GPGV presence: 94 accessions selected for their genetic diversity were analyzed. In both cases, GPGV detection was performed by RT-Q-PCR using the primers and probes developed by Bianchi et al (2015).The occurrence of the GLMD symptoms was visually surveyed in some vineyards selected by local advisers with potential "Pinot gris disease" problems in Champagne, Burgundy, Alsace and Mediterranean area.

Sequencing of GPGV variants. Seven vines (four symptomatic and three asymptomatic) from three different vineyards respectively located in Champagne, Vaucluse and Occitanie were selected for partial sequencing and GPGV phylogenetic analyzes. Total RNAs were extracted from leaves of asymptomatic or symptomatic plants using the Spectrum™ Plant Total RNA kit (Sigma). GPGV variants were analyzed by direct sequencing of the amplicons obtained with primer pair DetF/DetR, spanning the 3' end of the MP and the 5'' end of the CP genes (Morelli et al., 2014) respectively. Sequence comparisons and phylogenetic reconstructions were performed using the Mega6 software.

Symptom description. Within the collection, 21 varieties were identified showing GLMD-like symptoms. Amongst them, 10 vines from 6 varieties (Tempranillo, Muscardin, Zinfandel, Saperavi, Biancu, Gentile and Vermentino) were marked and repeatedly observed. The sanitary status of these vines was confirmed by specific RT-PCR tests against GPGV and three nepoviruses: all plants were GPGV positive and negative for *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV) and *Tomato black-ring virus* (TBRV). Precise description of the symptoms was performed and one leaf per vine was photographed each week to follow symptoms' evolution from 15 May to 09 June 2017.

RESULTS AND DISCUSSION

Vineyards' survey for GPGV presence. GPGV was globally detected in 65% of the 243 samples tested from this survey. This virus seems to be present in all the French viticulture areas, independently of the age of the

vineyards sampled, as it was detected in young vines as well as in century-old ones. GPGV was detected in a total of 18 different varieties. Concerning the grapevine collection, GPGV appears to be widespread as it was detected in 75% of the accessions tested, including wild accessions of *V. vinifera*. Typical GLMD symptoms were identified on Pinot noir and Pinot Meunier in Champagne and on Grenache in the Mediterranean area. In these plants GPGV was systematically detected. By contrast atypical symptoms were also observed in Burgundy and Alsace (on Pinot noir, Chardonnay, Aligoté, Gewurztraminer and Pinot gris). They were characterized by intense bushy growth and leaf deformation without chlorotic mottling: GPGV was not systematically detected in these plants.

Sequencing of GPGV variants. Phylogenetic analysis was performed using the sequence of the MP/CP region obtained from samples with typical symptoms of GLMD or from asymptomatic samples. Corresponding GPGV sequences retrieved from GenBank were included in the comparisons and phylogenetic reconstructions. According to the classification proposed by Bertazzon et al., (2016), the variants from the two symptomatic samples collected in Champagne clustered within the clade C, in which most of the variants from symptomatic vines were previously found. Interestingly, a GPGV variant from an asymptomatic sample collected in the same vineyard grouped in the clade A, which tends to cluster isolates from asymptomatic plants or from vineyards with a low disease incidence. Moreover, the mutation in the MP gene hypothesized to discriminate symptomatic variants from asymptomatic ones (Saldarelli et al., 2015) was found in the two Champagne variants from symptomatic plants but not in the variant from an asymptomatic plant. However, these correlations were not found for the four other pairs of variants isolated from symptomatic and asymptomatic samples and collected in Vaucluse (two pairs) or in Occitanie (two pairs). Indeed, all these GPGV variants were found to cluster within clade A, irrespective of whether the plant from which they were amplified displayed or not symptoms of GLMD.

Symptom description

A diversity of symptoms was observed on the GLMD vines including leaf deformation, chlorotic mottling, deformed leaves, bushy growth and even “enation-type” symptoms. Interestingly, quite variable symptoms could be observed in a same variety and even a same vine as shown as an example in Figure 1.

The symptoms did not evolve a lot from May to June on the affected leaves. In some cases, the whole vine was affected and remained stunted but generally, as previously described in Italy, the new leaves appeared normal and the general aspect of the vine showed eventually little impact. Overall, the symptomatology associated with GPGV appears to be very complex, variable and clearly warranting further studies. Further GPGV-hosts in-depth interactions studies are required to shed light on the putative pathogenic effects of GPGV and variants on *V. vinifera* cultivars. The latest results concerning methodological aspects and perspectives to help deciphering the Koch postulates for correlating GPGV and GLMD will be presented and discussed.

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O20: Grapevine virus diagnosis in the central valley of Chile through the implementation of a TaqMan-based system.

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INTRODUCTION

Viral diseases have become a problem of high impact for the wine industry. The establishment of pathogen-free propagation material is a critical component of the management of vine diseases. Conventional detection tools for these pathogens demand a lot of time and labor. In recent years, the ability to identify pathogens has improved greatly through methods of comparative analysis of RNA and / or DNA. On the other hand, quantitative assays can be performed through qRT-PCR and have been used to detect plant pathogens such as bacteria, fungi, oomycetes and viruses, as well as a simultaneous detection (Multiplex) approach to detect combinations of these pathogens.

Using these technologies, Viña Concha y Toro is working to establish the viral status of its main vineyards and in this way establish the bases of a mitigation and quality control system for their plant production.

MATERIALS AND METHODS

Four hundred and thirteen samples were analyzed from 5 vineyards located in different regions of the central valley (Maule, O'Higgins and Valparaiso). Total RNA extraction was performed using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). cDNA was synthesized using the iScriptTM Reverse Transcription Supermix for RT-qPCR kit (BioRad). The procedure for specific detection by RT-qPCR of each virus was performed according previously described primers, probes and simplex conditions (Osman et al., 2008; Bertolini et al., 2010; Rwahnih et al., 2011; Bianchi et al., 2015; Badher et al., 2016).

RESULTS AND DISCUSSION

We are working on the implementation of a panel of 15 viruses. For now, the four most relevant ones for the company have been implemented correctly (GFLV, GLRaV-1, GLRaV-2, GLRaV-3) and were prospected in the 413 samples. As is showed in Table 1, the highest incidence corresponds to GLRaV-3.

Table1: Virus detection in VCT grapevines. [a]% of positives related to the number of tested samples per variety. Total: % of positives versus all analyzed samples for each virus.

Grapevine variety	Virus[a]			
	GLRaV-1	GLRaV-2	GLRaV-3	GFLV
Red variety 1	0,8%	68%	62%	16%
Red variety 2	0%	5%	75%	40%
Red variety 3	0%	0%	25%	0%
White variety 1	0%	4,5%	39%	4,5%
White variety 5	0%	0%	87%	0%
Rootstock 1	0%	0%	0%	3,3%
TOTAL	0,4%	41%	52%	13%

To date, a 15% of the surfaces compromised have been analyzed for the 4 viruses considered for the first stage of implementation (We expect to cover the 50% at the end of 2019). Currently, 32 plants (Red variety 1) considered "clean" for this preliminary standard have been isolated in the vineyards to be considered for season 2018 of plant production. These are preliminary result but with a highest relevance in order to move forward in the implementation of a clean plant production system.

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O21: Development of antibodies against *Grapevine Pinot gris virus* (GPGV) in rabbits and Camelids.

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INTRODUCTION

After several common grapevine viruses were ruled out (Ampelo-, Nepo-, Clostero-, and Vitiviruses), the study of Pinot gris vines showing symptoms of leaf deformation, stunting, and chlorotic mottling by deep sequencing revealed the presence of a new *trichovirus* named *Grapevine Pinot gris virus* (GPGV, Giampetruzzi et al, 2012). The effects of GPGV infection on grapevines are still unclear and the link between virus infection and the occurrence of symptoms is still poorly characterized. Today GPGV has been confirmed to infect at least 28 grape varieties and has been reported in many countries in Europe and Asia as well as in Australia, Canada and USA. So far PCR is the only method available to confirm GPGV infection. A serology-based diagnostic tool is needed to perform cost-effective and robust large scale testing.

MATERIALS AND METHODS

A purified recombinant GPGV coat protein produced in *E. coli* was used to immunize rabbits as well as alpacas (*Camelidae*) to maximize chances to obtain useful detection reagents. In rabbits classical polyclonal antibodies were then purified to be used as DAS-ELISA reagents. In alpacas small antibody-like structures composed of heavy chain only (VHH, about 100 residues and 15kDa) were screened to isolate those binding to GPGV.

RESULTS AND DISCUSSION

So far classical IgGs purified from immunized rabbits were used in a prototype DAS-ELISA kit. The results obtained using infected grapevine material (GPGV positive; confirmed by PCR) are very promising. The reagents allowed the detection of different GPGV isolates from leaves and wood samples. In addition IgG-like small antibodies obtained from immunized alpacas were identified and are currently being characterized. These VHH-only antibodies have the potential to greatly improve the sensitivity and detection spectrum of the current test.
To our knowledge, this is the first DAS-ELISA for the detection of GPGV. Due to the worldwide distribution of the virus, this new reagent will be of great interest for certification programs and diagnostic laboratories.

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C2: High Throughput Sequencing as a tool for viral pathogen diagnosis and expedited release of quarantined propagative plant material- current prospect and challenges.

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High Throughput Sequencing (HTS) provides a rapid, robust approach for viral plant pathogen identification. Recent studies found HTS superior to conventional methods for detecting viruses of economic significance in grapevine and fruit trees. As such, Foundation Plant Services has an import permit that allows provisional release of propagative plant material that has been HTS screened for pathogens. When HTS is used in conjunction with current conventional methods, growers of certified and registered material can initiate propagative increase and virus elimination programs with new accessions years earlier. While HTS remains a powerful new technology with significant benefits for plant certification and quarantine programs, there are challenges to consider. Since detecting a given pathogen sequence does not mean that pathogen is responsible for the disease, establishing biological significance for viruses identified via HTS analysis is necessary. Biological effects are assessed by graft transmission, completion of Koch's postulates, spread and distribution studies, and determining the agronomic significance of the symptoms. In addition, efficient sample preparation methods for large scale application and bioinformatics algorithms to efficiently separate pathogen and host sequences must be developed, validated, and standardized across laboratories. No regulatory decision can be made on the importance of a novel virus without information on its biological effects.

C3: Current challenges in the application of High Throughput Sequencing for plant virus detection

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The application of High Throughput Sequencing (HTS) as a tool to resolve disease aetiology and virus discovery has been very successful in identifying viral (and other) agents in almost all agricultural crops and many natural environments. This laid the foundation to consider applying this technology for routine virus detection utilising the experience gained from earlier applications. The transition of the application of HTS for discovery to detection is filled with caveats that must be dealt with before implementation can be considered. To advance HTS beyond discovery, to detection will require additional validation and most likely the adoption of new technology specific guidelines.

The application of HTS as a detection tool, especially for the routine detection of known plant viruses, comes with the same challenges as any other new technology as well as some technology specific challenges. In this presentation I will unpack the advantages and limitations of HTS for plant virus detection and discuss the HTS specific issues from sampling, library construction, and sequencing through to bioinformatic analysis and interpretation that impact on the specificity, sensitivity, accuracy and reproducibility of an HTS assay. All these aspects need to be addressed prior to accepting the application of HTS as a routine plant virus detection assay.

O23: Sequence analysis reveals broad genomic variability of *Grapevine leafroll-associated virus 1* in three non-coding regions

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INTRODUCTION: *Grapevine leafroll-associated virus 1* (GLRaV-1) is a member of the genus *Ampelovirus*, family *Closteroviridae*. GLRaV-1 is one of several viruses that are associated with grapevine leafroll disease (GLRD) complex, one of the most widespread and destructive diseases afflicting the global grape and wine industry (Martelli et al. 2012). Despite its global distribution and economic importance, only limited work has been carried out on the genomics and genetic diversity of GLRaV-1 (Komenik et al. 2005; Alabi et al. 2011; Esteves et al. 2013; Fan et al. 2015). The complete genomes of two closely related isolates of GLRaV-1 were sequenced only recently (Donda et al. 2017). The objective of this study was to determine the genome sequence of an Ontario isolate and to probe into genome wide variability of GLRaV-1.

MATERIALS AND METHODS: The source materials for total RNAs used for NGS were cuttings of Riesling grape collected from a vineyard located in Niagara, Ontario. Vines exhibited typical leafroll symptoms. Total RNAs were isolated from cambium scrapings using a method we established. After removal of rRNAs, the resulting RNAs were used as template for a cDNA library. Single-end sequencing was run on an Illumina HiSeq 2500 sequencer. Sequence reads were assembled into contigs, followed by blast search against viral genome sequences available in GenBank. The sequences corresponding to both genome ends were obtained via RACE. An internal gap in the assembled near complete genome were obtained through cloning and sequencing of RT-PCR products using primers designed based on flanking sequences. To identify grapevine samples infected with GLRaV-1, primers LR1HSP502F & LR1HSP880R were used in RT-PCR. Nearly 400 leaf samples were tested. PCR products were cloned and positive clones were sequenced using M13 primers. Resulting sequences were subjected to phylogenetic analysis.

RESULTS AND DISCUSSION:

Genome Sequence and Structure: NGS of this library produced 82,891 reads matching GLRaV-1, accounting for 1.4% of total number of reads related to viruses and viroids in this sample. *De novo* assembly of reads specific for GLRaV-1 resulted in four contigs, with the size range of 1715-9599 nts. When mapped against the reference genome for isolate 1050 (NC016509), only a single internal gap of 118 nts was identified at position 9042-9159. Primers LR1.F8910 & LR1.R9850 were designed and used in RT-PCR to bridge this gap. As inherent to NGS-based genome sequencing, the 5' and 3' terminal sequences often are incomplete or unreliable. To this end, RLM-RACE was used to obtain the 5' terminal sequence. The 3' RACE used two strategies: tailing of genomic RNA with a poly(A) or poly(G). In total, 16 clones were obtained and used to determine the 3' end sequence of the genome.

Combining sequences from NGS and RACE, the complete genome sequence of RSL was determined. The RSL genome is 18,921 nts in length, with a 5' UTR of 857 nts, followed by nine ORFs, and a 3' UTR of 475 nts. The positions and sizes of these ORFs are identical to two other isolates, WA-CH and WA-PN. The RSL genome is 190 nts longer than WA-CH, and 25 nts shorter than WA-PN. It is 96.8% identical to WA-PN and 99.2% identical to WA-CH. Though it is over 90% identical to WA-CH, RSL shares with WA-PN in containing a 191 nts repeat between positions 9108–9300 (based on isolate RSL) that is absent in WA-CH.

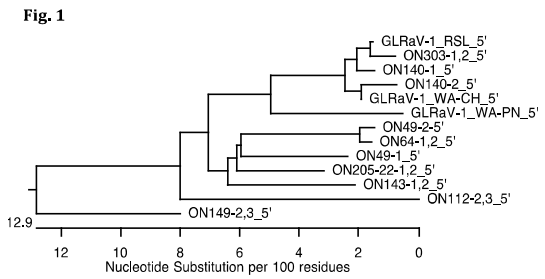
Genomic Variability: Comparison of these complete genomes and the near complete genome of isolate 1050 revealed a high degree of variability in three regions: the 5' UTR, the long intergenic UTR (LIN-UTR) between ORF1b and ORF2, and the 3' region. To probe into the genetic variability of GLRaV-1, we designed primers targeting these three regions. We also used primers targeting a 379 nt region of the HSP70h gene. The purposes of using HSP70h primers were to identify samples positive for GLRaV-1 and to attain an idea on its genetic variation in Ontario. Seventeen of the nearly 400 samples tested positive. The RT-PCR products were cloned, and 76 clones were sequenced. Phylogentic analysis of these 76 clones as well as other sequences retrieved from GenBank revealed seven groups (data not shown due to space limitations) as previously reported by others (Kominek et al. 2005; Alabi et al. 2011; Esteves et al. 2013; Fan et al. 2015). Most of the isolates from

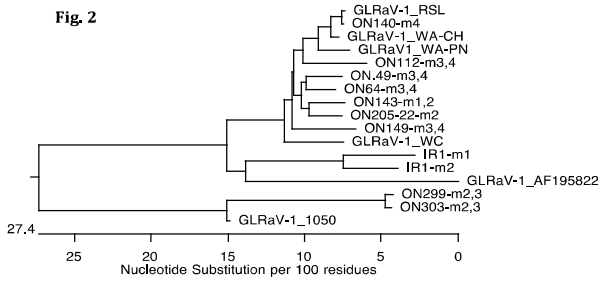
Ontario fall into Group 1, although belonging to different subgroups. Three isolates (ON231, ON299 and ON303) clustered with isolate 1050 in Group 2. Interestingly, the isolate IR belongs to group 4, together with the Australian isolate that also falls within this group.

We then set out to determine genome-wide variability of GLRaV-1 isolates using primers targeting the three highly variable regions. We selected a subset of isolates that represent different variant groups we have identified and have completed the analysis for the 5' UTR and the LIN-UTR. Four groups were identified for the clones corresponding to the 5' UTR (Fig. 1). These clones vary in size (875 to 942 nts) and sequence (84.5-99%). The two ON149 clones form a distinct cluster and are larger (939 or 941 nts) due to the presence of several insertions totaling of 55 nts. Clones from ON112 form a second group. The clones from ON303, ON140 and ON112 cluster with isolates WA-CH and RSL. The remaining clones from four other isolates form the fourth group. Interestingly, the two clones derived from ON49 differ by 8%, suggesting mixed infection of this isolate.

These samples were also subjected to genetic diversity analysis in the LIN-UTR, also resulting in four groups (Fig. 2). Clones from ON299 and ON303 form a distinct cluster that is distantly related to isolate 1050. The two clones from isolate IR1 formed a second cluster together with the Australian isolate. The remaining clones from six Ontario isolates fall within the largest group that also contained RSL, WA-CH, WA-PN, and WC. Overall, these clones differ substantially in sequence, with identities of 63.8-99.5%. Interestingly, the size of this region varies considerably among isolates, ranging from 812 (for ON299 and ON303) to 1125 (IR1-m1) nts. These size differences were due to a sequence repeat present in RSL, WA-PN, ON140 and IR1 but not in other isolates. Interestingly, the two clones from isolate IR1 differ in sequence as well as in size, with clone IR1-m1 being 1125 nts long while IR1-m2 being 1076 nts, indicating mixed infection of two distinct viral variants in this isolate.

In summary, data from this and previous studies by others demonstrate that GLRaV-1 is a truly unique virus. It has the second largest genome among all plant RNA viruses. GLRaV-1 exhibits unprecedented genomic variability among isolates, most prominently in the 5' UTR, the LIN-UTR, and the 3' region. Full understanding of the genetic variability, the mechanisms for such great variation, and function of these long and highly variable non-coding regions awaits further investigation.





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O24: Complete genome sequence analysis of three strains of *Grapevine leafroll-associated virus 4* from Washington State vineyards

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INTRODUCTION

The grape and wine industry contributes an estimated $5 billion annually to Washington State's economy (CAI Community Attributes Inc., 2015). Currently, wine grapes (*Vitis vinifera*) of both red- and white-berried cultivars are planted in approximately 60,000 acres, with 270,000 tons of grapes harvested in 2016 (Washington State Wine, 2017). Virus diseases are one of the major constraints, with grapevine leafroll disease (GLD) recognized as the most significant constraint to the sustainability of the grape and wine industry. Of the five species of *Grapevine leafroll-associated virus*es (GLRaVs, Family *Closteroviridae*), GLRaV-1, -3, and -4 (Genus *Ampelovirus*) and GLRaV-2 (Genus *Closterovirus*) were reported so far from Washington State vineyards (Naidu, 2011). Among the several strains of GLRaV-4 reported worldwide, strain 4, strain 5 and strain 9 were documented in wine and juice grape (*V. labruscana* 'Concord') cultivars in the State (Jarugula et al. 2008; Naidu, 2011; Bahder et al. 2013). Previously, the complete genome sequence of GLRaV-1, -2, and -3 isolates from Washington State vineyards was determined (Jarugula et al., 2010; Poojari et al., 2013; Donda et al., 2017). In this study, the full genome sequence of GLRaV-4 strains 4, 5, and 9 was determined and compared with genome sequences of GLRaV-4 strains reported from other grapevine-growing regions.

MATERIALS AND METHODS

Total RNA was extracted from *V. vinifera* cultivars Merlot, Sauvignon Blanc and Lagrein tested positive, respectively, for GLRaV-4 strain 4 (isolate WAMR-4), strain 5 (isolate WASB-5), and strain 9 (isolate WALA-9). RNA samples with >7.0 RIN value were subjected to high-throughput sequencing (HTS) using Illumina Hi-Seq 2500 platform. The quality filtered 125-base pair paired-end reads were assembled *de novo* into contigs using the CLC Genomics workbench 8.0 software (https://www.qiagenbioinformatics.com/) with default settings. The *de novo* assembled contigs were annotated against the non-redundant virus nucleotide database using BLASTn program (http://www.ncbi.nlm.nih.gov/blast). Internal gaps in genome sequence for GLRaV-4 strain 4, 5, and 9 were filled by RT-PCR using species-specific primers designed based on the HTS data and reference sequence corresponding to each strain obtained from GenBank. The 5' and 3' terminal sequence for GLRaV-4 strain 4, strain 5, and strain 9 was determined using protocols described previously (Donda et al., 2017). Pairwise sequence identities were carried out using the Muscle program (Edgar 2004) embedded in SDT v1.2 software (Muhire et al. 2014). SimPlot software was used to create plots of nucleotide similarity between the aligned sequences (Lole et al. 1999).

RESULTS AND DISCUSSION

The full-length genome of GLRaV-4 strain 4, strain 5, and strain 9 was determined to be 13,824 nucleotides (nt), 13,820 nt and 13,850 nt, respectively. Irrespective of differences in the overall size and sequence composition, the genome of GLRaV-4 strains 4, 5, and 9 characterized in this study encodes six predicted open reading frames (ORFs). The arrangement of these ORFs was identical between the three strains and their overall genome organization similar to GLRaV-4 strains 4, 5, 6, 9, Car, Pr, and Ob reported earlier (Aboughanem-Sabanadzovic et al., 2017). The genome of WAMR-4 isolate of GLRaV-4 strain 4 was smaller by 6 nt compared to 13,830 nt genome of LR-106 isolate of GLRaV-4 strain 4 reported from California (Accession number FJ467503). Both isolates shared 93.7% nucleotide sequence identity, indicating that they are more closely related to each other than to other strains of GLRaV-4. However, SimPlot comparison of the full-genome sequence of WAMR-4 and

LR-106 isolates showed more divergence in ORF1a compared to other ORFs. ORF-by-ORF comparisons between the two isolates showed greater than 96% amino acid (aa) sequence identity in all ORFs, except ORF1a that showed only 82% aa identity with corresponding sequence of LR-106 isolate. The genome of WASB-5 isolate showed ~94% identity with corresponding sequence of GLRaV-4 strain 5 reported from Brazil (isolate TRAJ1-BR, accession number KX828702), Canada (isolate 3138-03, accession number KX828702) and New York (Accession number FR822696). SimPlot comparison of the genome sequence of GLRaV-4 strain 5 isolates from Brazil, Canada, New York and Washington showed high degree of nucleotide sequence identity across the entire genome suggesting that they are genetically closely related to each other. However, it should be noted that the exact 5' and 3' terminus for GLRaV-4 strain 5 isolates from Canada and Brazil was not determined by RACE and the genome sequence of New York isolate is incomplete towards the 5'-end of the genome. The genome of WALA-9 isolate shared 94% nucleotide sequence identity with GLRaV-4 strain 9 reported from Spain (isolate Man086, accession number KJ810572). The genome size of WALA-9 isolate was smaller by 8 nt compared to 13,858 nt genome size of Man086 isolate. SimPlot analysis showed high sequence identity across the entire genome of WALA-9 and Man086 isolates, suggesting that they are distinct isolates of GLRaV-4 strain 9.

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O25: Detection and molecular characterization of newly emerging viruses in Greek vineyards

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INTRODUCTION

Grapevine is one of the most important crops in Greece, associated with wine and table grape production. It is affected by a variety of pathogens, among which viruses and viroids play a crucial role, causing important losses in vineyard production (Martelli, 2014). Recently, three newly identified viruses were detected in Greek vineyards: *Grapevine Roditis leaf discoloration-associated virus* (GRLDaV, genus *Badnavirus*, family *Caulimoviridae*), *Grapevine Pinot gris virus* (GPGV, genus *Trichovirus*, family *Betaflexiviridae*) and *Grapevine Syrah virus* 1 (GSyV-1, genus *Marafivirus*, family *Tymoviridae*) (Maliogka et al., 2015; unpublished data). In Greece, studies that involve newly discovered virus agents of grapevine are rather limited. Thus the objective of this research was to investigate the incidence of GRLDaV, GPGV and GSyV-1 in Greek vineyards as well as to molecularly characterize the obtained isolates.

MATERIALS AND METHODS

During 2009-2017, 192 samples originating from local and foreign varieties were collected from 11 vineyards (7 locations) of Greece (Table 1). Total RNA isolation was conducted according to Chatzinasiou et al. (2010). For the detection of GRLDaV, an RT-PCR was conducted using the primer set BADNAUP/BADNADO (5´-GAA GGA ATT GAA TCT CCA GCA GCA GG-3´, 5´-CTC TGC TAC ACC AAG TGA TAG ATT GTT GAG-3´) (Maliogka et. al, 2015) that amplifies a 261 bp part of the ORF2 gene, while for GPGV and GSyV-1 an RT-PCR was employed using the primers GPG-5637F/GPG-5939Do (5'-ATT GCG GAG TTG CCT TCA AG-3', 5'-CTG AGA AGC ATT GTC CCA TC-3') (Glasa et.al., 2014) and GSyV1F/GSyV1R (5'-CCA CCA TCT TCA CCG TYG ATC C-3', 5'-CCA TDG GRG AGG TTT CAG ATT TG-3') that target a 295 bp fragment of the movement protein and a 347 bp part of the coat protein (CP) of each virus, respectively. Ten isolates of GRLDaV, 5 from GPGV and 10 from GSyV-1 were selected for sequencing analysis. Amplified PCR products were purified from agarose gel and sequenced. The sequences were aligned with CLUSTALW available in MEGA7 and subjected to phylogenetic analysis using the Maximum Likelihood (ML) method. Sequence identities were calculated using Geneious (Biomatters Ltd., Auckland, New Zealand).

Ribosomal RNA depleted total RNA from two grapevine samples, that were found positive to GRLDaV, GSyV-1 (A2-1, grafted) and GPGV (GpFd, self-rooted), were further subjected to NGS using Illumina platforms (NextSeq, HiSeq2500) in order to acquire the full sequences of the viruses. The obtained paired-end read data were trimmed and deduplicated using PrinSeq-lite, and subjected to *de novo* assembly, after the removal of host reads, using the MIRA assembler plugin implemented in Geneious. The resulting de novo contigs and reads were mapped to the reference genomes to produce the complete genome of the isolates.

RESULTS AND DISCUSSION

Results showed the presence of GRLDaV, GPGV and GSyV-1 in both self-rooted and grafted local varieties (Table 1). Interestingly, GRLDaV was found in high incidence in the Greek varieties and especially in the self-rooted ones (44.9%) from the islands of Cyclades. On the contrary the virus was not detected in any of the foreign varieties tested thus indicating that it is endemic in Greece and probably in some neighboring countries. This notion is further reinforced by the fact that it was recently identified in local varieties in Italy and in Turkey. On the other hand, GPGV was found in high incidence in the foreign varieties tested (37.2%) and in lower in the local ones, a fact that highlights its putative introduction through the imported plant material. Finally, GSyV-1 was also identified in higher incidence in the foreign (15.7%) rather than the local varieties. Overall, GSyV-1 was identified in lower frequency compared to the other two viruses.

Table 1: Virus species incidence in Greek vineyards

	GRLDaV	GPGV	GSyV1
Foreign Varieties (%)	0/51 (0%)	19/51 (**37.2**%)	8/51 (**15.7**%)
Greek Grafted Varieties (%)	16/63 (**25.4**%)	5/63 (8%)	2/63 (3.2%)
Greek Self-Rooted Varieties From Cyclades Islands (%)	35/78 (**44.9**%)	1/78 (1.3%)	2/78 (2.3%)
TOTAL (%)	51/192 (26.6%)	25/192 (13%)	12/192 (6.25%)

Similarity analysis of Greek GRLDaV isolates revealed nucleotide (nt) identities between 78%-98%. Two of the isolates, from the self-rooted varieties, were the most divergent showing an identity between 78-88% in nt with already characterized isolates. Phylogenetic analysis of GRLDaV showed that the self-rooted varieties from Cyclades where clustered together, with the exception of the two divergent ones which formed a different group, while sequences from the Greek grafted varieties where closer to the Italian and the W4 isolates. Analysis of GPGV sequences revealed high identity ranging between 95-98% in nt. In the phylogenetic analysis the Greek GPGV isolates where clustered with the Slovakian and Turkish isolates (SK56, 11MP). Similarity analysis of the CP gene of GSyV-1 revealed high nucleotide identity among the Greek isolates as well as between Greek and foreign isolates deposited in GenBank (89-100% in nt). Subsequent phylogenetic analysis clustered the obtained Greek GSyV-1 isolates in two major groups according to the classification by Glasa et al. (2015).
Recovery of full genome sequences from GRLDaV and GPGV was made possible using the NGS data. However, only few contigs could be obtained from GSyV-1 possibly due to the low concentration of the virus, which could be also correlated with its low frequency detection. Full genome analysis of the GPGV isolate (GpFd) showed 99% identity with a Slovakian isolate (SK704) further confirming the low diversity of the virus. On the other hand, the GRLDaV isolate (A2-1) was 89.4-89.8% similar to the Italian Bobino Nero and the Greek W4 isolates, respectively. Interestingly, whole genomes analysis of the full sequences of GRLDaV also revealed the presence of a highly variable region with an indel polymorphism. Further analyses are currently underway in order to better analyse the diversity and evolutionary history of GRLDaV.

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O26: Grapevine Virus Diseases in Canada: Current Status and Future Perspectives

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INTRODUCTION

The Canadian wine and grape industry contributed over CAD$ 9 billion to the national economy in 2015 (Rimerman 2017). Grape production in Canada is confined to British Columbia (BC), Nova Scotia (NS), Ontario (ON), and Québec (QC) provinces. A nationwide survey conducted in 1994 reported the presence of several viruses in Canadian vineyards, including *Arabis mosaic virus, Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus-1* (GLRaV-1) and *Grapevine leafroll-associated virus-3* (GLRaV-3) (MacKenzie et al. 1995). However, Canadian viticulture has witnessed a significant increase in acreage and production of primarily *Vitis vinifera* cultivars and to some extent French-American and other interspecific hybrids since the mid-1990s. As has occurred in other grape-growing regions around the world, virus diseases have also recently been considered a major limiting factor to the sustainability of grape production in Canada reducing both yield and fruit quality. Accordingly, understanding the etiology and epidemiology of virus diseases as well as the status of their insect vectors in order to manage and thus mitigate the impact of these diseases have become a top priority for grape and wine industries across Canada. This study reports recent large-scale field surveys conducted in BC and limited surveys in ON and NS with the aim to demystify the current status of existing and emerging viral diseases and to identify their possible insect vector species. In addition, preliminary results on the effects of major virus diseases on fruit quality under the cool-climatic conditions of BC will be presented.

MATERIALS AND METHODS

Vineyard blocks were selected and surveyed for the presence of major grapevine viruses during the 2013 to 2017 growing seasons. In total, 3,056 random-composite and 1,256 targeted samples from 216 vineyard blocks were collected in BC as described by Poojari et al. (2017a). Similarly, 2,928 composite samples from NS (26 blocks) and ON (116 blocks) vineyards were collected. Leaves from the different samples obtained in BC and NS were tested for the presence of *Grapevine leafroll associated viruses*, viz., GLRaV-1, -2 -3 and -4, *Grapevine fanleaf virus* (GFLV), and *Grapevine Pinot gris virus* (GPGV) by single-step RT-PCR; and *Grapevine red blotch virus* (GRBV) by PCR using virus-specific primers (Rowhani et al. 2000; Poojari et al. 2016, Poojari et al. 2017a). Composite samples from ON vineyards were tested only for GLRaV-1, -2 and -3 and or GRBV. Similarly, insect vectors were monitored in the vineyard blocks and representative species were characterized based on partial sequencing of *mitochondrial cytochrome oxidase* c subunit 1 (*CO*1) gene (Poojari et al. 2017a). The PCR and RT-PCR amplicons of both viruses and insects were purified using the QIAquick PCR purification kit (QIAGEN) and cloned into pTOPO2.1 (Invitrogen) and sequenced. Nucleotide sequence analysis and evolutionary relationships were inferred using MEGA6. Field performances of healthy or GLRaVs infected vines and berry biochemical profiles were evaluated to determine the effects of GLRaVs on yield and fruit quality (Bogdanoff et al. 2017).

RESULTS AND DISCUSSION

Results of this study indicated the prevalence of GLRaVs and GRBV in BC vineyards (Poojari et al. 2017a, 2017b). Additionally, this study confirmed the presence of GFLV and reported for the first time GPGV in BC (Poojari et al. 2016). Among the GLRaVs, GLRaV-3 was the predominant species with a relative incidence of 16.7% (n=3,056). In the case of GRBV, a low incidence of 1.6% (n=2,000) was observed in BC vineyards. Significant differences in the relative incidence of GLRaV-3 were observed in BC vineyard blocks based on vineyard age as well as geographic regions. Phylogenetic analysis based on HSP70h gene sequences from

selected GLRaV isolates revealed close relationships with global isolates (Poojari et al. 2017a). Similarly, phylogenetic analysis of 35 GRBV isolates representing all three provinces grouped these isolates into two clades. Spatial distribution studies in six vineyard blocks in BC indicated the spread of GLRaV-3 ranged from 0 to 20% over three growing-seasons. In Ontario, during 2013-2017 growing seasons, the presence of GLRaVs-1, -2 and -3 was found in 57 out of 76 vineyard blocks with relative incidence ranging from 5 to 100%. Similarly, GRBV incidence was recorded in 36 out of 96 vineyard blocks with relative incidence ranging from 5 to 100%. Preliminary studies in NS have revealed that GLRaV-3 was recorded as the predominant virus species in that province with an incidence of 33.3% (n=501). GRBV incidence in NS was recorded at 3.8%. However, it should be noted that results from ON and NS represent a smaller number of vineyard blocks from a single growing season. Further surveys in ON and NS would provide valuable information for developing region-based management strategies for viruses and their insect vectors.

Insect monitoring studies showed the presence of *Pseudococcus maritimus* and *Parthenolecanium corni* in BC and ON vineyards. However, based on these results, other *Pulvinaria* species are suspected in Canadian vineyards. The presence of grape mealybug and soft-scale species in BC and ON vineyards emphasizes the importance of understanding the epidemiology of these insect vectors in order to develop management strategies to mitigate the secondary transmission within and between vineyards. Weekly observation of population dynamics in natural conditions indicated two generations of *P. maritimus* and one generation of *P. corni* in BC vineyards.

Studies on vine health and fruit composition in selected vineyard blocks in BC by comparing GLRaV infected versus healthy vines showed significant differences in total soluble solids, pH, anthocyanins and titratable acidity. Information generated through this research will assist federal and provincial regulatory agencies, grape growers of Canada, and diagnostic research laboratories to influence pest management policies and improve the sustainability of quality wine production in the cool climatic conditions of Canada.

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O27: Genetic diversity of Australian grapevine viroid isolates in Turkey

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INTRODUCTION

Many plant species are known as natural viroid hosts, but grapevine (*Vitis vinifera* L.) is one of the most permissive and oldest fruit trees in agricultural history. Five viroids infect grapevine: *Hop stunt viroid* in genus *Hostuviroid* (HSVd), *Citrus exocortis viroid* in genus *Pospiviroid* (CEVd), *Grapevine yellow speckle viroids* 1 and 2 (GYSVd-1 and GYSVd-2) and *Australian grapevine viroid* (AGVd) in genus *Apscaviroid* in the family *Pospiviroidae* (Sano et al. 1985; García-Arenal et al. 1987). Despite AGVd was the least studied among all other, the viroid was lately recovered from many grape producing countries such as Australia, Tunisia, USA, China, Italy, Iran and India (Rezaian 1990; Elleuch et al. 2002; Al Rwahnih et al. 2009; Jiang et al. 2009; Gambino et al. 2014). AGVd is 369 nt in length and appears to have originated from extensive RNA recombination involving GYSVd, CEVd, and ASSVd. Latest report in turkish viticultural areas was from Gazel and Önelge (2003) for the presence of GYSVd-1, -2, HSVd and CEVd-g as single or mixed infections. AGVd was only reported in one sample (cv. Menendi, GenBank accession number: KR706469) (Çandar et al. 2015) and little has been known about its distribution and population diversity in other grape growing regions. Here we report AGVd in autochthonous grapevine varieties in two major grape growing areas in Turkey, i.e. Eastern Mediterranean and Southeast Anatolia regions.

MATERIALS AND METHODS

A total of 22 autochthonous cultivars at >30 years old were investigated in 37 vineyards from Adana, Mersin, Hatay (Eastern Mediterranean, EM); Adıyaman, Gaziantep, Kilis, Mardin and Sanliurfa (Soth East Anatolia, SEA). Most samples were symptomatic, but not particularly induced by viroids. One-year-old canes were collected at the end of summer and the silica-capture extraction method (Foissac et al. 2000) was used with minor modifications to prepare the total nucleic acid (TNA). Two-step RT-PCR was performed with previously published primers (AGVd P7/P8) (Jiang et al. 2009). The amplified DNA products were custom sequenced by Medsantek, Turkey. The Blastn homology (Altschul et al. 1997) search revealed the sequences amplified were all similar to AGVd, suggesting the existence of AGVd in the surveyed areas. The MEGA6 software (Tamura et al. 2013) was used to estimate nucleotide diversity and phylogeny construction and evaluation.

RESULTS AND DISCUSSION

The DNA fragment with the expected size ca. 369 bp was successfully amplified by RT-PCR from 7 samples out of 200 tested autochthonous grapevine cultivars. The incidence of AGVd in the tested samples from both regions was 3.5% which was quite low comparing to the other grapevine viroids reported in Turkey (Gazel and Önelge 2003). Low incidence of AGVd in the Turkish grapevine accessions can be explained by self-rooted growing condition, low demand of the local ancient cultivars for commercial propagation and use of their fruits for fresh consumption and traditional sweets. The comparative analysis of five Turkish and the other AGVd isolates retrieved from GenBank with the type member (GenBank Acc.No. NC_003553) showed close relationship. The identity of the sequenced amplicons composed by 336 nt was more than 96% with each other and with the reference variant (Accession number NC_003553).

Three clustering patterns could clearly observed in the phylogenetic tree (Figure 1): one composed by the variants from China (EU743604, DQ362913), Iran (JN008865), India (KJ019302), Chile (unpublished data, KF007271), Tunisia (AF462155), Italia (KJ489019) and Australia (NC_003553) with three Turkish variants from SEA in this study (TR79: KY114493, TR182: KY114495, TR188: KY114496) and one from Aegean region (KR706469); the other two consisting of two individual Turkish isolates (TR: 189:KY114492; TR179:114494) from EM and SEA regions, respectively. Two isolates (TR 182: cv. Horoz karası and TR 188: cv. Antep karası) derived from the same province (Gaziantep, SEA), but different vineyard were closely related to each other and with the AGVd variants from Italy (KJ489019) and Australia (NC003553). The AGVd variant from 'TR79' isolate showed high nucleotide identity with the variant isolated from cv. Menendi in Aegean region and they were grouped with the variants from India (KJ019302) and Chile (KF007271). The localization on the phylotree of isolates 'TR189' (cv. Ceviz üzümü) and 'TR179' (cv. Antep karası) from EM and SEA, respectively indicates that they are slightly from all the others. Our study with the previous one has revealed a relatively low incidence of AGVd in three

regions in Turkey. Further research is needed to understand the evolution and adaptation of AGVd in Turkish viticulture.

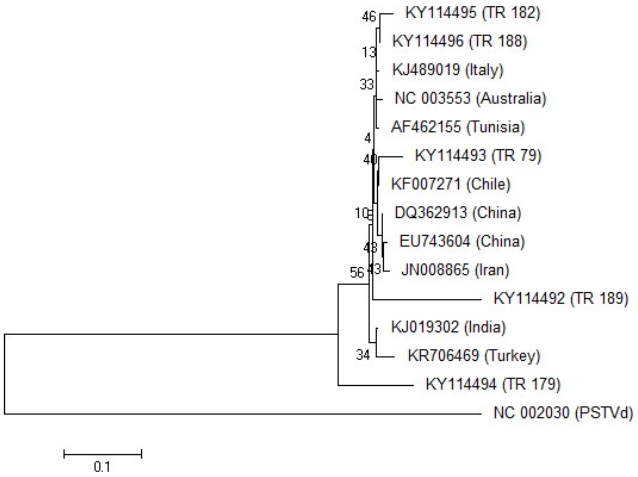

Figure 1. Phylogenetic analyses of representative variants of the AGVd isolates reported from several countries. PSTVd reference variant (NC002030) was adopted as an outgroup sequence.

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O28: *Grapevine line pattern virus*, an unknown but familiar grapevine virus

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INTRODUCTION

Grapevine can be affected by several diseases caused by viruses. Grapevine line pattern is one of these diseases has been reported only from Hungary. Its first report dates back to 1987 to the 9th IVG meeting at Kiryat Anavim, Israel, where Janos Lehoczky presented GLPV in a talk (Lehoczky et al 1987). Typical symptoms: chlorotic spots, rings or line pattern was reported from 3 different, susceptible varieties: Jubileum'75, Limberger and Irsai Oliver. The symptoms was connected to the presence of polymorphic virus like particles (ranging from 24 to 100nm) present in the symptomatic grapevines. Since that, based on these results the putative virus was thought as a possible member of the Ilarvirus genus of Bromoviridae. In the last 30 years there were no new report on grapevine line pattern disease, however it is always mentioned when grapevine diseases are listed (Lehoczky et al., 1992)(Martelli, 2014).

During his enthusiastic work, Prof Lehoczky established a pathologic garden where he collected all of the interesting grapevine viruses. GLPV was maintained on an interspecific hybrid Baco 22A, and although the executive administration of the viticulture research institute, owing this plot, changed several times, that vine, containing GLPV, is still exist there.

Evolution of sequencing techniques opened new avenues for virus research. With these new high throughput methods now it is possible to sequence the genetic material of all of the presented pathogens in the sample and describe viruses without any preliminary knowledge about their coding sequence (Massart et al., 2014). Small RNA NGS is a special field of NGS based virus diagnostics methods where small RNAs having identical sequences to the replicating virus, produced by the immune system of the plants, are sequenced and identified. From the host small RNA "pattern" sequences of the presented viruses can be described.

In our work we sampled the original host, the Baco 22A, growing in the pathogen garden at Kecskemét-Katonatelep in order to identify the line pattern causing agent.

MATERIALS AND METHODS

Plant material, sample preparation

In 2015 canes were collected from Baco 22A, sprouted and RNA was extracted from leaves, flowers, tendrils and shot tips. A pool containing RNA from each sampled organs was prepared and used for small RNA library preparation using Illumina Trusec small RNA library preparation kit. The library was sequenced, and the resulted reads (4,3 million) after adapter removal and trimming was analyzed by bioinformatics methods.

Pipeline for data evaluation of NGS results (bioinformatics)

Putative Ilarvirus specific contigs were built by 3 subsequent pipeline: 1/Non redundant small RNA reads without removing host based hits were used for *de novo* assembling of contigs using Velvet and Trinity followed by a sequenced similarity search with BLAST on the NCBI reference genome database.

2/Non-redundant small RNA reads were mapped using PatMaN on the Amazon lily mild mottle virus (AliMMV) Refseq genomes (NC_018402, NC_018403, NC_018404) with conditions permitting maximum 2 mismatches or 1 mismatch and 1 gap. Contigs were built by Velvet and Trinity and mapped to the reference genomes. 3/To minimize the number of small RNA reads for contig assembly host (*Vitis vinifera*) specific, prokaryote and eukaryote rRNA and tRNA sequences and known viral and viroid sequences were removed from the initial non-redundant small RNA list. Remaining 21-22nt long reads were used for contig assembly by Velvet and Trinity and annotated by BLAST.

Validation of predicted virus diagnostics by RT-PCR

To validate the presence of the putative GLPV primers were designed for each three genome of the virus. cDNA was synthetized from the pooled RNA extract, using random primer and Thermo Scientific Revert aid first strand kit. A ~2860 bp part of RNA1 was amplified by GLPVRNA1F1: ACACACCATTTCTCAGCGACGA and GLPVRNA1R1: GGCGTGTCAACCACGACTTC, a ~1080 bp product of RNA2 was amplified using

GLPVRNA2F2: ATTACATTTAGAGTTTCAACGGCTG, GLPVRNA2R1:CTGCGTTTCAGCAACGACTACAG while a ~1180 bp product of RNA3 was amplified using GLPVRNA3F1: GCACTGCTGTGGTGCCTGAGT and GLPVRNA3R1: GGGAAGAACGGATTCCTATACTC

RESULTS AND DISCUSSION

De novo contig assembly of the small RNA reads revealed the presence of GPGV, GRGV, GRSPaV, and two viroids: HSVd and GYSVd1 in the sample. Beside them 2 contigs were annotated as Amazon Lilly mottle virus (ALiMMV), a member of Anulavirus genus (Fuji et al., 2013). Anulaviridae is a new genus, the closest group to Ilarviridae, featured by the lack of 2b VSR on its RNA2 of the segmented genome. Contig assembly from collected ALiMMV familiar small RNA reads revealed 13/14/15 contigs mapped to the RNA1/2 and 3 of the putative genome and made us possible to design primers for RT-PCR validation. To increase the assembler sensitivity in our third pipeline the initial number of small RNA reads were reduced by removing host specific and other unrelated reads. As a result small RNA coverage by contigs increased to 73/54/74% of the RNA1, 2 and 3, respectively.

Primers designed according to the assembled reads were used for RT-PCR. Amplified product of both RNA 1, 2 and 3 were produced, cloned and sequenced by traditional Sanger sequencing. Phylogenetical analysis of the sequences of the amplified product showed that the virus present in Baco 22A belongs to Anulavirus genus, showing the closest similarity to ALiMMV. Beside ALiMMV two other viruses belongs to this genus: Pelargonium zonate spot virus (PZS)(Finetti-Sialer and Gallitelli, 2003) and Cassava Ivorian bacilliform virus (CIBV)(Scott et al., 2014), but none of them infects grapevine. Grapevine angular mosaic virus (GAMV)(Girgis et al., 2009) and Grapevine virus S (GVS) (JX513899) are grapevine infecting Ilarviruses but comparison of their available partial RdRP sequences showed that they are only distantly related to this putative virus.

Full genome sequencing and its phylogenetical analysis of this 3 partite virus will answer the question whether we have found a new virus or only a new host of an existing ones. However to verify the connection between this putatively new Anulavirus in the anciently GLPV infected grapevine and the originally described GLPV further investigation are needed.

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O29: *Grapevine fabavirus* and *Grapevine geminivirus A* in Japan.

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INTRODUTION

Grapevine fabavirus (GFabV) and Grapevine geminivirus A (GGVA) are emerging viruses detected using next-generation sequencing (NGS) analyses (Al Rwahnih et al., 2016, 2017). Al Rwahnih et al. (2017) detected GGVA from ten grapevines introduced from Japan. Also, GFabV and GGVA are detected from three Japanese original table grapes, 'Nagano Purple', 'Black Beet', and 'Shine Muscat', in South Korea (Al Rwahnih et al., 2016, 2017; Jo et al., 2017a, 2017b). 'Shine Muscat' (*Vitis labruscana* Bailey × *Vitis vinifera* L.) was developed in a breeding program of the NIFTS, NARO, and is becoming a very popular table grape in Japan. Here, we demonstrate that GFabV and GGVA are detected from 'Shine Muscat' trees in Japan.

MATERIALS AND METHODS

Total nucleic acids extracted from 'Shine Muscat' in a NIFTS orchard were analyzed by the NGS technique. Based on contig sequences, each of primer sets to detect GFabV and GGVA was designed. RT-PCR survey to detect GFabV, GGVA, *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine yellow speckle viroid 1* (GYSVd-1), and *Hop stunt viroid* (HpSVd) among 'Shine Muscat' trees in Japan were performed using the method of Nakaune and Nakano (2006).

RESULTS AND DISCUSSION

The RT-PCR survey detected GFabV and GGVA from various grapevines in Japan. Six 'Shine Muscat' trees showing different virus profiles were obtained during the survey (Table 1). GGVA was detected from all of them, and GFabV was detected from four of them. Trees Z1 and Z3 were GFabV-negative and GRSPaV-positive, and trees Z2, Z4, Z5, and Z6 were GFabV-positive and GRSPaV-negative, respectively (Table 1). In the orchards, they exhibited healthy looking, or mild to severe leaf malformations, chlorosis, and vein clearing as Jo et al. (2017a, 2017b) reported. Own-rooted individuals propagated from the six 'Shine Muscat' trees showed mild to severe leaf malformations, chlorosis, vein clearing and necrosis, and stunting. Al Rwahnih et al. (2017), Fan et al. (2017), and Jo et al. (2017a, 2017b) reported that GFabV and GGVA were detected from grapevines showing leaf malformations, ringspots, chlorosis, and vein clearing. Fan et al. (2017) reported that 'Beta' grapevines tested positive for GFabV and negative for other viruses using RT-PCR showed chlorotic mottling and leaf deformation. Influence of viral agents also should be further investigated for the case of 'Shine Muscat'.

Table 1. Six 'Shine Muscat' trees showing different virus profiles.

Trees	GFabV	GGVA	GLRaV-3	GRSPaV	GYSVd-1	HpSVd
Z1	–	+	–	+	+	+
Z3	–	+	–	+	–	+
Z2	+	+	+	–	–	+
Z4	+	+	–	–	+	+
Z5	+	+	+	–	+	+
Z6	+	+	–	–	+	+

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O30: The virome of Nigerian vineyards.

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INTRODUCTION

Grapevine was introduced to Nigeria in the 1970s and several table grape varieties are currently being grown primarily in the northern guinea savannah agroecological zone of the country encompassing parts of Bauchi, Gombe, Kaduna, Kano, Katsina and Plateau states. The berries serve as sources of essential minerals, vitamins and antioxidants for households whose diet is dominated by carbs. In addition, grape production serves as a source of livelihood for the largely small acreage growers thus contributing to poverty alleviation in the rural communities. Based on conservative estimates, a grape plant can generate seasonal revenue of about $60-$80 to the grower thus underscoring the economic potential of the viticulture industry in Nigeria. Virus diseases are a major constraint to grape production globally. Since grapevine is an introduced crop to Nigeria, viruses and other graft-transmissible agents (GTAs) could 'hitch-hike' contaminated propagules and pose a threat to an industry just at its infancy. The goal of this study was to investigate the virome of Nigerian vineyards and determine the incidence and distribution of identified GTAs.

MATERIALS AND METHODS

Surveys were conducted during the 2016 season and vineyard locations were identified through grower contacts. A total of 360 samples, consisting of 6-8 leaf tissue with intact petioles per vine, were collected from 40 vineyard locations spread across the six above-mentioned states. The samples were dried under $CaCl_2$ at room temperature, and then shipped under USDA-APHIS-PPQ permit to the Texas A&M AgriLife Research and Extension Center, Weslaco, TX facility for diagnosis. Equal amounts of leaf tissue were pooled together from all samples, mixed and distributed into two subsamples G1.1.164 and G2.1.165. Total nucleic acid (TNA) was isolated from each subsample (MagMAX-96 viral RNA isolation kit, Thermo Fisher Scientific, Inc.), then used for cDNA library construction after a ribosomal RNA depletion step (TruSeq Stranded Total RNA with Ribo-Zero Plant kit, Illumina, Inc.). Both libraries were subjected to highthroughput sequencing (HTS) on the Illumina NextSeq 500 platform at the Foundation Plant Services laboratory of the University of California, Davis, CA. The HTS reads were bioinformatically analyzed (Al Rwahnih et al., 2016) and pathogen-specific sequence reads assembled. The results were validated via two-step RT-PCR assays conducted on fresh TNA isolated from each of the 360 samples using published (Poojari et al., 2016; Ward et al., 2011) and newly designed primers (data not shown). Each sample was also tested by RT-PCR for the presence of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) and *Grapevine red blotch virus* (GRBV) using published primers (Meng et al., 2006; Poojari et al., 2016). Specificities of obtained DNA amplicons was confirmed by cloning (TOPO-TA Kit, Life Technologies) and Sanger sequencing of representative samples.

RESULTS AND DISCUSSION

A total of five table/juice grape varieties were encountered during the survey with the predominant variety being cv. Queen Golden (250/360), followed by cv. Bangalore Blue (80/360), Anab-e-Shahi (28/360) and one sample each for cv. Regina and cv. Israeli Blue. Analysis of samples by location showed that the majority were obtained from Kaduna (182/360) spanning all five cultivars, followed by Kano (67; 3 cultivars), Bauchi (52; 3 cultivars), Plateau (40; 2 cultivars), Gombe (11; 3 cultivars) and Katsina (8; 1 cultivar). Approximately 39.7 and 24.2 million raw HTS reads (each 76 nucleotides [nt] in length) were generated from subsamples G1.1.164 and G2.1.165, respectively. Bioinformatic analysis of the HTS reads resulted in the identification of reads specific to hop stunt viroid (HSVd), grapevine yellow speckle viroid 1 (GYSVd-1), and grapevine yellow speckle viroid 2 (GYSVd-2) in both subsamples G1.1.164 and G2.1.165. (Table 1). In addition to the three viroids, reads specific to *Grapevine leafroll-associated virus* 1 (GLRaV-1) were recovered only from subsample G1.1.164 (Table 1).

Table 1. Raw and assembled high-throughput sequencing (HTS) reads derived from dried leaf grapevine tissues obtained from different vineyards in the northern guinea savannah agroecological zone of Nigeria

cDNA Library	Total[a]	Virus and viroid-specific reads[b]			
		HSVd	GYSVd-1	GYSVd-2	GLRaV-1
G1.1.164	39.7M	700	1,854	1162	0
G2.1.165	24.2M	510	1239	1331	1767

[a]Total raw high-throughput sequencing (HTS) reads (each 76 nucleotides [nt] in length) expressed in million units (M).
[b]Assembled HTS reads specific to hop stunt viroid (HSVd), grapevine yellow speckle viroid 1 (GYSVd-1), grapevine yellow speckle viroid 2 (GYSVd-2), and *Grapevine leafroll-associated virus* 1 (GLRaV-1).

Screening of individual samples by RT-PCR revealed the occurrence of GLRaV-1 in ~24% (85/360) of samples, HSVd in ~53% (192/360), GYSVd-1 in ~33% (120/360), and GYSVd-2 in ~43% (155/360) while ~31% (113/360) were tested negative. In addition, GRSPaV was detected by RT-PCR in ~3% (10/360) of samples. Interestingly, GRSPaV was not detected by HTS perhaps due to its very low incidence in composited survey samples used for cDNA library construction. Further analysis of the results showed predominance of mixed infections of two to five different viruses (46.9% or 169/360) over single virus infections (21.7.1% or 78/360) versus no virus detected (31.4% or 113/360) (Fig. 1). None of the samples tested positive for GRBV.

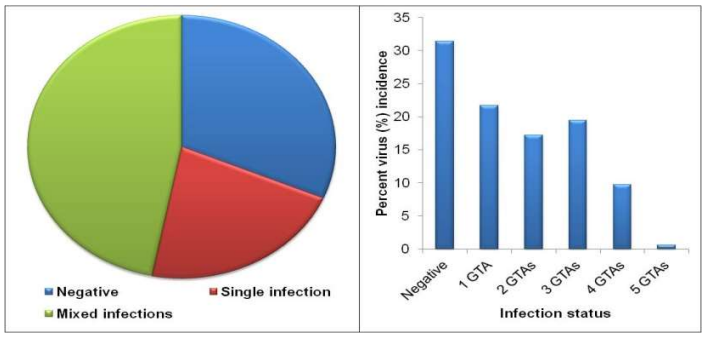

Figure 1. infection status of Nigerian vineyards based on surveys conducted across six states in the northern guinea agroecological zone of the country.

Our results documented the presence of two viruses (GLRaV-1 and GRSPaV) and three viroid species (HSVd, GYSVd-1 and GYSVd-2) in Nigerian vineyards (Zongoma et al., 2017a, 2017b). The likely pathway of introduction of these viruses into Nigeria is via inadvertent introduction of infected cuttings into the country. The results will be useful for educating growers on the importance of clean plant materials and the risk of inadvertent spread of viruses through sharing of non-tested grapevine propagation materials.

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O31: Evidence to support Syrah Decline is a non-infectious genetic syndrome in several Syrah selections

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INTRODUCTION

Syrah decline was first described as an emerging disease in France and reported in California soon after [3]. Symptoms appear in developed vines and include: leaf reddening and scorching, swelling at the graft union, superficial cracking and pitting of woody tissue, stem necrosis, and eventual death of the vine [1]. Biotic and abiotic factors, as well as host genetics, have been investigated as causation of the phenotype. High throughput sequencing (HTS) was performed on Syrah-FPS 06 (reported to be French clone 100), a selection exhibiting Syrah decline, as well as Syrah-FPS 08, an asymptomatic selection. HTS analysis on Syrah-FPS 06 revealed the presence of mixed infection with several viruses and viroids including: *Grapevine rupestris stem pitting associated virus, Grapevine rupestris vein feathering virus*, *Grapevine leafroll associated virus 4 strain 9, Grapevine Syrah Virus-1, Australian grapevine viroid* and *Grapevine yellow speckle viroid* 1 and 2 [1]. Genetic markers have been developed to segregate asymptomatic from moderate and severely symptomatic French Syrah selections [4]. HTS, was repeated for Syrah-FPS 06 as well as tissue culture treated vines produced from this selection. A survey was conducted for Syrah Decline symptoms and genetic screening was performed on all field planted Syrah and Shiraz selections in the Foundation Plant Services (FPS) collection at the University of California in Davis.

MATERIALS AND METHODS

Micro-shoot-tip tissue culture virus elimination therapy was performed on Syrah-FPS 06 in July of 1998. From apical shoot tips, 0.4 mm of meristematic tissue was excised and established *in vitro*. Explants were transferred to soil in 1999. RT-qPCR tests confirmed successful virus elimination. Total nucleic acids extracts were prepared and analyzed by HTS as previously described by Al Rwahnih [2]. Treated vines were initially field planted in 2003 and later propagates were field planted on own roots in two distinct vineyard locations in 2011 (2 vines) and 2014 (16 vines). For distinction, treated vines were assigned selection: FPS 06.1.

In 2017 a survey of all field planted Syrah and Shiraz selections was conducted to record symptom expression. During the 2017 season, HTS was performed twice on multiple field planted vines of Syrah-FPS 06.1 and repeated on propagates of the original Syrah-FPS 06 vine. Additionally, simple sequence repeat (SSR) based genetic screening was performed on all Syrah and Shiraz selections in the FPS collection using the SSR-containing marker, VMC5g7 [4]. Vines showing a genotype with an atypical third allele (198, 216, 218) were scored as positive. The genotype of vines scored as negative was 198, 216.

RESULTS AND DISCUSSION

HTS results confirmed the presence of the previously reported viruses and viroids in Syrah-FPS 06 and revealed the absence of viruses and viroids, in Syrah-FPS 06.1. This provides strong evidence that Syrah decline is unlikely the consequence of viral infection. In all but one case (Syrah-FPS 09) where field vines were 6 years established, red leaf symptoms corresponded with the SSR genetic screening results (Figure 1). In all but one case (Syrah-FPS 04) field vines are planted on own roots, refuting the hypothesis that Syrah decline symptoms are due to graft incompatibility. Symptom expression has been reported to be moderate in particular selections exhibiting the third allele at the VMC5g7 marker and may vary relative to soil and climatic conditions. Symptom expression was uniform in selections planted in multiple locations and consistent between mother vines and tissue culture treated vines. All Syrah-FPS 06.1 vines planted at distinct locations expressed symptoms; the only distinction being that symptoms in older vines were more severe. Monitoring vines for symptoms will continue for field planted Syrah and Shiraz vines within the FPS collection.

Figure 1. Syrah and Shiraz selections at FPS exhibiting Syrah decline symptoms. Selections including decimals underwent tissue culture treatment. The number prior to the decimal point indicates source selection.

		VMC5g7 Profile			
Cultivar	Selection	Allele 1	Allele 2	Allele 3	Symptoms Observed
Syrah	4	198	216	218	Yes
Syrah	4.1	198	216	218	Yes
Syrah	5	198	216	218	Yes
Syrah	5.1	198	216	218	**No**
Syrah	6.1	198	216	218	Yes
Syrah	7	198	216	218	Yes
Syrah	7.1	198	216	218	Yes
Syrah	8	198	216		No
Syrah	8.1	198	216		No
Syrah	9	198	216	218	**No**
Syrah	10	198	216		No
Syrah	11	198	216	218	Yes
Syrah	12	198	216	218	Yes
Syrah	13	198	216		No
Syrah	13.1	198	216		No
Syrah	14	198	216		No
Syrah	14.1	198	216		No
Syrah	15	198	216		No
Syrah	15.1	198	216		No
Syrah	16	198	216		No
Syrah	20	198	216		No
Syrah	20.1	198	216		No
Syrah	21	198	216		No
Syrah	21.1	198	216		No
Syrah	22	198	216	218	**No**
Syrah	22.1	198	216	218	**No**
Syrah	23	198	216	218	**No**
Syrah	23.1	198	216	218	Yes
Syrah	24	198	216	218	**No**

		VMC5g7 Profile			
Cultivar	Selection	Allele 1	Allele 2	Allele 3	Symptoms Observed
Syrah	24.1	198	216	218	**No**
Syrah	25	198	216	218	**No**
Syrah	25.1	198	216	218	**No**
Syrah	26	198	216		No
Syrah	26.1	198	216		No
Syrah	27.1	198	216		No
Syrah	34.1	198	216	218	**No**
Syrah	35.1	198	216		No
Syrah	37	198	216		No
Syrah	37.1	198	216		No
Syrah	38.1	198	216		No
Shiraz	1	198	216		No
Shiraz	1.1	198	216		No
Shiraz	2	198	216		No
Shiraz	3	198	216		No
Shiraz	3.1	198	216		No
Shiraz	4	198	216		No
Shiraz	5	198	216		No
Shiraz	6	198	216		No
Shiraz	6.1	198	216		No
Shiraz	7	198	216		No
Shiraz	7.1	198	216		No
Shiraz	8	198	216		No
Shiraz	8.1	198	216		No
Shiraz	9	198	216		No
Shiraz	9.1	198	216		No
Shiraz	10	198	216	218	Yes
Shiraz	10.1	198	216	218	Yes

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O32: Characterising the viral and fungal diversity in old and young grapevines

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INTRODUCTION

The economic life of the average South African vineyard is 20 to 25 years. However, a number of vineyards have remained profitable beyond their life expectancy, despite the prolonged exposure to environmental stresses. Recent years have seen growing interest and investment in old vines, and the clonal propagation thereof (Heyns, 2013). While 'old vine' is a common description on wine labels, its definition is open to interpretation. In South Africa, vines are generally regarded 'old' when they reach 35 years of age, while 'young' vines are less than ten years old. Anecdotes from sensory panels indicate that old vines produce wines of greater depth and complexity compared to young vines (Heyns, 2013). To date, limited research has been performed to establish which factors may be responsible for phenotypic differences between old and young vines, and how these differences influence wine character. Potential contributing factors include variations at the genome or transcriptome level, or differences in the viral and microbial component of the vines. The focus of the present study was to characterise the viral and fungal profiles of old and young vines, using next-generation sequencing in a metagenomics approach.

MATERIALS AND METHODS

Samples of four old and four young vines (*Vitis vinifera* cv. Pinotage) were collected from a wine estate in the Stellenbosch region of the Western Cape, South Africa. In this vineyard, several old vines displaying poor vigour were replaced with younger vines of the same clone. Prior to propagation, these vines were subjected to heat therapy to reduce the risk of virus transmission. Canes were sampled during the dormant season (July, 2014) before annual pruning of the vineyard. Old vines were 40 years old, while the young vines approximately seven years old.

To determine the viral diversity, double-stranded RNA (dsRNA) was extracted from phloem tissue to enrich for virus-specific nucleic acids, using a cellulose affinity chromatography extraction method (Burger & Maree, 2015). Sequencing libraries were prepared using the TruSeq RNA sample preparation kit, following a protocol adapted for dsRNA (Burger & Maree, 2015). The libraries were sequenced in two paired-end runs (2x250nt and 2x125nt) on the Illumina HiSeq 2500. Trimmomatic (Bolger *et al*., 2014) was used to trim reads for low-quality bases and to remove adapter sequences. The reads were assembled into contigs using CLC Genomics Workbench (https://www.qiagenbioinfor
matics.com/) and classified with command-line BLAST analysis (Camacho *et* al., 2009) against the NCBI database. Additionally, the reads were mapped to a database consisting of known grapevine-infecting virus and viroid genome sequences. Reverse-transcription PCR detection assays were performed to validate the presence of the identified viruses.

To characterise the fungal communities, total DNA was extracted from the vascular tissues of surface-sterilised cane material, using a cetyltrimethylammonium bromide method (unpublished). The internal transcribed spacer 2 was amplified with the ITS3F and ITS4R primer pair (White *et al*., 1990). Amplicon libraries were prepared and sequenced in a paired-end run (2x300nt) on the Illumina MiSeq. The initial data processing steps were performed following the UPARSE pipeline (Edgar, 2013). Read pairs were merged and filtered for quality, as recommended by Edgar and Flyvbjerg (2015). The variable ITS2 region was extracted from the flanking conserved ribosomal genes using ITSx (Bengtsson-Palme *et al*., 2013). High-quality ITS2 sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold. The OTUs were taxonomically classified against the UNITE database (Kõljalg *et al*., 2013) using the BLAST method, as implemented in QIIME (Caporaso *et al*., 2010). To measure the alpha diversity, multiple rarefaction analysis was performed using QIIME, and the Chao1 richness, observed OTUs and Shannon diversity index, calculated.

RESULTS AND DISCUSSION

Viruses of the families *Closteroviridae*, *Betaflexiviridae* and *Tymoviridae*, and four pospiviroids were detected. The virus community was more diverse in the old vines, with 31 and 16 virus variants detected in the old and young vines, respectively. This was expected, since old vines have been exposed to viral pathogens for a longer period. The economically important *Grapevine leafroll-associated virus* 3 was the most abundant species present in the samples, consistent with a recent survey of vineyards in the Western Cape (Jooste *et al*., 2015). Grapevine Syrah virus 1, and possibly *Grapevine rupestris vein feathering virus*, was identified for the first time in South African grapevines, expanding the global distribution of the virus(es).

The amplicon data revealed the presence of different filamentous and yeast-like fungal taxa commonly associated with grapevines, including species of *Alternaria*, *Aureobasidium*, *Cladosporium* and *Epicoccum*. Several pathogens of grapevine trunk diseases and postharvest rot, and endophytic species with biocontrol properties were detected. The young-vine sample group showed greater fungal diversity, as determined by three alpha diversity metrics, although not statistically significant. It may be speculated that the fungal community of old vines is more accustomed to the environment, and therefore less diverse. No differences were observed between the old and young vines, with regards to the community composition. The data generated in this study has contributed to research on the complex viral and fungal communities inhabiting old vines.

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C4: Grapevine phytoplasmas a 2014-2108 update

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PHYTOPLASMAS AND INSECT VECTORS

Grapevine yellows (GY) are often associated with molecularly differentiable phytoplasmas according to their geographic distribution and sometimes also to different varieties (Bertaccini, 2015; Dermastia *et al*., 2017) (Table 1). In Australia a grapevine disease reported as Buckland Valley grapevine yellows was further studied and deeper molecular characterization is in progress for its classification (Constable *et al*., 2003; D'Ercoli *et al*., 2018).

Table 1. Main phytoplasma ribosomal group identified in grapevine worldwide.

Continent	**Country**	**Disease name**	**Phytoplasma 16Sr group-subgroup**	**'*Candidatus* Phytoplasma' strain acronym**
Australia	**Australia**	**Buckland Valley grapevine yellows**	**16SrXXXIII-A**	**BVGY**
		Australian grapevine yellows	16SrII-A	'*Ca*. P. aurantifolia'
			16SrXII-B	'*Ca*. P. australiense'
America	USA	Virginian grapevine yellows	16SrIII-I	'*Ca*. P. pruni'
			16SrI-A	'*Ca*. P. asteris'
	Canada	**Grapevine yellows**	16SrI-B	
			16SrI-A	
			16SrIII	**'*Ca*. P. pruni'**
		"Bois noir"	16SrXII-A	'*Ca*. P. solani'
	Brazil	**Grapevine yellows**	**16SrIII-J**	**'*Ca*. P. pruni'**
	Chile		16SrVII-A	'*Ca*. P. fraxini'
			16SrI-B	'*Ca*. P. asteris'
			16SrI-C	
			16SrV-A	**'*Ca*. P. ulmi'**
			16SrXII-A	'*Ca*. P. solani'
	Peru		**16SrXV-A**	**'*Ca*. P. brasiliense'**
Europe	Germany	Palatinate grapevine yellows	16SrV-C	PGY
	Italy	**Grapevine yellows**	**16SrVI**	**'*Ca*. P. trifolii'**
	Italy		**16SrVII-A**	**'*Ca*. P. fraxini'**
	Italy, Hungary, Serbia		16SrX-B	'*Ca*. P. prunorum'
	Italy, Slovenia	Grapevine aster yellows	16SrI-C	'*Ca*. P. asteris'
		Grapevine yellows	16SrV-A	'*Ca*. P. ulmi'

	Italy; Portugal	Grapevine aster yellows	16SrI-B	'*Ca*. P. asteris'
	Italy, France, Switzerland, Serbia, Slovenia, Croatia, Austria, Hungary	"Flavescence dorée"	16SrV-C	FD-C
	Italy; France, Spain, **Portugal**, Slovenia, Croatia	**"Flavescence dorée"**	**16SrV-D**	**FD-D**
	Italy, France, Spain, Portugal, Germany, Serbia, Bosnia & Erzegovina, Austria, Hungary, Czech Republic, Bulgaria, Montenegro, Macedonia, **Moldova**, Georgia, Ukraine	**"Bois noir"**	**16SrXII-A**	**'*Ca*. P. solani'**
Africa	Tunisia	Grapevine yellows	16SrI-B	'*Ca*. P. asteris'
	South Africa			
		"Bois noir"	16SrXII-A	'*Ca*. P. solani'
Asia	Syria	Grapevine yellows	16SrVI	'*Ca*. P. trifolii'
	Turkey	Grapevine yellows	16SrIX	'*Ca*. P. phoenicium'
		Grapevine aster yellows	16SrI-B	'*Ca*. P. asteris'
	Israel,	"Bois noir"	16SrXII-A	'*Ca*. P. solani'
	Lebanon			
	Jordan			
	China			
	Iran	**Grapevine yellows**	**16SrIX-B**	**'*Ca*. P. phoenicium'**
			16SrVII-A	**'*Ca*. P. fraxini'**
			16SrII-B	**'*Ca*. P. aurantifolia'**
		"Bois noir"	16SrXII-A	'*Ca*. P. solani'

In the American continent studies in USA were carried out on North American grapevine yellows (NAGY) with a revision of 16SrIII phytoplasma classification indicating the presence of two 16SrIII-A sequevars, distinct from '*Candidatus* Phytoplasma pruni'. This disease was reported in Maryland, Missouri, southeast Pennsylvania, Ohio, Virginia, and in the Finger Lakes region of New York State infecting Chardonnay, Pinot Gris, Viognier, Petit Manseng, Cabernet Sauvignon, Malbec, and Black Malvasia (Davis *et al*., 2015). In Canada it was sporadically reported the presence of phytoplasmas belonging to the 16SrIII ribosomal group (Saguez *et al*., 2015), in particular the highest infection were observed in Sauvignon Blanc, Cabernet franc, Syrah and Cabernet Sauvignon cultivars (Vincent *et al*., 2015). In Chile the 16SrIII-J phytoplasma appears to be the most widespread, transmitted by *Bergallia valdiviana* it infects not only grapevine but several other woody and herbaceous species (Fiore *et al*., 2015; Quiroga *et al*., 2015); the draft genome sequence of this phytoplasma was also obtained (Zamorano and Fiore, 2016). In the same country grapevine resulted also infected by 16SrV-A phytoplasmas (Fiore *et al*., 2015). In southern regions of Brazil in Cabernet Sauvignon and Merlot showing downward rolled leaves, yellowing, reddening of leaf veins, witches' broom, abnormal development of stems, flowers and bunches referable to GY 16SrIII-J group with some SNP variations were identified (Dos Santos *et al*., 2017). In a GY disease in vineyards from Peru plants with leaf yellowing, vein necrosis, and in some cases berry shrivelling,

showed the presence of '*Ca*. P. brasiliense'-related phytoplasmas (16SrXV-A) (Wei *et al*., 2017). In Europe both "flavescence dorée" (FD) strains (16SrV-D: FD-D and 16SrV-C: FD-C) (Martini *et al*., 1999) are still present as epidemic or endemic diseases in all the countries where they were reported (Table 1). However in the northern Portugal 16SrV-D are inducing severe diseases in the local "vino verde" variety (Rebelo *et al*., 2016) and in Croatia new fast developing FD hotspots were observed showing different genotypes; some of them were also detected in the leafhopper *Phlogotettix cyclops* and in the invasive weed *Ailanthus altissima* (Plavec *et al*., 2018). *Orientus ishidae* was reported as a further occasional insect vector for 16SrV phytoplasmas in Italian vineyards (Lessio *et al*., 2016; 2017). In Germany further studies on 16SrV potential insect vectors allow to its detection in 65% of *O. ishidae* and *Allygus* spp., in 50% of *Lamprotettix nitidulus*, 20% of *Macropsis* spp. and 14% *O. alni* (Jarausch *et al*., 2017). The "bois noir" (BN), widespread in almost all European grapevine growing countries (Table 1) was reported from Moldova (Bondarciuc *et al*., 2018). In Italy *Euscelis incisus* and *Dicranotropis hamata* resulted able to transmit 'Ca. P. solani' to grapevine (Mori *et al*., 2018), the same phytoplasma was also detected in vineyard-collected *Euscelidius lineolatus*, *Mocydia crocea*, *Neoaliturs fenestratus* and *Psammotettix alienus* (Minuz *et al*., 2017). In Italian vineyards the 'Ca. P. fraxini' was detected in vineyards in Veneto and Puglia regions, while only in Veneto a 16SrVI phytoplasma was identified. In vineyards of this region *S. titanus* and *O. ishidae* were also found infected with 16SrXII-A, 16SrVII, and 16SrVI, and 16SrI-B phytoplasmas were identified in *O. ishidae* and *H. obsoletus*, and 16SrX-B in *S. titanus* (Zambon *et al*., 2018a; 2018b; Fiore *et al*., 2018). In the Asian continent there are new reports of phytoplasma from grapevine from Iran where '*Ca.* P. fraxini', '*Ca*. P. aurantifolia', '*Ca*. P. solani' and '*Ca*. P. phoenicium'-related strains were identified. In particular in the Marzaki province, '*Ca*. P. aurantifolia' strains were mainly detected, while in the other two provinces, all the four '*Candidatus* species' were identified with the prevalence of '*Ca*. P. solani'-related strains (Zamharir *et al*., 2017; Salehi *et al*., 2016).

PHYTOPLASMA - INSECT - GRAPEVINE INTERACTION

The most studied interactions are those of FD and BN phytoplasmas. For FD 347 sequences, corresponding to 215 annotated genes were assembled allowing to also tentatively filling the gaps of the FD draft genome already. Functional classification indicates that the most expressed genes were either related to translation and protein biosynthesis or hypothetical proteins with unknown function. Some of these latter were predicted to be secreted, acting as effectors with a potential role in modulating the interaction with the host plant. The qRT-PCR validation of the RNA-Seq expression values confirmed that a group II intron represented the FD genomic region with the highest expression during grapevine infection (Abbà *et al*., 2014). The early whole transcriptomic response of two grapevine varieties, one very susceptible to FD and the other tolerant to the disease was compared taking into account the plant constitutive features, the response to the vector infestation and the response to the phytoplasma presence. The presence of passive defense mechanisms in Tocai friulano, related to the higher constitutive expression of several defense-related genes compared to Chardonnay was observed together with the presence of FD phytoplasma signals that allow the repression of the jasmonate/ethylene-mediated response induced by *S. titanus* feeding (Bertazzon *et al*., 2018). The FD phytoplasma VmpA gene was expressed in *Spiroplasma citri* with a plasmid vectors in which the vmpA coding sequence was under the control of the *S. citri* tuf gene promoter and resulted in higher accumulation of VmpA than with the native promoter (Renaudin *et al*., 2015). Using *S. citri* mutants expressing the FD VmpA at the membrane level and fluorescent "beads" covered with VmpA studies were carried out to study interaction between these proteins and *Euscelidius variegatus* cells in adhesion tests *ex vivo* and *in vivo* ingestion. The results show the VmpA activity as adesin and a possible essential role in FD insect colonization (Arricau-Bouvery *et al*., 2017). Using leaf vein-enriched tissues of FD infected grapevines of cv. Modrafrankinja the seasonal transcriptional profiles of 14 genes showed an FD-specific plant response compared to other GY and associated with the SWEET17 vacuolar transporter of fructose. Non-targeted metabolome analysis identified 22 significantly changed compounds with increased levels during infection. Several metabolites corroborated the gene expression study. The dynamics of carbohydrate metabolism revealed significant accumulation of sucrose and starch in the mesophyll of FD-infected leaves and a significant up-regulation of genes involved in their biosynthesis. In addition, infected leaves had high activities of ADP-glucosepyrophosphorylase and sucrose synthase. The FD infection inhibits phloem transport, resulting in accumulation of carbohydrates and secondary metabolites (Prezelj *et al*., 2016). The role of six local grapevine

varieties as a source of FD inoculum for the insect vector *S. titanus* was investigated in Piedmont (Italy) comparing the FD load between red and white varieties with different susceptibility to the disease and using laboratory-reared *S. titanus.* The load for cv Arneis was significantly lower than for other varieties and although acquisition efficiency resulted to depend on grapevine variety and on FD load, even varieties supporting low FD multiplication resulted highly susceptible and good sources for vector infection, while poorly susceptible varieties results also to host high phytoplasma loads (Galetto *et al*., 2016). Extensive FD surveys in vineyards in France showed that Cabernet Sauvignon is highly susceptible, with a high proportion of symptomatic branches and phytoplasma titres, in contrast to Merlot. Localized insect transmissions and grafting showed that the phytoplasmas circulate in the whole plant in the Cabernet Sauvignon, but in Merlot they are restricted to the transmission point. Insect-mediated transmission under high confinement mimicking natural conditions allowed the classification of 28 *Vitis* accessions into three categories, according to the percentage of infected plants and their phytoplasma titres. Reduced symptoms, low phytoplasma titres, and low percentages of infected plants were found to be associated in the *Vitis vinifera* cultivars while the low susceptibility of Merlot was observed for one of its parents, i.e., Magdeleine Noire des Charentes. Rootstocks and their *Vitis* parents, although having high percentages of infected plants and intermediate to high phytoplasma titres, shared a symptomless response confirming that they can constitute a FD reservoir (Eveillard *et al*., 2016).

The application of the first droplet-digital-PCR-based absolute quantification of FD phytoplasma using the secY gene was reported. The sensitivity of the assay shown that it could be used for quantification and quality control of DNA based on in-house reference materials typically used in diagnostics and metrological laboratories. This new tool has great potential for monitoring phytoplasma kinetics, such as the progress of an infection, and variations of the phytoplasma titer through the season and, screening plants for resistance (Mehle *et al*., 2014).

MANAGEMENT

Analyses of space-time statistical features of a FD epidemic in *V. vinifera* plants were obtained in a vineyard of 17,500 m^2 surface area in the Piedmont region, Italy. Space-time dynamic point pattern analyses were applied to newly infected and recovered plants to highlight statistics of FD progression and regression over time. Results highlighted point patterns ranging from disperse (at small scales) to aggregated (at large scales) over the years, suggesting that the FD epidemic is characterized by multiscale properties that may depend on infection incidence, vector population, and flight behaviour. Dynamic analyses showed moderate preferential progression and regression along rows. Nearly uniform distributions of direction and negative exponential distributions of distance of newly symptomatic and recovered plants relative to existing symptomatic plants highlighted features of vector mobility similar to Brownian motion. (Maggi *et al*., 2017). Since Chile is free of FD and its vector *S. titanus* the consequences in case of accidental insect introduction in the Country were evaluated through a model using the BIOCLIM-DOMAIN tool, considering the current climatic conditions and the projections of estimated climate change in Chile. Results indicated that the establishment and the survival of the insect in Chile is possible considering current and projected climatic conditions (Quiroga *et al*., 2017).

During 2016, three field trials were conducted in organic vineyards to evaluate the efficacy of kaolin, orange oil, insecticidal soap and spinosad against *S. titanus* nymphs, in comparison with pyrethrins. The activity of kaolin was evaluated also in laboratory. In all field trials, kaolin had an efficacy against nymphs comparable to pyrethrins, while the other products were not effective. Laboratory results confirmed that kaolin increased nymph mortality. In organic vineyards, kaolin and pyrethrins are valuable tools in the management of FD. Nevertheless, their efficacy is lower compared to that of the synthetic insecticides used in conventional viticulture (Tacoli *et al*., 2017). Grapevine plants Glera and Chardonnay were treated for two years three times per year with (cold) plasma activated water (PAW) showed a reduction of symptoms and an increased fitness. Molecular analysis showed 40% reduction of phytoplasma presence in PAW treated plants (Zambon *et al*., 2017). Volatiles emission from BN recovered grapevine after treatment with acibezolar-S-methyl (BTH) and two glutathione oligosaccarin based products applications was evaluated in an Italian Chardonnay vineyard. These volatiles were repellents to *H. obsoletus* adults while one of them strongly attracted cixiids showing interesting potential in practical application for organic farming (Riolo *et al*., 2017).

Healthy *S. titanus* nymphs were allowed to feed on FD infected and recovered grapevines Chardonnay and Barbera and the recovered plants resulted not acting as inoculum sources. Moreover in Chardonnay the recovery percentage was very low and the wine produced was having a lower alcohol content and a low acceptability to the tasting; for Barbera the recovery was consistent and the wine parameters from recovered grapevine were similar to those of healthy plant produced wine (Galetto *et al*., 2017). '*Ca*. P. solani' was detected in the 30% of Chardonnay recovered grapevines moreover the phytoplasma populations in symptomatic and recovered plants were distinguishable on *vmp1* gene (Quaglino *et al*., 2017). A study aimed to understand whether salicylate- and jasmonate-defense pathways might have a role in the recovery from the BN disease was carried out using leaves from healthy, BN-infected and recovered plants, both in the presence (late summer) and absence (late spring) of symptoms. In symptomatic diseased plants (late summer), unlike symptomless plants (late spring), salicylate biosynthesis was increased and salicylate-responsive genes were activated. In contrast, jasmonate biosynthesis and signaling genes were up-regulated both in recovered and diseased plants at all sampling dates. Activation of the salicylate signaling pathway that is associated with the BN presence seems to antagonize the jasmonate defense response, by failing to activate or suppressing both the expression of some jasmonate responsive genes that act downstream of the jasmonate biosynthetic pathway, as well as the first events of the jasmonate signaling pathway. On the other hand, activation of the entire jasmonate signaling pathway in recovered plants suggests the potential importance of jasmonate-regulated defenses in preventing BN infections and disease (Paolacci *et al*., 2017).

Three media were evaluated for phytoplasma isolation and colony formation under microaerophilic growing conditions, using grapevine canes from plants showing yellows symptoms, and infected by FD, BN and AY aster yellows phytoplasmas as sources for two years at three sample collection times. Broad applicability and a good repeatability in supporting phytoplasma colony formation were obtained in Pivs® and CBs media. While the MB medium did not support phytoplasma isolation and growth, the CB media support a phytoplasma growth comparable to the one obtained in the previously reported media (Contaldo *et al*., 2012). This medium has a formulation that allows its modification to implement specificity towards selective phytoplasma growth (Contaldo *et al*., 2016). Following this report under the frame of a new H2020 project (TROPICSAFE) leaded by Italy the use of phytoplasma colonies from grapevine will be employed for antisera development and screening for phytoplasma susceptibility.

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O33: A potential new vector of aster yellows phytoplasma in vineyards in South Africa

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INTRODUCTION

Aster yellows phytoplasma (AY; 16SrI-B group) is associated with a severe disease in grapevine (*Vitis vinifera*) and is of phytosanitary concern in South Africa. It was recorded for the first time from vineyards in the Western Cape in 2006 (Engelbrecht et al., 2010). The phytoplasma has a broad host range (Hogenhout et al., 2008) and has been recorded from various wild and crop plants growing in and around vineyards in South Africa (Krüger et al., 2015). Several leafhopper species (Hemiptera: Auchenorrhyncha: Cicadellidae) are known to transmit AY (Weintraub and Beanland, 2006). The leafhopper *Mgenia fuscovaria* (Stål) has been identified as a vector of AY in South Africa (Krüger et al., 2011). However, the demonstrated transmissibility of AY to *Catharanthus roseus* (periwinkle) in the field is in contrast to the inability of *M. fuscovaria* to transmit the pathogen to grapevine(Krüger et al., 2015). This, together with the presence of AY in other leafhopper species, suggests that further taxa might be involved in the AY pathosystem in South African vineyards. In order to assist with the development of AY management strategies, leaf- and planthoppers were screened for the presence of AY, and transmission assays were carried out to identify vectors other than *M. fuscovaria.*

MATERIALS AND METHODS

Experiments were carried out in Vredendal in the Western Cape, South Africa, during different times of the year with leaf- and planthoppers collected in AY-infected vineyards. Transmission assays with field-collected insects were performed by giving one to six adults of the same species or species group access to an artificial sucrose feeding medium through Parafilm® for 24 to 48 hours. The media were screened after completion of feeding for the presence of AY phytoplasma following Bosco and Tedeschi (2013). In addition, transmission assays were carried out with *Nicotiana benthamiana, Triticum aestivum* (wheat, cultivar Duzi), and periwinkle as recipient plants. Groups of 3 to 20 adult leafhoppers per species were given inoculation access periods (IAPs) of 24 to 72 hours. Group size was determined by the number of insects collected in the field. In order to confirm that insects were collected from AY-infected vineyards and that recipient plants were AY-free prior to transmission assays, leaves from grapevine plants in vineyards and recipient plants were tested for the presence of AY. Nucleic acid extraction from leaf veins was done using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany). Nucleic acids from single intact leafhoppers were obtained using a non-destructive TNES buffer (1 M Tris-HCl, pH 7.4, 5 M NaCl, 0.5 M EDTA, 10 % SDS) extraction method adapted from a protocol provided by J. Peccoud and N. Sauvion (INRA Montpellier, France) based on Sambrook and Russell (2001) to preserve specimens for morphological identification. Field-collected insects, feeding media and plant samples were tested for the presence of AY with real-time PCR (Angelini et al., 2007).

Results and discussion

A total of 136 adult leaf- and planthopper samples were tested for the presence of AY, 80 of which were used in the artificial feeding assays. The leafhopper *Aconurella prolixa* (Lethierry) tested positive for AY and successfully transmitted the pathogen to the artificial feeding medium (Table 1). *A. prolixa* is a grass-feeding species, and controlled transmission assays were carried out with wheat. Eight wheat plant samples out of 51 used in transmission assays with this species tested positive for AY. One *Exitianus* sp. adult tested positive for AY but did not transmit the phytoplasma to the artificial feeding medium nor to *N. benthamiana* or periwinkle (*N. benthamiana*, n = 11; periwinkle, n = 2). Although *Cicadulina* spp. and *Toya* sp. tested positive for AY in an earlier study (A. de Klerk, pers. comm.) they did not transmit AY to the feeding medium nor to *N. benthamiana* (*Cicadulina* spp: n = 5, *Toya* sp.: n = 17). None of the other species tested positive for AY or transmitted the phytoplasma to the artificial feeding medium (Table 1).

Table 1. Presence of aster yellows phytoplasma (AY) in field-collected leaf- and planthoppers and transmission of AY to an artificial sucrose feeding medium used for screening for potential insect vectors.

Insect species	Insects tested/AY-positive insects	Feeding media tested/AY-positive media
Cicadellidae (leafhoppers)		
Aconurella prolixa	12/1	3/1
Cicadulina spp.	39/0	25/0
Exitianus sp.	45/2	19/0
Delphacidae (planthoppers)		
Toya sp.	3/0	2/0
Unidentified spp.	37/0	31/0

Previously, several plant species, including Poaceae, were identified as reservoir plants of AY in South African vineyards, and the leafhopper vector *M. fuscovaria* was able to transmit AY experimentally to several poaceous plant species (maize, wheat, triticale) (Krüger et al., 2015). It is not known whether *M. fuscovaria* or *A. prolixa* can transmit AY from Poaceae to grapevine. However, the identification of the grass-feeding *A. prolixa* as a potential vector suggests complex interactions between the phytoplasma, host plants and potentially more than one leafhopper vector species.

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O34: Potential role of *Euscelis incisus* Kirschbaum and *Dicranotropis hamata* Boheman in the transmission of '*Candidatus* Phytoplasma solani' to grapevine

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INTRODUCTION

In Euro-Mediterranean regions, "bois noir" (BN) phytoplasma strains ('*Candidatus* Phytoplasma solani', subgroup 16SrXII-A) (Quaglino *et al*., 2013) are transmitted to grapevine by *Hyalesthes obsoletus* Signoret, a polyphagous vector living preferentially on nettle (*Urtica dioica* L.), bindweed (*Convolvulus arvensis* L.), mugworth (*Artemisia vulgaris* L.), and chaste tree (*Vitex agnus-castus* L.) inside and/or around vineyards (Langer and Maixner, 2004; Sharon *et al*., 2015). Grapevine represents a dead-end host for this phytoplasma in grapevine, which is incidentally transmitted by *H. obsoletus* from other host plants (Weintraub and Beanland, 2006). In grapevine-growing areas where *H. obsoletus* is absent, the presence of BN implies the existence of alternative vectors. Recently, *Reptalus panzeri* Low and *Reptalus quinquecostatus* Dufour were reported as BN vectors in Serbia and France, respectively (Cvrković *et al*., 2014; Chuche *et al*., 2016). Several reports indicate that other Cixiidae and Cicadellidae captured within or near BN-diseased vineyards were found to contain the phytoplasma (Batlle *et al*., 2000; Palermo *et al*., 2004) therefore additional insect species could be capable of spread the disease (Mori *et al*., 2015). In the present study, transmission trials have been conducted to investigate the possible transmission of BN phytoplasma to grapevine from Cixiidae and Cicadellidae species prevalent in a vineyard in Franciacorta (Lombardy region, North Italy).

MATERIALS AND METHODS

Specimens of the 7 prevalent BN phytoplasma-harboring insect species, captured on 3 sampling days (June 11 and 25; July 7, 2015) in a BN-affected vineyard in Franciacorta, have been forced to feed on phytoplasma-free grapevine (cv Chardonnay) plants under controlled conditions. Dead insects were maintained at -30°C. '*Ca*. P. solani' was detected by nested PCR on *stamp* gene (Fabre *et al*., 2011) using as templates the total nucleic acids extracted from both the insect specimens (grouped in pools) and the petioles of grapevine plants collected in October 2015 and July 2016 (plants were kept in an insect-free greenhouse for one year).'*Ca*. P. solani' strains detected in insects and plants were characterized through nucleotide sequence analyses of *stamp* amplicons.

RESULTS AND DISCUSSION

Stamp gene amplification detected '*Ca*. P. solani' in *H. obsoletus*, *Euscelis incisus* and *Dicranotropis hamata* specimens and in grapevine plants on which they were allowed to feed, sampled in July 2016, one year after the transmission trials. Some *E. incisus* and *D. hamata* insects were negative but their host grapevine plants were positive to the PCR. The pooled insect analysis might have diluted the phytoplasmas to a too low titer for their detection. Only plants hosting *H. obsoletus* were infected by '*Ca*. P. solani' also in October 2015, at the end of transmission trials. No amplification was obtained from control plants kept in controlled conditions without insects in both years (Table 1).

Moreover, identity analysis of *stamp* gene nucleotide sequences evidenced that the same '*Ca*. P. solani' strain, identical to the prevalent strain in the examined vineyard in Gussago (Sanna *et al*., 2016), was found in the insect pools and grapevine plants used in the transmission trial (data not shown). These results indicated that *E. incisus* and *D. hamata* are able to transmit '*Ca*. P. solani' to grapevine. In previous studies *E. incisus* was found positive to '*Ca*. P. solani' (Škorić, 2013) and able to transmit "stolbur" from infected to healthy clover (Valenta *et al*., 1961); *D. hamata* was found infected by '*Ca*. P. solani' only in Franciacorta vineyards (Sanna *et al*., 2016). To the best of our knowledge, this is the first study reporting *E. incisus* and *D. hamata* as '*Ca*. P. solani' vectors to grapevines. Further studies are needed to investigate transmission efficiency in the open field and to accurately study the ecology of these insects.

Table1. Identification of '*Ca*. P. solani' in insects and grapevines used in transmission trials.

Insect species	Transmission date	'*Ca.* P. solani' detection			
		Insect pools (positive/total)	Grapevine plants		
			June 2015	Oct. 2015	July 2016
Control (no insects)	June 11	-	-	-	-
	June 25	-	-	-	-
	July 7	-	-	-	-
Dicranotropis hamata Boheman	June 11	0/11	-	-	+
	June 25	1/7	-	-	+
	July 7	0/6	-	-	-
Euscelidius variegatus Kirschbaum	June 11	0/3	-	-	-
	June 25	0/2	-	-	-
	July 7	0/2	-	-	-
Euscelis incisus Kirschbaum	June 11	2/3	-	-	+
	June 25	0/2	-	-	+
	July 7	0/2	-	-	+
Hyalesthes obsoletus Signoret	June 11	2/3	-	+	+
	June 25	1/3	-	+	+
Laodelphax striatella Fallén	June 25	0/4	-	-	-
	July 7	0/6	-	-	-
Psammotettix alienus Dahlbom	June 11	0/7	-	-	-
	June 25	0/7	-	-	-
	July 7	0/6	-	-	-
Reptalus panzeri Low	June 11	0/3	-	-	-

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O35: Spatial distribution of "bois noir" in four commercial vineyards cv Chardonnay

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INTRODUCTION

In Europe, one of the most recurrent grapevine yellows is "bois noir" (BN), which is becoming a real limiting factor for the productions (Maixner, 2011). The cv Chardonnay is particularly susceptible to BN infection, and a dramatic reduction of quantity of production was recorded, up to 50% in symptomatic plants, with consistent lower sugar content in the grape juice (Endeshaw et al., 2012). '*Candidatus* Phytoplasma solani' is the BN-associated phytoplasma (Quaglino et al., 2013) transmitted mainly by the polyphagous cixiid planthopper *Hyalesthes obsoletus* to a wide range of wild plants such as *Convolvulus arvensis*, *Calystegia sepium*, *Urtica dioica* and several other herbaceous hosts (Langer and Maixner, 2004). The analysis of spatial distribution of diseases may help to provide insights and hypotheses on aetiology and epidemiology of diseases and ecology of plant pathogens. Recently, several studies have focused on spatial analysis, improving the knowledge about BN epidemiology (Marchi et al., 2011; Murolo et al., 2014) and the possible role of host plants and insect vectors in the spread of the disease (Navratil et al., 2009; Mori et al., 2012; Landi et al., 2015; Mori et al., 2015) have been investigated. Aim of this work was to study the epidemiological pattern of BN in four commercial vineyards located in different pedoclimatic conditions in Marche region (central-eastern Italy).

MATERIALS AND METHODS

In four commercial vineyards cv Chardonnay, visual inspections were carried out at the late summer 2014, in order to evaluate the spread of BN. In order to estimate the disease severity, for the symptomatic plants an empirical scale (0-4) was used, where 0 = asymptomatic grapevine, 1 = plant showing 1–2 leaves with symptoms; 2 = plant showing more than 2 leaves with symptoms on one shoot; 3 = plant with leaves with symptoms on more than one shoot; and 4 = plant with more than 50% of canopy with symptoms. Bidimensional maps were elaborated for each vineyard by SYSTAT program, plotting symptoms and symptomless/healthy grapevines in order to spatially monitor the BN epidemiologyr. The occurrence of disease gradients within the vineyard was also studied. The percentages of diseased plants in each plot were plotted and the regression curve calculated in each vineyard. Using PASSAGE software, v. 2 (Rosenberg and Anderson, 2011), the aggregation of adjacent vines and the more complex spatial relationships over longer distances were examined by Morisita's index. The data of symptom severity (z) of each grapevine were defined with respect to plant position (x, y). The SYSTAT software first computes its own square grid of interpolated or directly estimated values. From this grid, contours were followed using the method of Lodwick and Whittle (1970), combined with linear interpolation. The plot automatically determines the number of contours to draw, so that the surface is defined and the contour labels can be characterized by different colors.

RESULTS AND DISCUSSION

In vineyard 1, 204 out 1,744 grapevine plants (11.7%) were symptomatic and most of them showed the disease severity belonging to class 2 (40.2%) and 3 (40.7%). In the vineyard 2, 259 out of 2,884 grapevine plants (9%) resulted symptomatic and mainly with a severity in class 2 (34.7%) and 3 (46,3%). In vineyard 3, 15.8% of surveyed plants (414 out of 2,614) showed BN symptoms, with a disease severity mainly in class 2 (26.8%) and 3 (41.1%). About 26% of symptomatic plants showed symptoms on more than 50% of the canopy (class 4). Finally, in vineyard 4 symptoms were recorded on about 13.1% of grapevine plants (734 out of 5,618), and most of them belonged to class 3 (37.6%) and 4 (33.5%), followed by severity class 2 (25.9%).

From the analysis of the dispersion index, the distribution of the grapevines with symptoms showed a clustered pattern. In particular, Morisita's index was >1 for all the vineyards. The indices of dispersion show that the distribution of the grapevines with symptoms follows a clustering of symptomatic plants or a clustering of healthy plants. Considering the distribution of grapevines in the vineyard according to the severity of the BN leaf symptoms, the construction of the two-dimensional contour maps provides a clearer graphical visualization of the grapevines that were more severely affected by BN along at least one of the borders of the vineyards, as reported

for the “flavescence dorée” disease (Pavan et al., 2012). The picture of disease severity appears to confirm a natural source of inoculum and the activity of potential vectors in spreading BN in this vineyard.
The monitoring of spatial distribution of BN in four commercial vineyards, analyzed with the data of molecular typing of ‘*Ca.* P. solani’, allowed to monitor the progression of the disease in the field, tracing the epidemic history, and understanding dispersal routes of pathogens (Maggi et al., 2017), as well as the impact and the efficiency of some control strategies (Romanazzi et al., 2013).

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O36: Results of the surveillance for "flavescence dorée" phytoplasma and *Scaphoideus titanus* in the Republic of Srpska (Bosnia and Herzegovina)

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INTRODUCTION

Since 2005 several surveys for grapevine yellows phytoplasmas and their putative insect vectors were conducted in Bosnian and Herzegovinian (BiH) vineyards (Delić et al., 2016; Đurić et al., 2017). The results of the surveys indicate the presence of '*Candidatus* Phytoplasma solani' in grapevine and in potential insect vectors and alternative hosts in Herzegovina vineyards (south part of the country with dominant grapevine production). Moreover in the same region a *Clematis vitalba* phytoplasma belonging to 16SrV-C subgroup was identified as well as the presence of *Scaphoideus titanus* Ball (Delić et al., 2011). In 2017 the first official special surveillance for the presence of "flavescence dorée" phytoplasma and *Scaphoideus titanus i*n Republic of Srpska, (constitutive part of Bosnia and Herzegovina) was announced with the main objective to verify the presence of "flavescence dorée" phytoplasma in grapevine, *C. vitalba*, *Alnus* spp. and *Ailantus altissima* and to monitor the presence and distribution of *S. titanus in all grapevine growing regions.*

MATERIALS AND METHODS

One hundred and five samples (81 grapevines, 6 *A. glutinosa*, 3 *A. altissima* and 14 clematis) were collected in 5 locations during July and August 2017. In addition, insects were captured on yellow sticky traps (Bug-Scan, Biobest, 25×40 cm) inside the same vineyards plots from July to September of the same year. Identification of leafhoppers was carried out using identification keys from Holzinger et al. (2003) and Biedermann and Niedringhaus (2004). Total nucleic acid extraction from leaf midribs was performed according to Green et al. (1999). All samples were tested for phytoplasma presence using PCR assays with universal primers P1/P7, followed by R16F2n/R2 in nested PCR and subsequent RFLP analysis (Lee et al., 1998). Later a multilocus sequence typing (MLST) approach was used to amplify *tuf*, *vmp1*, *stamp*, *secY*, *uvrB-degV* and *map* gene regions in phytoplasma positive samples (Schneider et al., 1997; Fialová et al., 2009; Fabre et al., 2011, Arnaud et al., 2007).

RESULTS AND DISCUSSION

Molecular analyses revealed phytoplasma presence in three grapevine and one *A. glutinosa* samples. RFLP with *Mse*I enzyme showed restriction profile specific for phytoplasmas from 16SrXII-A subgroup the grapevine samples and 16SrV group in *A. glutinosa* sample. Furthermore, MLST analyses of *tuf*, *vmp1* and *stamp* genes identified tuf-type b/V17/Rqg50 genotype in the grapevine samples (Trebinje locality), while map and uvrB-degV typing of AG92 isolate from *A. glutinosa* (Gradiska locality) indicate similarity with strains *map*-FD1 (FD70) (GenBank accession number MG551289) and PGY-A (GenBank accession number MG581414), respectively. This is the first report of the 16SrV phytoplasma group on *A. glutinosa* in BiH. The tree didn't exhibit symptoms. *A. glutinosa* trees and vineyards are often in close contacts in north part of Republic of Srpska where only 2% of grapevine cultivation is located. The 16SrV phytoplasma alternative hosts such as clematis and *A. altissima* are widely present around vineyards in Herzegovina and should be considered as a potential threat.

Investigation aimed to detect the presence of the vector *S. titanus* allow to detect its presence in four out of five examined vineyards in RS. Only in a previously insecticide treated vineyard taken as negative control the *S. titanus* presence was not recorded. From the middle of July to the end of August/beginning of September 1,028 *S. titanus* specimens were identified on yellow sticky traps (Table 1). Adults of *S. titanus* were collected from different grapevine varieties, different climatic regions such as mild Mediterranean climate in Trebinje and low continental climate in Prnjavor and Kozarska Dubica localities. *S. titanus* was identified also in vineyard (cultivar collection, Kozarska Dubica) located in a region where grapevine production is not common. These last hypotheses on this vector movement is that spreading of *S. titanus* is mainly due to human activities (Chuche and Thiery, 2014). From the different ratio between males in females in almost the same periods differences are observed between life cycles of the species present in south part (Trebinje) from those at north (Prnjavor, Kozarska Dubica) of RS (Table 1). Moreover *Dictyophara europaea* was identified on the yellow sticky traps from

Trebinje locality. The obtained results give useful direction for future studies, having in mind that “flavescence dorée” phytoplasma is well established in the neighboring countries Serbia, Croatia and Slovenia (EFSA, 2014) it is necessary to continue the surveys and testing of grapevines and wild plant potential reservoirs of this phytoplasma. Studies about life cycles of *S. titanus* in the north and south part of the state and on presence of other Auchenorrhyncha in and around vineyards are needed in order to design appropriate management for control strategy.

Table 1. *S. titanus* in selected vineyards in Republic of Srpska, BiH in 2017.

Trebinje					
Mokro polje*			Petrovo polje**		
15-27.7.2017	27.7-9.8.2017	15.8-6.9.2017	15-27.7.2017	27.7-9.8.2017	15.8-6.9.2017
52♂ 1♀	155♂ 7♀	5♂	177♂	200♂	2♂
Banjaluka					
Prnjavor***					
20.7-8.8.2017	8-25.8.2017				
139♂ 182♀	72♂ 86 ♀				
Prijedor					
Kozarska Dubica****					
19.7-9.8.2017					
14♂ 2♀					

Geographic position: *42,66615918,336115; ** 42,67264818,328083; ***44.87794317.752120; ****45,09645816,968187

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O37: Emergence of "flavescence dorée" in Croatia: distinct genetic clusters and new hotspots

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INTRODUCTION

"Flavescence dorée" (FD) phytoplasma comprising ribosomal subgroups 16SrV-C and –D, is one of the most important agents associated with epidemic and severe grapevine yellows (GY) diseases in the Euro-Mediterranean region. It is transmitted by the grapevine leafhopper *Scaphoideus titanus* Ball. The role of some other insect species as alternative vectors in FD and FD-related phytoplasma epidemiology has been studied (Filippin *et al.,* 2009; Mehle *et al.,* 2011; Casati *et al.,* 2016). The first identification of FD phytoplasma infecting grapevine in Croatia was recorded in 2009 (Šeruga Musić *et al*., 2011) in limited areas of continental regions of the country which initiated very intensive efforts in surveillance of all winegrowing regions. Thus, the objectives of this work were to give the overview of a decade of FD monitoring in Croatia with the emphasis on newly emerging hotspots, as well as to evaluate the advantage in using multilocus sequence typing (MLST) approach for tracing the disease emergence and spread. The distribution and prevalence of FD strains together with the finding of potential alternative plant hosts and insect vectors are also discussed.

MATERIALS AND METHODS

In the period 2008-2017, more than 1,200 samples of grapevine, wild herbaceous and woody plants as well as *S. titanus* and other potential insect vectors, were collected from different viticultural regions in the continental and coastal parts of Croatia. Phytoplasma detection and identification was performed by triplex real-time PCR (Pelletier *et al.,* 2009) enabling simultaneous detection of FD and "bois noir" phytoplasmas. Routine PCR/RFLP of 16S rDNA was also performed as reported (Plavec *et al*., 2015). MLST approach was applied for further characterization and genotyping of selected FD phytoplasmas by analyses of *secY*, *map* and *uvrB-degV* genes according to Arnaud *et al.* (2007). All amplicons were sequenced (Macrogen Europe, Amsterdam, the Netherlands), assembled and edited by using SequencherTM 4.7 (http://www.genecodes.com/) and Geneious software (http://www.geneious.com/), and aligned with ClustalX 2.0 (Thompson *et al.,* 1997). Phylogenetic analyses were performed by using MEGA 7 software (Kumar *et al.,* 2016) and followed by assignment of a comprehensive genotype.

RESULTS AND DISCUSSION

After the first detection of FD phytoplasmas in 2009 from 2 grapevine samples in the central continental Croatia, and the detection of infected *S. titanus* samples in 2011, the emergence of the disease and its epidemic trend in some of the important winegrowing regions was observed. In 2014, FD phytoplasma was for the first time identified in the northern coastal part of Croatia (Istria), where in the following year very high incidence of FD-infected grapevines of cultivar Malvasia occurred. New fast developing hotspots were observed in north-western continental region of the country with severe disease outbreaks in years 2016 and 2017 (Figure 1). MLST of selected FD phytoplasmas from grapevine, insect vectors and wild plants revealed the presence of distinct pathosystems and at least 16 different comprehensive genotypes. The greatest diversity of comprehensive genotypes was observed within mapFD2 cluster which was found to be prevalent in the north-western continental and the only one present in severely affected coastal region of the country clearly associated with the epidemic trend (Figure 1). In the eastern part of continental Croatia where no severe outbreaks have been observed, only FD phytoplasmas enclosed in mapFD3 cluster were found (Figure 1). Moreover, the finding of different genotypes belonging to mapFD2 and mapFD3 genetic clusters identified from the leafhopper *Phlogotettix cyclops* and the invasive species *Ailanthus altissima* Mill., respectively, suggests that alternative players might also be involved in FD epidemiology in Croatia.

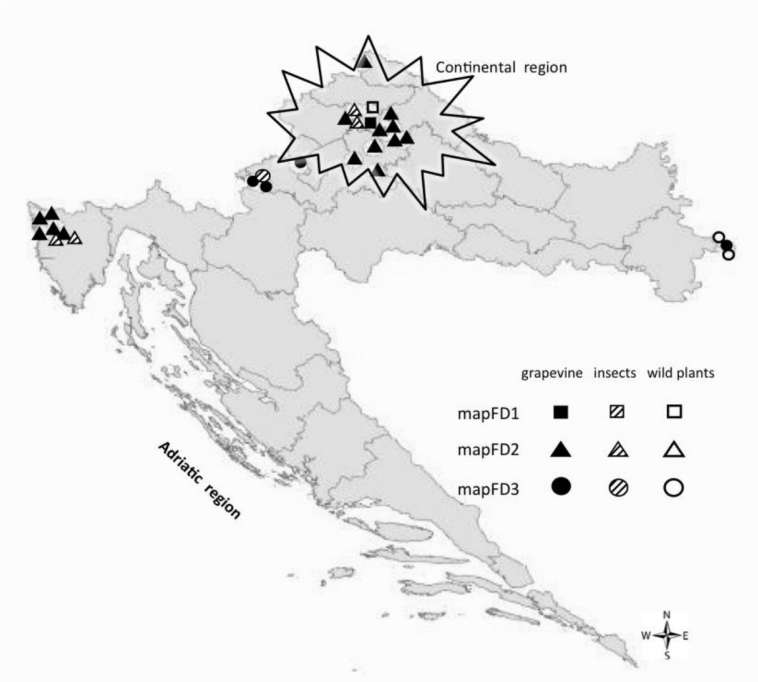

Fig. 1. Geographic distribution of *map* genetic clusters (mapFD) of "flavescence dorée" phytoplasma in Croatia. Marked is the area encompassing new hotspots with severe outbreaks in 2016 and 2017.

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O38: A teaching tool or how to calculate the "bois noir" disease severity

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INTRODUCTION

The disease triangle, a model in which the presence or severity of disease is the result of interactions among environmental, host and pathogen conditions, has been the basic concept used to describe the tritrophic relationships in plant pathology since the 1960s. However, except as a teaching tool for describing and easier understanding of interaction among pathogen, host and environment used in general plant pathology textbooks, it has never been constructed or used in practice. Although the disease triangle has been addressed in a Perspectives essay in Nature Reviews (Scholthof, 2007), this is the first actual disease triangle calculation and representation. The statistical model we have used for disease triangle construction is '*Candidatus* Phytoplasma solani' infection of cv Chardonnay grapevine plants, resulting in "bois noir" disease. "Bois noir" (BN) is the most common grapevine phytoplasma disease and has been, due to its potentially destructive outcome and thus agronomic impact, a subject of extensive research in recent years. It is also commonly used as a model system for studying grapevine yellows diseases.

MATERIALS AND METHODS

A proof of concept statistical model for disease triangle using original experimental data and different statistical and data mining approaches for a selected system of '*Ca*. P. solani' infection of cv. Chardonnay grapevine plants was generated. Phytoplasma content, the expression of 21 selected grapevine genes and environmental conditions were recorded and related to disease severity.

RESULTS AND DISCUSSION

The model (Fig. 1) predicts that in described conditions BN is a function of the expression of grapevine gen VVDMR6 (downy mildew resistance 6), summer rainfall and abundance of '*Ca*. P. solani'. The greatest impact among elements of the disease triangle is attributed to the pathogen, and is independent from the pathogen titer. DMR6, encodes oxidoreductase (2-oxoglutarate (2OG)-Fe(II) oxygenase) with unknown function (Van Damme *et al*., 2005, 2008; Zeilmaker *et al*., 2015). However, it is required for the susceptibility of *Arabidopsis* to *Hyaloperonospora arabidopsidis*, a causing agent of downy mildew in *Arabidopsis*. VvDMR6 has shown similar pattern during the infection of "flavescence dorée" that is associated with the most devastating grapevine phytoplasma disease. Based on collective evidence VvDMR6 has been suggested as a potential early marker of grapevine yellows diseases (Prezelj *et al*., 2016; Rotter *et al*., 2017).

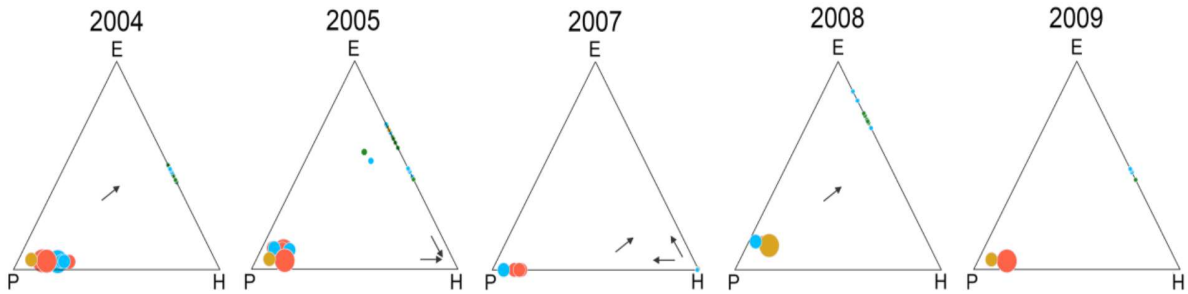

Figure 1: Disease triangles calculated for each growing season. Each bubble represents an individual plant. The position of a bubble within each triangle denotes the importance of each of the elements: pathogen (P), host plant (H) and environment (E). The plant sanitary status is represented by bubble color: green, plants that remained uninfected during the whole period; red, plants infected during the whole period; blue, plants tested positive to the pathogen in one season and negative in the subsequent; ochre, plants in which testing to pathogen was only temporary negative. The size of the bubbles denotes the disease severity group. The arrows indicate transitions

between the sanitary statuses of individual plants from one growing season to another. (Figure is taken from Rotter *et al*., 2017 and distributed under the terms of the Creative Commons Attribution 4.0 International License). This first *de facto* representation of the disease triangle is useful for showing disease dynamics over several years and could be applied to other plant-pathogen systems. It was create not only the system for disease triangle representation, but also a list of statistical and molecular biology methods needed to construct it. Additional added value of this study is a dynamic representation of the triangle, where using a newly designed R package, animatoR, it is possible to animate disease transitions between years. The overall results of this study contribute to understanding '*Ca*. P. solani' biology and its interactions with grapevine host plants.

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O39: New insights on Grapevine yellows disease in North-Eastern Italy

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INTRODUCTION

Grapevine yellows (GYs) is one of the most damaging phytoplasma-associated diseases that causes severe yield losses in every geographic area where grapevines are cultivated. The main yellows diseases in grapevine in Europe are “flavescence dorée” (FD, 16SrV-C/D ribosomal subgroups) (Martini *et al*., 1999) and “bois noir” (BN, '*Candidatus* Phytoplasma solani', 16SrXII-A ribosomal subgroup), transmitted by *Scaphoideus titanus* Ball and *Hyalestes obsoletus* Signoret, respectively. Recently the mosaic leafhopper *Orientus ishidae* (Matsumura) (Cicadellidae; Deltocephalinae) was found to be positive to 16SrV-C and -D phytoplasmas in Slovenia, Italy and Switzerland (Mehle *et al*., 2010; Gaffuri *et al*., 2011; Trivellone *et al*,. 2015); it was also shown as capable to transmit 16SrV phytoplasmas from broadbean to grapevine (Lessio *et al*., 2016). To verify the reasons of the continuous GY spreading in the Veneto region (North-Eastern Italy), “Prosecco areas”, the identification and molecular characterization of phytoplasmas in symptomatic and asymptomatic grapevine and insects captured in selected vineyards during a three year-survey was carried out.

MATERIALS AND METHODS

Total nucleic acids were extracted from 1 g of fresh plant tissue (leaf midribs) from 137 symptomatic and 24 asymptomatic grapevines belonging to four grapevine cultivars (Chardonnay, Glera, Pinot Gris and Perera) collected in 17 different vineyard, using a phenol/chloroform protocol. Following a CTAB- based DNA extraction procedure 29 batches of *S. titanus* (50 individuals), 26 of *H. obsoletus* (32 individuals), 69 of *O. ishidae* (89 individuals) and 2 of *H. hamatus* (4 individuals) were processed for molecular analyses to verify phytoplasma presence. Phytoplasma detection was carried out by nested-PCR using P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by $16R_{758}f/23SR_{1804}$ (Gibb *et al*., 1995; Padovan *et al*., 1995) and/or U5/U3 (Lorenz *et al*., 1995) primer pairs. Additional characterization was performed on *rp* gene with group specific primers (Lee *et al*., 2004; Martini *et al*., 2007). Direct sequencing of selected *16Sr* and *rp* gene amplicons was performed and assembled sequences were deposited in GenBank.

Table 1: Phytoplasmas detected in plants and insect samples during surveys in Treviso province vineyards. Insect numbers are referred to batches of 1 to 2 individuals.

Samples	Samples positive/collected	16Sr group/subgroup										
		V-C	V-D	XII-A	VII-A	VI	X-B	I-B	V-C + V-D	V-C + XII-A	V-C + VII-A	V-A
Grapevines	103/161	49	14	11	9	4	5	6	1	1	2	1
S. titanus	14/29	2		4	3	1	2	2				
O. ishidae	22/69	4		7	5	1		3		2		
H. obsoletus	10/27			6				4				
H. hamatus	0/2											

RESULTS AND DISCUSSION

The three years monitoring highlighted a significant percentage of phytoplasma positive plants in both, symptomatic (about 75%) and asymptomatic (about 40%) grapevine plants, with a prevalence of FD strains. During 2015 the presence of phytoplasma strains belonging to 16SrVI (4 samples) and 16SrVII ribosomal group (9 samples), both in single and in mixed infection was also detected in the 18% of the tested samples, mainly in asymptomatic plants. Moreover, phytoplasmas belonging to ribosomal groups 16SrI-B, 16SrV-A and 16SrX-B were occasionally detected in 12 samples (Table 1). Identification of phytoplasmas from insects showed the presence of 16SrXII-A, 16SrVII and 16SrVI in specimens of *S. titanus* and *O. ishidae*, while 16SrXII-A and 16SrI-B phytoplasma strains were identified in *O. ishidae* and *H. obsoletus*, and 16SrX-B in *S. titanus*. (Table 1). The results of this study confirm that GYs diseases in one of the most important viticultural areas in Italy are

associated with the presence of different phytoplasmas and diverse insects vectors. The number of *O. ishidae* captured in the selected vineyards is significantly higher than previously reported in North-West Italy and Switzerland, where the insect was quite uncommon and was collected under low density situations (Casati *et al*., 2017). Moreover the three insect species positive to phytoplasmas were carrying indeed different ribosomal groups reported as associated to GY diseases in Chile and Iran respectively (16SrVII; Gajardo *et al*., 2009; Zamharir *et al*., 2017) and occasionally in Syria (16SrVI; Contaldo *et al*., 2011). The 16SrVII-A and 16SrVI phytoplasmas were never detected before in Europe in grapevine, *S. titanus* and *O. ishidae* and their epidemiologic relevance is under further monitoring.

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O40: Detection of diverse phytoplasmas in grapevine in Apulia region, Italy

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INTRODUCTION

Grapevine yellows (GYs) are phytoplasma-associated diseases worldwide distributed in grapevine growing areas (Dermastia *et al*., 2017). "Stolbur" phytoplasmas were reported in several areas and in almost all Italian regions, where their presence is constantly increasing. During the last years, the presence, identification and molecular characterization of phytoplasmas in symptomatic grapevine (Fig. 1a), was carried out in samples collected from diverse provinces of Apulia region (South Italy) in the frame of the mandatory program of surveillance for the quarantine phytoplasma disease "flavescence dorée".

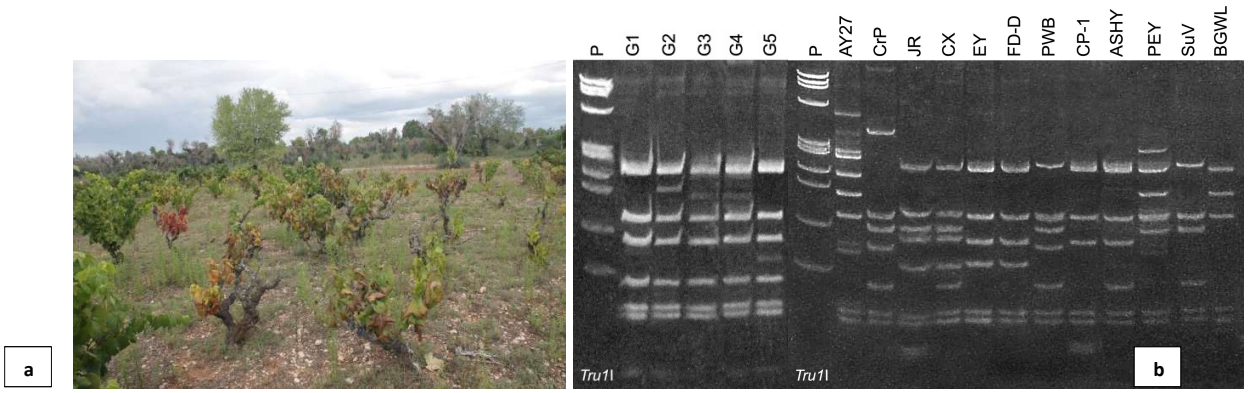

Fig. 1a. Vineyard in Apulia region of an unknown grapevine variety, showing typical GY symptoms and where the phytoplasma identified belong to the 16SrVII group. 1b. RFLP analyses in polyacrilammide 6.7% of U5/U3 amplicons from Apulian grapevine samples (G1-G5) and reference phytoplasma strains on the right. P, marker phiX174 DNA digested with *Hae*III length from top to bottom fragments in bp: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

MATERIALS AND METHODS

The surveys were carried out during the last 10 years (2006-2015) and a total of 910 samples were collected from diverse grapevine varieties in the whole Apulia region. Total nucleic acids were extracted from 1 g of fresh plant tissue (leaf midribs) using a CTAB-based protocol or a plant DNA extraction kit (Qiagen). Phytoplasma detection was carried out by nested-PCR using P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by R16F2n/R2 (Gundersen and Lee, 1996) or by $16R_{758}f/23SR_{1804}$ (Gibb *et al*., 1995; Padovan *et al*., 1995) and U5/U3 (Lorenz *et al*., 1995) primer pairs. The phytoplasma identification in positive samples was achieved by RFLP analyses with informative enzymes (*Tru*1I, *Taq*I) and comparison with classified phytoplasma strains (Bertaccini, 2014) and/or by direct sequencing of selected R16F2n/R2 and U5/U3 amplicons with at least two time coverage per sample.

RESULTS AND DISCUSSION

The different molecular assays employed confirmed the presence of phytoplasmas belonging to 16SrXII-A and 16SrVII groups (Fig. 1b) in symptomatic grapevine plants. In particular, 233 samples were found to be positive to 16SrXII-A ('*Candidatus* Phytoplasma solani', "bois noir") phytoplasmas and 5 samples positive to the presence of

16SrVII ('*Ca*. P. fraxini', ash yellows) phytoplasmas. The locations where these phytoplasmas were detected covered the whole viticultural growing areas of Apulia: Manduria and Francavilla Fontana (Taranto province), Campi Salentini, Ugento and Veglie (Lecce province), Cisternino (Brindisi province), Locorotondo (Bari province), Incoronata, Manfredonia and Cerignola (Foggia province). Both white and red cultivars from grapevine for wine and grape production resulted infected: Primitivo, Chardonnay, Uva di Troia, Negramaro, Malvasia nera, Sangiovese, Montepulciano, Pinot noir and Trebbiano; moreover some infection was detected in unknown local cultivars. In 2013, *Scaphoideus titanus*, vector of "flavescence dorée" was identified in Apulia through the capture of 19 adult specimens in Loconia (Barletta-Andria-Trani province), which had resulted negative to the presence of "flavescence dorée" when were assayed by nested PCR (Digiaro *et al*., 2014). Noteworthy mentioning that no other individuals of *S. titanus* were captured in the following years. The 16SrVII ribosomal group of phytoplasmas was reported in the last fifteen years as associated to GY diseases in Chile and Iran respectively (Gajardo *et al*., 2009; Zamharir *et al*., 2017), however this group was recently also detected in Europe (North Italy) in grapevine, *S. titanus* and *Orientus ishidae* (Zambon *et al*., 2018). The epidemiological relevance of '*Ca.* P. fraxini' phytoplasmas in Italian vineyards, in spite of their detection in a few locations and in a few samples, is therefore to be kept under further monitoring.

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O41: Comparative early transcriptome profiling of two grapevine varieties with very divergent susceptibility to "flavescence dorée"

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INTRODUCTION

"Flavescence dorée" (FD) is the most serious grapevine yellows disease in Europe. It causes big losses in quality and quantity production in all the infected areas, and often can lead to the death of the plants. The associated agents are phytoplasmas belonging to phylogenetic subgroups 16SrV-C and -D. They are transmitted from grapevine to grapevine by the leafhopper *Scaphoideus titanus*. Differences in susceptibility among grapevine varieties do exist, both in field and laboratory conditions. For instance, the cv Chardonnay is known in the entire world to be a very susceptible variety, which dies in a few years if control strategies against the insect vector are not undertaken. On the opposite, it is very difficult to find out symptomatic plants of some varieties, such as the Italian Tocai friulano, which is also very easily subjected to recovery phenomena. These data suggest that specific genetic features are present in the different grapevine cultivars associated to resistance to the phytoplasma and/or possibly to its vector. The aim of this work was to compare the early whole transcriptomic response of two grapevine varieties, one very susceptible to FD and the other tolerant to the disease. Comparisons were made taking into account the plant constitutive features, the response to the vector infestation and the response to the phytoplasma presence.

MATERIALS AND METHODS

The trials were carried out in a climatic chamber under controlled conditions. The experimental design included 12 theses, each with three biological replicates. The variables were: two varieties (Chardonnay and Tocai friulano), three conditions (absence of vectors, healthy vectors, and infective vectors), two time points (3 and 6 days post insect infestation). *Ex-vitro* micropropagated plants and vectors reared in controlled conditions and experimentally infected were used. The sources of inoculum were FD-infected grapevine leaves, where half of the insects were allowed to feed for one month. DNA extraction and quantitative (q)PCR were used to verify the phytoplasma presence in insects and plantlets (Angelini et al., 2007).
Sequencing of the whole transcriptome (RNA-Seq) of the grapevine plantlets was carried out by means of NGS technology (Illumina). Read counts were generated from Bam alignment files with HTSeq software. Data normalization and call of differentially expressed genes (DEGs) was implemented with DESeq2 version 1.2.8 Bioconductor (R) package by setting to local, False Discovery Rate (FDR) threshold to 0.05 and enabling independent filtering. GO enrichment analyses were conducted with the GOseq Bioconductor package.

RESULTS AND DISCUSSION

The qPCR confirmed the phytoplasma presence or absence in all insects and plantlets used in the trials. At first the comparison of the constitutive transcriptome of Chardonnay and Tocai friulano was performed taking into consideration the mock treatments. They were largely different, as expected. Several genes encoding pathogenesis-related (PR) proteins showed constitutively higher expression levels in Tocai friulano compared with Chardonnay. A constitutive different modulation of some branches of the secondary metabolism was observed between Chardonnay and Tocai friulano, in particular concerning genes involved in the phenolic metabolism, such as monolignol and stilbene biosynthesis. Significant differences were also observed in the constitutive expression of genes involved in the terpenoid metabolism. Finally, significant transcriptional differences between the two varieties were observed for a group of genes involved in hormone biosynthesis, mobilization and signal transduction. In particular, many genes involved in the jasmonic acid (JA) biosynthetic pathway showed higher transcript level in Tocai friulano. It is interesting to note that decreased JA levels are crucial for the success of the vector of aster yellows phytoplasma infestation in *Arabidopsis* (Sugio et al., 2011). Secondly, for each grapevine cultivar and the two time points analyzed, two kinds of contrasts were set, namely mock vs. grapevine with healthy *S. titanus* and mock vs. grapevine with FD-infected *S. titanus*, in order to identify the plant response to piercing and sucking by *S. titanus* (HSt) and the plant response in the three-trophic interaction between grapevine-phytoplasma-insect (FDSt), respectively. As far as signaling pathways is concerning, a large number of

genes coding different groups of protein kinases, mainly receptor-like kinases (RLKs), were modulated in response to HSt and FDSt in both varieties. Calcium-mediated signaling was significantly activated 3 days after HSt feeding only in Chardonnay, while very few genes were modulated in Tocai friulano. Otherwise, in the two cultivars, during the 6 dpi response to FDSt, a decreased abundance of transcripts for genes involved in calcium signaling was observed. Hormone-related pathways were strongly modulated. In Chardonnay upon HSt feeding many DEGs involved in the JA, ethylene (ET) and abscissic acid (ABA) synthesis and signal transduction pathways were detected, most of those upregulated, while a lower number of DEGs was observed upon FDSt infestation. In Tocai friulano the feeding of both healthy and infective insects did not modify extensively the expression of the genes involved in hormone signaling pathways. The analyses of the transcription factors showed that in Chardonnay many genes with important roles in the regulation of the transcriptional reprogramming associated with plant stress responses were differentially expressed during the time-course of the experiment. On the opposite, no clear response was observed in the Tocai friulano. Many genes implicated in the control of the cellular redox state were modulated in response to HSt or FDSt feeding in both cultivars, but particularly in Tocai friulano. Activation of these pathways has been observed in grapevine varieties partially resistant to phytoplasmas (Margaria and Palmano, 2011). The transcription of several genes involved in cell wall metabolism was affected after HSt or FDSt attacks, at different timing in both varieties. In Tocai friulano upon HSt feeding a number of protease genes were induced, in particular some subtilin-like protein genes, which have been reported to be involved in both immune responses and programmed cell death (Figueiredo et al., 2014). In the secondary metabolism the transcriptomic response of the two varieties was very different. In Chardonnay, several genes involved in the general phenylpropanoid pathway, genes encoding stilbene synthase (STS) and putative laccases were upregulated in the 6 dpi response to FDSt infection. In Tocai friulano three days after HSt feeding the flavonol metabolism was upregulated, whereas genes encoding STS and laccases were strongly downregulated. Moreover, the response of Tocai friulano to FDSt infection was characterized at both timings by the upregulation of several genes involved in synthesis of terpenoids, in particular carotenoids, at different levels. As far as the primary metabolism is concerning, several DEGs implicated in photosynthesis were downregulated in Chardonnay in response to HSt or FDSt feeding. Contrariwise, upregulation of a number of genes involved in photosynthesis was observed in Tocai friulano.

In conclusion, this work showed: (i) the existence of passive defense mechanisms in Tocai friulano, related to the higher constitutive expression of several defense-related genes compared to Chardonnay; (ii) a late activation of the defense mechanisms against FDSt in Chardonnay, that can be also associated to its high susceptibility; (iii) FD phytoplasma provides signals that allow the repression of the JA/ET-mediated response induced by *S. titanus* feeding. Thus, it is reasonable to suppose that FD phytoplasma enhances its success on Chardonnay plants also by suppressing the effectual JA-regulated defenses induced by *S. titanus* feeding.

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O42: A multilocus sequence typing strategy to further characterise the Buckland Valley grapevine yellows phytoplasma

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INTRODUCTION

The Buckland Valley grapevine yellows (BVGY) phytoplasma has only been reported from grapevine yellows affected grapevines in two vineyards of an isolated area of the Buckland Valley alpine region in Victoria, Australia (Gibb *et al*., 1999, Constable *et al*., 2003). Phylogenetic analysis of the 16S rRNA gene indicated that it was a molecularly distinct phytoplasma most closely related to phytoplasmas in the 16SrI group (Gibb *et al*., 1999, Constable *et al.,* 2002). It has subsequently been allocated as the type member of the 16SrXXIII group (Zhao *et al*., 2010). Analysis of the intergenic spacer region showed that the BVGY phytoplasma was even less closely related to other phytoplasmas (Constable *et al*., 2002). In this study the sequence of the 16S rRNA gene and 16S/23S intergenic spacer (ITS) region was confirmed. An analysis of other gene regions has been undertaken to further determine the relationship between the BVGY phytoplasma and other phytoplasmas.

MATERIALS AND METHODS

Twelve samples were collected from grapevine yellows affected Chardonnay grapevines from one vineyard in the Buckland valley of Victoria, Australia. DNA was extracted from each sample using a DNeasy plant DNA extraction kit (Qiagen) according to the method of Green *et al*., (1999). Detection of the region containing the 16S rRNA gene, the 16Sr/23Sr intergenic spacer (ITS) region and part of the 23S rRNA gene was done as described previously (Gibb *et al*., 1999). PCR was also done to amplify the two ribosomal protein (rp) genes, *rplV (rpl22)* and *rpsC (rps3)* (Martini *et al*., 2007), the elongation factor Tu (*tuf*) gene (Schneider *et a*l., 1997; Makarova *et al*., 2012), *secY* gene (Lee *et al*., 2006; 2010), *secA* gene (Hodgetts *et al*., 2008) and genes within the *gro*L-*stam*p-*nad*E locus (Fabre *et al*., 2011; Mitrovic *et al*., 2011). PCR fragments were purified and then cloned using a pGEM-T Easy kit (Promega). Three clones per PCR product were sequenced to generate a consensus sequence. Molecular analyses were performed using MEGA6.0 tools (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Eleven of the 12 grapevine samples tested positive for phytoplasmas using the nested PCR to detect 16Sr-16Sr/23Sr ITS-23Sr gene regions. Sequence analysis indicated that sequenced products were identical to the same gene region of BVGY phytoplasma that was previously reported (Constable *et al*., 2002). One strain was chosen for PCR detection and sequence analysis of other phytoplasma genes and the results are given in Table 1.

Table1. Multilocus detection of the BVGY phytoplasma and highest percentage of sequence similarity between each gene and other most closely related phytoplasmas.

Gene region	Detection by PCR	Range of sequence similarity (%) to other phytoplasmas	Phytoplasma group (% sequence similarity)
16Sr	Yes	89-97%	16SrI-aster yellows (97%)
16Sr/23Sr ITS	Yes	71-81%	16SrI-aster yellows (81%)
rplV (rpl22)	Yes	76-83%	16SrI-aster yellows (83%)
rpsC (rps3)	Yes	71-81%	16SrI-aster yellows (81%)
tuf	Yes	81-86%	16SrXII-"stolbur" (86%)
secY	No	-	-
secA	Yes	73-86%	16SrXII-B-Cordyline phyt. (86%)
*gro*L-*stam*p-*nad*E	No	-	-

These results indicate that the BVGY phytoplasma bears greatest relationship to the 16SrI – aster yellows group of phytoplasmas, but is distant enough to justify the distinct phytoplasma group (16SrXXIII) assigned by Zhao *et al*., (2010). The sequences of the other genes and the unique sequences previously identified by Constable *et al*.,

(2002) will be used to allocate a '*Candidatus*' species name to this unique grapevine infecting phytoplasma. To further analyze and improve knowledge of the BVGY genome a novel chromosome isolation and next generation sequencing strategy is being developed and this information may also be used to further characterize the BVGY phytoplasma.

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O43: The use of transcriptome analysis to identify plant defense responses associated with grapevine leafroll disease in greenhouse vs. field plants.

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INTRODUCTION

Research into plant-pathogen interactions can lead to the identification of plant defence response triggers. RNA silencing, mediated by small RNAs (sRNAs), is a defense mechanism of plants to prevent virus replication and disease development. Grapevine leafroll disease (GLD), caused by the phloem-limited virus *Grapevine leafroll-associated virus 3* (GLRaV-3), is an economically important disease affecting wine and table grape cultivars, as well as rootstocks (Maree et al., 2013). The phenotypic symptoms associated with the disease were described extensively (Almeida et al., 2013; Maree et al., 2013; Naidu et al., 2014, 2015), however the molecular plant responses elicited are still poorly understood. In this study, a next-generation sequencing (NGS) and RT-qPCR approach was followed to identify sRNAs and genes associated with GLD in three *Vitis vinifera* cultivars (Chardonnay, Chenin blanc and Cabernet Sauvignon).

MATERIALS AND METHODS

Six one-year-old *V. vinifera* plants each of three cultivars, Chardonnay, Chenin blanc and Cabernet Sauvignon, were obtained from a commercial nursery. *Grapevine leafroll-associated virus* 3 variant group II was graft-inoculated onto three plants from each cultivar, using infected Cabernet Sauvignon buds. High quality total RNA was extracted from phloem material from each of the 18 plants using a modified cetyltrimethylammonium bromide (CTAB) protocol (Bester et al., 2014). Both sRNA and mRNA sequencing libraries were prepared from the same RNA extract for each plant sample (Fasteris, Switzerland) and Illumina next-generation sequencing (NGS) data was generated. Known and novel miRNAs were identified as previously described (Bester et al., 2017a). The Tuxedo pipeline (Trapnell et al., 2012) was used for the analysis of the mRNA NGS data. Differentially expressed known and novel miRNAs identified through the bioinformatics analysis were validated using stem-loop RT-qPCR assays. Differentially expressed transcripts were validated using SYBR green RT-qPCR assays. Samples from 40 Cabernet Sauvignon plants (20 symptomatic and 20 asymptomatic plants based on phenotypic evaluation) were collected from two farms in the Stellenbosch region. Phloem material was collected from the canes and RNA extracted as for the NGS plants. These samples were subjected to the same stem-loop RT-qPCR and RT-qPCR protocols as the NGS samples to evaluate plant responses under field conditions.

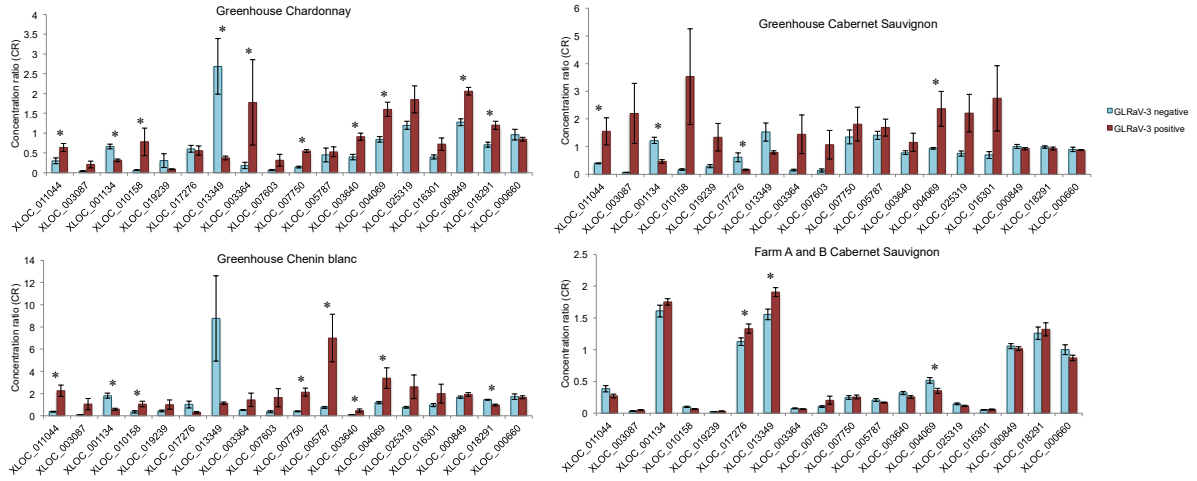
RESULTS AND DISCUSSION

Six and three miRNAs were identified in the sRNA NGS data to be significantly differentially expressed in infected Chardonnay and Chenin blanc, respectively. The expression of these miRNAs and 12 miRNAs from a previous study in Cabernet Sauvignon (Bester et al., 2017b) were included in the stem-loop RT-qPCR screening panel. Fourteen, seven and five of these miRNAs were found to be differentially expressed in the qPCR data of infected Chardonnay, Chenin blanc and Cabernet Sauvignon samples, respectively. MicroRNAs c141107, c141224 and c187937 were down-regulated in all three cultivars. The same panel of miRNAs used for the NGS samples were used to screen the Cabernet Sauvignon samples from the two wine farms, to evaluate miRNA expression under field conditions. In total, 12 miRNAs were significantly down-regulated and three miRNAs were significantly up-regulated. The novel miRNAs c141107 and c141224 were also down-regulated in the field-grown grafted Cabernet Sauvignon samples on both farms. This can imply a potential role for these miRNAs in the plant's response to GLRaV-3 infection.

The expression of 15 genes differentially expressed in all cultivars in the mRNA NGS data and three additional genes (XLOC_000849, XLOC_018291 and XLOC_000660) identified to be potentially modulated by miRNAs in a previous study (Bester et al., 2017b) were assessed with RT-qPCRs. The differential expression of ten, eight and four genes was validated with RT-qPCR for Chardonnay, Chenin blanc and Cabernet Sauvignon, respectively. Both the NGS and RT-qPCR data (Fig. 1A) demonstrated that two genes (XLOC_011044 and XLOC_004069) were consistently up-regulated (p-value < 0.05) in all GLRaV-3 infected samples, in all cultivars. The expression of the 18 genes was also evaluated in the Cabernet Sauvignon samples from the two wine farms. Only three of

the differentially expressed genes identified in the greenhouse NGS data, had significant differential expression in the field plants when all samples were evaluated. Only one gene (XLOC_004069) was consistently significantly differentially expressed in GLRaV-3 infected plants according to the greenhouse sRNA NGS data, greenhouse RT-qPCR data and the field RT-qPCR data, however this gene was up-regulated in the greenhouse and down-regulated in the field data. Even though the greenhouse provides an ideal environment to answer specific biological questions, the results from the field study highlights the difficulties in extrapolating the greenhouse evaluations to field conditions. Even though a greenhouse trial can eliminate the influence of environmental factors, the same response can be masked in the field and be difficult to detect. However, the cultivar-specific and universal viral responses identified here, contribute to elucidating the molecular mechanisms underlying the GLRaV-3 stress responses in grapevine.

Fig. 1: Gene expression profiles evaluated with RT-qPCR. The mean concentration ratio (CR) ± standard error (SE) of three biological replicates, with each replicate an average of three technical replicates is displayed (A: Greenhouse data; B: Field data). Statistically significant differences between GLRaV-3 negative and positive samples are indicated by asterisks (*p-value < 0.05).



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O44: A single amino acid of *Grapevine fanleaf virus* determines symptomatology.

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is the major causal agent of fanleaf degeneration, one of the most devastating viral diseases of grapevine worldwide (Andret-Link et al., 2004; Martelli, 2017; Schmitt et al., 2017). GFLV belongs to the genus *Nepovirus* (subgroup A) in the family *Secoviridae* and has a bipartite (+) sense RNA genome expressed by monocistronic translation followed by proteolytic processing (Fuchs et al., 2017). Phenotypes of GFLV strains can be quite distinct *in planta* with, for instance, strain GHu eliciting systemic vein clearing and strain F13 causing a symptomless infection in the model host *Nicotiana benthamiana*. Recently, it was shown that the RNA-dependent RNA polymerase (RdRp) encoded by GFLV-GHu RNA-1 carries a strain-specific symptom determinant module at its 3' terminus (Vigne et al., 2013); however, the nature of this module (nucleotide position 2065-2473 of the RdRp coding region) is not known. Therefore, we produced several chimeric RNA-1 constructs between strains GHu and F13 to identify the minimal GFLV-GHu RdRp component necessary for symptom expression in *N. benthamiana.*

MATERIALS AND METHODS

Genomic cDNAs of GFLV strains F13 and GHu in a cauliflower mosaic virus (CaMV) 35S expression cassette were amplified by PCR from pGA482G plasmids (Gottula, 2014) using specific primers and Phusion polymerase (New England Biolabs). Amplicons were cloned by restriction enzyme digest and ligation into the multiple cloning site of pCLEAN-G181 (Thole et al., 2007). The integrity of recombinant clones was verified by plasmid sequencing at the Center of Computational and Integrative Biology DNA Core Facility at Massachusetts General Hospital (Cambridge, MA). Sequencing-confirmed, purified (Omega Bio-tek E.Z.N.A. plasmid mini kit) plasmids were electroporated separately into electrocompetent *A. tumefaciens* GV-S161 cells [GV3101::pMP90 cells (Hellens et al., 2000) harboring the pCLEAN-S161 helper plasmid (Thole et al., 2007)] (Osterbaan et al., 2017).
Plasmids pCLEAN-GHu-1 or pCLEAN-F13-1 were then used as templates to engineer several GFLV chimera to replace 66-90bp segments of the 3' end of RdRp of pCLEAN-GHu-1 with the homologous sequence from strain F13 or vice-versa. Additionally, single amino acid mutants in the RdRp of GFLV-GHu were produced. Chimeras were generated by site-directed mutagenesis using the Q5 Site-directed mutagenesis kit (New England Biolabs) with specific primers designed using the NEBaseChanger software (v1.2.6). Resulting chimeric plasmids were screened for the proper mutations by Sanger sequencing (Cornell University Biotechnology Resource Center). One correctly mutated plasmid clone of each construct was then fully sequenced. Subsequently, fully sequenced plasmids were analyzed for *in planta* infectivity by agroinoculation using pCLEAN-GHu-2 and p24 from *Grapevine leafroll-associated virus* 2 (GLRaV-2) (Vigne et al., 2013) as a viral silencing suppressor (VSR) (Li et al., 2017).
For agroinoculation of *N. benthamiana*, agrosuspensions carrying pCLEAN-GHu-1 (wild-type or chimera) or pCLEAN-F13-1 (wild-type or chimera), pCLEAN-GHu-2 and pGA-p24 were adjusted to OD_{600nm} = 1.0 and combined in a 1:1:1 ratio to form GFLV inocula. Each experiment with 10 seedlings was duplicated. Agroinoculated plants were maintained in growth chambers (25°C, 50% relative humidity, 16-hour photoperiod) and monitored for symptom expression (Osterbaan et al., 2017). GFLV infection was assessed by DAS-ELISA in apical leaves of agroinoculated *N. benthamiana* using specific antibodies (Bioreba, Reinach, Switzerland). Samples were considered positive if the absorbance (OD_{405nm}) of both duplicate wells were greater than three times the average absorbance of healthy *N. benthamiana* tissue. Fidelity of virus progeny was verified by sequencing of reverse-transcription (RT) polymerase chain reaction (PCR) products (QIAGEN OneStep) with specific primers and total RNA extracted from apical leaves (E.Z.N.A. Plant RNA kit).

RESULTS AND DISCUSSION

Preliminary agroinoculation experiments revealed that the genomic make-up of the viral inoculum (G1-G2 and reassortant F1-G2), the nature of co-infiltrated VSR (p24), and temperature at which plants were maintained (25°C) significantly increased systemic GFLV infection, while high optical densities of infiltration cultures (OD_{600nm} of 1.0 or 2.0) increased the consistency of systemic infection *in planta* (Osterbaan et al., 2017). Binary plasmids pCLEAN-GHu-1 and pCLEAN-F13-1 in this optimized system were amenable to rapid and reliable manipulation

by site-directed mutagenesis using the Q5 site-directed mutagenesis kit. Varied staggered chimeric constructs were engineered and used to exclude nucleotides (nt) 2065-2310 which are part of the 408nt stretch of the GFLV-GHu RNA-1 RdRp that were previously identified to carry a symptom determinant in *N. benthamiana* (Vigne et al., 2013). Additional staggered chimera further narrowed the symptom determinant to a stretch of 21nt. Indeed, inclusion of the corresponding 21nt from F13 into pCLEAN-GHu-1 abolished symptom expression. Of the seven amino acids coded by the 21nt of interest, only two (K and S) are distinct between strains F13 and GHu. Single amino acid mutants that swapped residues between strain GHu and F13 indicated that one of the two distinct amino acids is necessary for symptom expression while the other has no role in symptomatology. Double mutants confirmed these results. The GFLV-GHu amino acid necessary for symptom expression is basic with electrically charged sides chains. Substituting this amino acid in pCLEAN-GHu-1 with residues from any of the other amino acid classes abolished symptom expression. This research provided insights into the critical role of a single GFLV-GHu RNA1 amino acid in determining systemic vein clearing symptoms in *N. benthamiana*.

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O45: Metagenomic-based impact study of GM grapevine rootstock: Importance of transgenic sequence insertions onto infecting-GFLV population diversity.

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is responsible for one of the most damaging disease of grapevine known as fanleaf degeneration. GFLV belongs to the *Nepovirus* genus in the *Secoviridae* family and is specifically transmitted from plant to plant by the dagger nematode *Xiphinema index*. The viral genome is constituted of two positive-sense single-stranded RNA molecules (RNA1 and RNA2) each coding for polyprotein. GFLV has been documented to display a broad range of molecular variants in several part of the world and that mixed infection with genetically distant variants and recombination are legion in vineyards (Andret - Link et al., 2004). Fanleaf degeneration disease is present in all temperate regions where grapevine is cultivated. A plethora of symptoms affecting both vegetative and reproductive organs can be attributed to GFLV infection, such as leaves distortion and mosaic, canes malformation with short internodes and fruit quality being impacted causing severe yield reduction (up to 80%). Traditionally, control measures rely mainly on monitoring the spread of the disease focusing on the vector, plant certification, regulating infected budwood and soil transfer. As of yet, no sources of natural genetic resistance against GFLV have been identified in the *Vitis* spp genetic pool, making genetic engineering a valuable alternative.

Following the pathogen derived resistance (PDR) concept (Sanford and Johnston, 1985), we obtained a few genetically modified (GM) grapevine lines, carrying the full length of the *GFLV-coat protein* gene (*cp*, strain F13) under the constitutive 35S promoter and the selective marker (*nptII*) gene. Previous studies indicated no evidence of undesired impact of GM grapevine cultivation by looking at the genetic diversity of viruses and associated bacteria communities as well as on the incidence of horizontal gene transfer (HGT) events (Hily et al., 2017; Vigne et al., 2004a).

In this present work, using state of the art metagenomics, we confirmed that all GM lines, cultivated in a vineyard soil under greenhouse conditions, were infected by the virus within the 4th year of plantation. Nonetheless, one GM line (GMR68), impacted the infecting-GFLV population diversity, underlying a potential resistance mechanism. To better understand such feat, our GM lines were further characterized and copy number of transgenic sequence/organization within grapevine genome were identified. Also, DNA methylation, mRNA and siRNA detection were analyzed.

MATERIAL AND METHODS

Plant material, sampling and nucleic acid extractions

In this work, wild-type rootstock (WTR, cultivar 41B) and five GM grapevine rootstock lines (Vigne et al., 2004b) were used, with 3 GM lines (GMR68, GMR206 and GMR240) being studied in depth. Six repetitions of each GM lines and untransformed controls were randomly planted under greenhouse conditions in September 2006. Grapevines were grown in a vineyard soil naturally infested with nematodes and virus, under a high density set-up (Hily et al., 2017).

For the GFLV virome study and high-throughput DNA sequencing, leaves were sampled in June 2013, when the virus titer is believed to be at its peak (Walter and Etienne, 1987). In June 2017, another round of sampling was performed for downstream methylation, siRNA and mRNA detection study. DNA extraction was performed following either DNeasy plant mini kit (Qiagen) or a modified CTAB extraction procedure. Total RNAs were extracted using Concert Plant Reagent (Invitrogen).

High-throughput sequencing, NGS data and sequences analyses, genetic diversity study

For GFLV diversity analyses, we focused solely on encapsidated GFLV sequences for which an immuno-capture (IC) step was performed followed by an RT-PCR (Reverse Transcrition Polymerase Chain reaction) step (Hily et al., 2017) targeting either part of the RNA1 or the *cp* gene. Both RNA and DNA libraries were prepared at the GeT-Genotoul platform facility (INRA-Toulouse, France) according to Illumina's protocols. All downstream

analyses were performed using Workbench 8.5.1 software (CLC bio Genomics, Qiagen) as previously described (Hily et al., 2017). For GM lines genotyping, left and right borders of the T-DNA were identified after a '*de novo* assembly'. Insertion sites were confirmed after designing specific primers.

DNA methylation, mRNA and siRNA detection

McrBC-PCR analysis of the methylation levels was performed using 0.5µg of genomic DNA digested with the enzyme in a final volume of 50µl according to the manufacturer's instructions. PCRs were then achieved using GoTaq® Flexi DNA polymerase Kit (Promega, Madison, WI, USA), targeting different genes. Small RNA detection was performed as described by Garcia et al., 2017 (Poster ICVG Chile).

RESULTS

Emergence and spread of the disease was monitored by DAS-ELISA (Bioreba AG) every year until 100% of infection was reached, which was achieved within the 4th year post-plantation, confirming that none of the GM line were resistant to GFLV (Hily et al., 2017).

Following metagenomics analyses, a genetic diversity study on GFLV was performed. Many viral sequences were obtained after '*de novo'* assembly. All sequences derived from the RNA1 segregated in 3 clades, with no genetic differentiation between populations (*i.e.* GM or non-GM rootstocks). On the other hand when focusing on the *cp* gene sequences, genetic differentiation was observed between populations, all involving GMR68 which was lacking *cp* sequences from clade I and II.

To better understand such specific resistance of GMR68 to clade I and II, number and location of transgenic sequence insertions were detected within each GM line genomes. While GMR206 and GMR240 accounted for only one complete transgenic sequence insertion, two distinct insertion sites with 're-arrangement' of the transgenic sequence were found in GMR68 genome. Such transgenic structure might activate gene silencing activity (Morino et al., 1999). To further decipher GMR68 singularity, hallmarks of gene silencing (*e.g.* mRNA accumulation, genome methylation, siRNA) were tested and they will be presented and discussed.

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O46: Transcriptomic analysis of grapevine cv. Gewurztraminer infected by two strains of *Grapevine fanleaf virus* with distinct phenotypes

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INTRODUCTION

Grapevine fanleaf virus (GFLV) causes fanleaf degeneration disease, one of the most detrimental viral diseases of *Vitis* spp worldwide (Andret-Link *et al.*, 2004). Infection can sometimes lead to early death of vines. Little is known about GFLV/grapevine interactions and the molecular mechanisms of symptoms development. However, environmental conditions, the scion and rootstock genotypes, as well as the GFLV isolate play key roles in their expression (Vuittenez, 1956). To gain knowledge of pathogenesis, Gewurztraminer (GW) grapevines graft-inoculated with either GFLV strain F13 or B844 were selected for a comparative transcriptomic analysis. GFLV-F13 infected GWs exhibit discrete foliar symptoms, whereas GFLV-B844 infected GWs display a severe stunting (Vigne *et al.*, 2015). A next generation sequencing approach (RNA-Seq) was performed to identify transcriptional changes associated with the distinct phenotypes. To this end we compared gene expression profiles of non-infected GW vines and GW vines infected with GFLV-B844, or GFLV-F13. Differently affected pathways were identified to be distinctly altered and their possible contribution to contrasting symptoms will be discussed.

MATERIALS AND METHODS

Experimental plot:

Experimental GW grapevines were planted in 2006 in a vineyard site free of *Xiphinema index*, the soil-borne vector of GFLV; it included grapevines inoculated either with strain GFLV-F13 or GFLV-B844, and non-inoculated control plants. Singly-GFLV infected vines were achieved by heterologous grafting of certified Kober 5BB onto infected *Chenopodium quinoa* and subsequent grafting of GW cultivar onto infected Kober 5BB. We selected three grapevines per condition at the cluster emergence stage for the collection of young apical leaves on May 21, 2016. Collected tissue were flash frozen in liquid nitrogen.

RNA extraction and RNA-Sequencing:

Total RNA was extracted from 200 mg of leaf tissue using an adapted protocol of the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Libraries were prepared following instructions of the TruSeq Stranded mRNA kit (Illumina, SanDiego, California), and sequenced (paired-end, 150 pb) on a HiSeq3000 (Illumina) at the GeT-PlaGe platform facility (INRA, Toulouse, France).

Data analysis:

The differentially expressed genes (DEGs) analysis was performed with a data processing pipeline consisting in the following steps: reads mapping with TopHat2 (v.2.0.11) / Bowtie2 (v.2.2.1) on the reference *V. vinifera* PN40024 genome (Jaillon *et al.*, 2007), gene expression quantification with HTSeq-count (v.6.0.0), counts normalization and detection of differentially expressed genes using R (v.3.3.2), and the DESeq2 package. Finally we used TopGo R package and blast2GO tool for Gene Ontology (GO) enrichment analysis.

Real-time RT-PCR:

RT-qPCR was used to validate both the RNAseq results and the deregulation of targeted *V. vinifera* genes. Reverse transcription was performed on 1 µg of total RNA using Superscript III enzyme (Thermo Fisher Scientific, Walthman, Massachusetts). qPCRs were carried out on a LightCycler 480 thermocycler (Roche, Basel, Switzerland) using SYBR GREEN Master Mix (Roche) following the manufacturer recommendations. The relative expression of the targeted genes was calculated with $2^{-\Delta\Delta Ct}$ using the control samples as calibrators.

RESULTS AND DISCUSSION

Phenotype description:

Canes and leaves of GFLV-B844 infected GW vines were shorter and their development was impaired compared to non-infected plants (Fig. 1). GFLV-F13 infected GW vines showed an intermediate phenotype between control vines and GFLV-B844 infected vines.

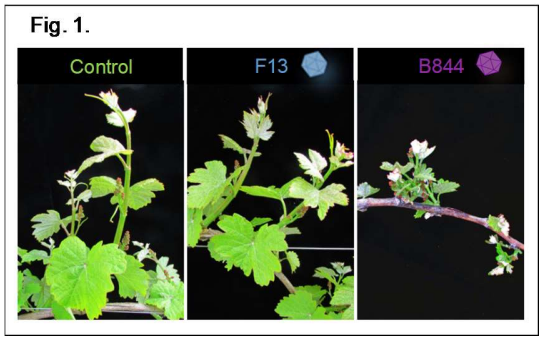

Fig.1. GFLV symptoms of secondary shoots of GW. Compared to control vines, GFLV-B844 infected vines show stunted shoots and GFLV-F13 infected shoots show mild symptoms. Photographs were taken when leaf samples were collected.

High throughput RNA-Sequencing and differentially expressed gene (DEG) results:

Close to 50 million reads were obtained by RNA-Seq for each of the nine samples sequenced. Among the 31,842 annotated genes for which reads mapped to the *V. vinifera* reference genome, 25,255 were expressed in the nine samples. From these 25,255 genes, the low counted genes (4,407) were filtered out and a DEG analysis was performed. Out of the 20,848 remaining genes, 3,981 were deregulated (false discovery rate or FDR<0.05) in GFLV infected GW vines compared to the control vines (Fig. 2.). Some deregulated genes were common to both viral strains, thus representing a pool of genes involved in GFLV infection in general. Other genes were specifically deregulated by either GFLV-B844 or GFLV-F13 (Fig. 2).

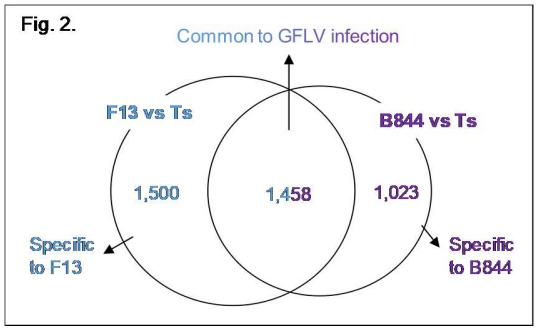

Fig. 2. DEG Analysis. Of the 20,848 expressed genes identified, 3,981 are deregulated (FDR<0.05) by GFLV infection. In addition, 2,958 and 2,481 genes are deregulated by GFLV-F13 (blue) and GFLV-B844 (purple), respectively. Among these deregulated genes 1,458 are common to both GFLV strains, corresponding to a broad deregulation network during GFLV infection.

To validate the transcriptomic results, RT-qPCR was performed on a series of randomly chosen genes. Using GO enrichment method different pathways modified in a strain specific manner were identified. The validation of these specific responses in biological replicates will be presented and the potential role of the deregulated pathways in symptom expression will be discussed.

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O47: Interactive responses of *Vitis vinifera* L. plants infected by *Grapevine virus B* (GVB) in field conditions leads to ameliorate berry secondary metabolism.

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INTRODUCTION

Grapevine represents one of the most economically important fruit crops in the world with very old origins and it is potentially infected by many viral entities. *Grapevine virus B* (GVB), a member of the genus *Vitivirus*, family *Betaflexiviridae*, is closely associated with the Corky bark disorder, one of the syndromes of the Rugose Wood complex (Martelli, 2014). Although not particularly widespread in grapevine, GVB is generally considered harmful and it potentially affects graft unions. In this study, combined agronomic, molecular, ecophysiological and biochemical approaches were used to analyze the interaction between GVB and *Vitis vinifera* wine red cultivar 'Albarossa', in order to characterize specific responses triggered by GVB in the host. This work aims to deepen understanding of the multifaceted grapevine-virus interactions in field condition.

MATERIALS AND METHODS

The study was carried out in a vineyard located in North-West Italy, at the Cannona experimental station (Piedmont Region) during two consecutive seasons (2015 and 2016). *V. vinifera* 'Albarossa' plants, grafted onto Kober 5BB, were obtained by vegetative propagation from a single mother plant originally infected only by GVB, further subjected to sanitation, but leaving some lines still infected by the virus. Six GVB-infected and six GVB-free plants of 'Albarossa' were randomly selected along two parallel rows in the vineyard. In 2015 and 2016, leaf gas exchange rates were monitored using a portable apparatus LCpro+ ADC (Analytical Development Company, Hoddesdon, UK) and the transcriptional profiles of specific candidate genes were quantified by RT-qPCR in leaf and berry tissues. Analytical separation of anthocyanins was performed using HPLC-DAD system (1260 Infinity, Agilent Technologies) and Rubisco contents were evaluated by densitometry on SDS-PAGE gel.

RESULTS AND DISCUSSION

The analysis of agronomical parameters monitored during the two years did not highlight significant differences between free and GVB-infected plants (Table 1). Yield, vegetative vigor and soluble sugars in berries were similar for both the sanitary conditions, but they were influenced by the seasonal climatic conditions. Conversely, net photosynthesis (P_n) (Fig. 1), stomatal conductance (g_s) and substomatal CO_2 concentrations (c_i) were affected by GVB infection, particularly at the end of the season. However, GVB-infected grapevines showed a less relevant decrease in physiological performances than other grapevine-virus combinations (Mannini et al., 2012; Montero et al., 2017). The overall picture of gas exchange measurements indicated a moderate metabolic, non-stomatal, photosynthetic limitation in GVB-infected plants in the absence of either environmental or water limitation. Regarding the biochemistry of photosynthesis, Rubisco contents were higher in GVB-infected plants, mainly at the end of August and consistent to what observed for *VvRubisco activase* expression levels. At the end of the season, together with lower P_n levels, slightly higher concentrations of total soluble carbohydrates were measured in leaves of GVB-infected vines, in parallel with the up-regulation of genes encoding a callose synthase (*VvCAS2*), an acidic vacuolar and a cell wall invertase (*VvGIN2* and *VvCWINV*, respectively) and a sucrose synthase (*VvSUSY4*). Taken together, these findings support the hypothesis that GVB infection impaired phloem loading and transport, by callose deposition as a defense response mechanism, resulting in carbohydrate accumulation in leaves and, in turn, inducing a moderate inhibition of photosynthesis without heavy detrimental phenotypic effects.

Interestingly, several genes (*VvMybA1*, *VvUFGT*, *Vv3AT*, *VvF3'5'H* and *VvF3'H*) involved in anthocyanin biosynthesis showed higher expression levels in GVB-infected berries over the ripening period reflecting the

higher concentration of total anthocyanins, particularly tri-hydroxylated form (Fig. 1), and acylated anthocyanins in infected mature berries respect to GVB-free ones (Table 1).

Table1: Agronomic features, total anthocyanins (TA), berry weight, concentration and profile of anthocyanins grouped per class in healthy GVB-free and GVB-infected berries of *Vitis vinifera* cv Albarossa. Values represent averages of three biological replicates. Significance among averages was evaluated by the Duncan's test (* = P ≤ 0.05; ** = P ≤ 0.01; ns= non-significant).

	Yield/plant (Kg)	Vigor (pruned wood/plant) (Kg)	Weight of 10 berries (g)	Soluble sugars in berries (g L^{-1})	Total anthocyanins (mg g $skin^{-1}$)	Total free anthocyanins (mg g $skin^{-1}$)	Free tri-hydroxylated forms (mg g $skin^{-1}$)	Free di-hydroxylated forms (mg g $skin^{-1}$)	Total acyl derivatives
GVB-free	3.91 ± 0.41	0.56 ± 0.06	18.0 ± 0.3	141 ± 23,27	32.0 ± 0.4	27.6 ± 1.7	21.9 ± 0.1	2.5 ± 0.1	7.6 ± 0.2
GVB-infected	3.37 ± 0.63	0.61 ± 0.14	16.4 ± 0.3	145 ± 29,44	36.4 ± 1.8	24.4 ± 0.3	24.9 ± 1.4	2.8 ± 0.3	8.8 ± 0.3
Sign.	ns	ns	**	ns	**	*	*	ns	**

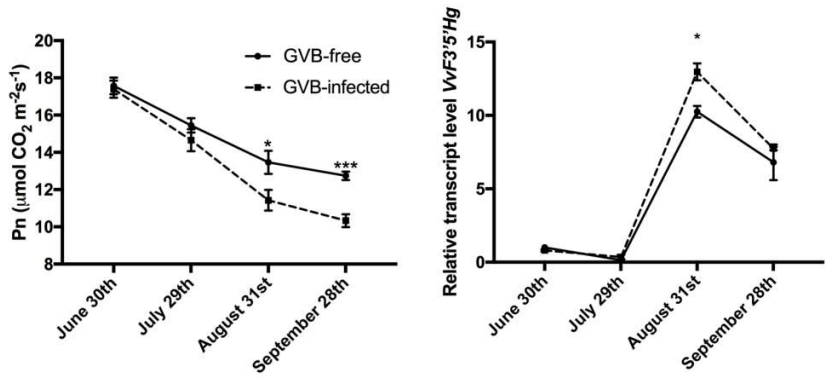

Figure 1: Seasonal time course of carbon assimilation rates (P_n) and transcriptional profile of *VvF3'5'H* gene in GVB-free (solid lines, circles) and GVB-infected (dashed line, squares) 'Albarossa' plants. Data are mean values ± SE (n=3). Asterisks denote significant differences attested by Student's *t* test (P<0.05).

Higher levels of soluble carbohydrate stimulate anthocyanin biosynthesis, and the slight increase of carbohydrate concentration measured in both GVB-infected berries and leaves could support the activation of berry anthocyanin biosynthesis. The higher contents of anthocyanins and acylated anthocyanin forms (which confers color stability in wine) resulted in positive sensorial effects on the wine produced from GVB-infected 'Albarossa' berries, as attested by the overall judgment of testers (Mannini et al., 2015). Noteworthy, as a consequence of the source-sink transition, GVB infection in berries caused metabolic changes involving genes and compounds of the anthocyanin branch that enhance the production of the more stable acyl derivative forms. Other studies are needed to further explore this fascinating plant-virus interaction using different GVB strains and *V. vinifera* cultivars grown in other environments or under stressful conditions. Besides providing a comprehensive ecophysiological, biochemical and molecular picture of grapevine response to GVB in field conditions, these results would open new perspectives in the multifaceted world of plant-virus-environment interactions.

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O48: Variable concentration ratios of *Grapevine leafroll-associated virus 3* genetic variants.

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INTRODUCTION

The economic importance of grapevine leafroll disease (GLD) in table and wine grapes warrants continuous research to manage and limit its impact. The phenotypic symptoms associated with GLD have been described extensively (Naidu et al., 2014, 2015), but limited studies have been performed on grapevine's defence response or the potential role of GLRaV-3 genetic variant or virus titre on disease expression. Several genetic variant groups have been identified of which five have been detected in South African vineyards. In a previous study, we reported the validation and application of an RT-qPCR assay able to detect and quantitate these five (I, II, III, VI and VII) genetic variants (Bester et al., 2014). Here we report the influence of GLRaV-3 genetic variant group and grapevine cultivar on the GLRaV-3 virus concentration ratio (VCR), calculated using a relative quantitation model with efficiency correction, in both graft-inoculated and naturally infected plants.

MATERIALS AND METHODS

GLD symptomatic Cabernet Sauvignon plants were field-collected and established and maintained in an insect-free greenhouse since 2006. Individual plants were evaluated and determined to each be infected with a single genetic variant of GLRaV-3 (Bester et al., 2012). These plants were used in subsequent analyses as is, or used to graft-inoculate additional plants. Three cultivars (Chenin blanc, Cabernet Sauvignon and Chardonnay), established on rootstocks, were graft-inoculated with GLRaV-3 variant group II to evaluate the influence of cultivar on the VCR. GLRaV-3 variant groups I, II, III, VI and VII were graft-inoculated on Cabernet Sauvignon plants to evaluate the influence on VCR within a single cultivar. A field survey was conducted in 2016 to collect GLD symptomatic plant material from vineyards that were established with certified material. A CTAB buffer extraction protocol (Carra et al., 2007), as modified in Bester et al. (2014), was used for total RNA extraction from two grams of phloem scrapings. DNase treatment was performed using RQ1 RNase-free DNase (Promega). The GLRaV-3 VCRs of all samples were determined using a SYBR Green RT-qPCR assay on the Rotor-Gene Q thermal Cycler (Qiagen) (Bester et al., 2014). All reactions were performed in triplicate. Virus concentrations were quantified by comparing the expression of the ORF1a gene of GLRaV-3 to the geometric mean of three reference genes, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH), actin and alpha-tubulin. All calculations were performed using the web application Harbin (Bester et al., 2017).

Results and discussion

Initially VCRs were determined for GLRaV-3 variant group II infections in three cultivars (Fig. 1A). Both own-rooted and grafted Cabernet Sauvignon (symptomatic red cultivar) plants were compared to Chardonnay (symptomatic white cultivar) plants and no significant difference was observed. However, when compared to Chenin blanc (asymptomatic white cultivar) significantly lower VCRs were observed. The observation that the asymptomatic cultivar could tolerate a high GLRaV-3 variant group II titre is interesting and poses the question if this is true for all variant groups.

The VCRs of five South African GLRaV-3 variant groups (I, II, III, VI, VII) were directly compared in graft inoculated Cabernet Sauvignon plants (Fig. 1B). In the course of three sampling rounds over an 18-month period, variant groups I and II were consistently found to have higher VCRs compared to groups II, VI and VII. No difference in foliar symptom expression was observed.

In 2016 (autumn) a field survey was conducted on three GLD-affected vineyards and GLRaV-3 VCRs calculated for each vine collected. The GLRaV-3 genetic variant composition for each vine was also determined and single variant infections found to be rare. Up to four variants were observed to be present in a single vine. Due to the complexity of the mixed-variant populations, and limited numbers in some combinations, comparisons between all combinations were not possible. In Fig. 1C, mixed infections containing groups I and/or II are compared to other combinations, and substantial higher VCRs observed.

Collectively, it can be concluded that the genetic variants characterised for GLRaV-3 are most likely biologically distinct. It remains to be seen if they have variable economic impacts.

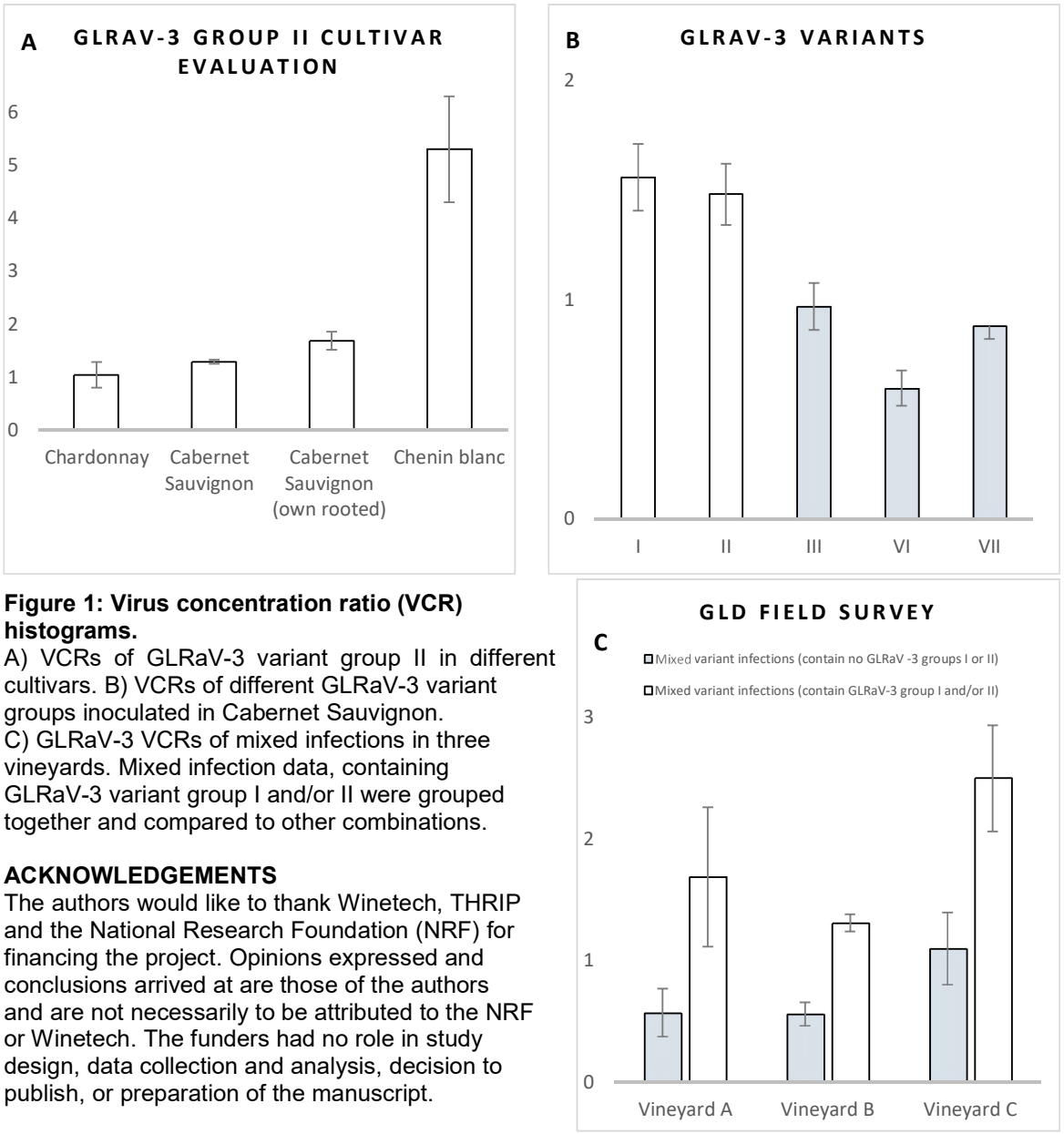

Figure 1: Virus concentration ratio (VCR) histograms.
A) VCRs of GLRaV-3 variant group II in different cultivars. B) VCRs of different GLRaV-3 variant groups inoculated in Cabernet Sauvignon.
C) GLRaV-3 VCRs of mixed infections in three vineyards. Mixed infection data, containing GLRaV-3 variant group I and/or II were grouped together and compared to other combinations.

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O49: The influence of *Grapevine leafroll-associated virus 3* genetic variants on management initiatives in New Zealand

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INTRODUCTION

Grapevine leafroll disease threatens the sustainability and future growth of grape and wine production worldwide, which is caused predominately by *Grapevine leafroll-associated virus* 3 (GLRaV-3) in New Zealand. This viral disease reduces vine vigour, and fruit yield and quality. The New Zealand wine industry actively manages GLRaV-3 by addressing three critical elements: minimising mealybug populations, removing (roguing) GLRaV-3-infected vines (that can act as sources of virus in the vineyard) and replanting with certified GLRaV-3-free grapevines. To identify virus-infected vines for roguing, it was demonstrated that for red berry cultivars visual inspections for the classical symptoms (leaves with dark red inter-vein, green veins and rolling margins) was a cost-effective method (Bell et al. 2017). Importantly, these authors showed the efficacy of visual diagnostics was comparable with ELISA testing.

Genetic variants of other plant viruses have been shown to cause different symptoms across different host plant species/cultivars and can also cause differential symptoms in the same host plant species/cultivars (Lee & Keremane 2013). With the high genetic variation identified in the GLRaV-3 population in New Zealand and worldwide (Maree et al. 2013), could this genetic variation influence the reliability of visual canopy assessments for disease management in New Zealand? To better understand the vine-virus relationship, a study was initiated involving the establishment of a tri-regional field trial studying the Group I, Group VI and NZ2 GLRaV-3 genetic variants.

MATERIALS AND METHODS

Source plant material used as inoculum for the establishment of the field trial was screened using ELISA, conventional and real-time RT-PCR assays using virus-specific primers and next generation sequencing. Four grapevine cultivars (Merlot, Pinot noir, Pinot gris, and Sauvignon blanc) were green grafted with single infections of GLRaV-3 genetic variants representative of groups I, VI and NZ2. Twenty biological replicates were planted for each treatment (plus a healthy control) and cultivar at each of three grape-growing regions (Auckland, Hawke's Bay and Marlborough).

Over three successive Southern Hemisphere growing seasons (2014–15 to 2016–17), assessments of foliar symptoms were made. The visual symptoms on vines were monitored fortnightly to monthly, as required, throughout the growing season (i.e. late December/early January to mid-April). For red berry grapevines, foliar symptoms were assessed based on a symptom score ranging from 0 (no symptoms) to 3 (severe symptoms). White berry grapevines were assessed based on "yes" or "no" criterion.

RESULTS AND DISCUSSION

From all 3 years, no noticeable symptoms were observed at any sites on white varieties or uninfected (healthy control) red variety grapevines. Generally, early season inspections typically revealed greater numbers of Merlot vines showing foliar symptoms of GLRaV-3 compared with Pinot noir-infected vines. In addition, at two of the regional sites, a larger proportion of both red variety vines were observed with leafroll symptoms earlier in the second and third seasons compared with the first season (Figure 1a). This observation supports previous research showing Cabernet sauvignon vines infected for at least 24 months expressed symptoms up to 2 months earlier than vines infected for no more than 12 months (Bell et al. 2015).

While all three GLRaV-3 genetic variants expressed foliar symptoms in the red berry cultivars, we did compare and contrast these symptoms. Observations from all three growing seasons revealed that vines infected with NZ2

showed GLRaV-3 symptoms later in the growing season compared with vines infected with Groups I and VI, with the progression of NZ2 foliar symptoms slower to spread through the vine canopy, particularly to the higher portions of the vine canopy (representative results shown in Figure 1b; GLRaV-3 infected Merlot grapevines in Hawke's Bay).

To ensure most sources of GLRaV-3 inoculum among red berry cultivars are identified and rogued as quickly as possible, it is important that visual assessments be undertaken by trained and experienced assessors, and that inspections occur at least twice late in the growing season before leaf fall. To do so minimises the risk of non- or misidentification of foliar changes due to factors like environmental stress or the delayed appearance of foliar symptoms.

Further research is still required to investigate what physiological and biochemical affects GLRaV-3 and its genetic variants have on the grapevine, particularly following the observed differences in foliar symptom expression between grapevine cultivars and the virus genetic variants.

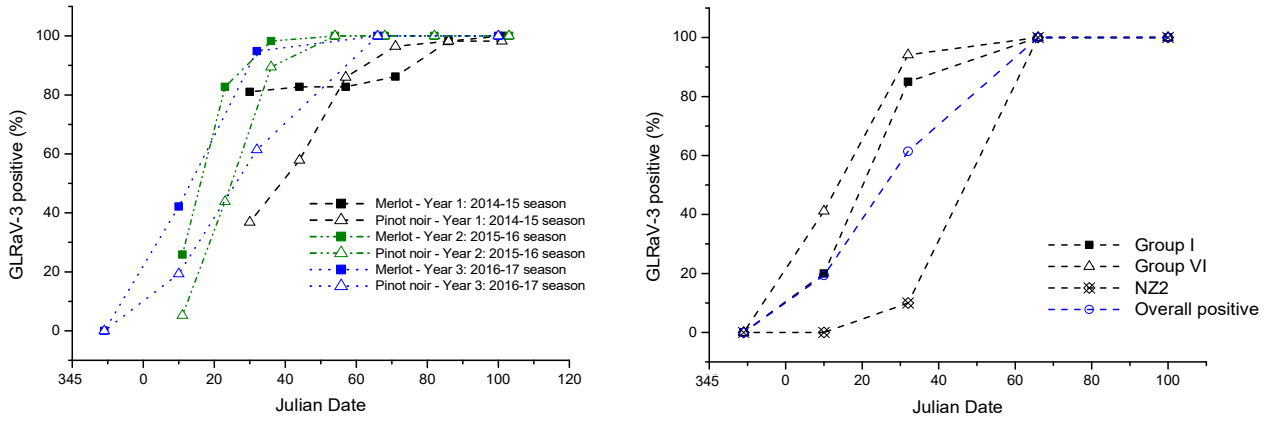

Figure 1. Visual identification of *Grapevine leafroll-associated virus* 3 (GLRaV-3) at the Hawke's Bay field site. (a) Overall percentage of Merlot (solid square) and Pinot noir (open triangle) vines singly infected with any of the three GLRaV-3 genotypes that were positively identified by foliar symptoms for 2014–15 (long dash black line), 2015–16 (dash dot dot green line) and 2016–17 (dotted blue line); (b) Percentage of Merlot vines singly infected with Group I (solid square), Group VI (open triangle) or NZ2 ('x' centred diamond) for the representative 2016–17 season. The delayed symptom expression observed in NZ2 infected vines compared with the expression of Group I and Group VI infected vines reduced the overall percentage of GLRaV-3-infected vines (open circle blue line) positively identified by foliar symptoms.

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O50: Effects of *Grapevine leafroll-associated virus 2* infection on leaf sugar metabolism in V*itis vinifera* cv. Malbec

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INTRODUCTION

In Argentinian viticulture, the occurrence of the leafroll disease (LD) has been well documented; however, the identification of the associated viruses has started only a few years ago. At least four viral species have been related to this disease, being the incidence of Grapevine leafroll associated virus 2 (GLRaV-2) the most important in Mendoza vineyards (Gómez Talquenca *et al.*, 2009; Lanza Volpe *et al.*, 2010). The GLRaVs are phloem-limited viruses that can reduce leaf net photosynthesis, and consequently berry soluble solids and vine productivity (Bertamini *et al.,* 2004; Basso *et al.,* 2010; Endeshaw *et al.,* 2014). Studies on Nebbiolo vines infected with GLRaV-3 showed that the leaf net photosynthesis was variable along the season, but differences between infected and non-infected vines where significant at the end of the growing season (Guidoni *et al.,* 1997). Reductions in leaf net photosynthesis in infected vines are generally correlated with an increase in the anthocyanin concentration of the leaf, typically seen as an inter-venal reddening in red cultivars. These effects are associated with other physiological processes such as disturbances in the carbon transport and accumulation of assimilates, which in turn, may reduce the sugar levels (Brix) in the berries and grape quality (Basso *et al.,* 2010). While most of the studies have been conducted on GLRaV-3, the GLRaV-2 effects on leaf net photosynthesis, sugar transport and yield components, have not been studied in depth.

MATERIALS AND METHODS

The experiment was conducted during season 2016/17 on *Vitis vinifera* L. cv. Malbec (clon ENTAV® COT-598) at INTA Mendoza Experimental Station, Argentina (32°59' S; 68°52' W). Four virus-free (non-infected; NI) and four GLRaV-2 infected (infected; I) plants were selected for the experiment. The sanitary status regarding GLRaV-2 in every vine was previously assessed by DAS-ELISA using commercial coating and conjugate antibody preparations (Bioreba AG, Reinach, Switzerland). The absence of other important viruses and the ELISA results were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) as described by Gambino (2015). Daily dynamics of leaf net photosynthesis (P_n) were performed on days with clear sky at four phenological stages: pre-veraison, veraison, berry harvest-ripe and post harvest. The leaf net photosynthesis was measured on two adult East exposed, basal leaves per vine, every three hours, starting at 06:00 h and finishing at 21:00 h using a portable open-circuit infrared gas analyzer (CIRAS-2, PP Systems, Hertfordshire, U.K.) equipped with an automatic cuvette (PLC6 (U), CRS121, PP Systems, Hertfordshire, U.K.). At the dates and in the same leaves were photosynthesis dynamics was performed, 10 mm disks were collected at predawn and after dusk. The leaf disk were processed to extract soluble sugars, and the amount of glucose, fructose and sucrose was quantified by means of an enzymatic test.

RESULTS AND DISCUSSION

The virus infection had no effects on the leaf net photosynthesis at early stages of the season (pre-veraison and veraison), where I and NI vines had similar values of P_n along the day (Figure 1 a,b). In contrast, the virus decreased P_n at harvest in I vines as compared to those NI, with significant differences at 09:00 h and 12:00 h (Figure 1 c). After harvest, the effect of the virus was still evidenced on the P_n of I vines, that showed significantly lower values at 09:00 h as compared to NI vines (Figure 1 d). These results are in agreement with previous studies that have observed a reduction in the leaf net photosynthesis of adult leaves at the final stages of the season, in coincidence with the symptoms appearance (Guidoni *et al.,* 1997, Montero *et al.,* 2016). However, other studies have also been observed reductions in P_n of infected vines at early stages of the season, even before the development of symptoms (Endeshaw *et al.,* 2104). After midday (12:00 h), the P_n of both treatments decreased as they were East exposed leaves and thus, no longer exposed to the sun during the afternoon. In both plants (I and NI) the P_n values were higher at the pre-veraison and veraison stages, and then decreased along the season, as the leaves get older (Zufferey *et al.,* 2000). The soluble sugar concentration did not differ between I and NI at any moment. GLRaV-2 infected Malbec grapevines had lower leaf net photosynthesis at the

harvest-ripe berry stage compared to non-infected vines, suggesting that the transport of sugar to the berries was significantly affected by the presence of the virus. These observations suggested that GLRaV-2 virus can play an important role as a biotic stress for the sugar transport metabolism, and consequently for the quality and quantity of yield in Malbec grapevines.

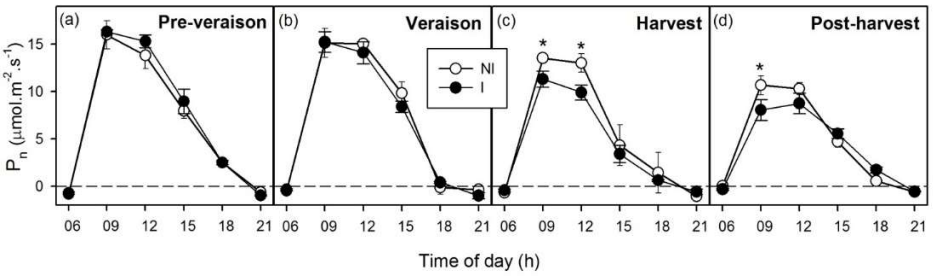

Figure 1. Diurnal dynamics of leaf net photosynthesis (P_n) measured from 06:00 h to 21:00 h at different phenological stages: (a) pre-veraison, (b) veraison, (c) harvest and (d) post-harvest in leaves of non-infected (NI; open symbols) and infected (I; closed symbols) with GLRaV-2 Malbec grapevines. Season 2016/17, Luján de Cuyo.

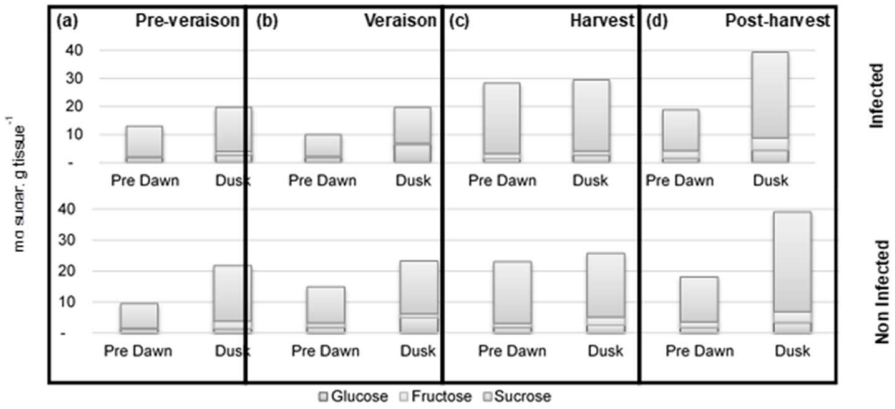

Figure 2. Diurnal dynamics of leaf soluble sugars measured at predawn and after dusk at different phenological stages: (a) pre-veraison, (b) veraison, (c) harvest and (d) post-harvest in leaves of non-infected (NI; lower graphic) and infected (I; upper graphic) with GLRaV-2 Malbec grapevines. Season 2016/17, Luján de Cuyo, Mendoza.

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O51: Is *Grapevine leafroll-associated virus 7* associated with leafroll disease?

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INTRODUCTION

More than 60 viruses have been reported so far to infect grapevines. However, grapevine leafroll (GLR) is one of the most economically important viral diseases of grapevine. Its serious impacts on yield and fruit quality have been documented for several grapevine cultivars. GLR symptoms on red cultivars are typically observed in autumn and comprise red discolorations in the interveinal parts of leaves and leaf margins rolling downward. For white cultivars, GLR symptoms are visually less evident; however, infected grapevines may show chlorotic mottling and leaf curling.

GLR has a complex etiology associated with different filamentous viruses referred to as *Grapevine leafroll-associated virus*es (GLRaVs). All GLRaVs identified to date belong to the family *Closteroviridae*. In total, 5 different GLRaV species have been identified: one in the genus *Closterovirus* (GLRaV-2), three in the genus *Ampelovirus (*GLRaV-1, GLRaV-3 and GLRaV-4*)* and one in the genus *Velarivirus* (GLRaV-7). GLRaV-7 was originally reported in a symptomless white-berried accession from Albania (AA42) that induced leafroll symptoms when grafted onto Cabernet Sauvignon indicator (Choueiri et al., 1996). However, several authors reported that GLRaV-7 may cause incertain leafroll-like symptoms (Al Rwahnih et al., 2012a, Reynard et al., 2015). The association between GLRaV-7 and leafroll disease is thus not conclusive. To address this question, we investigated the etiological role of GLRaV-7 in leafroll disease. Herein, we report the monitoring of Swiss vineyards and *Vitis* repositories for GLRaV-7. We present our efforts to identify grapevine accessions infected by GLRaV-7 and for which the presence of other GLRaVs was ruled out.

MATERIALS AND METHODS

Reference samples were collected from Agroscope grapevine virus collection (Switzerland) (Gugerli et al., 2009). A GLRaV-7 survey was conducted in the National *Vitis* germplasm repository established in Pully (Switzerland) (Fahrentrapp et al., 2015).
Total RNA was extracted following a rapid CTAB procedure. Two assays were tested in parallel for detecting GLRaV-7 using the conditions described in the original publications: a RT-qPCR assay (Al Rwahnih et al., 2012b) and a conventional RT-PCR assay (Lyu et al., 2014). The presence of other coinfecting closteroviruses was assessed by ELISA using reagents from Bioreba AG and reference antisera developed at Agroscope.

Accessions were graft-inoculated onto the leafroll-specific indicator *Vitis vinifera* cv. Gamay Rouge de la Loire. Eight replicates were planted in the field in 2017. Graft-inoculated GLRaV-1,-2,-3-infected vines were grown as positive controls.

RESULTS AND DISCUSSION

GLRaV-7 was not found in a previous monitoring of commercial Swiss vineyards (Reynard and Gugerli, 2012). We therefore monitored the grapevine viral collection and the National *Vitis* germplasm repository for the presence of GLRaV-7. We tested over 700 grapevine accessions. Finally, we were able to identify 13 accessions infected by GLRaV-7. However, most accessions were coinfected by other leafroll viruses. Only three accessions were identified from which the presence of other GLRaVs could be ruled out: Pinot noir 23, New York Muscat and Sultanine blanc. GLRaV-7 from Pinot noir 23 was described elsewhere (Al Rwahnih et al., 2012a). We confirm that under our conditions GLRaV-7 PN-23 did not induce any leafroll symptoms on Pinot noir and Gamay Rouge de la Loire. Sequencing of the amplified PCR products (518 nt) from these three isolates revealed an nt-identity between 91 and 93 percent. Sultanine blanc was assayed in biological indexing. Infection by GLRaV-7 was verified. We did not observe any leafroll specific symptoms after the first year in the field. Work is in progress to get the complete genome of this isolate using high-throughput sequencing.
In conclusion, our findings suggest that GLRaV-7 isolate from Sultanine blanc do not induce leafroll symptoms. Further studies are needed to evaluate the association between GLRaV-7 and leafroll disease.

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O52: Improvement of a protocol for sanitation and early diagnostic validation of autochthonous grapevine germplasm

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INTRODUCTION

Early detection of infections in programs of production of virus-tested vine propagating material is of paramount importance to reduce costs of maintaining sanitized plants. In Apulia (Italy), the Regional Project "Re.Ge.Vi.P.", aiming to the recovery, conservation and sanitary improvement of autochthonous vine cultivars, gave the cue to assess the minimum period of time required after sanitation to detect virus infections with molecular techniques.

Previous results of this work, relatively to the period 2013-2015, were given (Morelli et al., 2015) and regarded the analysis of the sanitary status of *ca.* 150 grape cultivars from which *ca.* 100 apexes were obtained by thermotherapy and *in vitro* culture. The work, continued in 2016-2017, allowed to confirm the previous findings which showed the good efficacy of the sanitation protocols and the reliability of applied diagnostic techniques in the whole process of production of vine propagating material.

MATERIALS AND METHODS

The sanitary status of 43 autochthonous cultivars, collected in the 2016-2017 period, was assessed by ELISA on phloem tissues scraped from dormant cuttings.

According to the detected virus(es), each vine accession was submitted to specific sanitation protocols, already in use in our laboratory (Bottalico et al., 2003). These protocols consist in the application of *in vitro* culture of meristematic apexes when vines are infected by phloem-limited clostero and vitiviruses, and, limitedly to nepovirus infections, a preliminary thermotherapy treatment is also included.

After sanitation, three groups of 89, 60 and 49 plantlets, were submitted to qRT-PCR diagnostic assay (Faggioli et al., 2013), following, respectively, 70-100, 100-200 and more than 200 days the acclimatisation stage in the greenhouse. Molecular detection was repeated 5-6 months and again 2 years later, for those plantlets resulting virus-free at the first assessment.

RESULTS AND DISCUSSION

The initial sanitary status of the 43 accessions before sanitation is reported in Table 1.

Virus	GVA	GVB	GLRaV-1	GLRaV-2	GLRaV-3	GFLV	GFkV	ArMV
N. infected/tested accessions	22/43	1/43	10/43	4/43	28/43	16/43	22/43	0/43

Table 1. Sanitary status of the field collected vines

Comparison with the sanitary status of vine accessions recovered in 2013-2015 (Morelli et al., 2015) showed a similar virus distribution pattern characterized by the prevalence of *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine virus A* (GVA) and *Grapevine fleck virus* (GFkV).

After sanitation treatment, the qRT-PCR analysis showed that 62 out of 198 meristematic apexes excised from 43 different cultivars were still infected, which corresponds to a 70% of successful sanitation process (Table 2).

Number of infected/tested apexes							
GVA	GVB	GLRaV-1	GLRaV-2	GLRaV-3	GFLV	GFKV	ArMV
19/113	1/1	0/37	0/18	3/114	30/79	19/105	0/0

Table 2. Sanitary status of vines after sanitation

The lower efficiency of sanitation treatment on GFLV-infected vines was due to the failure of detecting this virus by ELISA before sanitation. Supposed lack of GFLV infection wrongly directed sanitation to be solely performed by *in vitro* culture of meristematic apexes, thus skipping thermotherapy, which is indeed more efficient in eliminating nepoviruses.

Days after acclimatization	Number of infected/tested apexes
70-100	34/89
100-200	20/60
>200	8/49

Table 3. Results of qPCR detection performed on apexes, at different times following sanitation treatment

Comparison among the qRT-PCR results obtained at the three periods of assessment (Table 3) showed that as early as 70-100 days after acclimatisation, the assay is able to detect infected vines which escaped the sanitation, despite their young age and reduced size. Moreover, plantlets found virus-free at this first assay confirmed their status in the second and third assays, performed 5-6 months and 2-3 years later. A confidence interval sufficient to assess the sanitary status of sanitized vines requires performing ELISA for two vegetative seasons whereas the adoption of qRT-PCR provides consistent results on 3-months old greenhouse-transferred plantlets. These data show that qRT-PCR is an efficient tool for the early detection of grapevine viruses regulated by the Italian legislation, therefore allowing to dramatically reduce the number of sanitized vines to be maintained in the greenhouse, after their transfer from the growth chambers.

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O53: The PAThOGEN project: a new approach to improve grapevine virus knowledge and management

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INTRODUCTION

France, Italy and Spain represent together about 80% of total wine production in Europe. Beyond the well-known fungal pathologies, *Grapevine virus A*nd virus-like diseases such as fanleaf and leafroll are of major economic and agronomic importance because they are present in most vineyards in Europe. One of the reasons of the spread of these diseases is the lack of knowledge concerning grapevine viruses by the main actors of the sector. To face this problem, five partners worked together to develop the PAThOGEN project, a training program aimed to improve grapevine virus knowledge and management. The partnership gathers one French technical center (IFV), one Spanish university (USC), one Italian applied research center (CREA), one Spanish foundation specialized in training and technology transfer (FEUGA) and one Italian SME specialized in the development of informatics tools and in knowledge transfer in the vine sector (HORTA).
The objectives of PAThOGEN are: (i) to develop a high-quality work-based Vocational and Education Training (VET) program, (ii) to develop basic and transversal skills using innovative methods and (iii) to improve the skills of professionals of the wine sector. The long-term perspective of this project is to ensure the economic sustainability and durability of worldwide vineyards by decreasing the impact of virus diseases.

MATERIAL AND METHODS

Development of the courses' contents
The partners developed an innovative e-learning platform containing three levels of training: basic level (four modules) mainly for growers and nurseryman, advanced level for technicians and advisors (six modules), and a third level for future "trainers". Courses content were redacted by IFV, CREA and USC : Module 1 is an introduction to main virus diseases, and their agronomic and economic impact. Module 2 focuses on virus transmission. Module 3 introduces the detection methods available to identify grapevine viruses. Module 4 broaches the control and the management of virus diseases and their vectors. These four modules are common to all the three levels. Module 5 brings up the thematic of the emerging viral diseases. Finally, module 6 takes stock of research on natural engineered resistance, tolerance and cross protection. A first revision of all these modules was carried out by the partners; a second revision was done by some advisory boards (AB) of the three countries. AB are local stakeholders of the wine sector. They had to evaluate the adequacy of the contents to the target audience. A final scientific revision was carried out by different specialists from France, Spain, Italy, USA and South Africa.

Development of the e-learning platform
The definitive versions of the e-learning were created using Microsoft PowerPoint software in each language and were then uploaded on the platform. Courses were published using Forma.LMS, an open source and free Learning Management System. Modules and chapters are HTML5 multimedia objects compliant with the standard SCORM and compiled with Articulate Storyline. Supplementary materials (audios, videos, documents, glossary, photo gallery…) were added to improve the global richness of the course. The flow of navigation is sequential with restrictions driven by intra-module questions. A final test was set-up at the end of each module in order to assess the knowledge just acquired. Trainees who reach 75% of correct answers had access to the next module.
Field session and evaluation of pilot courses

In addition to the e-learning courses available on the platform, the training is completed with two practical sessions in field, one in spring and one in autumn. Once passed this part, successful trainees had access to the practical identification of grapevine virus disease symptoms in vineyard. A satisfaction questionnaire was provided to the students at the end of both e-learning and practical session to evaluate this pilot course.

RESULTS AND DISCUSSION

Since the beginning, the interest in PAThOGEN project has been high (more than 300 pre-registered to date). Thus, for the pilot course, trainees were selected to represent the different professional categories of the wine sector (**Fig. 1**).

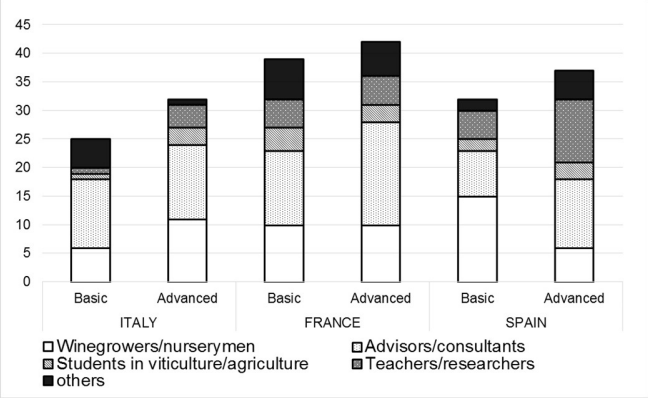

Fig. 1 *Number and professional categories of trainees in the pilot courses for each country.*

However, not all the subscribers had completed the e-learning; **table 1** resumes the numbers of the pilot course for each partner country.

TABLE 1. SELECTED TRAINEES´ PERFORMANCE IN THE PILOT COURSE.

	ITALY		FRANCE		SPAIN	
NUMBER *(%)* OF TRAINEES	**BASIC**	**ADVANCED**	**BASIC**	**ADVANCED**	**BASIC**	**ADVANCED**
ENROLLED AFTER SELECTION	**25**	**32**	**39**	**42**	**32**	**37**
FINISHED THE E-LEARNING	**17*(68%)***	**26*(81%)***	**21*(51%)***	**22*(52%)***	**21*(66%)***	**26*(70%)***
ATTENDED THE FIELD SESSION	**8*(47%)***	**19*(73%)***	**13*(65%)***	**15*(68%)***	**17*(81%)***	**20*(77%)***

The satisfaction questionnaires showed a very good response of students to the e-learning pilot course in terms of contents, interest and achievement of the expectations. In each country and for both basic and advanced levels, the average response was over 4 out of 5 (very good). The field session was crucial to finalize the training and was well appreciated by students (60% find it excellent and 40% good) because it allowed them to identify the symptoms of virus diseases *in vivo*.

We are currently improving the three versions of the e-learning courses taking into account the evaluations and the remarks done by the trainees. The English version will be soon available.

The short term prospect of the project is to open the courses in a paying version in the different regions of our three countries. In a longer term perspective, we would like to spread this training to other wine-growing countries all over the world, without forgetting to adapt the contents to local needs!

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P1: Field survey and molecular characterization of Californian isolates of Grapevine Pinot gris virus.

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INTRODUCTION

Grapevine Pinot gris virus (GPGV) was first discovered in 2012 by high-throughput sequencing (HTS) in Italian Pinot gris vineyards (*Vitis vinifera* L.) (Giampetruzzi et al., 2012). This new member of the genus *Trichovirus*, in the family *Betaflexiviridae*, was first reported in the US in a study of the collections at Foundation Plant Services (FPS) in Davis, California (Al Rwahnih et al., 2016). Very recently, GPGV was also reported by a private plant diagnostic laboratory, which was the first detection of GPGV in Napa Valley vineyards (Angelini et al., 2016). The virus can cause chlorotic mottling, leaf deformation and stunting symptoms in different cultivars. However, diagnosis of GPGV based on specific symptoms can be challenging because a) in some cases the virus appears to be asymptomatic, and b) in other cases, it occurs as a mixed infection with other symptomatic viruses. Recent transmission studies indicated that the eriophyid mite, *Colomerus vitis*, a pest that infests grapevine and has been reported in California, is a suspected vector (Malagnini et al., 2016), which opens the possibility of horizontal spread of the virus. Additionally, GPGV was reported in herbaceous hosts (Gualandri et al., 2016).

MATERIALS AND METHODS

To assess the prevalence and spread of GPGV infection, 687 samples were collected from symptomatic and asymptomatic vines in 284 vineyards across 10 counties in California in 2016 and 2017. Additionally, a collection of 1075 samples of stored total nucleic acid (TNA) from a previous study (Arnold et al., 2017) was included in the assessment. TNA was isolated from leaf petiole tissue using the MagMax Express-96 and later analyzed for the presence of GPGV by quantitative RT-PCR (RT-qPCR). This novel RT-qPCR was developed using an alignment of all GPGV sequences available in the GenBank, aiming to detect all variant strains of the virus. To determine the mixed infection status of GPGV-positive plants, RT-qPCR analysis of known grapevine viruses (Grapevine Disease Testing Protocol 2010, http://fps.ucdavis.edu/fgr2010.cfm) was performed on 121 samples. Additionally, selected samples from different regions and varieties underwent further virome analysis using HTS. Briefly, TNA from source plants were subjected to ribosomal RNA depletion and complementary DNA library construction using a TruSeq Stranded Total RNA with Ribo-Zero Plant kit. Sequencing was performed on the Illumina NextSeq 500 platform. DNA libraries generated an average of 41 million reads per sample and a pipeline was developed to assemble reads and search for plant pathogens and filtered to only contain viral candidates. The viral census of infected vines generated by HTS described the make-up of mixed infections and the full genome of California isolates of GPGV; such sequences were employed in phylogenetic analysis to characterize viral strains and determine the phylogenetic relationships among symptomatic and asymptomatic isolates.

RESULTS AND DISCUSSION

In Napa County, the percentage of 275 vines testing positive for GPGV per infected location ranged from 8.7% to 100%, and the virus was found to be present in multiple grape varieties, including Chardonnay, Cabernet Sauvignon, Sauvignon blanc, Cabernet franc, Merlot, Petit Verdot, Malbec, Sauvignon Musque, Viognier, 1616 C, Schwarzmann, Pinot Noir, Carmenere, and Zinfandel. No vines tested positive for GPGV in Santa Barbara (66 vines), San Luis Obispo (47 vines), Monterey (55 vines), San Joaquin (49 vines), San Benito (2 vines), Merced (8 vines), Fresno (37 vines), Madera (4 vines), and Sonoma (144 vines) Counties. Similar results were obtained from the stored samples (960 vines from Napa County, 55 vines from Sonoma County, and 60 vines from Santa Cruz County). The only GPGV-positive vines were in Napa County where the number of samples that tested positive for GPGV per infected location ranged from 2.9% to 100%. Of all the stored TNA from Napa County, 29.8% of samples tested positive for GPGV**.** Most vineyards where GPGV was detected did not show symptoms. When symptoms were observed, samples were co-infected with *Grapevine fanleaf virus* (GFLV) and symptoms were consistent with those of GFLV, including stunting, chlorotic mottling and leaf deformation. Using HTS, 16 California isolates of GPGV were fully sequenced. Such isolates share 95-99% nucleotide homology with

asymptomatic reference isolates and 98-100% homology with each other. Except for one isolate, all California isolates cluster together according to the phylogenetic analysis (Figure 1).

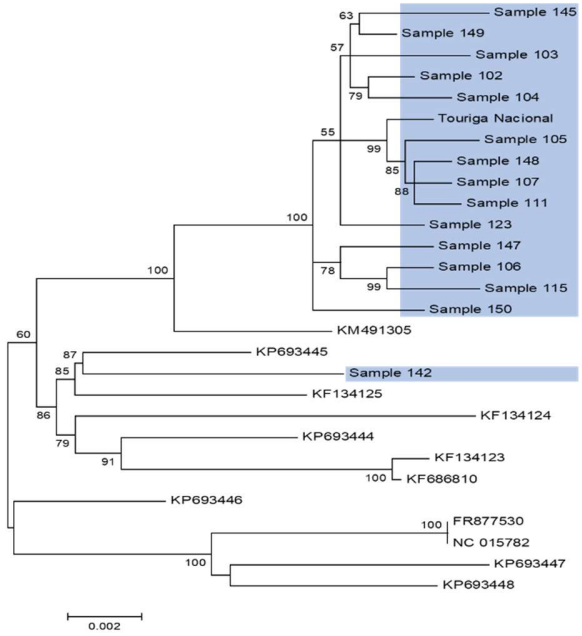

Figure 1. Maximum likelihood tree of GPGV isolates. GenBank accession numbers of sequences employed for the phylogenetic analysis. Shaded area: California isolates.

Newly sequenced California isolates and all GPGV sequences available in the GenBank were aligned and specific primers and probes (RT-qPCR) were designed. The developed RT-qPCR assay was efficient for the detection and monitoring of all known GPGV isolates. Preliminary results showed that the RT-qPCR assay detected all conventional PCR-positives with 100% accuracy.

In summary, GPGV is relatively widespread in Napa County. Worldwide, GPGV has been identified in symptomatic and asymptomatic wine and table grapevines and can occur as a mixed infection with other viruses. GPGV isolates share high homology with asymptomatic reference isolates (GenBank). Vines that showed typical GPGV symptoms were all also positive for GFLV. The new RT-qPCR assay detected all known variant strains of the virus efficiently.

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P2: Status of grapevine viruses in Texas vineyards.

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INTRODUCTION

Viruses are major constraints to sustainable production of wine grapes worldwide. Of the over 70 viruses infecting grapevine, those associated with grapevine leafroll disease (GLD), fanleaf degeneration and decline, rugose wood disease complex, and recently red blotch disease are considered most economically important. Texas is one of the top 10 wine producing states in the USA but little is known about the sanitary status of vineyards in the state with regards to major viruses of grapevine. In a previous study, the occurrence of *Grapevine leafroll-associated virus 2* (GLRaV-2) and GLRaV-3 was reported from vines showing symptoms of grapevine leafroll disease (GLD) in one vineyard site (Jones et al., 2014). Since then, there is no information on the occurrence and distribution of both viruses and other major grapevine viruses across vineyards within the thriving industry in the state. The objective of this study was to determine the incidence and distribution of 10 major grapevine viruses across Texas vineyards.

MATERIALS AND METHODS

Surveys were conducted during 2016 and 2017 seasons and prospective vineyards were identified through grower contacts and viticulture advisors based on a suspected history of virus problems. Petiole samples were collected individually from a total of 196 red and white fruited grapevine varieties in 42 vineyards distributed across 23 counties. Total RNA extracted from each sample (Spectrum Plant Total RNA Kit) was subjected to two-step RT-PCR using published diagnostic primers targeting DNA fragments of the 10 major viruses including GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, *Grapevine fanleaf virus* (GFLV), *Tobacco ringspot virus* (TRSV), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine virus A* (GVA), GVB, and *Grapevine red blotch virus* (GRBV) (Rowhani et al., 1993; Minafra and Hadidi, 1994; Fuchs et al, 2010; Poojari et al., 2016). The virus specificities of DNA amplicon obtained for each virus was confirmed by cloning (TOPO-TA Cloning Kit) and Sanger sequencing of representative samples.

RESULTS AND DISCUSSION

The results showed the occurrence of eight (GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, TRSV, GVA, GRSPaV and GRBV) of the 10 viruses as single or mixed infections of different proportions in the samples. The three most prevalent viruses were GRSPaV (57% or 112/196), GRBV (46% or 91/196), and GLRaV-3 (30% or 58/196) while those with the least occurrences were GLRaV-4 (5% or 10/196), GVA (4% or 8/196) and GLRaV-2 (0.5% or 1/196) (Fig. 1). Mixed infections of two to five different viruses was predominant (2006 = 57.4%; 2017 = 36.5%) over single virus infections (2006 = 31.1%; 2017 = 35.1%) versus no virus detected (2006 = 11.5%; 2017 = 28.4%) (Table 1). None of the samples tested positive for GFLV and GVB.

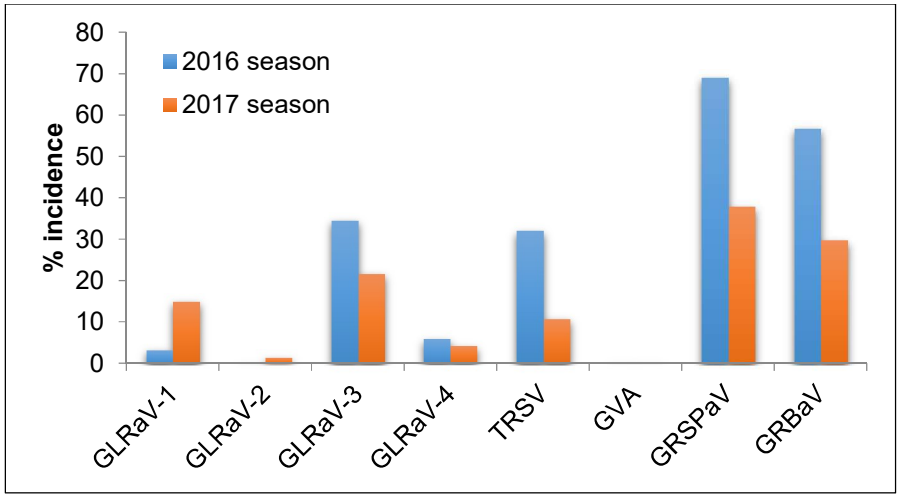

Figure 1. Incidence of eight viruses detected during surveys conducted in 42 Texas vineyards in 2016 and 2017 growing seasons.

Table1: Sanitary status of Texas vineyards based on RT-PCR screening of 196 field-collected samples for 10 major grapevine viruses†.

2016 season:			2017 season:		
Infection status	**No. Positive**	**% Positives**	**Infection status**	**No. Positive**	**% Positives**
Negative	14	11.5	Negative	21	28.4
Single virus	38	31.1	Single virus	26	35.1
Two viruses	27	22.1	Two viruses	18	24.3
Three viruses	23	18.9	Three viruses	9	12.2
Four viruses	17	13.9	Four viruses	0	0
Five viruses	3	2.5	Five viruses	0	0
Total	122	100	Total	74	100

†Samples analyzed in 2016 and 2017 seasons were obtained from different vineyards.

Further analysis of the results showed presence of single and mixed virus infections in both vinifera (e.g. Chardonnay, Merlot, etc.) and non-vinifera (e.g. Blanc du Bois, Black Spanish) grapevine varieties (data not shown) indicating widespread occurrence of viruses in native and non-native grapevine cultivars in Texas.

The Texas wine grape industry contributes more than $13.1 billion of economic value to the state (https://www.txwines.org/texas-wine/texas-wine-industry-facts/). In a previous study, the occurrence of two GLD-associated viruses was reported from a single vineyard location in Texas (Jones et al., 2014). In this study, a more robust survey conducted across several vineyards in all eight America Viticultural Areas (AVAs) in the state confirmed the presence of both viruses in Texas in addition to six other previously unreported viruses in the state. Interestingly, TRSV was documented for the first time on Blanc du Bois, an American hybrid grape cultivar, showing decline symptoms (McBride et al., 2017). Against the background of documented negative impacts of viruses (Alabi et al., 2016), it is important to determine the virus landscape of the fledging Texas wine grape industry particularly since most of planting stock grown in the state are sourced from California and Washington. Also, a significant amount of the acreage is planted to non-vinifera varieties due to their resistance to Pierce's disease but very little information is available about the sanitary status of these vines. Thus, results obtained in this study have established a "baseline" for the maturing industry that will be invaluable as the acreage expands and new problems are encountered. Furthermore, the results have shown that the Texas industry is not "immune" to the same disease problems that are experienced in different grape growing regions worldwide (Martin et al., 2005; Fiore et al., 2008; Liu et al., 2013; Jooste et al., 2015; Poojari et al., 2017). From a practical standpoint, the results will be valuable in educating growers in the state about the importance of clean plant materials and the risk of inadvertent spread of viruses through sharing of non-tested grapevine propagation materials.

ACKNOWLEDGEMENTS

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P3: Digital image analysis of leafroll symptoms and damage assessment for different GLRaV-3 isolates

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INTRODUCTION

Leafroll symptoms are best observed in indicators as Cabernet franc, Cabernet sauvignon, Pinot noir, and others, and therefore the indexing of selected clones is traditionally done by grafting onto any of them. The detection of GRLaV-3 by biological indexing is reliable and as far as we know there are no references concerning indicators which have showed no symptoms. But even in indicators the leafroll symptoms could be either stronger or milder depending on the season, year or leafroll virus specie/s. In several studies, the leafroll infected plants had diminished photosynthetic activity and other changes in physiologic processes that explain the yield and quality losses and the delay in ripening (Manini et al., 2012). The precocity and strength of leafroll symptoms that would explain the damages could be used to predict them and to compare the virulence of isolates.
The objective measurement of the percentage of symptomatic Pinot noir leaf area has been used for further evaluation and classification of several GLRaV-3 isolates, whose molecular variability had already been studied (Pesqueira et al., 2016).

MATERIALS AND METHODS

Beginning in 2012, several of the GLRaV-3 isolates characterized by Pesqueira et al. (2016) were grafted (chip budding) onto Pinot noir in an experimental vineyard in Portomarín (Lugo, Spain) planted in 2009; the experimental design consisted in three blocks of three plants of 10 GLRaV-3 isolates randomly assigned to two terraces. Plants were analyzed the next year after having been grafted and re-grafted if negative.
Digital raw pictures of all plants were taken every week, from June to September in 2016 and three times in 2017. All the photos were standardized at the same size to show the same section of the plant, and the percentage of red colored leaves was measured using the Image Analysis Software for Plant Disease Quantification (Assess 2.0, APS Press; Lamari, 2008). Nine plants grafted with buds from a healthy plant were used as control and “0” for symptoms. At harvest, the following data were recorded for each plant: number and weight of clusters, weight of 100 grapes, pH, ºBrix and total acidity (mg/L tartaric acid) of must obtained from 50 grapes randomly picked at harvest.

RESULTS AND DISCUSSION

The digital image analysis was quick and very consistent in the estimation of the main leafroll symptoms in Pinot noir as long as the plant did not show any other disease symptoms. In 2016 a poorly controlled downy mildew attack made the assessment difficult due to the presence of necrotic areas: just after veraison, the program overestimated symptoms, and at the end of the season they were underestimated; some adjustments had to be done to improve the assessment. On the contrary, in 2017, symptoms were clean and clear, and the digital analysis was able to assess the percentage of symptomatic area without further adjustments.
The different isolates induced different precocity and intensity of leafroll symptoms (Figure 1), to the point that it was possible to distinguish most of them in the field. F49BR6 showed the highest precocity and intensity of symptoms; MP4.11 did not show leafroll symptoms, not even at the end of the season in any of the 4 years following the grafting. The other five isolates appear in two groups, with higher symptoms in F31BR8, MP8.28 and TMF6.1 and lower ones in F35RE8 and F2CA1; that rating was significant in 2016 and 2017 (Figure 2).
The relation between symptoms and damages is shown in Figure 3: the Kg*Brix for MP4.11 were not significantly different from the control for any of the parameters analyzed in 2015 and 2016; F49BR6 performance was worse than that of any other isolate, with yield losses up to 65%, and an average of 17.2 ºBrix (2013-2016), while for the same years MP4.11 and 120A had had an average of 21.5 ºBrix.
The different virulence of these GLRaV-3 isolates was responsible for significant differences in the leafroll symptoms and performance when grafted onto the same cultivar, with same rootstock and in the same location. MP4.11 is an interesting isolate that did not show symptoms in the source cultivar (Mencía) - neither in the original location, nor on its own roots in this plot - or in Pinot noir several years after grafting. MP4.11 is detected by ELISA with different antibodies and by RT-PCR with primers LC1/LC2 y CP3U/CP3D (Turturo et al., 2005) and

is included in the molecular group II (Pesqueira et al., 2016). The unique pathosystem of leafroll viruses includes many variants (Naidu et al., 2015), some of which are mild or hypovirulent (Habili et al., 2009; Rast et al., 2012), so further molecular studies are in course to investigate the GLRaV-3 MP4.11.

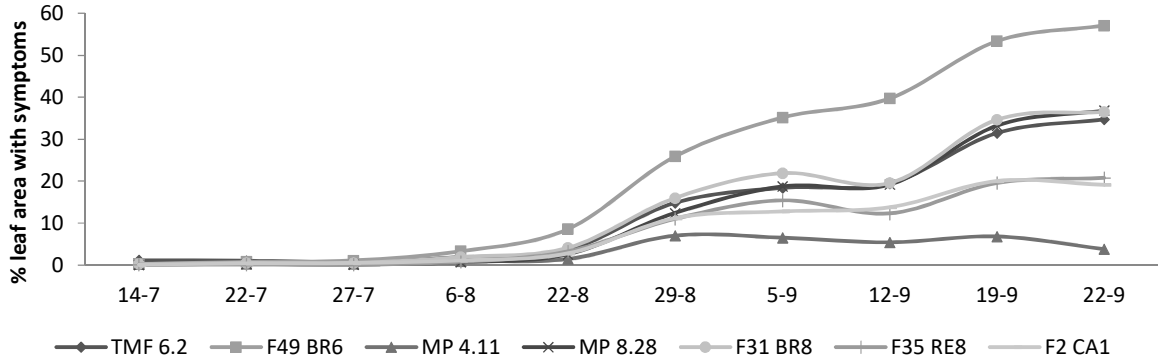

Figure 1: Leafroll symptoms evolution in Pinot noir for several GLRaV-3 isolates (2016): percentage of red leaves given by Assess 2.0 digital analysis program.

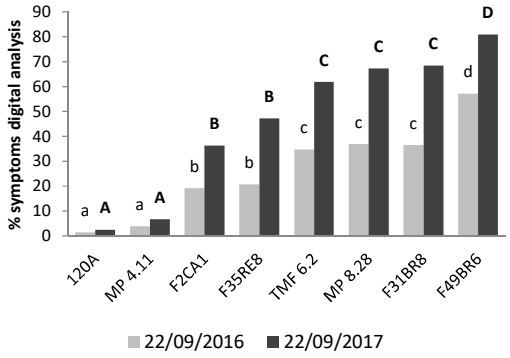

Figure 2: Percentage of red leaves given by Assess 2.0, for 7 GLRaV-3 isolates and one healthy control after harvest in 2016 and 2017. Different letters mean significant differences with p<0,05 (Tuckey b test).

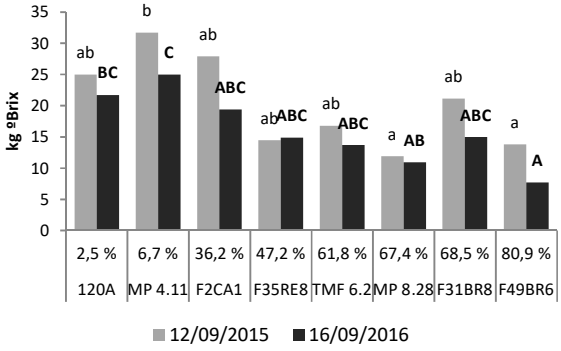

Figure 3: Kg*Brix in 2015 y 2016 for 7 GLRaV-3 isolates and one control. In the X axis: percentage of red leaves at harvest. Different letters mean significant differences with p<0,05 (Tuckey b test).

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P4: Detection of *Grapevine fanleaf virus* (GFLV) by tissue printing ELISA

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INTRODUCTION

Tissue Printing Enzyme-linked Immunosorbent Assay, (TP-ELISA) has been used, with different names, for the detection of viruses in several crops (Lin et al., 1990; Hsu and Lawson, 1991); it is currently used for *Citrus tristeza virus* in countries such as Spain and central California (USA) which maintain zero-tolerance policies for CTV that require sensitive, specific, and reliable pathogen-detection methods (Vidal et al., 2012). Grapevine leafroll associated viruses can be detected by TP-ELISA during the summer (Couceiro et al., 2006). Most authors found quite similar sensitivity and reliability between DAS and TP-ELISA for the detection of plant viruses (Whitworth et al., 1993; Couceiro et al., 2006). With TP-ELISA there is not sample processing because no sap extraction is needed; in addition, there are less steps and the incubation time is shorter than in DAS-ELISA, and therefore, the time and cost per sample is considerably lower (Couceiro et al., 2006). Tissue printing is especially useful when many plants are to be tested: in nurseries, in epidemiological studies to check virus presence and transmission in the field (Cabaleiro et al., 2008) or in clonal selection, to make a first quick and cheap screening to reduce the number of plants to be analyzed by the most sensitive molecular methods. The prints can be done *in situ* and the membranes sent to the lab to be analyzed later. As far as we know, grapevine nepoviruses as GFLV have not been detected by tissue printing.

MATERIALS AND METHODS

Samples from fanleaf infected Albariño plants from two locations were analyzed by TP- ELISA during summer 2016 and 2017 in order to check the reliability of the detection of GFLV. One of the locations is close to the sea and warmer than the other which is 200 km inland.

Antibodies conjugated with alkaline phosphatase from Bioreba AG (Basel, Switzerland) were used at the same dilution as in DAS-ELISA (1:1000). Leaf petioles from young and mature leaves (from main or lateral shoots), tendrils and shoot tips are printed onto nitrocellulose membranes with 0.45 µm pore size (Sartorius, Goettingen, Germany). The membranes are blocked in dry skimmed milk at 1-2% in the extraction buffer for grapevine (the same as for DAS-ELISA) during one hour at room temperature, or overnight at 4-6ºC. The membranes are then dipped directly in alkaline phosphatase conjugated antibody solution (in same conjugated buffer as for DAS-ELISA) and incubated for 2 h at room temperature or overnight at 4-6 ºC. After incubation the membranes are washed carefully 3-4 times for at least five minutes each while stirring in a saline buffer (0.085% NaCl, 0.05% Tween 20 in distilled water). Finally, the membranes are covered with BCIP-NBT ready-to-use liquid substrate (SIGMA B-1911). Purple color starts to develop on the prints after 30 minutes, but the reaction is only stopped with tap water when the membrane starts getting dark and the positive controls are clear. A dissecting microscope can be used to observe the purple color in doubtful samples.

RESULTS AND DISCUSSION

The best season for the detection of GFLV was, as for DAS-ELISA, during spring: in June adult leaf petioles from main shoots (ALMS) were the best (100% positive); later on, the detection in those leaves decreased progressively while in young leaf in main shoots (YLMS) the virus could be detected during all summer, and it only wasn´t reliable in September when the main shoot growth had stopped. Tender leaves from young lateral shoots (LLS) are always a good plant material to be tested, all summer (Figure 1). In other plant tissues, as tendrils or shoot tips, the detection of GFLV was less consistent. Symptomatic leaves, rolled and cut to be printed, were good samples for TP, but the use of petioles is easier, prints occupy less room in the membranes, they can be kept for further analysis and the purple color develops over all the printed area giving easy-to-read results. There were no differences between the plants in the two locations in both years; the key factor for the detection was the age/maturity of the tissue, being tender ones the best; but it is recommended to analyze at least two leaves per plant.

When tissue printing was followed by RT-PCR detection instead of serological analysis of CTV, the sensitivity increased, and the combination of both methods gave the best results, making the indexing onto indicators unnecessary (Vidal et al., 2012). Work is in progress to check whether that would work for the main regulated grapevine viruses.

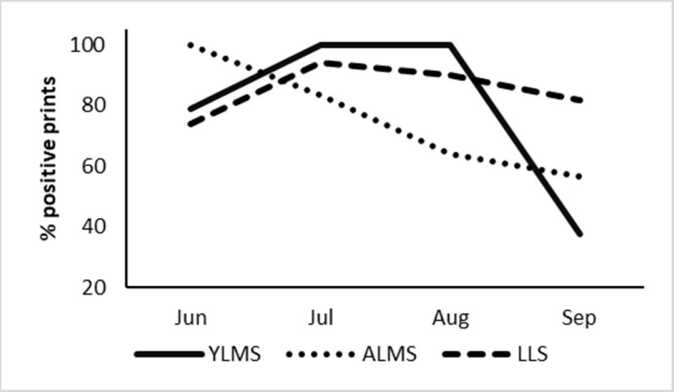

Figure 1. GFLV detection by TP-ELISA along the summer in: Young leaves from main shoots (YLMS), Adult leaves from main shoots (ALMS) and leaves taken from lateral shoots (LLS).

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P5: Serotyping *Grapevine leafroll-associated virus 3*

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INTRODUCTION

The negative impact of the *Grapevine leafroll-associated virus* 3 on the production of quality wine has been long acknowledged. In order to mitigate its spread in the field three actions are essential, the control of the vectors by a suitable spraying regime, the removal of infected plants when possible (Bell et al 2017) and, at the base of the disease management is the assurance that only virus-free grapevines are planted. In New Zealand, the grafted grapevine standard (GSS) ensures that new planting material is of a good quality and GLRaV-3-free. Fulfillment of this condition is based entirely on ELISA testing by testing laboratories accredited by an ILAC member. It is therefore critical that the GLRaV-3 assay is sensitive and detects all known strains of the virus. We describe here the results of the detection of highly variable strains of GLRaV-3 with a range of antibodies.

MATERIALS AND METHODS

Plant material was selected for single virus infections of different GLRaV-3 strains. Representative members of GLRaV-3 from New Zealand included: Group I, Group VI (isolates NZ1 and NZ1vm), and NZ2 (Cohen et al 2012; Chooi et al 2013), and from USA included: Group 1, GLRaV-3e, GLRaV-3f and GLRaV-3 NZ2. ELISA was performed as described previously (Blouin et al., 2017) in either double antibody sandwich or triple antibody sandwich ELISA. In order to compare the effect of the antibodies, the same coating antibody, a polyclonal antibodies (pAbs) from goat (kindly supplied by Darius Goszczynski), was used for all the assays. The following capture antibodies were then used: monoclonal (mAb) mAbNY1.3 (Ling et al., 2000) conjugated with alkaline phosphatase (AP) kindly supplied by Bioreba, pAbs As163 (Ling et al., 2000) kindly supplied by Marc Fuchs mixed with anti-rabbit AP (Sigma) and a mAb GLRaV-3 antibody, developed by Dr. Adib Rowhani at Foundation Plant Services (FPS), University of California, Davis, mixed with anti-mouse AP conjugate (Sigma).

RESULTS AND DISCUSSION

All GLRaV-3 strains by were detected with the pAbs As163 together with anti-rabbit–AP. Conjugated mAbNY1.3 showed variable affinity between the virus isolates with a weaker detection of the divergent group VI and NZ2. Isolate NZ1vm, that is genetically very similar to NZ1, was only very weakly detected and the Californian isolate GLRaV-3f was not detected (Table 1). Results using the FPS mAb together with anti-mouse-AP were comparable with the results using pAbs As163. Both pAbs As163 and mAb mAbNY1.3 were prepared at Cornell University against isolate NY1, at a time when the wide diversity of sequence variants of GLRaV-3 was unknown (Ling et al., 2000). The FPS mAb was developed against recombinant protein synthetized with a much broader knowledge of the diversity of the virus. In our tests the FPS mAb detected all samples in a similar manner to the Cornell pAbs. These results are reassuring that the commercially available ELISA reagents for GLRaV-3 are satisfactory for the detection of various virus strains as all the commercial ELISA kits rely on pAbs or a mixture between pAbs and mAb (Blouin et al., 2017). These results demonstrate that reliance on mAbNY3.1 or similar mAbs for detection may lead to false negatives in results. However, they also show that the reactivity ratio between generic antibodies such as the Cornell pAbs or the FPS mAb against mAbNY3.1 can be used to identify new divergent isolates of GLRaV-3.

Table 1: Reaction rate of ELISA assay expressed in milli OD per min in three different ELISA with a polyclonal goat antibody used as coating, against three types of capturing antibody: monoclonal MAbNY1.3 AP conjugated; polyclonal As163 (with anti-rabbit AP); monoclonal FPS mAb (with anti-mouse AP)

	mAbNY1.3	pAbs As163	FPS mAb
Gp1 (NZ)	38.10	10.36	8.65
Gp1 (US)	23.45	12.03	6.76
Gp1 (US)	9.24	5.11	2.82
GpVI-NZ1	9.46	9.36	6.99
GpVI-NZ1	13.74	14.96	9.51
GpVI-NZ1vm	0.27	9.30	8.17
GpVI-NZ1vm	0.28	9.09	8.04
GpVI-NZ1vm	1.45	13.76	10.68
NZ2 (NZ)	8.38	10.54	6.74
NZ2 (NZ)	8.56	10.67	7.09
NZ2 (NZ)	9.28	8.52	7.90
NZ2 (US)	12.16	8.03	4.50
GLRaV-3e (US)	4.43	11.09	6.32
GLRaV-3f (US)	0.04	7.35	4.54
buffer	0.01	0.03	0.01

ACKNOWLEDGEMENTS

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P6: Field performances and grape quality of the same clone of 'Nebbiolo' (*Vitis vinifera* L.) when infected by GLRaV-1+GVA, GVA or healthy

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INTRODUCTION

Leafroll, one of the most important virus diseases in grapevine, is mainly due to single or mixed infection of the *Ampelovirus Grapevine Leafroll associated Virus 1* (GLRaV-1) and *3* (GLRaV-3). *Grapevine virus A* (GVA) is a *Vitivirus* associated with the aetiology of Kober Stem grooving. According to the available literature, GLRaV-1, often in mixed infection with GVA, is reported to reduce growth and yield, generally without affecting fruit maturity, and resulting in a reduction of leaf net photosynthesis and chlorophyll content (Mannini and Digiaro, 2017). The aim of this study was to add information on the effect of GLRaV-1 alone or in mixed infection with GVA on grapevine agronomic and grape qualitative parameters. The trial was conducted with a clone of 'Nebbiolo' (*Vitis vinifera* L.), one the most important red wine cultivar of Piedmont (North-west Italy).

MATERIALS AND METHODS

A clone of 'Nebbiolo', originally infected by GLRaV-1 and GVA and tested free from GFLV, GLRaV-3 and GFkV, was heat-treated obtaining a healthy line and another line GLRaV-1 free but still infected by GVA. The three lines of the clone, GLRaV-1+GVA, GVA and Healthy, were then grown potted under screen-house conditions until they were adult vines. Buds were collected from the mother plants of the three lines, propagated by grafting on healthy Kober 5BB rootstocks and planted in three parallel and facing rows. Along the rows, 5 vines were selected for each sanitary status in order to carry out field assessments. The vineyard was located in a typical area for 'Nebbiolo' cultivation and vines were vertically trained and single-cane pruned. The plantation density was 5000 vines per hectare. The virological status of every single selected vine (5 + 5 + 5) was controlled by DAS-ELISA on dormant cane samples collected during 2014-2015 winter time and using commercial kits according to the manufacturer's instructions (Agritest Srl, Valenzano, Bari, Italy and Sediag, INRA, France). The tests confirmed that the progenies of the three lines were respectively GLRaV-1+GVA infected, GVA infected and GLRaV-1+GVA free. In addition the three progenies resulted free from ArMV, GFLV, GLRaV-2, GLRaV-3 GFkV and GVB. In 2016 and 2017, the main agronomic and juice qualitative parameters were assessed on each single selected vines. At the first harvest a sample of 300 berries was collected from the same vines in order to carry out analyses of berry anthocyanin concentration by HPLC-DAD (1260 Infinity, Agilent Technologies). In addition, for each sanitary status, eco-physiological assessments were carried out by mean of an open gas exchange apparatus LCpro+ ADC (Analytical Development Company, Hoddesdon, UK) with a broad-leaf chamber in four dates (June, July, August and September) during 2016 growing season. Field data were statistically elaborated by ANOVA.

RESULTS AND DISCUSSION

The presence of GLRaV-1+GVA induced an evident decrease of vine vigor and of the amount of crop/vine. The lower crop was consequent to the lower number of clusters/vine with smaller size (Table 1). Not so when only the GVA was present. In this case all those parameters resulted comparable to the ones of the healthy vines. Juice sugar concentration did not differ among the three sanitary status; however the GLRaV-1+GVA diseased vines produced grapes with a higher content of organic acids. Grape composition did not differ very much between GVA infected and healthy vines. Among grape qualitative parameters, total anthocyanins (responsible of wine colour) were increased by virus elimination on a per berry basis, although without the support of statistical significance. However, expressing data per kg of berry weight, healthy vines produced less anthocyanins due to the higher berry weight respect to GLRaV1+GVA infected vines. When only GVA was involved, the results were closer to the ones with double infection.

In terms of eco-physiological performances, the presence of the double infection highly penalized all the parameters involved when compared to healthy vines: photosynthesis (Pn), transpiration (E) and substomatal Co_2 concentration (Ci) (Figure 1). The reduction in photosynthetic activity was evident since the early assessment (June) and increased over the season. When only GVA was present, the Pn reduction was delayed to July and

became particularly evident in August (when climatic condition induced water stress). Nevertheless the gap between GVA infected and healthy vines in plant physiological behaviour was rather less important compared to the one when the double infection was involved.
In conclusion the eradication of the mixed infection of GLRaV-1+GVA from vines induced important positive modifications in the agronomic performances (i.e. increase of vigor and yield) but without clear effects on grape quality. The field response of the vines infected only by GVA did not differ significantly from the one of healthy plants confirming the positive effect of GLRaV-1 elimination; however bunch weight, yield, and vigor of these plants resulted lower in comparison to the values of healthy vines. Regarding eco-physiological parameters, the data clearly indicated that the vines infected by both the viruses were dramatically low-performing compared to healthy plants: this would explain their poor agronomic behaviour. The single GVA infection affected vine physiology in a milder way and later in the season then resulting in a lower impact on vine field features.
These results, although limited to the conditions of this trial, showed a strong negative synergistic effects on the overall vine performances due GLRaV-1 and GVA mixed infection and a lower impact on vines due to GVA alone.

Table 1 - Field performances and grape composition

NEBBIOLO	Healthy	GVA	GLRaV-1 and GVA	Sign.
Fertility (n°. inflor./vine)	0.73 a	0.80 a	0.50 b	*
Yield (kg/vine)	1.72 a	1.57 a	0.75 b	*
Bunch wt (g)	239 a	209 a	119 b	*
Bunches/vine (n.°)	7.7 a	7.7 a	5.8 b	*
Pruning wood wt (g/vine)	480 a	450 a	270 b	*
Soluble solids (°Brix)	25.3	25.3	25.2	ns
Titratable acidity (g/L)	6.53 b	6.46 b	7.24 a	*
pH	3.11 b	3.17 a	3.17 a	*
Tartaric acid (g/L)	5.92	5.85	6.21	ns
Malic acid (g/L)	1.11 b	1.23 ab	1.38 a	*
Tot. anthocyanins (mg/kg)	939.9	1031.1	1055.6	ns
Tot. anthocyanins (mg/berry)	26.8	23.6	22.3	ns
Berry wt (g)	1.6 a	1.6 a	1.5 b	*

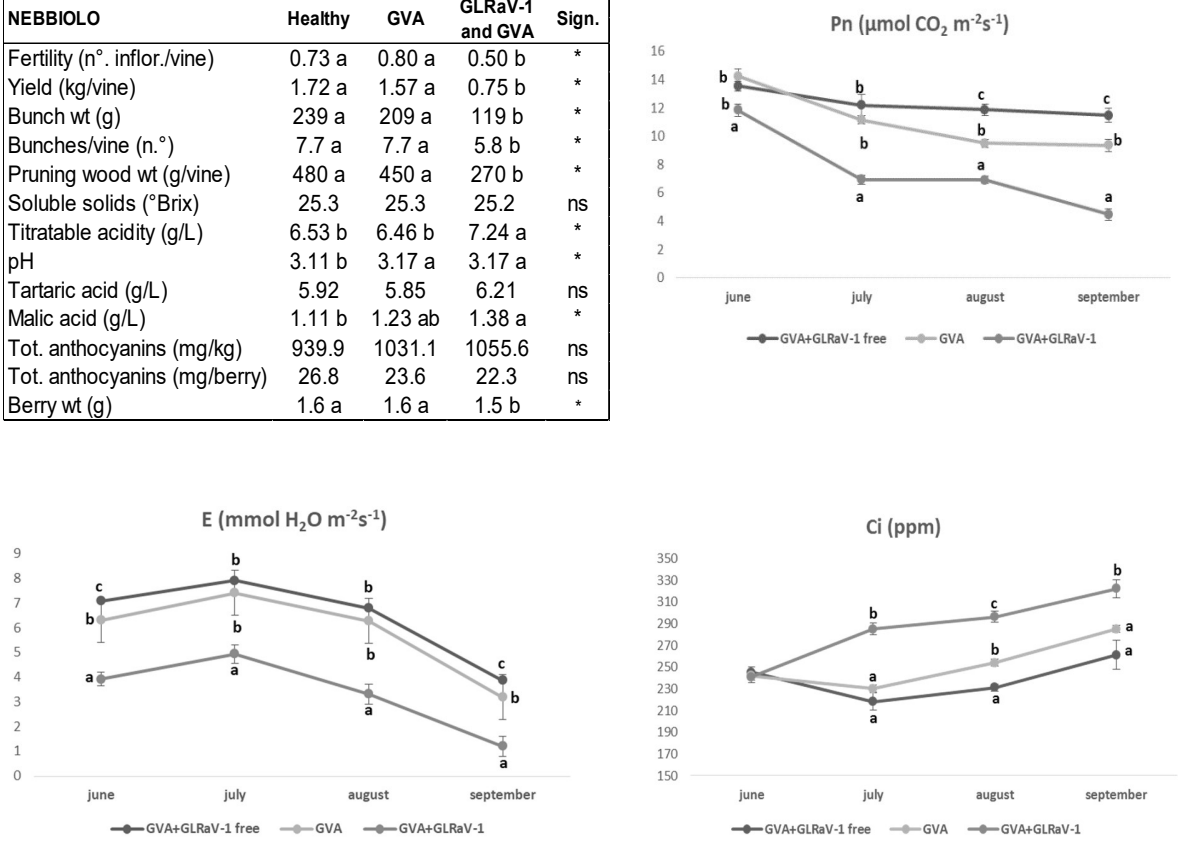

Figure 1: Photosynthesis (Pn), transpiration (E) and substomatal Co_2 concentration (Ci) trends throughout the vegetative season in healthy, GVA and GLRaV-1+GVA infected vines.

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P7: Detection and sequence analyses of vitiviruses in the Mediterranean

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INTRODUCTION

Rugose wood (RW) is one of the most important diseases of grapevine and is associated with graft-transmitted viruses that affect the wood and decrease the vigor and life span of plants (Credi, 1997). One foveavirus (Rupestris stem pitting-associated virus, RSPaV) and five vitiviruses [*Grapevine virus A* GVA), GVB, GVD, GVE and GVF] are reported to be involved in the complex disease (Boscia *et al*., 1993, Abou-Ghanem et al., 1997; Nakaune et al., 2008; Al Rwahnih et al., 2014). In particular, GVA and GVB have been tightly correlated with Kober stem grooving and corky bark (Garau et al., 1994; Boscia et al., 1992), whereas the specific etiological role of GVD, GVE and GVF on grapevine remains still unknown (Rosa et al., 2011). Currently, serological techniques are available for the GVA and GVB detection, but not for GVD, GVE and GVF. For the latter viruses the molecular techniques appear to be the only tools for their identification. Moreover, there is an obvious lack of data regarding the distribution of vitiviruses and their genetic variability in the Mediterranean region, in particular for the two newly discovered vitiviruses GVE and GVF. This study reports the presence of vitiviruses that were detected in vines originated from different Mediterranean countries and from China through the application of RT-PCR assays, using newly designed primers specific to the coat protein (CP) gene and the analysis of their genetic relationships.

MATERIALS AND METHODS

Phloem tissue from mature canes (218 samples), collected from two collections plots (Locorotondo and Valenzano, south of Italy) of grapevine varieties from different Mediterranean countries, were used for total nucleic acids (TNAs) extraction (Foissac et al., 2001) and first strand cDNA synthesis. For RT-PCR, and to the aim of this study, the CP viral sequences of all isolates for each virus species were recovered from the NCBI public database and aligned using ClustalX1.8 (Thompson et al., 1997). All nucleotides degeneracy at both primers sites were taken into consideration and accordingly universal species-specific primers were constructed. Primers used, i.e. GVA [CPU-s(5'- ATGGSWCANWCGCMARGAGRGKVGA -3') CPU-a (5'- CTATATYTCRACAGCYTGYTCVCC -3')]; GVB [CPU-s(5'-ATGGAAAATATATCCCGGATGGC-3') CPU-a (5'- CTATATYTCRACAGAYTGCTC-3')], GVD [CPU-s (5'- ATGTAYCTKAGGACSCTSTTCGG-3') and CPU-a (5'- TTATATCTCAACTGCCTGCTCTCC-3')], GVE [CPU-s (5'- ATGGAGTCAAAAGCGATCMGRTC-3') and CPU-a (5'- CTAGACYTCCACCGAGYTTTC-3')] and GVF [CPU-s (5'- ATGGCTCAGATATCAAGAAGGATG-3') and CPU-a (5'- TCAGATCTCAGCTGCTTGTTCACCG-3')], amplify PCR products of 597 bp, 594 bp, 480 bp, 605 bp, 600 bp and 597 bp in size for GVA, GVB, GVD, GVE and GVF, respectively. RT-PCR cycling consisted of an initial denaturation at 94°C for 4 min, and then repeated 40 cycles of denaturation, annealing and elongation, respectively at 94°C for 30 sec, 55°C for 40 sec and 72°C for 40 sec. After visualization in agarose gel TAE1x, the PCR products were ligated into plasmid StrataClone™ PCR Cloning vector pSC-A (Stratagene, USA) and sequenced (Eurofins Genomics, Germany). CLUSTALX1.8 was used to generate the multiple sequence alignments and identity matrix, whereas the phylogenetic trees were constructed using the neighbor-joining (NJ) method in the MEGA6 analysis package (Tamura et al., 2013).

RESULTS AND DISCUSSION

RT-PCR assays, using the primers designed on the CP gene, successfully amplified the expected products from 91 out of 218 tested samples (41.7%). In particular, 57 samples were infected by a single vitivirus specie (62.6%), 22 by double infections (24.2%) and 12 by triple infections (13.2%). GVE was the most widespread vitivirus and was detected in 14.7% of samples, followed by GVF (11.5%), GVB (6.9%), GVA (5.5%) and GVD (2.8%). Nucleotide sequence identity matrix of CP of the virus isolates sequenced in this study and of those retrieved from Genbank showed that GVA presents the widest variability between isolates, ranging from 75% to 98.8%. Contrarily to other reports, GVE showed a consistent variability (77.2% to 100%), very similar to that of GVB (77.1% to 99.4%). GVD and GVF were the most conserved viruses showing a variability that ranged from 82% to 100% and 86.4% to 99.8%, respectively. Sequences obtained were all deposited in the Genbank and the release of their accession numbers is in progress. The phylogenetic trees showed that GVF isolates are grouped in two clusters, both grouping all Mediterranean isolates (Fig. 1a), whereas the phylotree of GVE generated three

clusters, with all Mediterranean isolates allocated in the group II, except for the isolates HT52-1 from Tunisia (in the group I) and H188-2 from Malta (in the group III) (Fig. 1b). GVD isolates were distributed in four clusters although the sequences of only few isolates were available in Genbank. All clusters showed a narrow variability between them; nonetheless, it is easily noticeable that the isolates originating from Tunisia and Jordan are closer to the Italian isolates and slightly distant from the Brazilian ones (Fig. 1c). The phylogenetic tree of GVB showed the presence of five clusters and also in this case, all isolates coming from China and Japan clustered in the same group III, whereas all isolates from USA and Brasil grouped in the same group I (Fig. 1d).

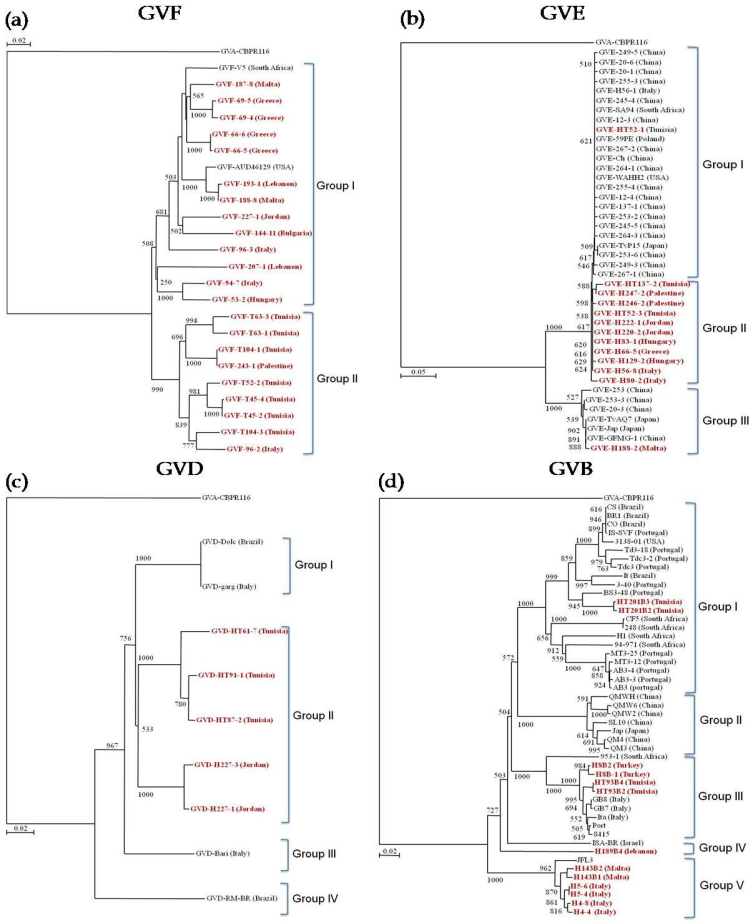

Figure 1: Phylogenetic trees constructed on nucleotides sequences of the CP gene of GVA, GVB, GVD and GVF. Sequences obtained in this study are highlighted in red.

CONCLUSIONS

The genus *Vitivirus* seems to be one of the most important viral groups infecting the grapevine, both for the importance of the symptoms that induce and for their widespread. The recent discovery of GVE and GVF has effectively expanded the potential importance of this group of viruses, although still little is known on their effective impact on grapevine. The survey conducted in this study, which examined hundreds of vines from different European and non-European countries, allowed to draw some indications, although only preliminary, on the potential sequence variability of vitiviruses from all the world. Upon confirmation of the already known widespread of GVA and GVB, this study showed that significantly prevalent in different countries are also GVE (11%) and GVF (11.5%), while on the other hand GVD seems to be quite rare, found only in a few Italian, Tunisian and Jordanian vines. The newly designed primers on the CP gene of all five vitiviruses have demonstrated to be reliable for the use in RT-PCR assays to detect several isolates from different origins, contrarily to those designed on the RdRp gene (ongoing experiment, unpublished data). In addition, this study has expanded the knowledge on the distribution of vitiviruses in the Mediterranean basin and other non-European countries, and has provided the first report on the presence of GVD in Jordan, of GVE in Greece, Hungary, Italy, Jordan, Malta and Palestine, and of GVF in Afghanistan, Bulgaria, China, France, Greece, Hungary, Italy, Jordan, Lebanon and Malta. This study has also highlighted a high genetic variability within different vitivirus species, in particular in GVE genome, contrarily to previous notes reporting for this virus a high sequence conservation level (Nakaune *et al.*, 2008). The phylogenetic trees constructed on the CP gene of vitiviruses have proven to be much more reliable than those built on the RdRp gene for grouping the viral isolates according to their geographic origins.

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P8: Discovery and partial characterization of a novel virus, tentatively named Grapevine virga-like virus

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INTRODUCTION

The families *Bromoviridae* and *Virgaviridae* are composed of viruses with a single-stranded positive sense RNA genome possessing an alpha-like replication complex and a 3'-t-RNA-like structure. The main difference between these two families is that while virions in the family *Virgaviridae* are rod-shaped, those in the family *Bromoviridae* are bacilliform or icosahedral (Adams et al., 2009; Bujarski et al., 2012). Phylogenetic analysis of these viruses further supports the distinction between these two groups (Adams et al., 2009). By using high-throughput sequencing (HTS), we encountered a novel virus, provisionally named Grapevine virga-like virus (GVLV) in three out of seventeen grapevine samples. This virus also possesses an alpha-like replication complex and depending on the genomic region, shows low identity to viruses belonging to either families *Virgaviridae* or *Bromoviridae*. It is, however, more closely related to a newly described, unassigned virus, Citrus virga-like virus (CVLV) (Matsumura et al, 2017). So far, 4,620 nucleotides (nt) have been sequenced, enabling partial characterization of this divergent virus.

MATERIALS AND METHODS

To characterize the viromes of 17 grapevine samples, collected from the south, southeast and northeast regions of Brazil, dsRNA extracts were subjected to HTS on the Illumina HiSeq 2000 platform at Macrogen (Seoul, South Korea) or Eurofins Genomics (Huntsville, USA). Following a typical metagenomic pipeline, we previously identified a novel virus, Grapevine enamovirus-1, infecting four different cultivars. Reanalysis of the data using the most up-to-date viral RefSeq database from the NCBI revealed the presence of GVLV. Reads were trimmed and host derived sequences subtracted with Trimmomatic (Bolger et al., 2014) and BWA (Li and Durbin, 2010) before *de novo* assembly with SPAdes (Bankevich et al., 2012) and taxonomic assignment directly from the reads with the Kaiju webserver (Menzel et al, 2016). Reads that aligned to CVLV in the Kaiju analysis were extracted and *de novo* assembled. Contigs built by SPAdes were subjected to tBlastX (Altschul et al, 1990) searches against the most up-to-date viral RefSeq from NCBI. GVLV was found at very low coverage depth in three different grapevine samples: *Vitis flexuosa* (sample 2M-VF; 12 reads), *V. vinifera* cv. Semillon (sample S16-S; 26 reads) and *V. vinifera* cv. Cabernet Franc (sample S19-CF; 2 reads). In total, eight contigs were assembled for GVLV in the S16-S sample. Blastx searches aligned these contigs to different regions of the alpha-like replication complex of CVLV, with 45-68% of amino acid identity. Four sets of primers were designed to sequence the gaps between these contigs and confirm the infection of GVLV on the S16-S sample. PCR amplicons were sequenced yielding two contigs (GVLV-Met-Hel and GVLV-RdRp). To eliminate ambiguous characters from these sequences, GLVL-Met-Hel and GVLV-RdRp were reassembled with contigs previously built with the CLC Bio workbench assembler (CLC Bio, Qiagen, USA), also extending those sequences to a total of 4,620 nt. Phylogenetic trees for the methyltransferase and partial helicase domains were built by maximum likelihood on MEGA 7 (Kumar et al, 2016). The best-fit substitution model was estimated, and trees were built under the LG + G + I model (Le and Gascuel, 2008) with 5 gamma categories and 1,000 bootstrap replicates. This analysis included viruses from the families *Virgaviridae*, *Bromoviridae* and the genus *Idaeovirus*.

RESULTS AND DISCUSSION

Phylogenetic analysis of the methyltransferase domain positioned GVLV and CVLV as outgroups of the families *Virgaviridae* and *Bromoviridae*, whereas in the case of the partial helicase domain, GVLV and CVLV were both more closely related to the family *Virgaviridae*. This incongruence in the phylogenetic trees when considering

distinct genomic regions suggests the occurrence of early recombination events in the alpha-like replication proteins of the *Virgaviridae*, *Bromoviridae* and related viruses (Codoñer and Elena, 2008). However, it may also reflect inaccuracy in these phylogeny reconstructions since they were based on small genomic regions, especially in the partial helicase tree, that showed the worst bootstrap values. Whether GVLV and CVLV should be included as members of either one of these families depends mostly on the virion particles they form, as well as other genomic features, which description would require knowledge of the full genome sequence. GVLV shows low similarity with viruses in the families *Virgaviridae* and *Bromoviridae*, and a great portion of the putative polymerase shows no similarity to any known virus besides CVLV, indicating that these two viruses may be part a novel group. Based on Blastx alignments, GVLV-Met-Hel and GVLV-RdRp contigs show 26% and 32% identity with Bacopa chlorosis virus (*Bromoviridae*; query cover = 48%) and *Rehmannia mosaic virus* (*Virgaviridae*; query cover = 40%), respectively. Attempts to amplify the genomic region located between the GVLV-Met-Hel and GVLV-RdRp contigs have failed, suggesting that they may be located on distinct genomic segments. To further characterize this virus, 3' and 5' rapid amplification of cDNA ends (RACE) and visualization of the viral particles of GVLV by transmission electron microscopy (TEM) are currently underway. Additionally, RNA extracted from semi-purified viral particles will be subjected to HTS.

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P9: Detection of *Grapevine leafroll-associated virus* 9 (GLRaV-9) in plant host and vector insect (*Planococcus ficus*) in Turkey

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INTRODUCTION

Grapevine leafroll disease (GLRD) is one of the most diffused among viral diseases for the grapevine (*Vitis vinifera* L.) (Almeida *et al*., 2013; Maare *et al*., 2013). To date, 11 *Grapevine leafroll associated virus*es (GLRaVs) have been described: GLRaV-1, -2, -3 -4, -5, -6, -7, -9, -Pr, -De, and GLRaCV, all belonging to the *Closteroviridae* family. Of these viruses, GLRaV-2 is the only member of the *Closterovirus* genus; a tentative genus called *Velavirus* has been created to classify GLRaV-7, while the rest of GLRaVs belong to the *Ampelovirus* genus. GLRaV-1 and GLRaV-3 are apparently the most common ampeloviruses in vineyards and, together with GLRaV-2, the main viruses responsible for GLRD damages. The others ampeloviruses (GLRaV-4, -5, -6, -9, -Pr, -De, and GLRaCV) belong to a common phylogenetic clade (subgroup II) in the *Ampelovirus* genera and may be classified as divergent variants of one virus, GLRaV-4, instead of distinct species, according to recent taxonomical classification (Martelli *et al*., 2012). The presence of some GLRaVs were reported from many viticultural areas in Turkey in the past. GLRaV-4 (Kaya *et al*., 2012) and -5 (Buzkan *et al*., 2010), the first GLRaV-4-like viruses, have been lately detected in some locations in Turkey. Therefore, a study was carried out to investigate the presence of GLRaV-9 in the subgroup II in Turkish autochthonous varieties and potential vector insects.

MATERIALS AND METHODS

A total of 116 vineyards in two viticultural areas (eastern Mediterranean and southeast Anatolia) was visited for the presence of leafroll-like symptoms and mealybug vector investigation. Grapevines were essentially self-rooted table varieties. Plant tissues and *Planococcus ficus* samples were collected from July to the end of August and were stored at +4°C for PCR (mealybugs preserved in 70% ethanol solution). About 100 mg of leaves/petioles was processed for total nucleic acid (TNA) isolation with silica-capture method (Foissac *et al.,* 2005). Five or ten insects, depending upon the size, were also used for TNA isolation (Singh *et al*., 1995). All TNAs from plants and insects were stored at -20°C before cDNA synthesis. Two-step reverse transcription polymerase chain reaction (RT-PCR) was performed using primers LR9-F/LR9-R (Alkowni *et al*., 2004). PCR amplicons were custom-sequenced directly with both primers by Medsantek (Turkey). Alignments of the obtained sequences with additional homologous sequences retrieved from GenBank after using the Blastn program (Altschul *et al*., 1997). A Neighbor-Joining method (Saitou and Nei, 1987) with bootstrap validation was applied to draw a phylogenetic dendrogram (from MEGA6 package).

RESULTS AND DISCUSSION

A total of 423 grapevine samples from 33 autocthonous varieties was tested by RT-PCR assay. GLRaV-9 was detected in 16 samples from both regions. The highest number of GLRaV-9 infected sample was obtained from southeast Anatolia region (approx. 3%). Comparative analysis of 10 Turkish (TK) and other GLRaV-4-like isolates showed close relationship. Two clustering patterns could clearly be observed in the phylogenetic tree (Fig. 1). Group one contained five TK isolates (TK 51, 53, 54, 55, 65) from three autochthonous varieties (cv. Yalova incisi, Pafu and Hönüsü) from different locations and their nucleotide identity ranged between 82% to 85% with those of GLRaV-4 strain 9 (KJ810572, AY297819). Four TK isolates grouped with the US GLRaV-4 isolate (FJ467503) (Abou-Ghanem Sabanadzovic et al., 2012) in the same cluster (Group II). The nucleotide identity of the isolate TK48 and the US GLRaV-4 isolate was 97%. The TK 78 remained out of two clusters having nucleotide identity as one of other GLRaV-4 variants. To our knowledge, This is the first report of GLRaV-9 in turkish vineyards. PCR reaction with all the *P. ficus* samples did not result positive for the presence of GLRaV-9. Plant samples from the same mealybug infested areas were also negative for GLRaV-9 detection.

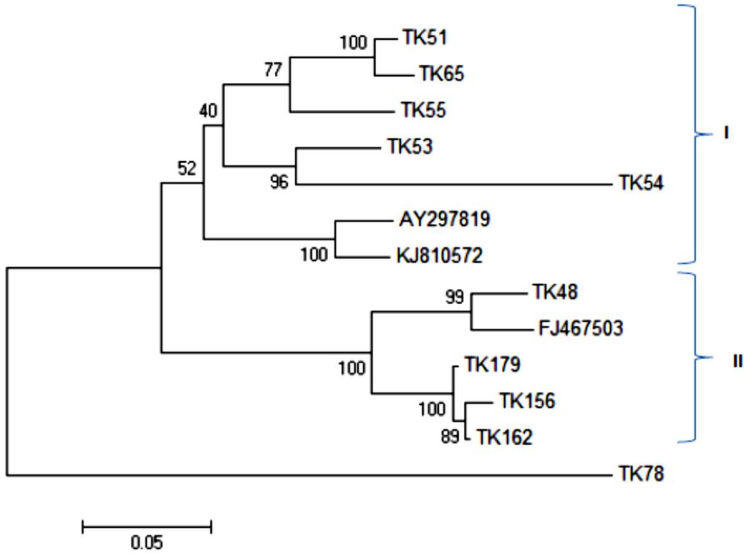

Fig.1. Phylogenetic comparison of the Turkish GLRaV-9 isolates with other homologous sequences from GenBank.

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P10: Survey on grapevine yellows and their vectors in the Republic of Moldova

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INTRODUCTION

In the Republic of Moldova, the area of grapevine plantations is 140,000 hectares. Between 2004 and 2014, 40,000 hectares were planted with imported seedlings from European nurseries. However, in the same period, a very harmful grapevine disease appeared. Currently, the disease is common in all the vineyards. Symptomatic plants are found in autochthonous grapevine varieties such as Rara neagra, Feteasca neagra, Moldova, as well as in vineyards older than 35 years. This suggests that Moldova has very effective and mobile phytoplasma insect vectors. The aim of this work was to identify the agents associated with the disease by molecular analyses. Moreover, monitoring of vineyards was conducted to provide more detailed information on the spread of the disease. Finally, a preliminary survey of possible insect vectors was carried out.

MATERIALS AND METHODS

Visual survey. Plantations were inspected in various zones of cultivation of grapevines from July to September 2017 on seven plots. In each plot five to 40 rows of plants (400 to 10,000 plants) were observed, depending on the area of the plantation. The line of visually observed plants passed along the diagonal of the plantation.
Insect collection. The catching of insects in the vineyard was carried out using sticky yellow traps, from July to September 2017. Three traps were placed in 4 plots and replaced once a month, for a total of 36 traps.
Molecular analyses. The extraction of DNA was carried out according to Angelini *et al*. (2001). Identification of phytoplasmas was performed by nested and quantitative (q)PCR. Nested PCRs were carried out with universal primer pair P1P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995))followed by 16r758f/M23Sr (Martini *et al*., 1999) or by primers R16(V)F1/R1 and R16(I)F1/R1 (Lee *et al*., 1995; 1995), whose specificities are universal, and only for 16SrV and 16SrI, 16SrIX, 16SrXII, and 16SrXV phytoplasma groups, respectively. RFLP analysis with the restriction enzymes *Taq*I and *Tru9*1 was performed to identify the phytoplasmas. The qPCR was carried out on phytoplasma ribosomal genes according to Angelini *et al*. (2007).

RESULTS AND DISCUSSION

Symptoms. In Moldova the first symptoms of the disease appear in early July. In white varieties a weak chlorosis appears on the leaves of one or several shoots of the plant. With the development of the disease, these leaves become golden yellow with metallic luster, crispy and curly downward. Along the main veins, chrome yellow spots appear, which subsequently become necrotic. By the end of the growing season, the leaves twist into a triangular shape. In varieties with red berries, redness of the leaf blades occurs. Redness often concerns only one sector, limited to two or three veins. Affected leaves, unlike healthy ones, survive the first light frosts, so in late autumn infected plants are visible from afar. Infected shoots are distinguishable by short internodes and stunted growth. On the surface of the symptomatic canes numerous pustules appear. In autumn, the affected canes show lack of lignification and with the onset of low temperatures, often die. Affected canes, as a rule, are not harvested due to the fact that the inflorescences dry up and fall off. The characteristic symptoms indicate that this disease could be associated with phytoplasma presence.
Visual survey. The inspected vineyards in the Republic of Moldova are highly affected by the disease (Table 1). Infection varies from 2% in Syrah to 69% in Chardonnay. In 4-years old vineyards, the percentage of symptomatic plants ranges from 29% in Sauvignon to 35% in Chardonnay, which poses a serious threat to the viticulture. Both international grapevine varieties and autochthonous varieties Feteasca Neagra, Feteasca Alba and Rara Neagra are affected by the disease.
Molecular analyses. In the summer 2017, 17 symptomatic plants of three varieties (one Traminer, eight Sauvignon, eight Feteasca neagra) were sampled for phytoplasma detection by molecular analyses. Nested PCR with universal primer pair 16r758f/M23Sr yielded 16 positive samples, all positive also to the R16(I)F1/R1 primer pair. RFLP analyses of the amplicons revealed that the samples were containing 16SrXII-A phytoplasmas,

'*Candidatus* Phytoplasma solani', the agent associated with grapevine "bois noir". The data were confirmed by qPCR.

Insect survey. In order to survey for the potential insect vectors of the disease, yellow sticky traps installed in the vineyards were used for insect sampling. The identification of captured insects revealed the presence in the vineyards of known and potential vectors of phytoplasmas, such as *Scaphoideus titanus, Hyalesthes obsoletus, Orientus ishidae*, and other leafhoppers, such *as Philaenus spumarius* and *Euscelidius variegatus*. *S. titanus* had been previously identified in Moldova in 2013 (Timus, 2015). The role of these insects, in particular of *H. obsoletus*, in the transmission of the BN phytoplasma under the Moldova conditions is being studied.

Table 1. Percentage of symptomatic plants observed in the 2017 survey in Moldovan vineyards.

Grapevine cultivation area	Variety	Year of planting	Total number of plants observed	Symptomatic plants (%)
Central zone	1. Chardonnay	2005	1,600	51,68
	2. Cabernet Sauvignon	2004	7,230	9,66
	3. Pinot noir	2004	4,301	11,54
Southern zone	1. Feteasca Neagra	2008	4,277	6,91
	2. Rara Neagra	2008	4,600	14,31
	3. Syrah	2008	3,382	1,99
	4. Malbec	2008	3,700	7,91
	5. Chardonnay	2013	1,180	35,4
	6. Sauvignon	2013	1,180	29,2
	7. Feteasca Alba	1982	400	22,7
South-eastern zone	1. Cabernet Sauvignon	2004	8,512	19,55
	2. Pinot noir	2004	6,920	16,21
	3. Merlot	2004	10,240	6,48
	4. Chardonnay	2004	3,800	68,71

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P11: Molecular characterization of "bois noir" phytoplasma strains in a single vineyard in central Serbia

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INTRODUCTION

Grapevine "bois noir" (BN) is a phytoplasma disease widely present in large numbers of viticultural regions of the world, and it can result in heavy reductions in yield and grape juice quality. BN is associated with the presence of '*Candidatus* Phytoplasma solani' (BN), transmitted from herbaceous plants to grapevine by polyphagous insect vectors. In the Euro-Mediterranean Region, phloem-feeding Hemiptera of the Cixiidae family are the vectors of "stolbur" phytoplasmas (Weintraub and Beanland, 2006) and in particular, the plant hopper *Hyalesthes obsoletus* Signoret is the main responsible for BN transmission to grapevine (Sforza *et al*., 1998). Adult of *H. obsoletus* can feed on various herbaceous plants, but only a few species are selected to complete their life cycle. The different ability to induce epidemic outbreak is often associated with molecularly differentiable phytoplasmas usually enclosed in the same taxon (Bertaccini *et al*., 2014). A study was therefore carried out to verify the presence of molecular variability of BN strains in a restricted environment such as a vineyard located in the central Serbia planted with different grapevine varieties.

MATERIALS AND METHODS

DNA was extracted from 100 mg of fresh leaf midribs and petioles collected from symptomatic grapevines, belonging to four cultivars (Chardonnay, Pinot Gris, Cabernet Sauvignon and Riesling), in a young vineyard located in Velika Plana, Serbia, using the DNeasy Plant Mini Kit (Qiagen). Phytoplasma detection was carried out by nested-PCR on 16S rRNA gene using P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by R16F2n/R2 (Gundersen and Lee, 1996) and/or U5/U3 (Lorenz *et al*., 1995) primer pairs. The amplicons were preliminary subjected to RFLP analyses with the restriction enzyme *Tru1*I to verify phytoplasma identity. Samples that have been tested positive for BN phytoplasma presence were analyzed on *tuf*, *stamp* and *secY* genes using primers specific for 16SrXII-A phytoplasmas, when available, and following reported procedures (Langer and Maixner, 2004; Fabre *et al*., 2011; Lee *et al*., 2010). As a positive controls BN and "flavescence dorée" (FD) strains originating from Serbia and PTV, from tomato from Italy (16SrXII-A group) (Bertaccini, 2014) were used, while sterile deionized distilled water was employed as a negative control.

Table 1. Results of multigene analyses on selected grapevine samples (nc, not classified).

Sample	Geographical origin	Grapevine cultivar	Year of planting	PCR/RFLP results			
				16S rRNA	Tuf	Stamp	SecY
F1	Velika Plana	Chardonnay	2007	16SrXII-A	-	B	A
F2	Velika Plana	Pinot Gris	2010	n.c.	-	-	-
F3	Velika Plana	Chardonnay	2010	16SrXII-A	B	A	A
F4	Velika Plana	Cabernet Sauvignon	2010	16SrXII-A	B	-	A
F5	Velika Plana	Cabernet Sauvignon	2008	16SrXII-A	-	A/B	-
F6	Velika Plana	Cabernet Sauvignon	2009	16SrXII-A	C	A	A
F7	Velika Plana	Riesling	unknown	16SrXII-A	B	-	A
F8	Velika Plana	Cabernet Sauvignon	2006	16SrXII-A	B	A	A
F9	Velika Plana	Chardonnay	2006	16SrXII-A	B	-	A
F10	Velika Plana	Chardonnay	2007	16SrXII-A	-	A/B	A
PTV (control strain from tomato Italy)				16SrXII-A	B	B	A

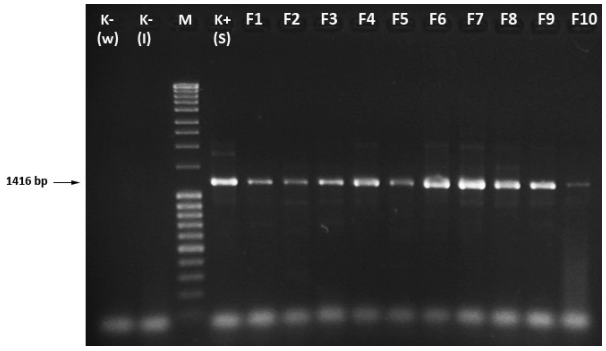

Fig. 1. Nested-PCR with primers P1/P7 (first step) and R16F2n/R16R2 (second step). Sample designations (Table 1) are indicated above the respective lanes. Lane K- (w), negative control (nuclease free water); Lane K- (I), negative control from the first PCR step used as a template for second PCR step. Lane M, molecular size marker GeneRuler DNA Ladder Mix (#SM0333; Thermo Scientific, Vilnius, Lithuania); K+, "stolbur" phytoplasma strain from grapevine in Serbia.

RESULTS AND DISCUSSION

The grapevine samples resulted positive in nested PCR with primers R16F2n/R2 (Fig. 1) and the RFLP analyses confirmed the presence of BN phytoplasmas (16SrXII-A, '*Candidatus* Phytoplasma solani'-related) in the symptomatic grapevine plants, belonging to the cultivars analyzed. In one of the samples (F2, from Pinot Gris) the BN presence was not detected by RFLP analyses of the amplified product and the identity of the phytoplasma is under study. The multigene analysis approach was carried out on selected samples (Table 1) and showed the presence of some variability in the BN strains identified (data not shown). Sample F2 resulted not amplifiable with any of the primers selected (all specific for 16SrXII and/or 16SrI phytoplasmas). Also, the 3 genes could not be amplified in all the remaining samples. The most frequently amplified was the *secY* gene, followed by *tuf* and *stamp* genes. RFLP analyses with informative enzymes indicated the presence of polymorphisms in all the amplicons but not in those of *secY* gene (Table 1).

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P12: First report of *Clematis vitalba* and *Ailanthus altissima* infected with 16SV-C subgroup phytoplasmas in Bulgaria

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INTRODUCTION

In Europe, two type of the grapevine yellows (GY) diseases are present: "bois noir" (BN), associated with the presence of '*Candidatus* Phytoplasma solani' (16SrXII-A subgroup) transmitted by *Hyalesthes obsoletus* Signoret; and "flavescence dorée" (FD), a quarantine disease in the European Univion, associated with the presence of FD phytoplasmas (16SrV-C and –D subbroups) (Martini *et al*., 1999) transmitted by *Scaphoideus titanus* Ball. In Bulgaria the presence of BN and its vector *H. obsoletus* has been reported (Avramov *et al*., 2008). FD have not been yet detected, but its vector *S. titanus* was recently found in the country (Avramov *et al*., 2011). Moreover, FD and its insect vector have been present at least since 2003 in the nearby Serbia (Duduk *et al*., 2004) with very serious economical losses to grapevine growers, whereas since 2009 the presence of *S. titanus* has been recorded In Romania (Chireceanu, 2014). The aim of this study was to verify the presence of FD phytoplasmas in symptomatic grapevines and plants and insects in vineyards environments in Bulgaria.

MATERIALS AND METHODS

In 2016, in routine surveys for identification of phytoplasma associated with GY, symptomatic grapevines together with *Clematis vitalba* and *Ailanthus altissima*, species known as host of FD-like phytoplasmas (Angelini *et al*., 2004, Filippin *et al*., 2011), were collected. Five grapevines were collected from vineyards in the regions of Targovishte and Shumen (Northern Bulgaria); other 14 grapevine samples, 11 *C. vitalba* and 11 *A. altissima* plants were collected in the experimental vineyards, near to a germplasm collection and mother plant fields of the Institute of Viticulture and Enology in Pleven (Northern Bulgaria). Grapevines were from both of local and international varieties.

In the same year, eight yellow sticky traps were located inside those vineyards to monitor the presence of potential and known GY insect vectors, from June 20th to September 1st and changed every 14 days. Moreover 28 specimens of *S. titanus* were captured alive in vineyards by sweepnet.

Total nucleic acids were extracted from the leaf veins and from the insects collected by sweepnet with a CTAB method (Angelini *et al*., 2001). Quantitative (q)PCR analysis on ribosomal genes was carried out in order to detect the presence of phytoplasmas of the 16SrXII and 16SrV groups (Angelini *et al*., 2007). The samples were amplified also by nested PCR using the universal primer pair P1/P7 (Deng and Hiruki, 1991) followed by 16r758f/M23Sr (Martini *et al*., 1999) primers. RFLP analysis for phytoplasma identification was performed with the restriction enzyme *Taq*I.

RESULTS AND DISCUSSION

The qPCR showed that nine out of 19 grapevines were positive to '*Ca*. P. solani' presence while seven out of 11 *C. vitalba* and one out of 11 *A. altissima* were infected with 16SrV group phytoplasmas. The nested PCR followed by RFLP confirmed the results on grapevine and revealed that *C. vitalba* and *A. altissima* harboured phytoplasmas belonging to 16SrV-C subgroup (Table 1).

Table 1. Results of the PCR/RFLP analysis of the plant samples.

Species	Number of samples	positive	negative	Phytoplasma	Positive samples (%)
Vitis vinifera	19	9	10	16SrXII-A	47.37
Clematis vitalba	11	7	4	16SrV-C	63.6
Ailanthus altissima	11	1	10	16SrV-C	9.1

Monitoring potential and known GY vectors through sticky traps revealed the presence of many leafhopper species such as *Empoasca vitis, Eupterix aurata, Psammotettix sp., Zygina sp.* and *Zyginidia sp.*, for a total of 936 insects; among those 513 were *S. titanus*, five *H. obsoletus* and 17 *Reptalus* spp. (Table 2). PCR results on 28 *S. titanus* specimens were negative.

Table 2. Insects collected by yellow sticky traps in 2017.

Leafhoppers	Yellow sticky traps								Total
	№ 1	№ 2	№ 3	№ 4	№ 5	№ 6	№ 7	№ 8	
Empoasca vitis	11	114	20	30	58	13	9	17	**272**
Eupterix aurata	7	0	4	23	8	5	6	1	**54**
Hyalesthes obsoletus	0	0	0	0	1	1	1	2	**5**
Psammotettix sp.	2	3	2	7	0	0	0	0	**14**
Reptalus spp.	1	4	0	2	9	1	0	0	**17**
Scaphoideus titanus	120	31	49	85	79	72	37	40	**513**
Zygina sp.	5	13	3	4	0	4	0	0	**29**
Zyginidia sp.	1	1	4	2	18	6	0	0	**32**
								Total:	**936**

Further molecular analysis are needed in order to better characterize the 16SrV phytoplasmas infecting *C. vitalba* and *A. altissima*. The observations made during the survey revealed that *C. vitalba* and *A. altissima* are widespread everywhere, and are very common on the hedges around and also into the vineyards. Considering the possibility of transferring FD phytoplasmas from *Clematis* and *Ailanthus* plants to the grapevines by occasional vectors and overall the high *S. titanus* populations present in the vineyards, the monitoring and strict control of weeds, insects and propagation material should continue to prevent the entrance and spread of FD in the vineyards.

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P13: Robotic tools towards a new sustainable and eco-friendly IPM to control grapevine "flavescence dorée"

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INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one the most economically important crops worldwide due to the high value of the grape and the importance of wine making sector. In Portugal, it plays an essential role on the national economy. Indeed, Portugal was classified as being the 5th largest European producer and the 10th having the largest global wine makers group (Instituto da Vinha e do Vinho - IVV, 2015).

Portugal has a great biodiversity of grapevine cultivars specific to each wine producing areas that have intrinsic, unexplored, differential susceptibilities to the "flavescence dorée" disease (FD) (Sousa *et al*., 2003; 2009; 2010; 2011). A great concern is that many of these cultivars are highly susceptible to the FD, compromising the existing biodiversity.

FD is a quarantine disease in Europe, included in the 2000/297/CE Directive and in A2 EPPO (European and Mediterranean Plant Protection Organization) list, it may lead to drastic yield loss, and even the death of the grapevine, if efficient control measures are not applied in a timely manner. Using healthy propagating materials, applying chemical treatment against to the insect vector, and eliminating infected plants are methods to control the spread of the disease, but there is no strong evidence that these methods are fully effective, since FD has been slowly spreading in EU. The dissemination of the disease is made through the leafhopper vector, *Scaphoideus titanus* Ball, and by infected propagation material.

Current management strategies to control the diseases associated with phytoplasmas like FD are mainly based on uprooting of symptomatic plants, using of insecticides to reduce the number of insect vectors, and applying hot water treatment to vegetative propagation material like rootstocks (very often asymptomatic host of phytoplasmas).

Diagnostics methods like PCR (Polymerase chain reaction) for early pathogen detection in plants and vectors have been developed. However, there is a need for further technological advances aiming to develop screening methods sensitive but applicable on large scale surfaces in very short time frames.

MATERIAL AND METHODS

PARRA project [Consortium - Leader: TEKEVER; Partners: INIAV (Instituto Nacional de Investigação Agrária), IPVC (Instituto Politécnico de Viana do Castelo), UTAD (Universidade de Trás-os-Montes e Alto Douro), AGRICIÊNCIA) has been exploring the concept of equipping UAVs (*Unmanned* Aerial Vehicle) commonly known as *drone,* with hyperspectral sensors in order to collect FD disease data samples and develop an early detection algorithm for FD symptoms ("spectral signature" of the disease). The proposed solution explores different technologies to collect data such as different types of sensors as EO (Electro-optical), IR (Infrared) and hyperspectral installed onboard the UAVs (Snow, 2017; Hou *et al*., 2016).

Different degrees of symptoms from two portuguese cultivares, Vinhão (red) and Loureiro (white), were compared in order to develop the FD algorithm and establish a pattern according to the environment light condition of data collected from the UAV.

RESULTS AND DISCUSSION

The collected data to identify the infected plants with FD are currently under analysis. These environmental friendly tools will contribute to the development of new sustainable and eco-friendly Integrated Pest Management strategies adapted to the new paradigm of Crop Protection that are very fast climate change, reduced number of pesticides in the European market, urgent need to protect the environment and an increasing circulation of plant

materials around the world that is linked to the introduction of new pests and diseases in Europe.

ACKNOWLEDGEMENTS
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P14: Comparative analysis of six diagnosis methods for *Grapevine fanleaf virus*

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is a small icosahedral plant virus with a bipartite genome composed of positive-stranded RNA molecules, named RNA1 and RNA2 (Schmitt-Keichinger et al., 2017). GFLV is specifically transmitted from vine to vine by the ectoparasitic nematode *Xiphinema index*. This virus is the principal causal agent of fanleaf degeneration, one of the most severe diseases of grapevine worldwide. GFLV reduces yield by up to 80% and infected vines often exhibit a reduced productive lifespan. *Vitis* spp. are the major natural hosts of GFLV. Two distinct types of symptoms are generally associated with fanleaf degeneration: malformations and yellow mosaic (Vuittenez, 1956). Symptoms in a diseased vineyard vary with the GFLV isolate, cultivar and rootstock variety, soil composition and environmental factors. Due to the soil-borne nature of the disease, the presence of the virus is a significant problem in established vineyards in France.

Control strategies of GFLV are primarily based on cultural practices and soil disinfection to reduce the population of the nematode vectors. Since the 2000's, most chemicals used as nematicides have been prohibited in Europe, making the profitable cultivation of grapevine challenging in fanleaf infected vineyards. One of our research objectives is to control fanleaf degeneration by cross protection with mild GFLV isolates (Ziebell and Carr, 2010). The identification of mild isolates in vineyards requires extensive surveys. In 2016 we started out a survey in highly diseased field plots in Champagne area to select some infected vines with mild symptoms and surrounded by vines with severe symptoms (Fig. 1). Surprisingly a substantial proportion of grapevines exhibiting faint symptoms was tested negative in DAS-ELISA. In order to know if this result could be explained by a potential low viral concentration due to virus silencing or to a so far undescribed serological diversity of the virus, we looked for the best diagnostic assay by comparing six GFLV diagnosis methodologies, i.e. DAS-ELISA, RT-qPCR, IC-RT-PCR, RNAseq (long and small RNAs), Northern blot for the detection of viral small-interfering RNAs (vsiRNAs).

Fig. 1. Overall view of one of the surveyed GFLV-infected vineyards in the Champagne region of France. Grapevines (cv. Chardonnay) were planted in 1985 in a highly GFLV-infected vineyard site. The arrow points to a vine with mild symptoms surrounded by vines exhibiting strong yellowing symptoms

MATERIALS AND METHODS

Plant material. Samples of young apical leaves and canes were collected from selected vines (30 years old in average) in early spring and winter, respectively. Tissues were ground into a fine powder with a mortar and pestle in liquid nitrogen. Aliquots of ground tissue were stored at -80°C until further testing.

DAS-ELISA

Frozen leaf and cane tissues were ground in presence of buffer for DAS-ELISA using commercial antibodies as per the manufacturer's recommendations (Bioreba AG, Reinach, Switzerland). The detection limit of DAS-ELISA was determined with serial dilutions of purified virus preparations.

RNAseq and sRNAseq

Total RNA, extracted with an adapted protocol of the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), was used for deep sequencing analyses on long RNAs (RNAseq) or small RNAs (sRNAseq) separately. RNAseq analysis was performed as previously described (Hily et al., 2017). For sRNAseq, we focused mainly on the 21 to 24 nt size classes, from which mapping and *de novo* assembly were performed. Analyses were conducted using CLC Genomics Workbench 8.5.1 software (Qiagen, Aarhus, Denmark), as well as the online version of VirusDetect (Zheng et al., 2017).

RT-qPCR and IC-RT-PCR

Degenerated primers were designed within the most conserved regions after alignment of 32 complete GFLV genomes. RT-qPCR was carried out in triplicates. A melting curve analysis was performed to ascertain that a single product was produced in each case. Absolute quantification was determined from standard curves using plasmid serial dilutions. For IC-RT-PCR, GFLV particles were immunocaptured on microtiter plates and denatured before RT-PCR (Vigne et al., 2004).

Northern blot for the detection of vsiRNAs

Total RNA was extracted from frozen tissue using Concert Plant Reagent (Invitrogen, Carlsbad, CA, USA). For siRNAs detection, low molecular weight RNA was analyzed using 30 □g of total RNA separated by denaturing polyacrylamide gel electrophoresis and transferred onto Hybond N+ membranes (GE Healthcare, Piscataway, NJ, USA). Following chemically crosslinking, low molecular weight RNA was hybridized with a mix of five radiolabeled DNA probes amplified from five genetically distinct GFLV isolates.

RESULTS AND DISCUSSION

Our preliminary work focused on DAS-ELISA and Northern blot for vsiRNAs using six grapevine samples (Fig. 2). For sample H12, the only DAS-ELISA positive sample, vsiRNAs derived from GFLV RNA1 and RNA2 were detected, in both leaves and canes. These results indicate consistency between DAS-ELISA and Northern blot for vsiRNAs. Results were also coherent between DAS-ELISA and Northern blot for samples L11 and L13 for which no GFLV was detected by both techniques.

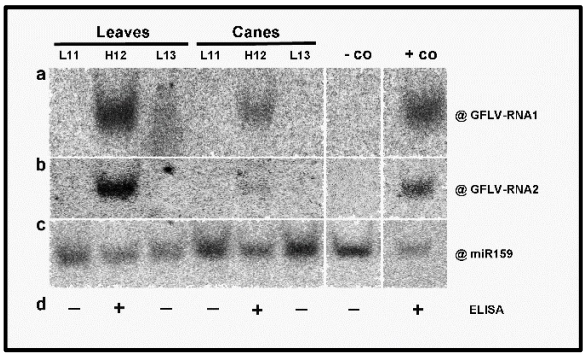

Fig. 2. Detection by Northern blot of GFLV siRNAs from RNA1 and RNA2. Total RNA was separated on a 17% polyacrylamide gel and hybridized with ^{32}P-labelled DNA probes corresponding to (a) GFLV-RNA1, (b) GFLV-RNA2 and (c) miR159 used as loading control. Results correspond to samples from leaves or canes of three grapevines (L11, H12, L13), a negative control from healthy leaves (-co) and a positive control from GFLV-F13 infected *Chenopodium quinoa* leaves (+co). The bottom line (d) indicates DAS-ELISA results.

Twenty samples will be comparatively analyzed according to the six afore mentioned diagnosis methods and the viral accumulation will also be quantified. This work will identify the most sensitive and reliable diagnosis assay to quantitatively follow the spatio-temporal distribution of GFLV in grapevines. This method will be applied as a tool to screen for candidate mild strains that will be used in cross-protection experiments.

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This work was supported by Moët & Chandon, le Comité Champagne (CIVC), le Bureau Interprofessionnel des Vins de Bourgogne (BIVB), and le Conseil Interprofessionnel des Vins d'Alsace (CIVA).

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P15: Genetic diversity of *Grapevine leafroll-associated virus*-2 isolates in Turkey

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INTRODUCTION

Grapevine leafroll-associated virus-2 (GLRaV-2) was described since 1984 and was associated with the grapevine leafroll disease, the most widespread disease of *Vitis vinifera* worldwide (Gugerli *et al*., 1984). GLRaV-2 is a member of the genus *Closterovirus* in the family *Closteroviridae*. GLRaV-2 is known to be transmitted by grafting with infected material. However, some isolates of GLRaV-2 were mechanically transmitted to herbaceous hosts like *Nicotiana benthamiana (*Goszczynski *et al*., 1996*)*.. To date, six complete genomic sequences of GLRaV-2 have been deposited at GenBank. Phylogenetic studies have suggested that the virus occurs as divergent molecular variants, segregating into five (Bertazzon *et al*., 2010) or six distinct lineages (Jarugula *et al*., 2010). The viral genome of six isolates was completely or nearly completely sequenced: PN, Sem and OR1 from the USA (Abou Ghanem *et al*. 1998; Zhu *et al*. 1998; Liu *et al*. 2009), RG from California (Rowhani *et al*., 2000), 93/955 from South Africa (Meng *et al*., 2005) and BD from Italy (Bertazzon *et al*., 2006). Partial sequences were obtained from other GLRaV-2 isolates, but only three of them were new atypical variants (Beuve *et al*., 2007). Routine detection of GLRaV-2 in Turkish vineyards has mainly performed by serological assays; however, information on variability and pathological properties of its strains has not been available yet. Therefore, we started a study to document genetic variability of GLRaV-2 in autochthonous cultivars from two economically important grape regions of Turkey.

MATERIALS AND METHODS

The field study and sample collection were conducted in late autumn 2014 and 2016 on table grape collected in eastern Mediterranean (EM) and southeast Anatolia (SEA) regions of Turkey. A total of 206 samples were collected from symptomatic and asymptomatic vines from 33 autochthonous cultivars at seven locations. Phloem scrapings was used for total nucleic acid isolation with silica-capture method (Foissac *et al.,* 2005) and two-step reverse transcription polymerase chain reaction (RT-PCR) was performed with primer pair which encompass 543 bp located in the coat protein gene (Gambino and Gribaudo, 2006). Direct sequencing of the amplified products was performed with the same primers used for PCR (Medsantek, Turkey). To investigate the different variants of GLRaV-2 population, multiple alignments of nucleotide sequences were performed using CLUSTAL W (Thompson *et al*., 1994), respectively, with default settings from MEGA6 software (Tamura *et al*., 2013) and comparison at the nucleotide level for CP regions of Turkish isolates and representative sequences of the different phylogenetic groups described up to now was conducted.

RESULTS AND DISCUSSION

A total of four samples tested positive for GLRaV-2. The low incidence of GLRaV-2 might be due to the self-rooted condition of autochthonous cultivars that has prevented dispersion of the virus. Three novel sequences obtained from EM in this study were in good quality to deposit in the GenBank database with the accession numbers MF281987-281989. Comparative analysis of the Turkish isolates and other GLRaV-2 isolates in GenBank showed close relationship. The identity of nucleotides of the sequenced amplicon among the isolates ranged between 92% and 99%. Two clustering patterns could clearly be observed in the phylogenetic tree (Figure 1). Results revelaed that the Turkish isolates were closely related to the reference strains PN, Sem, OR1 and 93/955. The strain 93/955 and varial variant from isolate '185' were grouped in a separate cluster of in the group I having high nucleotide identity (99%). The PN and H4 clades in this study proved to be sister groups as indicated by Bertazzon et al. (2010). The refence strains BD, RG, PG11 created group II with less identity from all others. This study represents the preliminary data on the genetic diversity conducted in two major grape-growing regions of Turkey including autochthonous germplasm collection with first sequencing of Turkish isolates. The isolates provided from distant vineyards showed an important similarity and cluster in the same lineage. However, a large survey on autochthonous and commercial foreign cultivars is needed to have better understandings for genetic variability and pathological propertes of GLRaV-2 isolates.

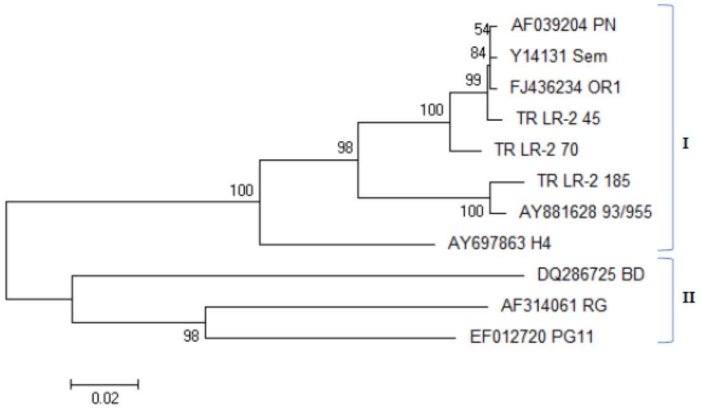

Figure 1. Phylogenetic tree comparing genetic variability of nucleotide sequences in the amplified fragment among of Turkish GLRaV-2 isolates and the type strains of the virus.

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P16: *In silico* detection of a defective genomic RNA of *Grapevine leafroll-associated virus 4* strain 5 in High-Throughput Sequencing data

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INTRODUCTION

Grapevine leafroll disease (GLRD) is one of the most economically important viral diseases affecting grapevines (Maliogka et al., 2015). Most viruses associated with this disease belong to the family *Closteroviridae*, and are designated *Grapevine leafroll-associated virus*es (GLRaVs). The family *Closteroviridae* consists of viruses with a positive-sense ssRNA genome of ~15 kb and rod-shaped particles. GLRaV-1, -3 and -4 belong to the genus *Ampelovirus*, while GLRaV-2 and -7 belong to the genera *Closterovirus* and *Velarivirus*, respectively (Maliogka et al., 2015). These viruses exist within its host as a diverse cloud of related but distinct genomes known as a quasispecies (Lauring and Andino, 2010). The complexity of a quasispecies population is augmented by recombination events, which are also associated with the emergence of novel genotypes and defective genomic RNA molecules (dRNAs) (Bar-Joseph and Mawassi, 2013). In this work, we report the detection of a dRNA from a GLRaV-4 strain 5 in *Vitis vinifera* cv. Trajadura, containing multiple virus infections. This GLRaV-4 strain 5 dRNA is 9,295 bp long and is composed by the polyprotein, RNA-dependent RNA polymerase (RdRp) and a partial truncated divergent coat protein (CPd) ORFs, similar to a class 3 *Citrus tristeza virus* (CTV) dRNA. These dRNAs are composed by a large 5' terminus portion of the genomic RNA, containing the entire replicase complex, and usually a truncated 3' terminus ORF (Bar-Joseph and Mawassi, 2013).

MATERIALS AND METHODS

Seventeen grapevine samples collected from Brazil have been previously subjected to High-throughput sequencing (HTS) at Macrogen (Seoul, South Korea) or Eurofins Genomics (Huntsville, USA). The cDNA libraries were constructed from double-stranded (dsRNA) extracts, and sequencing was performed on the Illumina HiSeq 2000 platform (2 x 100 bp). The GLRaVs infecting these plants have already been described (Fajardo et al., 2017). For each GLRaV positive sample, the sequencing reads were aligned to the complete genome of the corresponding GLRaV with the program BWA (Li and Durbin, 2010). Variant calling was performed with LoFreq (Wilm et al., 2012). To screen for the presence of GLRaV derived dRNAs on these samples, we used the program ViReMa (Routh and Jonhson, 2014). This analysis revealed the presence of a dRNA from a GLRaV-4 strain 5 on a multiple virus-infected *V. vinifera* cv. Trajadura (S18-TRAJ).

RESULTS AND DISCUSSION

Due to vegetative propagation, grapevines often present mixed infections and complex viral pathosystems. By using HTS, we identified a dRNA from a GLRaV-4 strain 5 in *V. vinifera* cv. Trajadura coinfected with GLRaV-3, *Grapevine Red Globe virus* (GRGV), *Grapevine Syrah virus 1* (GSyV-1), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine virus A* (GVA), *Grapevine fleck virus* (GFkV) and *Grapevine rupestris vein feathering virus* (GRVFV) (Fajardo et al., 2017). Recombinant dRNAs of GLRaVs are thought to be generated by template switching mechanisms, and are dependent on the parental sequence for essential viral functions, such as replication, encapsidation or systemic movement (Bar-Joseph and Mawassi, 2013). While retaining the entire replicase complex, the p5, HSP70h, HSP90h, coat protein (CP) and part of the CPd ORFs are absent in the GLRaV-4 strain 5 S18-TRAJ dRNA. Both parental full length GLRaV-4 strain 5 and GLRaV-3 genomes, as well as the other viruses infecting this host, may be providing the function of these proteins for efficient infection of GLRaV-4 strain 5 dRNA. The junction site of the GLRaV-4 strain 5 dRNA is located at nucleotides 8,785 and 13,313 of the genomic RNA. Similar dRNAs, composed by the entire replicase complex and a truncated 3' terminus ORF, have been described for the closterovirus CTV. These dRNAs can be mechanically transmitted to citrus plants and inoculated into *Nicotiana benthamiana* protoplasts, and are possibly self-replicating (Che et al., 2002). A high number of single nucleotide variants (SNVs) was annotated in our analysis (Fig. 1), suggesting a

diverse intrahost population of GLRaV-4 strain 5. However, it is not clear whether these variants are present in the full length or defective genome of GLRaV-4 strain 5. To our knowledge, this is the first report of a dRNA of GLRaV-4 strain 5.

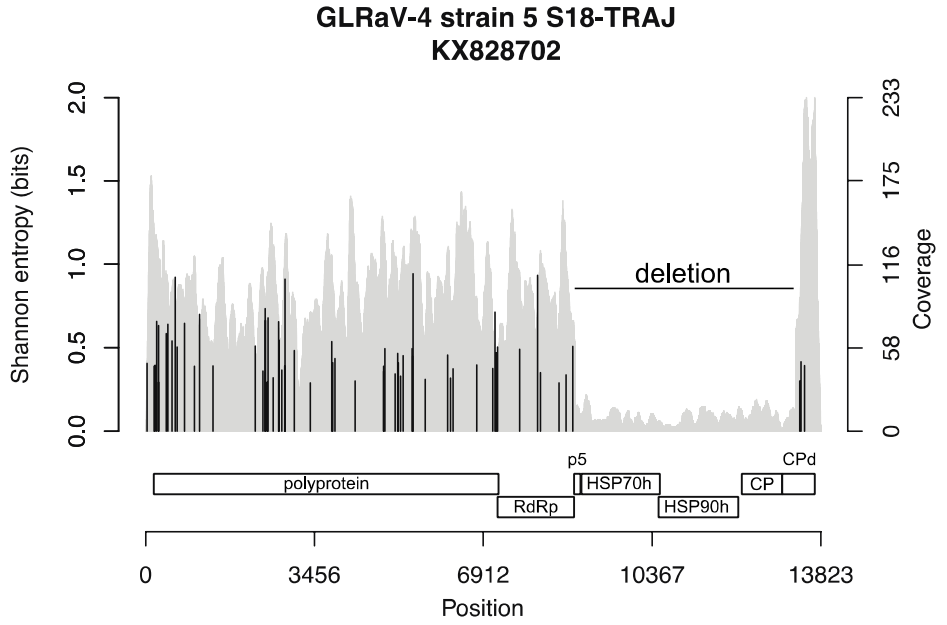

Fig. 1. Shannon entropy (left) of each single nucleotide variant (SNVs) annotated and coverage depth (right) of the full length genome of GLRaV-4 strain 5 (KX828702) on the S18-TRAJ sample, along with a schematic representation of the genome organization of this virus (below), showing the polyprotein, RNA-dependent RNA polymerase (RdRp), p5, HSP70h, HSP90h, coat protein (CP) and diverged coat protein (CPd) ORFs.

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P17: Deep sequencing analysis of *Grapevine pinot gris virus* (GPGV) isolates from Lazio and Sardinia

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INTRODUCTION

Grapevine pinot gris virus (GPGV) was first described on 'Pinot gris' in Northern Italy associated with symptoms of stunting, chlorotic mottling and leaf deformations (GLMD: Grapevine Leaf Mottling and Deformations) (Giampetruzzi *et al.,* 2012). Successively, several reports revealed a widespread occurrence of GPGV and the associated symptoms in many European and non European countries and on different varieties (Gualandri *et al*., 2017). These studies also reported the presence of GPGV in symptomless plants, making uncertain the relationship between GPGV and the symptoms (Bianchi *et al*., 2015; Bertazzon *et al*., 2016).
A recent investigation in central and southern Italy (Lazio and Sardinia regions) showed the presence of GPGV in varieties never described to host the virus, among them 'Vermentino' and 'Cabernet Sauvignon' (Gentili *et al*., 2017). In order to study the epidemiology of the disease and the correlation between symptoms and virus isolates, tissues from three vines of cv 'Vermentino', showing or not GLMD symptoms and a vine of cv 'Cabernet Sauvignon' with not typical GLMD symptoms were analyzed by Next Generation Sequencing (NGS).

MATERIALS AND METHODS

Two cv 'Vermentino' symptomatic vines from Sardinia and one asymptomatic vine from Lazio plus one 'Cabernet Sauvignon' with a mild stunting not typically associated with GPGV were collected and used for the analyses. Total RNAs extracted from leaf tissues according to Giampetruzzi *et al*. (2016) were used to synthesize mRNAs libraries with the TruSeq RNA Sample Prep kit (Illumina). Libraries were sequenced in paired end 2x150bp by Genewiz Europe (www.genewiz.com). Evaluation of quality, trimming and screening by subtraction of grape genome sequences (CRIBI, http://genomes.cribi.unipd.it/grape/) were carried out to obtain mRNAs that were successively assembled into larger contigs using the Velvet software with two different k-mer (61 and 71). The virome of each sample was analyzed for homology by BLAST search of contigs against the GenBank Virus Reference Sequence database (Ref-Seq; NCBI).
GPGV genomes from the four vines were finally assembled using Geneious 10.2 software by mapping reads to a GPGV full-length sequence (NCBI accession number KF134123.1). GPGV consensus sequences were phylogenetically analyzed with Mega 7.0 software using 1000 Bootstrap and the Maximum Likelihood method of analysis.

RESULTS AND DISCUSSION

BLAST analysis of the *de novo* assembled contigs showed that all vines were infected by GPGV and *Grapevine rupestris stem pitting associated virus* (GRSPaV). *Grapevine fanleaf virus* (GFLV) was present in the two symptomatic 'Vermentino' and in the 'Cabernet sauvignon' vines, whereas the symptomless 'Vermentino' vine was free from all other viruses (Table 1).
Reference mapping analysis allowed to assemble GPGV genomes from the four vines with very high coverage depths and reads covering over the 99% of the reference genome (Table 1).
Comparison of the assembled genomes showed that the GPGV sequence obtained from the 'Cabernet sauvignon' is significantly different at nucleotide and amino-acid level in all the three GPGV genes (RNA dependant RNA polymerase – RdRp; Movement protein – MP and Capsid protein – CP) from the three sequences from 'Vermentino'(data not shown). These findings were confirmed by a phylogenetic analysis including all available GPGV complete genomes (retrieved by National Center of Biotechnology Information database). The Phylogenetic tree, obtained using Maximum Likelihood method, showed that the three 'Vermentino' genomes clustered in the larger group containing all other worldwide-reported GPGV isolates whereas the 'Cabernet sauvignon' has a deviating topology which is more close to the related *Grapevine berry inner necrosis virus* (Figure 1).
The present study does not allow to draw a conclusion about GPGV association with GLMD symptoms since the vines are infected by multiple viruses. Similarly, no association of the four GPGV isolates with their geographic

origin was observed, quite the two isolates from Lazio ('Vermentino' symptomless and 'Cabernet sauvignon') cultivated in the same vineyard, were significantly different from each other, which induce to suppose that they originate from independent introductions and identify the infected propagation material as major responsible for GPGV spread. A major importance assumes the unusual topology of the isolate from Cabernet sauvignon since it could represent the missing link between GPGV and GINV.

Sample	Raw reads	Unmapped Vitis Vinifera	Symptoms	Virome	GPGV (KF134123.1) reference sequence			
					Mapped reads	Bases with coverage	Average coverage	Max coverage
Vermentino 1-2 Sardinia	11,541,603	8,509,328	Strong GLMD Symptoms	GVA, GVE, GFLV, GRSPaV , GLRaV3	3,443	99.93%	35X	85X
Vermentino 1-3 Sardinia	33,592,204	6,022,975	Strong GLMD Symptoms	GRSPaV, GFLV	93,536	99.83%	959X	1,964X
Cabernet sauvignon Lazio	41,226,706	8,479,497	No tipical GPGV Symptoms	GVA,GFLV, GRSPaV	13,909	99.82%	142X	358X
Vermentino Lazio	26,938,101	7,165,198	None	GRSPaV	5,931	99.94%	61X	169X

Table 1. Summary of symptoms and viromes of the four analyzed vines. Details of GPGV percentage of genome coverage and coverage depth are given.

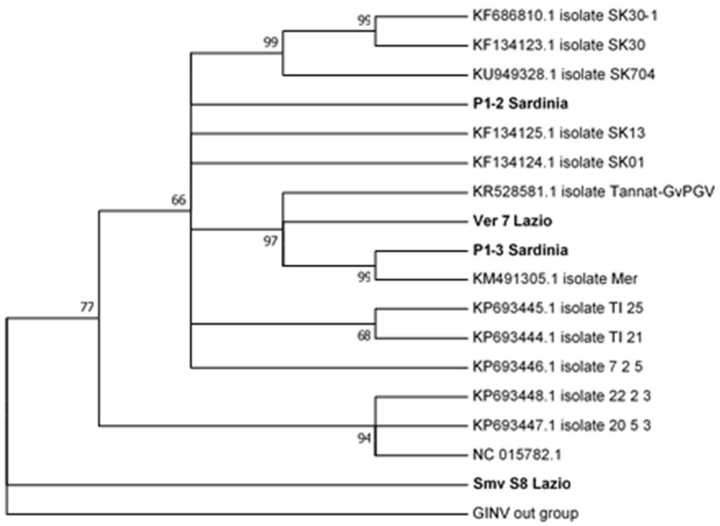

Figure 1 Molecular Phylogenetic analysis by Maximum Likelihood method of complete genome sequences of GPGV of isolates identified in this study (highlighted in bold) and available in NCBI genBank.

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P18: Group II variants of *Grapevine virus A* are associated with Shiraz Disease in Australia

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INTRODUCTION

Shiraz Disease (SD) is a highly destructive disease syndrome in Australia affecting Shiraz, Merlot, Malbec and a number of other red varieties including a Catalonian variety called Sumoll. It causes a yearly damage of around AU$70,000 ht^{-1} (Habili et al., 2016). Sensitive varieties show stunted growth in spring and red leaves with a leathery texture, downward rolling and unlignified cracked wood in the autumn (Habili and Wu, 2015). All rootstocks and white varieties as well as some red varieties, e.g. Cabernet Sauvignon, Grenache, Sangiovese and Nero d' Avola, are tolerant to SD. While *Grapevine virus A* (GVA) and GRSPaV have always been detected in SD affected vines, the co-infection of GVA with GLRaV-3 and other Ampeloviruses has also been reported (Goszczynski and Habili, 2012).

Three molecular groups of GVA (groups I-III) have been proposed for the South African GVA variants based on a variable short sequence of 293 nt. at the 5'-end of the replicase gene (Goszczynski and Habili, 2012). Only members of group II are associated with SD in South Africa as well as in Australia (Goszczynski et al., 2008; Goszczynski and Habili, 2012). Here, we report that the GVA isolates detected by Next Generation Sequencing (NGS) in Crimson seedless table grape and Malbec wine grape are phylogenetically assigned to group II when a segment of the CP gene was used. Our study confirmed the previous work which allocated Australian GVA variants in group II.

MATERIALS AND METHODS

Total nucleic acids from dormant canes of Crimson Seedless (CSL) clone 314 table grape sampled in late autumn in Western Australia and from Malbec clone MAT 3 grafted onto Richter 110 in South Australia were extracted and subjected to NGS. The screening for GVA was done by RT-PCR as described previously (Habili et al., 2003). A pair of primers specific for Groups I+II of GVA was used: GVAH587SPI-II: 5'-GACAAATGGCACACTACG and the reverse primer GVAC995SPI-II: 5'-AAGCCTGACCTAGTCATCTTGG. NGS was carried out using total nucleic acid extracts from bark tissues as described by Al Rwahnih et al., 2015.

RESULTS AND DISCUSSION

The NGS analysis of the GVA sequence in CSL and Malbec showed that both belong to Group II variants of GVA which are associated with Shiraz Disease. We previously reported an unclear group status for the GVA in CSL based on the nt sequence of the 5' region of the polymerase, a variable region which has been used for grouping of GVA variants (Goszczynski and Habili, 2012). Here, we report that GVA in symptomless and SD tolerant CSL belongs to Group II variants, and is phylogenetically related to the GVA in SD sensitive Malbec. In Australia, to date, 10 isolates of GVA from Shiraz, Merlot, Malbec and CSL have been partially sequenced and interestingly all grouped with variant II (Fig. 1). The sequencing of the RT-PCR products using group I+II specific primers can confirm these findings.

Wine grape growers in Australia perform top-working, a practice which involves grafting popular varieties onto existing unwanted varieties. If a sensitive variety is used as the scion it will develop SD in two years and it should be removed. It is worth noting that Group III of GVA variants also exists in Australia, but it is always mixed with group II, which appears to be the dominant group (Goszczynski and Habili, 2012). Since GVA is weak to act as a suppressor of gene silencing, in the case of Shiraz Disease, it has usually been detected with an Ampelovirus which may provide the required suppressor (Goszczynski and Habili, 2012). In South Africa, of all the leafroll viruses, only GLRaV-3 is present with GVA in SD affected vines, while in Australia GLRaV-1 and GLRaV-4/9 have also been detected.

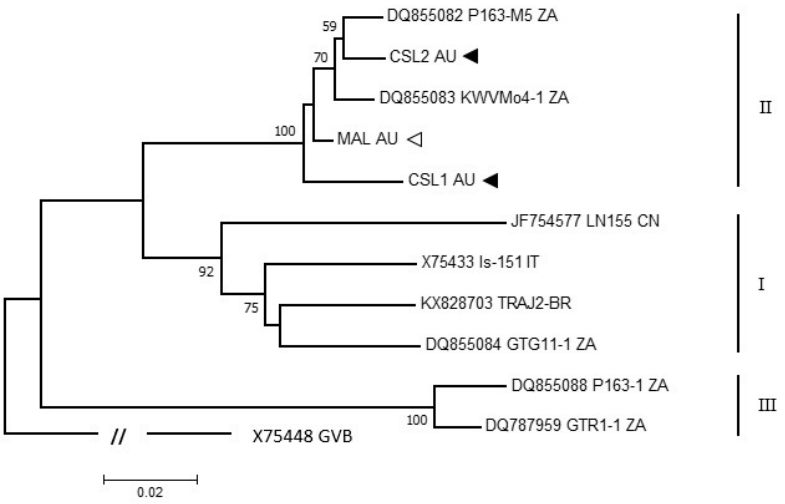

Fig. 1. Phylogenetic tree of GVA showing selected members of the three variant groups of the virus. Ten of the Australian GVA isolates associated with Shiraz Disease which have been studied so far are in Group II. These are represented by two isolates from Crimson Seedless (black arrow-head) and Malbec (clear arrow-head), respectively. These have been sequenced by NGS. A 328 nt segment of the CP from position 6489 to 6816 (Accession # DQ855083) was used to construct the tree. GBV was chosen as an out group. Bootstrap values (1000 replicates) are depicted at the branch nodes

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P19: Occurence of *Grapevine virus D* (GVD) in Turkish vineyards

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INTRODUCTION

Rugose wood (RW) is a graft-transmissible disease affecting grapevines (*Viti*s spp.) worldwide (Graniti and Martelli, 1966) and causing severe reduction of growth and yield of affected grapevines (Goheen, 1988; Du Preez *et al.,* 2011). At least six different viruses belonging to the genera *Vitivirus* and *Foveavirus* in the family *Betaflexiviridae* are associated with the disease which is distributed worldwide (Martelli, 2015). Among these viruses, rupestris stem pitting-associated virus (RSPaV), *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB), associated with the etiology of the RW disease, were reported in Turkish vineyards (Martelli, 2014; Buzkan *et al*., 2015), whereas no information is available on the occurrence of *Grapevine virus D* (GVD). Therefore, the presence of GVD was investigated in autochthonous grapevine cultivars from two viticultural areas in Turkey, i.e. Eastern Mediterranean and Southeast Anatolia.

MATERIALS AND METHODS

A total of 32 autochthonous varieties was investigated in 19 vineyards (Table 1). No clear symptoms attributable to rugose wood disease were observed on the trunks and this not common situation could be mainly explained with prevalence of self rooted vineyards/plants visited. One-year old cuttings were collected for GVD detection. Phloem scrapings was used for total nucleic acid isolation with silica-capture method (Foissac *et al.,* 2005) and two-step reverse transcription polymerase chain reaction (RT-PCR) was performed using primers CP7V/CP471C (Abou-Ghanem *et al*., 1997). PCR amplicons were custom-sequenced directly with both primers by Medsantek (Turkey). Alignments of the obtained sequences with additional homologous sequences retrieved from GenBank after using the Blastn program (Altschul *et al*., 1997). Phylogenetic tree was made by neighbor-joining (NJ) method (Saitou and Nei, 1987) using MEGA v.06 software (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Total RNA was extracted from 142 samples and tested for the presence of GVD by RT-PCR. A 474 bp-product corresponding to a fragment of the coat protein gene was amplified from 13 samples, accounting for a prevalence of 9%. The cultivars with GVD infection were cvs. Hönüsü, Horoz karası, Rumi, Antep karası, Besni. BLASTn analysis could be carried out with the nucleotide sequences of two isolates from cv. Besni. The Turkish isolate "TR167" showed 90% nucleotide sequence identity with a GVD isolate from Italy passaged onto a herbaceous host (*Nicotiana occidentalis*) (GenBank accession No. Y07764) and 98% with GVD isolates from Brazil (GenBank accession No. JQ031715, JQ031716). The isolate "TR202" had nucleotide identity with a GVA isolate from USA (Fig. 1). This might be due to high sequence homology in coat proteins of both viruses. GVD was only detected in grapevines from Southeast Anatolia, where it is a very common practice to establish vineyards with propagation material exchanged among growers without any pathogen testing. To our knowledge, this is the first report on the occurrence of GVD in Turkish grapevines.

Further study is necessary to carry out with more isolates and bigger genome portion for a better understanding of genetic diversity of the isolates. New primers are needed for an efficient detection of GVD in infected plant tissue.

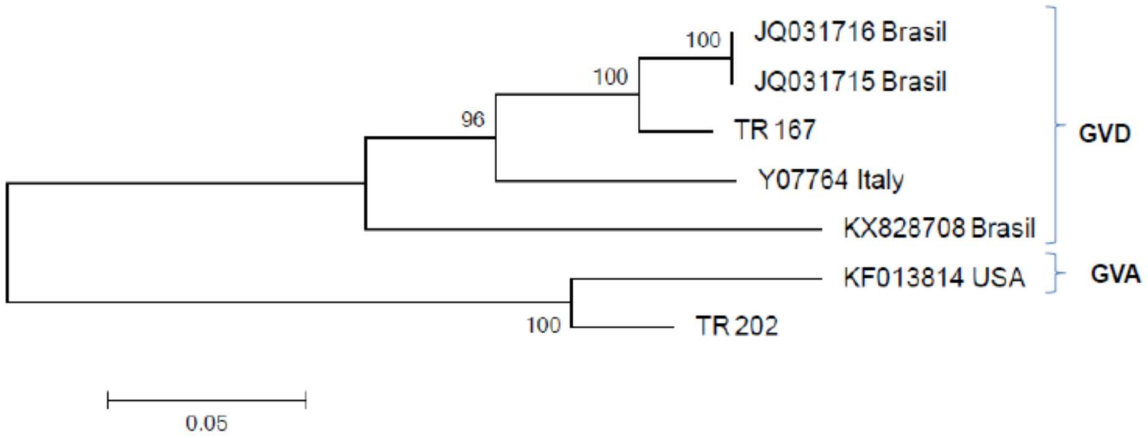

Fig.1. Phylogenetic comparison of the Turkish GVD isolates with other homologous sequences from GenBank.

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P20: Genomic characterization of Grapevine virus J, a novel virus identified in grapevine

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INTRODUCTION

The grapevine (*Vitis vinifera*) is considered one of the oldest crops in the history of humanity (Terral et al. 2010), and as a result of this early domestication brought a long exposure to different pests and pathogens. These pathogens include the vitiviruses (genus *Vitivirus*, subfamily *Trivirinae*, family *Betaflexiviridae*), whose type species is *Grapevine virus A* (GVA) which is associated with the etiology of rugose wood disease in grapevine (Minafra et al. 2017). Species in the genus *Vitivirus* have a positive-sense RNA genome with five open reading frames (ORFs), including a distinctive 20K ORF between the ORF1 (polymerase) and the ORF3 (movement protein) (Adams et al. 2004; Martelli et al. 1997). To date nine viruses are formally classified as vitiviruses (https://talk.ictvonline.org/), five of which are reported in grapevine: GVA, Grapevine virus B, *Grapevine virus D* (GVD), *Grapevine virus E* and *Grapevine virus F* (GVF) Minafra et al. 2017). Three recently discovered viruses, *Grapevine virus H* (GenBank: MF521889; Candresse et al. 2017), *Grapevine virus G* (GenBank: MF405923; Blouin et al. 2017) and Grapevine virus I (GenBank: MF927925; A. G. Blouin, personal communication), are proposed as members of the *Vitivirus* genus. Additionaly, a new variant of GVD was incorrectly named *Grapevine virus K* (GVK; GenBank: MF072319) (Blouin et al. 2017). In this abstract we are reporting a new vitivirus that we detected during the characterization of a new selection of white grape, *Vitis vinifera* cv. 'Kizil Sapak' from Turkmenistan, and tentatively named *Grapevine virus J* (GVJ).

MATERIALS AND METHODS

As part of the characterization process of new accessions introduced to the Foundation Plant Services (FPS, UC-Davis), the above-mentioned vine was analyzed by high-throughput sequencing (HTS). Briefly, total nucleic acid extracts from leaf petioles were used as a template for DNA library construction (Al Rwahnih et al. 2016). Later, the library was sequenced using the Illumina NextSeq 500 platform at the FPS. Contiguous consensus sequences (contigs) were generated from the Illumina reads and compared against the GenBank database of viruses via tBLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The previous analysis revealed nine contigs that ranged in size between 4,743 to 7,375 nucleotides (nt), and showed a distant relationship (average identity: 52%) with several vitiviruses. Consequently, the longest contig was extended to obtain the full genome (7,390 nt) of the putative virus; later, the complete genome was analyzed using the ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Once the coding regions were identified, they were investigated independently using the SmartBLAST (https://blast.ncbi.nlm.nih.gov/smartblast/smartBlast.cgi).

RESULTS AND DISCUSSION

A *Vitivirus*-like sequence was identified from the 'Kizil Sapak' grapevine by HTS, and later the putative virus was named GVJ (GenBank accession number MG637048). The genomic arrangement of GVJ, consisting of five ORFs, resembles other known vitiviruses. Thus, ORF1 encoded a polypeptide of 1,702 amino acids (aa) and was similar (52% identity and 99% query coverage) to the polymerase gene of GVA. A 17.7 kDa protein of unknown function was encoded by the ORF2 (164 aa). ORF3 (273 aa) showed 52% identity (95% query coverage) with the movement protein of GVA. A predicted coat protein (CP) was identified from the ORF4 (197 aa), based on 86% identity (81% query coverage) with the CP of GVD. Finally, the 103 aa integrating the ORF5 were associated with a putative RNA binding protein present in different vitiviruses.

Considering the genomic organization and sequence homology, we propose that GVJ should be placed within the genus *Vitivirus*, subfamily *Trivirinae*, family *Betaflexiviridae*; which was later confirmed by phylogenetics. GVJ was within the genus *Vitivirus* (Fig. 1) with GVD and GVK as the sister taxa in the two generated trees (replicase and CP genes). Even more, based on the current species demarcation criteria inside the genus *Vitivirus* (King et al. 2011), GVJ should be considered a new species, because of the low aa identity (less than 80%) with polymerase genes present in other vitiviruses. We are currently developing a detection test for GVJ, with the intention of

conducting a survey in different grape growing regions in California and the USDA National Clonal Germplasm Repository (NCGR) in Winters, CA.

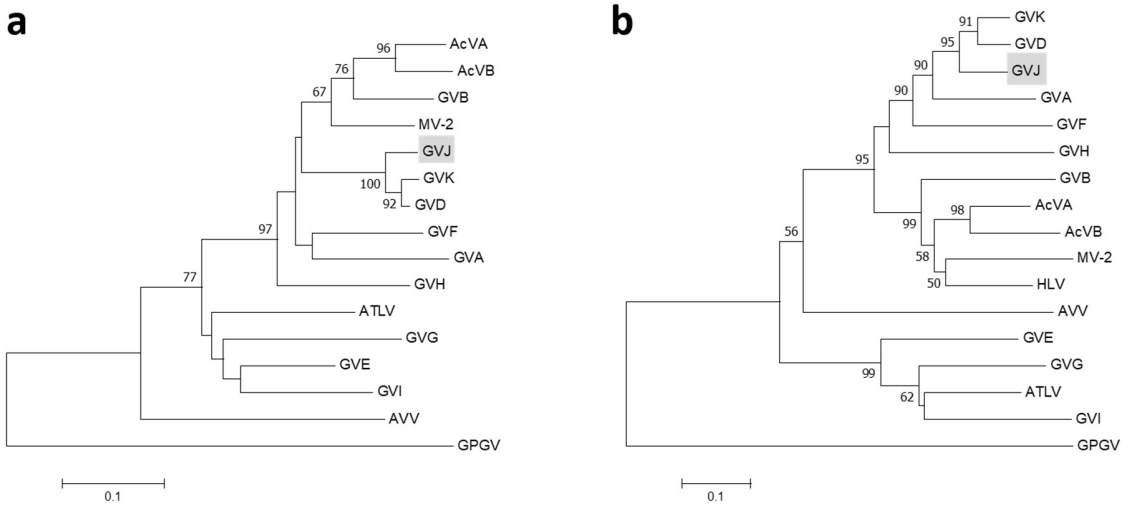

Fig. 1 Phylogenetic inference of *Grapevine virus J* (GVJ) in relation to members of the genus *Vitivirus*. Actinidia virus A (AcVA, JN427014); Actinidia virus B (AcVB, NC_016404); Mint virus 2 (MV-2, AY913795); *Grapevine virus B* (GVB, NC_003602); *Grapevine virus H* (GVH, MF521889); *Grapevine virus F* (GVF, NC_018458); *Grapevine virus A* (GVA, NC_003604); *Grapevine virus K* (GVK, NC_035202); Arracacha virus V (AVV, NC_034264); *Grapevine virus E* (GVE, NC_011106); Agave tequilana leaf virus (ATLV, NC_034833); *Grapevine virus D* (GVD, KX828708, Y15892); *Grapevine virus G* (GVG, MF405923); Grapevine virus I (GVI, MF927925); Heracleum latent virus (HLV, X79270); and Grapevine Pinot gris virus (GPGV, NC_015782, outgroup). Neighbor-Joining tree based on the amino acid sequences of the replicase **(a)** and coat protein **(b)**. Horizontal branch length is proportional to genetic distance; the scale bars represent changes per site. Bootstrap values less than 50% are not shown.

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P21: The past and future of *Grapevine fanleaf virus*

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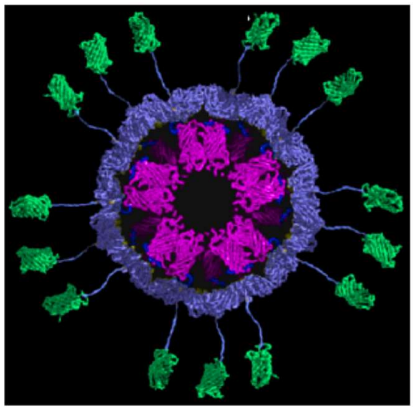
Fanleaf degenerative disease is often considered to be the most detrimental and widespread viral disease of grapevine. It affects vineyards worldwide, in particular those of high-added value in which grapevines have been cultivated for centuries. The disease is characterized by a range of symptoms that include yellow mottling and distortion of the leaves that can resemble a fan, malformed canes with exceedingly short internodes, smaller than normal clusters and overall stunted vines of reduced vigor (Schmitt-Keichinger et al., 2017).
Grapevine fanleaf virus (GFLV) and to a lesser extent *Arabis mosaic virus* (ArMV) are the major causal agents of fanleaf degenerative disease. As members of the genus *Nepovirus* within the family *Secoviridae,* these viruses are transmitted in nature by ectoparasitic dagger nematode vectors of the genus *Xiphinema* that primarily feed on root tips (Andret-Link et al., 2017). GFLV and ArMV possess a bipartite positive-strand RNA genome. Their icosahedral capsid with *T* = pseudo3 symmetry is composed of 60 copies of approximately 54 kDa coat protein (CP) that play essential functions in transmission by nematodes (Lai-Kee-Him et al., 2013; Marmonier et al., 2010; Schellenberger et al., 2010; Schellenberger et al., 2011). Empty particles are frequently found upon GFLV purification from infected plants, suggesting that the CP of GFLV is able to self-assemble into virus-like particles (VLPs). We recently confirmed the VLP self-assembly capacity of the CP upon its transient expression in *Nicotiana benthamiana* leaves. In addition, we found that the Nand C-terminal ends of the GFLV CP are compatible with the genetic fusion of large proteins such as fluorescent proteins and the plantbased production of nucleic-acid free VLPs (Belval et al., 2016). In this respect, pending N- or C-terminal fusion, up to sixty recombinant proteins can be exposed to either the inner cavity or outer surface of VLPs, respectively (Figure 1). Such properties are unique for a single viral structural protein and are of biotechnological interest.

Figure 1.
Molecular modeling of A GFLV-derived VLP In which 60 GFP Are exposed At the Outer surface And 60 Red fluorescent Protein are encaged.



With the initial aim to gain further insights into virus movement in plant and transmission by nematodes, we recently also produced Nanobodies (Nbs) against GFLV and ArMV. Nbs are single domain peptides derived from heavy chain only antibodies naturally found in camelids (Muyldermans, 2013). Because of their unique biochemical properties combining monomeric structure, small size and high stability, they have proven to be of outstanding biotechnological interest yet their use in agro-biotechnology remains scarce. Among the different Nb directed against GFLV that were isolated, Nb23 displayed remarkable properties as it conferred strong resistance to GFLV upon stable expression in the model plant *Nicotiana benthamiana* and also in grapevine rootstock, the natural host of the virus. We showed that resistance was effective against a broad range of GFLV isolates independently of the inoculation method including upon nematode transmission but not against its close relative, *Arabis mosaic virus*. We also demonstrated that virus neutralization occurs at an early step of the virus life cycle, prior to cell-to-cell movement (Hemmer et al., 2017). Our findings may pave the way for the generation of novel antiviral strategies in plants based on Nbs.
In addition to their antiviral activity, we also investigated the potential of Nbs as reagents for ELISA, in particular their performance for the detection of a wide range of natural GFLV and ArMV isolates from different grapevine

collections. Our result show that Nbs outcompete the classical kits available on market in terms of sensitivity and spectrum and present the additional advantage to be easily produced in *E. coli*.
Finally, the capacity of Nbs to recognize with high specificity and affinity GFLV-derived VLP was exploited to expose various proteins at the outer surface of particles. Using a set of three different Nbs we managed to expose up to 180 recombinant proteins such as fluorescent proteins at the surface of a single VLP. Combined with the encaging capacity of VLP.

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P22: *Grapevine Syrah virus-1* and *Grapevine Pinot gris virus* prevalence and variability in Turkish Grape Varieties

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INTRODUCTION

Turkey is an important grapevine (*Vitis vinifera* L.) producing country; and the production was 4 175 356 ton in 2014 marketing fresh, dry or processed form (Anonymous, 2014). Grapevine (*Vitis vinifera* L.) is one of the most important cultivated crops in the world and susceptible to several graft-transmitted agents that cause several diseases (Martelli, 2014). A lot of viruses can cause diseases on grapevines. Nowadays, more than sixty viruses belonging to different genera and families have been reported in grapevines (Martelli, 2014). Vegetative propagation can cause worldwide spread of these pathogens, favoring the emergence of complex diseases. Recently, *Grapevine Pinot gris virus* (GPGV) in the genera of *Trichovirus* and *Grapevine Syrah virus*-1 (GSyV-1) of *Marafivirus* have been characterized in the grapevines (Al Rwahnih *et al.,* 2009; Giampetruzzi *et al.,* 2012) and also in Turkey grapevines (Gazel *et al.,* 2016; Çağlayan *et al.,* 2017). The objective of this research was to search the prevalence of these two viruses in Turkey and characterization the isolates provided from different locations of the country in order to evaluate the historical presence and the route of the dissemination.

MATERIALS AND METHODS

In June-July and September of 2015-2016, viral symptomatic grapevine leaves were collected from the most important grapevine producing areas of Turkey. Total RNAs were extracted using ZR RNA MiniPrep™ (Zymo Research, USA) and cDNA were synthesized by using EasyScript Plus™ cDNA Synthesis kit (Abm, Canada). A real-time PCR assays or PCR assays were evaluated using the primer pairs of SY_5922F 5'-CCA ATG GGT CGC ACT TGT TG-3' and SY_6295R 5'- ACT TCA TGG TGG TGC CGG TG-3' amplifying 374 bp of partial coat protein (CP) of GSyV-1 genome (Glasa *et al.,* 2015) and GSyV-1 Det-F 5'- CAA GCC ATC CGT GCA TCT GG-3' and GSyV-1 Det-R 5'- GCC GAT TTG GAA CCC GAT GG-3' amplifying 295 bp of 230 kDa polyprotein of GSyV-1genome (Al Rwahnih *et al.,* 2009). To test GPGV in grapevine samples GPG_6609F 5'-GAG ATC AAC AGT CAG GAG AG-3' and GPG_7020R 5'- GAC TTC TGG TGC CTT ATC AC-3' primer pair amplifying 411 bp of partial CP of GPGV genome was used (Glasa *et al.,* 2014). SYBR green-based real-time PCR assays were performed with the mixture of 2 µl cDNA and 18 µl of amplification mixture including 10 µl of 2X Roche FastStart Essential DNA Green Master (Germany), 0.2 µl of each primer (20 pmol) and 7.6 µl of RNase-DNase free sterile water. Amplification condition was performed in Roche LightCycler® Nano Real-Time PCR system. The melting analysis was carried out for evaluation of the specificity of qPCR results with 0.1°C increase per second from 65°C to 95°C. The provided amplicons were sequenced directly from both ends and consensus sequences were analyzed with the corresponded genome sequences available in the Genebank by Mega 7 software (Kumar *et al.,* 2017).

RESULTS AND DISCUSSION

More than thousand grapevine plant samples were tested by qPCR methodologies and GPGV infection was found more prevalent than GSyV-1 in Turkish vineyards. Interestingly, not only foreign cultivars but also local varieties were also infected with the virus species which could be the indication of long term presence of viruses in the country. GPGV was prevalent at 9.08% rate and GSyV-1 was 5.17%. Two Mediterranean and two Aegean Region isolates of GSyV-1 (Acc.no: KY594000, KY594001, KY593998, KY593999); four Aegean and six East

Anatolian Region isolates of GPGV were sequenced and analyzed. This study showed a clear clustering of GPGV isolates in two groups. Turkey isolates clustered together with most of the Asian isolates like China, South Korea and an Australian isolate, an isolate from Canada, Czech Republic and Slovakia. GSyV-1 isolates were clustered in three groups with their partial coat proteins and two groups with their 230 kDa polyprotein region. According to both regions, Turkey isolates clustered together with the common isolates. No big diversity observed among the all Turkish viral isolates either on GPGV or GSyV-1.

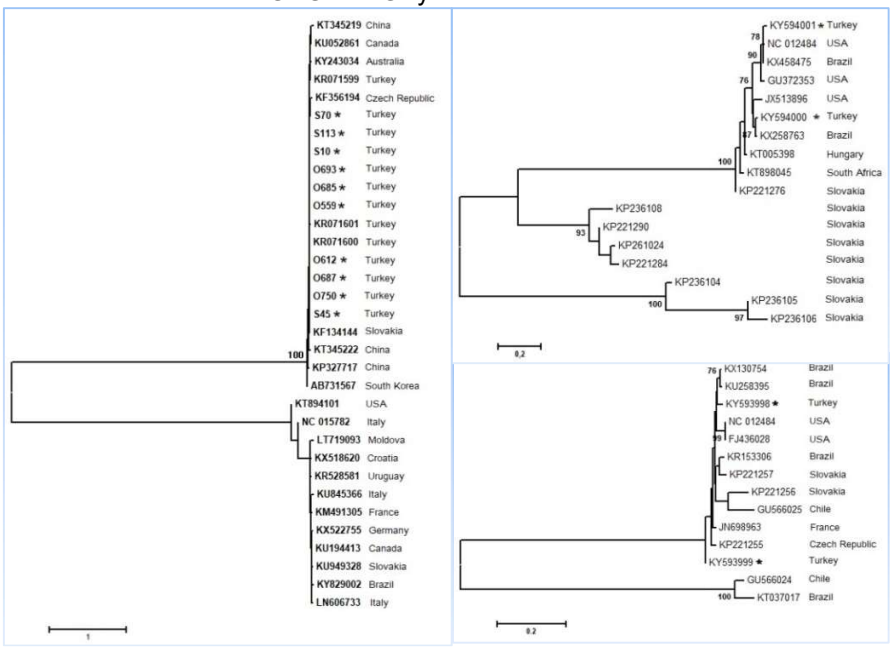

Figure 1. The neighbor joining tree (bootstrap 1000) of sequenced *Grapevine Pinot gris virus* (GPGV) and *Grapevine Syrah virus*-1 (GSyV-1) isolates from Turkey and the sequences of the isolates available at genebank. A) GPGV partial coat protein (CP), B) GSyV-1 partial CP, C) GSyV-1 partial 230 kDa polyprotein. Asterisk represents the Turkish isolates in the study.

To evaluate the real genetic diversity of the isolates, the necessity of more isolates researches was clearly observed on the tree, especially on GSyV-1. The more isolates and the more gene region should be studied to understand the genetic variation and distribution of the viruses.

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P23: Proteomic analysis of a GRBV-infected grapevine

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INTRODUCTION

Grapevine red blotch is a recently identified viral disease that was first recognized in 2008, when foliar symptoms similar to leafroll were observed in Napa Valley (California) on vines testing negative for known *Grapevine leafroll-associated virus*es. In 2012, *Grapevine red blotch virus* (GRBV) was independently discovered in California and New York and was later demonstrated to be the causal agent of red blotch disease (Fuchs et al., 2015). Due to its wide occurrence in the US, vector transmission and impacts on grape industry, this virus has the potential to cause serious economic losses. Despite numerous attempts, it was not possible to isolate, nor visualize, viral particles from GRBV infected plants. Consequently, this has hampered the development of a serological assay that would facilitate GRBV detection in grapevine. We therefore decided to explore a proteomics approach in order to quantify GRBV proteins in infected plants and to identify potential biomarkers for viral infection.

MATERIALS AND METHODS

Grapevine materials used for this study were collected from the grapevine virus collection at Agroscope in Nyon (Switzerland). Grapevine material was ground in a RETSCH planetary ball mill PM-100. Aliquots of 50 to 200 mg plant tissue were used subsequently for each of the experiments. Proteins from leaves were extracted with SDS buffer, from petioles essentially as described elsewhere (Wang et al., 2006). Gel chromatography (SDS-PAGE) was used to assess successful extraction of proteins. Extracted proteins were digested to completeness with Lys-C and trypsin. Shotgun data-dependent nano-LC-MS/MS data was acquired on an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific). The data was interpreted by MaxQuant software using the latest UniprotKB Vitis vinifera protein database, including protein sequences from *Vitis vinifera* associated species, and viral proteins from RNA-Seq experiments. RNA-Seq analyses were performed with RNA-Seq libraries prepared from total leaf RNA and sequencing on an Illumina HiSeq 4000 to obtain paired-end 75 nt reads.
Protein digests of leaves and leaf petioles were spiked with accurately defined amounts of three GRBV coat protein specific peptides, heavily labeled on the C-terminus with 13-C and 15-N isotopes. The non-labeled native and the heavily labeled standard peptides were quantified by a scheduled PRM approach using again nano-LC-MS/MS on a QExactive HF Orbitrap (ThermoFisher Scientific). The chromatographic peak intensities of five, respectively eight, most intense fragments per peptide were integrated to the corresponding peptide intensity and used for absolute protein quantification.

RESULTS AND DISCUSSION

Our study included an initial phase of protocol optimization for grapevine tissue preparation and analysis by mass spectrometry. It was not possible to identify GRBV by shotgun nano-LC-MS/MS data in leaf lamina extracts. However, it was possible to identify the V1 (coat protein) and V2 proteins of GRBV in GRBV-infected petioles, both with in-solution shotgun and in-gel digested 20-30 kDa gel slices, based on a total of 10 and 11 different peptides, respectively. Again, it was not possible to identify any GRBV peptides when analysing by nanoLC-MS/MS gel slices from leaf lamina extracts. This might indicate that GRBV is rather expressed in petioles. This is not surprising given geminiviruses are known to be restricted mainly to phloem of an infected plant (Hanley-Bowdoin et al., 2013). Comparative, semi-quantitative proteomics between healthy and GRBV-infected leaves and petioles revealed higher protein extraction efficiencies from tissues of infected plants. This bias in extraction efficiency rendered the biomarker discovery impossible. GRBV CP load determination revealed that the CP protein made up roughly 0.04% of the extractable protein mass in petioles, while this value is about 100-times lower in leaves based on the PRM-based detection of one peptide. Grapevine plants used in the proteomic study were also analyzed by high-throughput sequencing (RNA-Seq) to obtain a full indexing of its viruses. Besides

GRBV, *Grapevine rupestris stem pitting-associated virus* (GRSPaV) was shown to be infecting the plants. GRSPaV related peptides of ORF5 (capsid protein) were as well identified in petiole extracts but not in leaves. Thus, mass spectrometry can represent a tool for virus detection in plant as already proposed by Blouin and colleagues (Blouin et al., 2010).

In conclusion, we show that GRBV protein expression in grapevine leaves is too low for detection without sub fractionation of protein extracts. Therefore, a serology-based diagnostic tool seems to be unlikely to work. Finally, we are still studying the effect of GRBV infection on the grapevine proteome.

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P24: Nanobodies against GFLV and ArMV: ready for the diagnostic of fanleaf in vines?

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INTRODUCTION

Grapevine fanleaf virus (GFLV) and *Arabis mosaic virus* (ArMV) are the major viruses responsible for grapevine fanleaf degeneration. GFLV is often considered as the most detrimental and widespread grapevine viral disease since it affects vineyards worldwide and specifically old ones (Basso et al., 2017). These nepoviruses cause substantial crop losses, reduce fruit quality and shorten longevity of grapevine. To prevent their dissemination, the European certification regulates the production and marketing of grapevine propagation material especially concerning the sanitary status.This certification relies mainly on the detection of viruses by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) using commercial immunochemical reagents composed with polyclonal or monoclonal antibodies.

Alternative means to produce antibodies have recently emerged through the production of Nanobodies (Nbs). Nbs are small peptides derived from heavy chain only antibodies found in camelids (Muyldermans, 2013). Nb is a monomeric structure with remarkable physicochemical characteristics, such as solubility and stability, and a high production yields in *E. coli* or yeast. More importantly, the capacity of tailoring such Nb in order to make fusions with specific tags represents a great advantage to produced Nbs-based detection reagents.

Previous work led us to produce two collections of Nanobodies recognizing reference strains of GFLV and ArMV (Ackerer, 2016, Hemmer et al., 2017). Anti-GFLV and -ArMV Nbs were tailored to produce detection reagents and their capacity to use them as substitutes to classical antibodies was previously described (Ackerer et al., 2015 and Hemmer et al, 2017). Here we present the performance of home-made Nb-based detection reagents towards GFLV and ArMV onto 474 grapevine samples taken from different vineyards and compared them to some commercial detection kits.

MATERIALS AND METHODS

Production of the GFLV and ArMV nanobodies.

The production and the screening of GFLV- and ArMV-Nbs were described by Ackerer (2016) and Hemmer et al. (2017).

DAS-ELISA tests with commercial kits and Nbs-based detection reagents.

The detection was performed with two commercial DAS-ELISA detection kits for GFLV (Suppliers A and B) and one DAS-ELISA detection kit for ArMV (supplier B) according to the manufacturer's instructions. For Nbs, a DAS-ELISA test was also developed both for GFLV and ArMV. The coating step was done with tailored Nbs (anti-GFLV or anti-ArMV) and the detection was performed with alkaline phosphatase-tagged Nbs as described by Ackerer et al. (2015). Samples were considered positive when the absorbance values exceeded those of the negative control samples (healthy vines) by at least a factor of 2.2.

Evaluation of the GFLV kits and GFLV Nbs reagents onto positive controls

Three grapevine leaves samples known to be infected by GFLV were used to evalute the sensitivity of the GFLV Nbs-based detection reagents compared with two commercial GFLV kits. Crude extracts were diluted in extraction buffer from 1/80 to 1/250. The absorbances at 405 nm were measured after 30 minutes, 1 and 2 hours of substrate hydrolysis.

Selection of vineyards and sampling

Sampling was done in spring during the years 2016 and 2017. One sample was constituted of about 10 leaves taken from one individual vine. In 2016, 280 samples were taken from 5 vineyards in southern (Vaucluse) and northern France (Alsace) showing typical fanleaf symptoms. Both symptomatic and asymptomatic vines were sampled. In 2017, analyses focused more towards northern France vineyards where the prevalence of ArMV is supposed to be higher compared to GFLV. Altogether, 194 samples were then selected from 5 vineyards. Again both symptomatic and asymptomatic vines were sampled.

RESULTS AND DISCUSSION

Comparison of the GFLV Nbs-based detection reagents with commercial detection kits

The results obtained two hours after adding the substrate (as indicated in the French official method) are indicated in Figure 1. Whatever the detection reagents used (GFLV-Nbs, GFLV-A (supplier A) and GFLV-B (supplier B)), all three positive controls revealed GFLV-positive at the 1/80 dilution. By contrast, differences in detection between kits appeared at higher dilutions (1/120 and above). Hence, whereas the performance of both GFLV-Nbs and the commercial kit GFLV-B were maintained at all dilutions (100% of detection), the GFLV-A kit progressively failed to detect GFLV. At 1/250 dilution GFLV was totally undetectable in all the three samples with this kit. We also observed that the absorbance increased faster with the Nbs-based reagents than with all other kits as observed after 30 minutes (results not shown).

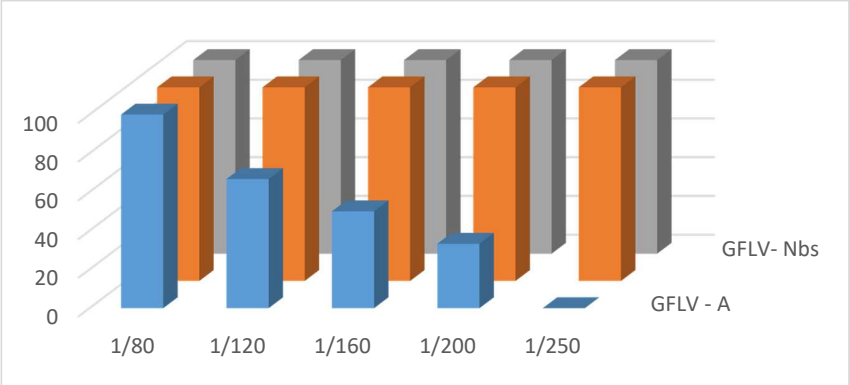

Figure 1: Percentage of positive samples in relation to the dilution (2 hours of substrate digestion). GFLV-A and GFLV-B: DAS-ELISA from commercial suppliers and A and B; GFLV-Nbs: home-made Nb-based detection reagents develop for a DAS-ELISA.

Performance of GFLV and ArMV Nbs-based reagents for virus detection with samples taken from the vineyard.

The samples were analyzed by DAS-ELISA successively with Nbs home-made detection kits and detection kits from supplier B. The results are given in table 1.

Tab 1: Correlation between GFLV-and ArMV-Nbs home-made detection reagents (Nb) and commercial kits from supplier B (B)

		Concordances			**Discordances** (number of samples)		
		%	Numbers of samples				
		(Nb-/B-) & (Nb+/B+)	Nb-/B-	Nb+/B+	Nb-/B+	Nb+/B-	**Vineyards / Area**
2016	GFLV	**100**	108	172	0	0	5 (Alsace & Vaucluse)
2017	ArMV	**98,9**	114	78	2	0	5 (Alsace)
2017	GFLV	**95,3**	135	50	7	2	5 (Alsace)

In 2016, 100% of concordance was obtained between the extracts tested with GFLV-Nbs compared to the GFLV-B onto the 280 samples independently of the localization. In 2017, very good results were also obtained even if some discordances could be observed with 7 GFLV samples and 2 ArMV samples. A new test will be realized towards these discording samples to confirm these results and if so, the capsid proteins of these samples will be sequenced and analyzed for structural differences that could explain the observed discrepancy.

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P25: Survey of grapevine viruses in Perú

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INTRODUCTION

Grapevine (*Vitis vinífera L.)* is one of the most cultivated species across the world and a host for numerous patogens as well. It hosts a high number of infectious agents amog viruses, viroids y Phytoplasms (Martelli, 2014). The plant viral diseases cause serious economic losses in many of the main crops, diminishing their yields and quality (Byoung-Cheorl et al., 2005). Therefore, the knowledge about these patogens and the optimization of techniques for diagnostic have experienced a considerable increasing in the last years (Digiaro et al., 2007). In relation to grapevine viruses, they have been described and clustered between more than 21 distinct genders (Martelli, 2014), and only some of them are significant in different regions over the world where it is cultivated. Between the main grapevine viruses are: *Grapevine Leafroll associated Virus* (GLRaVs*), Grapevine fleck virus* (GFkV), *Grapevine fanleaf virus* (GFLV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB) that are frequently found in vineyards all over the world (Hewitt et al., 1972; Kim et al., 2004; Sabanadzovic et al., 2000). In the case of Peru, it has been reported the presence of various grapevine viruses, as GFkV, GFLV, GLRaVs and ToRSV, which affect different cultivars of grapevine (Fribourg, 2007). Furthermore, during the 2008 - 2009 period it has been performed a prospection of viruses in Ica region, for determining the existence of the following viruses: GFkV, GFLV, GLRaV-1 and GLRaV-3 in vineyards; in this work DAS-ELISA technique was applied (Tenorio et al., 2009). Those previous backgrounds, are the only ones available about the existence of grapevine viruses in Peru. Taking into account the economic losses that viral diseases cause in grapevine crops, it is of great importance to develop reliable and effective diagnostical systems. For those reasons, the principal purpose of this work was performed a prospection about the phytosanitary situation of the main grapevine-producing areas, Ica, Arequipa and Tacna regions, with respect to viral infections of 7 viruses by DAS-ELISA and RT PCR techniques.

MATERIALS ANS METHODS

A total of 1207 samples were collected (with their respective repetitions) corresponding to quebranta, moscatel, Italia, torontel and negra criolla cultivars from 28 vineyards of the evaluated regions. All the samples were serologically analized by DAS-ELISA, according to Clark and Adams (1997). Using specific comercial antiserum for detection of antigens of the following viruses: *Grapevine fleck virus* (GFkV), *Grapevine fanleaf virus* (GFLV), Grapevine Leafroll associated Virus type 1, 2, 3 (GLRaV-1, GLRaV-2, GLRaV-3), Tomato ringspot virus (ToRSV) and Arabis mosaic virus (ArMV); with their respective controls belonging to BIOREBA Company.

To adjust the Incidence and Prevalence values with respect to viral infection, 295 samples were selected, those samples were evaluated by RT-PCR technique. The total ARN was obtained following the CTAB extraction protocol. Then, synthesis of the respective cDNA for each sample was conducted using the M-MLV Reverse Transcriptase kit (Invitrogen, Life Technologies). The Polymerase Chain Reaction (PCR) was fulfilled using primers already reported that are specific for the Coat Protein of the viruses in study. In addition, it was verified the quality of RNA using primers for grapevine rRNA 18S which amplified for a band of 844 bp (Gambino et al., 2006).

Finally, the Incidence (percentage of leaves infected by the diesease or pest in relation to the total collected leaves) and Prevalence (percentage of the infected grapevine – producing areas in relation to the total sampled grapevine – producing areas) values were calculated.

RESULTS AND DISCUSSION

Regarding the evaluation of viral infection by DAS-ELISA, the major value of total incidence was 7.04% for the GFkV infection, while the remaining viruses did not exceed the 3.5% (Table1). Regarding the highest values of prevalence, these were 71,4 % for GLRaV-2 and 67.9 % for GFkV (Table 2).

Table 1.- Viral prevalence in the three regions.

Viruses	TEST DAS-ELISA			Total
	Ica	Arequipa	Tacna	
% (+) GLRaV-1	3.64	3.52	1.01	2.73
% (+) GLRaV-2	3.64	2.51	3.27	3.15
% (+) GLRaV-3	2.18	1.76	3.02	2.32
% (+) GFLV	1.46	2.01	4.28	2.57
% (+) GFkV	9.95	6.53	4.53	**7.04**
% (+) ArMV	1.21	3.52	2.02	2.24
% (+) ToRSV	1.46	4.27	2.27	2.65

Table 2.- Viral Prevalence (infected vineyards / total sampled vineyards)

	Infected Vineyards % - TEST DAS ELISA						
	GLRaV-1	GLRaV-2	GLRaV-3	GFLV	GFkV	ArMV	ToRSV
N° Infected Vineyards	15	20	15	16	19	12	14
%	53.6	71.4	53.6	57.1	67.9	42.9	50.0

Preliminary results indicate until now that GLRaV-2 and GFkV are significant, considering the parameters of incidence and prevalence.

This research is the first report of a wide study about the incidcence, prevalence, and distribution of grapevine viruses in the principal grapevine – producing areas. It included 1207 samples taked, ramdomly, from 28 vineyards, the data obtained shows an average percentage of viral infections of 19.5% by DAS-ELISA. By this way, it is demonstrated that the most important grapevine viruses across the world are present in our regions, with more or less incidence, depending mainly on the origin of the propagated material. The farmer utilizes a traditional technology: he resorts to a material for propagation that comes from existent vineyards, without technical or sanitary criteria (MINCETUR, 2004). And, in case he has access for acquiring material from the nurseries, those can only certify that the material is obtained from vigorous mother plants and, apparently, free of diseases, on the basis of a visual inspection. With the results obtained at this moment, it is confirmed the presence of 5 viruses (GFkV, GFLV, GLRaV-1, GLRaV-3 and ToRSV) already reported in our country by previous reports; Castillo (1973) delivered the presence of GFLV in the valleys of Ica, Chincha, Cañete, Mala and Lima; for his part, Fribourg (2007) reported 4 viruses that attacked cultivars of grapevine as GFkV, GFLV, GRLV and ToRSV. Finally, Tenorio et al (2009) achieved an evaluation for GFkV, GFLV, GLRaV-1 and GLRaV-3 viruses in the most important farms and nurseries of grapevine in Ica region. Considering this last study, it agrees with the present work that the virus with more incidence in grapevine - producing fields in Ica continue being GFkV. In regards to GLRaV-2 and ArMV, this study is the first one on demonstrating their presence on vineyards in Peru. The two viruses found, considered so far the most important, are GFkV and GLRaV-2. These viruses have a particular feature, both does not have vectors of transmission reported (Constable et al., 2012; Maliogka et al., 2015). However, they have been found in all the grapevine-producing countries of the world (Martelli et al., 2006). The prevalence of the virus is very high, in simple infectious as well as multiple ones with other dangerous viruses (Cretazzo et al, 2010). On the other hand, although ELISA is utilized habitually for detection of plant viruses, it has been demonstrated that it lacks of sensibility for viral detection at low concentration. In the attempt for overcoming this matter, many molecular tests and primers for amplification of the nucleic acid through PCR, for various grapevine viruses as nepovirus (Martelli, 1993), closterovirus (Martelli et al., 1997), vitiviruses (Boscia et al., 1997) and GFkV (Sabanadzovic et al., 1996) have been developed. Currently, it is being carried out the evaluation of the viral infection for the agents mentioned in this study, using 295 samples, by RT-PCR, in order to adjust the values of viral incidence and prevalence in grapevine in Peru. To this effect, the genes of the viral coat protein corresponding to the viruses in study, are being amplified using primers already reported (findings not published yet).

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P26: Grapevine viruses in three provinces of Pakistan

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INTRODUCTION

Grapevines (*Vitis vinifera* L.) were grown on 15,360 ha in Pakistan producing 66,036 tons of grapes in 2014 (FAO 2017). Grapes ranked 10th among fruit crops grown in Pakistan (Jaskani et al. 2008). Grapes are grown in all four provinces (Balochistan, Khyber Pakhtunkhwa, Punjab, and Sindh) but the majority of grape production occurs in Balochistan (GoP 2011). The important grapes varieties grown in Balochistan are Haita, Kishmish, Sunderkhani, Sahibi and Shekhali (Aujla et al. 2011). In Pakistan, 87% of grape production is used as table grapes and 13% as dried fruit (Aujla et al. 2011). Important cultivars of table grapes are also grown in some districts of Khyber Pakhtunkhwa and annual production is 122 thousand tons with an average yield of 19 tons per hectare against the potential of 25 tons per hectare (Bashir et al. 2012). Popular cultivars of table grapes (Italia, Cardinal, Flame Seedless, White Kishmish, Thompson Seedless, Perlette and King's Ruby) can be grown in central area of Punjab (Uddin et al. 2011). In 2008-2009, Pakistan exported 184,256 kg grapes to Bahrain, Bangladesh, Germany, United Arab Emirates and United Kingdom, which were valued 9,054,000 rupees (approximately $141,850 USD) (Reisch et al. 2012). Due to the lack of information about grapevine viruses in Pakistan, the objective of this survey was to provide knowledge about the occurrence and prevalence of the grapevine viruses in different grape-growing regions of Pakistan.

MATERIALS AND METHODS

To analyze the prevalence of grapevine viruses, 249 grapevines were collected during 2014-2016 from 13 vineyards (in three provinces) were tested for 18 viruses, phytoplasmas and *Xyllella fastidiosa* using RT-qPCR. Total RNA extraction was performed by grinding 300 mg petiole material in guanidine buffer according to Osman et al. (2012) and using GeneJET Plant RNA Purification Mini kit (Thermo Scientific Fisher) according to the manufacturer's directions. Viruses included in the survey were: *Grapevine leafroll-associated virus*es (GLRaV-) 1, -2 (including 2 Red Globe), -3, -4 (including GLRaV-4 strains 5, 6, 9, Pr, Car), and GLRaV-7; *Grapevine fanleaf virus* (GFLV); Tomato ringspot virus (ToRSV); Tobacco ringspot virus (TRSV); Arabis mosaic virus (ArMV); Grapevine viruses A (GVA), B (GVB), D (GVD), E (GVE), and F (GVF); *Grapevine red blotch virus* (GRBV); *Grapevine fleck virus* (GFkV); *Grapevine rupestris stem pitting-associated virus* (GRSPaV); and Grapevine Pinot gris virus (GPGV). Primers and probes used for the detection of these viruses were: GLRaV-1, GLRaV-2, GLRaV-4, GLRaV-4 strain 5, GLRaV-4 strain 9 (Osman et al. 2007), GLRaV-2RG, GFkV, ToRSV (Osman et al. 2008), and GLRaV-3 (Osman and Rowhani 2006). For all other viruses, primers developed at UC Davis were used (V. Klaassen, unpublished). Positive and negative controls for all grapevine viruses were also included. RT-qPCR was carried out in 394-well plates using the TaqMan® Fast virus 1-step Master Mix kit (Applied Biosystems). All samples were amplified in 10µl reaction using 2µl RNA and reaction mixtures and cycling conditions according to the one step protocol by Osman et al. (2012). The cycle threshold (CT) value was calculated in a Microsoft Excel format and graphically by an amplification plot. The positive Ct value was set up to 36.

RESULTS AND DISCUSSION

The most prevalent viruses were: GVA (49.4%), GLRaV-2 (38.6%), GRSPaV (37.3%), and GFkV (36.1%). Other viruses detected were: GLRaV-1 (2.4%), GLRaV-2RG (5.2%), GLRaV-3 (7.2%), GLRaV-4 and its strains 5, 6, and Pr (17.3%), GLRaV-7 (4.4%), GFLV (12%), GVB (4.4%), GVD (0.8%), GVE (1.2%), and GPGV (2%) (Rasool, et al. 2017). Mixed infections were detected in 75.9% of samples. Viruses tested for, but not detected include GLRaV-4 strain 9, GLRaV-4 strain Car, GRBV, ToRSV, TRSV, ArMV, and GVF. *Xylella fastidiosa*, the causal agent of Pierce's disease, and phytoplasmas were also tested for but not detected. This is the first survey for grapevine viruses and thus the first report of the above viruses in Pakistan. Grapevine viruses are economically important and present a major threat for the grapevine industry. To the best of our knowledge the presence of grapevine viruses was confirmed for the first time in Pakistan. It is evident from the results that Punjab and Pakhtunkha provinces having more infection as compared to Baluchistan. Results of this study provide stakeholders with the current knowledge of the status of grapevine viruses in Pakistan. In addition, this research

demonstrates the use benefit of diagnostic tests for the detection of viruses of grapevines which can continue to be used for routine virus screening.

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P27: Detection of grapevine viruses, viroids and "stolbur"-group phytoplasma '*Candidatus* Phytoplasma solani' in grapevine using next-generation sequencing

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INTRODUCTION

Next generation sequencing (NGS) technologies are applied to a greater extent for detection of plant pathogens in the last years (Adams *et al*., 2009, Loconsole *et al*., 2012, Rott *et al*., 2017). Besides transcriptome analysis, NGS is used for diagnostics of virus, viroid and phytoplasma diseases of grapevines (*Vitis vinifera* L.) (Abbà *et al*., 2014, Nicolaisen *et al*., 2011). NGS data confirmed the presence of viruses in grapevine samples, moreover, mixed virus infections have been revealed and new viruses were discovered (Coetzee *et al*., 2010, Pantaleo *et al*., 2010, Giampetruzzi *et al*., 2012). RNA sequencing combined with metagenomic analysis enables an unbiased analysis of infected plant samples. Plant pathogens will be detected by their RNA genomes respectively by their transcripts (Loconsole *et al*., 2012). Virus, viroid and phytoplasma diseases of grapevine cause severe crop losses in viticulture (Basso *et al*., 2017, Bisztray *et al*., 2012, Martelli, 2014). "Bois noir" (BN), the prevalent grapevine yellows disease in Europe, is associated with '*Candidatus* Phytoplasma solani' ("stolbur"-group; Quaglino *et al*., 2013). BN disease has been reported in almost every grapevine producing region in Germany, but with the prevalence in the viticultural areas Mosel, Middle Rhine and Nahe (Langer and Maixner, 2004). In 2006, the emergent symptoms of grapevine enation disease (GED) in Germany have been reported, mainly for the wine-growing regions Rhine-Hesse and Nahe. However, the etiology of GED, causing formation of enations on the underside of basal leaves and growth depression of infected plants, still remains unknown (Bisztray *et al*., 2012, Martelli, 2014). No correlation of Reverse Transcription (RT)-PCR detected virus species and occurrence of disease has been found so far.For developing and implementing effective control strategies detection of viruses and virus-like diseases associated with serious grapevine diseases is essential. Therefore next-generation sequencing (Illumina) was applied in this study for detection of virus and phytoplasma presence in total RNA extracts of two grapevine samples.

MATERIALS AND METHODS

An asymptomatic shoot from "bois noir" infected grapevine (*Vitis vinifera* L. cv. Riesling) was collected in a sloping and terraced vineyard at the Dienstleistungszentrum Ländlicher Raum (DLR) Mosel (Bernkastel-Kues, Germany). Leaf material from grapevine cv. Dornfelder, exhibiting growth reduction was sampled in a vineyard in Worms-Herrnsheim (Rhine-Hesse, Germany). Total nucleic acids were extracted from leaves using a CTAB extraction method (Jakovljevic *et al*., 2016). Total RNA, treated with DNase, was subjected to a NGS pipeline, conducted at the DSMZ Plant Virus Department (Braunschweig, Germany) comprising the following steps (Knierim *et al*., 2017): depletion of plant ribosomal RNA, subsequent random cDNA and second strand synthesis, preparation of Nextera XT library (Illumina), paired-end 2x301 sequencing on Illumina MiSeq platform. NGS data were analyzed using the bioinformatic software Geneious 9.1.8. Raw reads were trimmed (1%), paired reads were set and merged. After subtraction of host genome-specific reads (*Vitis vinifera* genome including plastide and mitochondrion), processed reads were mapped to databases containing virus and viroid reference genomes and phytoplasma sequences (from NCBI, Genbank: viral genomes, filter: host=plants; two genomic drafts of '*Ca.* P. solani' strains, GenBank nucleotide sequences of '*Ca.* P. solani', "bois noir"/"stolbur" phytoplasma) by Blastn search and use of a map to reference tool. Additionally, processed reads were *de novo* assembled and consensus sequences of obtained contigs were mapped to reference sequences.

RESULTS AND DISCUSSION

Two grapevine samples were subjected to NGS pipeline starting from total RNA extract for generating an untargeted metagenome dataset. Therefore untargeted and unknown pathogens may be identified and a parallel detection of a variety of pathogens (viruses, viroids and phytoplasmas) using a single technology is feasible. Sequencing of the library prepared from asymptomatic sample (tested negatively by "stolbur"-specific PCR assay, Maixner *et al*., 1995) of "bois noir" diseased grapevine, generated a total of 1,279,589 reads. 58.1% of these reads were successfully processed. 91.2% of trimmed reads were mapped to host genome, finally obtaining 68,423 unaligned reads. *De novo* assembly produced 9,444 contigs (27,840 reads), of which in total eleven contigs aligned to three "stolbur" phytoplasma sequences ("bois noir" phytoplasma strain CH-1 16S rRNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer and tRNA-Ile gene, complete sequence; and 23S

ribosomal RNA gene, partial sequence, HQ589193.1; strain 284/09 “stolbur” phytoplasma draft, FO393427.1; strain 231/09 “stolbur” phytoplasma draft, FO393428.1). This NGS approach enables the detection of low titre infections in tissues. Beside phytoplasma sequences, genomes of grapevine pathogens were found to be present: *Grapevine leafroll-associated virus* 1 (GLRaV-1; NC_016509), *Grapevine rupestris stem pitting associated virus*-1 (GRSPaV-1; NC_001948), *Grapevine Pinot gris virus* (GPGV, NC_015782), Grapevine satellite virus (isolate AUD46129, NC_021480) and Hop stunt viroid (HSVd-g; NC_001351), that is detected in grapevine with high prevalence (Sano et al., 2001). Infection by GRSPaV, which is widely distributed in European vineyards and can be reliably tested all year round in leaf samples (Weber *et al*., 2002), was confirmed in RT-PCR, in contrast to GLRaV-1 presence. For GLRaV-1 detection in fall, it is recommended to sample canes for phloem scrapings (Weber *et al*., 2002), this could increase detectability compared to leaf samples. For GED symptomatic sample a set of 738,170 MiSeq-reads were obtained, thereof 610,012 reads (82,6%) were trimmed and subsequently mapped to the host genome (97.5%). 15,455 unaligned reads were *de novo* assembled to 2,421 contigs (8,349 reads). 14 contigs were mapped to GRSPaV-1 genome, covering 85.6% with 75.7% pairwise identity. Both, GRSPaV-1 and *Grapevine virus A* (GVA; NC_003604, seven contigs mapped) were positively tested in RT-PCR analysis (Minafra and Hadidi, 1994). Further contigs were aligned to Grapevine yellow speckle viroid-1 (GYSVd-1; NC_001920) and to HSVd-g. Further sequence homologies were identified for both NGS samples by blastn and map to reference analysis of unassembled reads as well as produced contigs, representing hints for the presence of additional viruses. However, putative contaminations during extraction step and NGS sample prepa-ration pipeline, including the fact that other samples are parallel sequenced (e.g. incorrect assignment of library indices), must be considered in the evaluation of NGS data. Low depth of sequences and genome/sequence coverage were achieved for these additional matches. Low abundance of patho-gen-derived reads might correlate with the high percentage of host genome-mapped reads. The latter can be improved by developing a depletion method, that specifically removes ribosomal RNA from grapevine. In general, the validation of hits via alternative detection assays should be performed. Further analysis of contigs shall address the presence of unknown viruses. In this study NGS technology (RNA-Seq) was applied for the parallel detection of phytoplasmas, viruses and viroids in two single grapevine samples.

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P28: The Davis Grapevine Virus Collection – A Current Perspective

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INTRODUCTION

Research on grapevine virus biology is dependent on reference isolates to design antibody and nucleic acid probes, to confirm successful transmission, and verify assays [1, 7]. Many grapevine viruses and presumed viral diseases cannot be artificially preserved as purified preparations; and therefore, require maintenance of virus positive grapevine type collections [3]. Foundation Plant Services (FPS) serves as the primary quarantine facility for importation and virus screening of foreign grapevine cultivars and selections into the United States (US). The department maintains two virus source vineyards at the UC Davis Plant Pathology Farm which serve as a library of references for domestic, identified grapevine viruses [3]. The vineyards house diverse isolates of viruses and virus-like-agents (VLAs) utilized in routine diagnostics as well as historic and current grapevine virology research. To prevent the loss of this invaluable collection due to aging, efforts began in 2016 to collect, test, and propagate grafted and own rooted vines for initiation of an improved Davis Grapevine Virus Collection (DVC).

MATERIALS AND METHODS

Twenty dormant cuttings were collected from 540 vines in 2016, as available. Bark scrapings were processed, and subject to RT-qPCR/qPCR testing for 36 pathogens: 34 viruses with 3 sets of primers designed to detect variants of *Grapevine leafroll associated virus* 3 (GLRaV-3), and two bacteria: *Xylella fastidiosa* and a general set of primers to detect phytoplasmas [7]. Results were evaluated and designated as positive (POS), negative (NEG), or inconclusive.

Two hundred vines were selected and propagated. St. George FPS 20.1 was chosen as the rootstock cultivar due to described virus tolerance and phylloxera resistance [6]. High throughput sequencing (HTS) was performed on a composite sample of 12 field vines and detected only *Hop stunt viroid,* (HSVd). Two buds of each source vine were chip budded, 2 buds per vine, onto 4 container grown St. George 20.1 rootstocks. In cases where initial grafting efforts were unsuccessful, four additional vines were chip budded. Each selection was also propagated under mist bench conditions. Where production of 2 grafted field vines was unsuccessful (Table 1), selections were field planted on own roots.

Four acres were selected as the location for the DVC. The field was fumigated with methyl bromide (98%) and chloropicrin (2%) at a rate of 400 lb/acre; after which, oats were sown. Steel, adjustable trellising with three incremental extended cross arms and drip line irrigation were installed. Vines were planted on 10'x15' spacing. Initial planting occurred on April 25, 2017 and confirmation qPCR testing for a panel of 16 pathogens was performed in November of 2017.

RESULTS AND DISCUSSION

Of 540 vines tested, single infections were only identified in 28 selections, which tested positive for *Grapevine rupestris stem pitting associated virus* (GRSPaV). Only two vines tested negative for all 36 pathogens. The virus collection naming convention identifies selections based upon disease symptom expression [3]. In most cases PCR testing confirmed the presence of causal agents for each designated disease. The vines that tested negative or positive only for GRSPaV included selections considered to be infected with grapevine leafroll disease: LR111, LR113 LR121, LR125, corky bark: CB125, and latent viruses: LV92-11, LV92-15, LV93-02, and LV93-03. The PCR panel included only a portion of the 70+ reported grapevine viruses, of which 36 were tested [4]. Through recent advancements in diagnostic technologies, methods for further investigation of these selections is available. HTS can detect both well-characterized and novel viruses [1]. Within the DVC, novel viruses have been discovered and identified by HTS: *Summer grape latent virus* [2] and *Grapevine geminivirus A* (GGVA) [2].

Where vines within a selection exhibited distinct disease profiles, propagations were made from multiple source vines. Uniform disease status within a selection was expected, as vines were propagated from a single source vine. This preliminary variation could suggest vector transmission within the vineyard, propagation escape, or unspecific laboratory detection.

Efforts to establish grafted vines were unsuccessful for 12 selections. Graft incompatibility can result from virus infection of scion material. Symptoms are described as bud failure, young vine decline, or collapse. Latent viruses in scion sources have been demonstrated to cause a hypersensitive response when grafted to specific rootstocks [5]. Particular combinations of vitiviruses and grapevine leafroll strains have been associated with vine

collapse on certain rootstocks [6]. Where graft incompatibility was observed, we successfully established own rooted vines from two node cuttings.

Interpretation of survey results provokes discussion and provides insight for future evaluation. Further biological and sequence evaluation of those selections which tested negative for suspected disease may lead to discovery of novel VLAs or variants of known viral pathogens, improved diagnostic assays, or identification of non-viral grapevine syndromes. Through establishing a disease profile of each selection, we enable future epidemiology studies of the collection. Furthermore, investigation into the viral agents and disease profiles with incidence of graft incompatibility may expose the biological impact of otherwise considered inconsequential infections.

Table 1. Selections Exhibiting Graft Incompatibility

Cultivar	Selection	Quantity of cuttings collected	Quantity Successfully Grafted	Quantity Successfully Propagated	Virus Profile
Hunisa	VM-5	11	**0**	4	GLRaV 3, GRSPaV, GVA
Italia	LR101	15	**0**	4	GLRaV 3, GRSPaV, GVA
Grand noir	GFLV106	20	**0**	2	GFkV, GFLV, GLRaV 2, GLRaV 3, GLRaV 5, GRSPaV, GVA, GVFV
Zinfandel	LR129	12	**0**	4	GAMV, GRBaV, GRSPaV
Black Seedless	CB103	20	**0**	4	GLRAV 1, GLRAV 7, GRSPAV, GVA, GVFV
Seedless Emperor	LR128	18	**1**	4	GLRaV 3, GLRaV 3e, GLRaV 4, GRSPaV, GVFV
Gharibi	W017	20	**1**	4	GFLV, GLRaV 3, GVA
Katta Kourgane	CB123	4	**1**	4	GLRAV 2, GLRAV 3, GLRAV 5, GRSPAV, GVA, GVB, GVF, GVFV
Koshu	W018	20	**1**	3	GLRaV 3, GRBaV, GRSPaV, GVB
Neirbetta	W019	20	**1**	4	GLRaV 2, GLRaV 3, GRSPaV, GVA, GVB
Thomson Seedless	GFLV107	17	**1**	3	GLRaV 5, GVA, GVFV
Negrita	CB111	20	**1**	4	GLRAV 1, GLRAV 7, GRSPAV, GVA, GVFV

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P29: Deep sequencing analysis of viruses infecting grapevines: Discovery of two new and highly distinct variants of *Grapevine leafroll-associated virus 3*

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INTRODUCTION: The grapevine and wine industry in Canada is new, first established in the 1970s with Ontario being the largest producer followed by British Columbia. The situation of grapevine viruses and viral diseases and their economic impact to grape and wine production in Canada is largely unknown. However, since 2013 there have been sudden outbreaks of viral diseases in the Niagara Peninsula, the center of grape production region of Ontario. In an effort to understand the distribution of viruses and their impact to the grape and wine industry, we have initiated large-scale survey work since 2014. We have witnessed severe losses and even total crop failure in numerous vineyards we visited. One of the vineyards severely infected with viruses was an organic vineyard established in the early 1980s where Riesling grapes were grown. The infected vines looked considerably small with only few clusters of grapes and with yellowing and downward rolling of leaves. To identify the viruses present in these Riesling plants, we conducted a global analysis of the virome of the infected Riesling plants. Through the use of the Illumina HiSeq 2500 platform and total nucleic acids isolated from the infected vines, we identified five species of viruses and three viroids in the infected vine. For three of the viruses identified, we revealed the presence of multiple viral variants. Interestingly, two of the variants of *Grapevine leafroll-associated virus 3* (GLRaV-3) represent novel isolates as they are only distantly related to GLRaV-3 isolates whose genome sequences are available in GenBank.

MATERIALS AND METHODS: The severely diseased vine, sample 8415, was collected from an organic Riesling vineyard in Niagara, Ontario. Total nucleic acids were isolated from cambium scrapings by using Spectrum™ Plant Total RNA Kit (Sigma) with our modified method (Xiao et al. 2015). After removal of rRNAs, the RNA prep was used as template for construction of a cDNA library using TruSeq RNA Sample Prep Kit. NGS was carried out on an Illumina HiSeq 2500 sequencer generating 51-bp single-end reads. The sequencing data set was analyzed in iPlant Discovery Environment and CLC Genomics Workbench. To obtain complete or partial genome sequences for the viruses and viral variants, viral sequence contigs derived from *de novo* assembly were manually compared to known viral genome sequences and assembled into various variant-specific sequences. Confirmation of novel variants of GLRaV-3 was done by RT-PCR and Sanger sequencing. RLM-RACE kit was used to determine the 5'- and 3'-termini of the genome of novel variants. Phylogenetic analyses were performed by using Neighbor Joining method (MEGA 7).

RESULTS AND DISCUSSION:

Multiple viruses and viral variants are detected in a single vine:

A total of 73,385,886 sequence reads were obtained from the cDNA library. Among them, 23,026,032 reads were not mapped to the *Vitis vinifera* genome when analyzed in iPlant. These reads were then used to map against the complete reference sequences of viruses and viroids(http://www.ncbi.nlm.nih.gov/genome/viruses/) to identify viruses and viroids. Among these reads, 5,930,512 hits aligned with sequences of five viruses and three viroids, which are *Grapevine leafroll-associated 1* (GLRaV-1), GLRaV-3, *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine virus A* (GVA), GVB, and three viroids. The total number of virus and viroid reads is 8.1% of the total sequence reads, indicating heavy infection of this vine by multiple viruses and viroids, of which GLRaV-3 is the major component, accounting for 85% of the total virus and viroid reads.

Virus contigs generated from *de novo* assembly using CLC Genomics Workbench were manually compared with known viral genome sequences and assembled into various virus variant sequences (**Table 1**). Near complete genome coverage with a small internal gap of 118 nt as compared with the reference isolate WA-CH (Donda et al. 2017) was generated for the GLRaV-1 isolate in this sample (Li et al., this proceedings). A number of contigs were identifies as GRSPaV and belong to four groups, which are similar to isolate VF1, SY, MG and GG, respectively (Lima et al. 2006; Morelli et al. 2011; Meng et al. 2013). GVA contigs were assembled into three variants and have only 83, 82 and 79% sequence identity with isolate I327-5, GTG11-1 and 3138-03, respectively (Goszczynski 2007, 2014), indicating they are new variants. Complete genome coverage was obtained for GVB, which is 97% identical to X75448 (Saldarelli et al. 1996). Near complete genome sequences of three distinct GLRaV-3 isolates were assembled. The first has 99% nt sequence identity with isolate 623 (Jooste et al. 2010);

the second, designated here as 8415A, is 76.2% identical to isolate GH24 (Maree et al. 2015) with 99.1% of genome coverage and consisted of 18,320 nt with no gap; the third isolate, which we designate as 8415B, has 67.1% nt sequence identity when compared to PL-20 with 99.1% of genome coverage and consisted of 18,373 nt without a gap.

8415A and 8415B represent two novel and distinctive variants of GLRaV-3: The sequences of GLRaV-3 isolates 8415A and 8415B generated from the *de novo* assembly were confirmed with RT-PCR and Sanger sequencing while their 5'- and 3'-terminal sequences have been obtained via RACE. The genome of 8415A (KY073323) is 18,573 nts long and contains 12 ORFs with ORF2 being absent. Based on the number of ORFs and phylogenetic analyses using full-length genome sequences (**Fig. 1**), it is evident that 8415A is more closely related to GH24 than to any other viral variants. However, it is still very distinct from GH24. For example, when the entire genome sequence was used in the comparison, 8415A is only 76.2% identical to GH24 (**Table 1**). Nucleotide sequence identities between homologous ORFs between the two isolates vary among the ORFs, ranging from 64.9% for ORF11 to 84.4% for ORF1b (not shown). The genome of 8415B (KY073324) is 18,564 nts long and contains 13 ORFs. Isolate 8415B seems to be distantly related to variants of groups I-V, all of which were classified into Supergroup A more recently (Maree et al. 2015). When full genome sequences were compared, sequence identities of 8415B to other isolates range from 65.2% (to 139) to 72% (to WA-MR, 623, PL-20) (Jarugula et al. 2010) (data not shown). Therefore, both isolates identified here represent novel and distinct variants of GLRaV-3.

This study not only revealed two new distinct variants of GLRaV-3, but also further demonstrates the power of NGS in the global detection and identification of viruses and viroids and their variants in grapevine.

Table 1. Summary of assembled contigs from sample 8415 and their mapping to viruses and viral variants

Viruses	GenBank accession no.	Reference isolates	Sequence identity (%)	No. of contigs	Genome coverage (%)
GLRaV-1	KU674796.1	WA-CH	99	2	99.9
GLRaV-3	GQ352632.1	623	99	3	96.6
	KM058745.1	GH24	76.2	2	99.1
	GQ352633.1	PL20	67.1	3	99.3
GRSPaV	KT948710.1	VF1	94 - 96	3	97.3
	HE591388.1	Syrah	96 - 97	3	96.1
	FR691076.1	MG	98	2	96.3
	JQ922417.1	GG	97 - 98	5	17.8
GVA	KC962564.1	I327-5	83	5	96.1
	DQ855084.2	GTG-11-1	82	9	77.9
	JX559641.1	3138-03	79	1	93.3
GVB	X75448.1	Italy	97	1	100

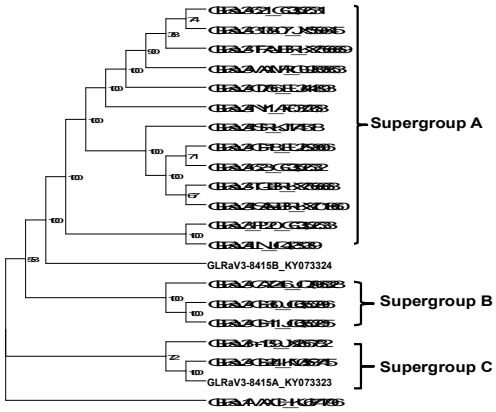

Figure 1. Phylogeny of full-length genomes of GLRaV-3 isolates. Neighbor Joining was used to construct the tree. GLRaV-1 isolate WA-CH was included as an outgroup.

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P30: Development of a quantitative PCR assay for the detection of *Grapevine red blotch virus*.

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INTRODUCTION

Grapevine red blotch virus (GRBV) is an emerging grapevine virus causing a non-curable and spreading disease. Typical symptoms caused by GRBV infection on red cultivars is reddening of leaf blade. Fruit quality on diseased vines is also impacted compared to healthy controls (Reynard and Gugerli, 2015). *Grapevine red blotch virus* is widespread in North America (Krenz et al., 2014). Due to its wide occurrence, transmissibility and impacts on grape quality, this emerging virus has the potential to cause serious economic losses.
Diagnostic reagents are needed for this pathogen since GRBaV will be tested in certification program in North America and this virus will be monitored in the rest of the world.

MATERIALS AND METHODS

All 27 complete GRBV genomes available at NCBI (stand May 2016) were aligned using Geneious R10. Several quantitative PCR assays (TaqMan) were designed in conserved regions. A host gene from *Vitis vinifera* was used in duplex as internal PCR control. All combinations were tested using healthy and infected grapevine samples as well as positive and negative controls available in the grapevine virus collection at Agroscope. DNAs from these samples were extracted using a rapid CTAB method. The best primers/probe setting based on sensitivity and specificity of detection was chosen to be deeper validated with the help of key laboratories in USA. Sampling will also be studied through the year to determine the best period and material (e.g. bark scrapings, young vs mature leaf) to be used for detection (ongoing work at Agroscope).

RESULTS AND DISCUSSION

DNA was extracted from different infected and healthy cultivars using the CTAB method and analysed by qPCR with the chosen assay at Agroscope and BIOREBA. The assay allows the detection of GRBV from each infected accession with a great sensitivity (limit of detection between dilutions of the extracted DNA 10e5 and 10e6). Ongoing study of material (wood, leaves) collected at different periods of the year will reveal which part of the plant to collect and at which season in order to get the most reliable detection of the virus.

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P31: Occurrence of Grapevine Viruses in Western Caucasus region of Russia

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INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most important cultures in the southern regions of Russia. The yield of grape is highly depends on the phytosanitary state of vineyard. Viruses cause yield reduction, graft union-incompatibility, negatively affect flowering, shoot and leaves growth.

MATERIALS AND METHODS

About 500 samples were collected from 16 vineyards of Western Caucasus region. RNA was extracted according to the protocol described Rott and Jelkman (1990). Viruses were tested by RT-PCR with specific primers followed by sequence of PCR-products.

RESULTS AND DISCUSSION

The results of diagnostics showed infection with the most common grapevine viruses. *Grapevine fleck virus* (GFkV) was detected in 4.2% samples, *Grapevine virus A* (GVA) in 2.5% samples, *Grapevine leafroll-associated virus*es-1 (GLRaV-1) in 1% samples, GLRaV-3 and *Grapevine rupestris stem pitting-associated virus* (RSPaV) in 0.5% and 0.4% samples respectively. 1.5% of samples had mixed GRSPaV and GFkV infection.

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Annexed Table 1

Table 1. Grapevine-infecting viruses. (from Martelli, 2017. Updated)

Family	Genus	Species
Viruses with isometric particles (+)ssRNA genome		
Secoviridae	*Fabavirus*	*Broad bean wilt virus* (BBWV), *Grapevine fabavirus* (GFabV)*;
	Nepovirus	*Artichoke Italian latent virus* (AILV), *Arabis mosaic virus* (ArMV), *Blueberry leaf mottle virus* (BBLMV), *Cherry leafroll virus* (CLRV), *Grapevine Bulgarian latent virus* (GBLV), *Grapevine Anatolian ringspot virus* (GARSV), *Grapevine deformation virus* (GDeV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine fanleaf virus* (GFLV), *Grapevine Tunisian ringspot virus* (GTRV), *Peach rosette mosaic virus* (PRMV), *Raspberry ringspot virus* (RpRSV), *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV), *Tomato black ring virus* (TBRV)
	Unassigned in the family	*Strawberry latent ringspot virus* (SLRSV)
Bromoviridae	*Alfamovirus*	*Alfalfa mosaic virus* (AMV)
	Cucumovirus	*Cucumber mosaic virus* (CMV)
	Ilarvirus	*Grapevine line pattern virus* (GLPV), *Grapevine angular mosaic virus* GAMoV)
Tombusviridae	*Carmovirus*	*Carnation mottle virus* (CarMV)
	Necrovirus	*Tobacco necrosis virus D* (TNV-D)
	Tombusvirus	*Grapevine Algerian latent virus* (GALV), *Petunia asteroid mosaic virus* (PAMV)
Tymoviridae	*Marafivirus*	*Grapevine Syrah virus 1* (GSyV-1)*, *Grapevine asteroid mosaic-associated virus* (GAMaV), *Grapevine rupestris vein feathering virus* (GRVFV), *Blackberry virus S* (BVS), unnamed putative marafivirus-like virus,
	Maculavirus	*Grapevine fleck virus* (GFkV) *Grapevine redglobe virus* (GRGV)
Luteoviridae	*Enamovirus*	*Summer grape enamovirus* (SGEV), *Grapevine enamovirus-1* (GEV-1)*
Viruses unassigned to families	*Idaeovirus*	*Raspberry bushy dwarf virus* (RBDV)
	Sobemovirus	*Sowbane mosaic virus* (SoMV)
Viruses with isometric particles dsRNA genome		
Reoviridae	*Oryzavirus* (?)	*Summer grape latent virus* (SGLV) = *Grapevine*

		Cabernet Sauvignon reovirus (GCSV)
Endornaviridae	*Endornavirus*	*Grapevine endophyte endornavirus* (GEEV), three unnamed grapevine-associated endornaviruses
Partitiviridae	*Deltapartitivirus*	*Grapevine cryptic virus 1* (GCV-1) = *Grapevine partitivirus 1* (GPV-1); an unnamed Grapevine-associated partitivirus
Amalgaviridae	*Amalgavirus*	An unnamed amalgavirus
Viruses with enveloped particles (-)ssRNA genome		
Bunyaviridae	*Tospovirus*	*Tomato spotted wilt virus* (TSWV)
Viruses with filamentous particles (+)ssRNA genome		
Closteroviridae	*Closterovirus* *Ampelovirus* *Velarivirus*	*Grapevine leafroll-associated virus 2* (GLRaV-2), *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine leafroll- associated virus 4* (GLRaV-4), *Grapevine leafroll-associated virus 13* (GLRaV-13) *Grapevine leafroll-associated virus 7* (GLRaV-7),
Alphaflexiviridae	*Potexvirus*	*Potato virus X* (PVX)
Betaflexiviridae	*Foveavirus*	*Grapevine stem pitting-associated virus* (GSPaV); *Grapevine virus T* (GVT)*
	Trichovirus	*Grapevine berry inner necrosis virus* (GINV), *Grapevine Pinot gris virus* (GPGV)*
	Vitivirus	*Grapevine virus A* (GVA); *Grapevine Virus B* (GVB); *Grapevine virus D* (GVD); *Grapevine virus E* (GVE); Grapevine virus F (GVF); *Grapevine virus G* (GVG)*; *Grapevine virus H* (GVH)*; *Grapevine virus I* (GVI)*; *Grapevine virus J* (GVJ)*
Potyviridae	*Potyvirus*	*Bean common mosaic virus* (BCMV) peanut strain; An unidentified *Potyvirus*-like virus isolated in Japan from a Russian cultivar
Viruses with rod-shaped particles (+)ssRNA genome		
Virgaviridae	*Tobamovirus* Undetermined	*Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV), *Grapevine virga-like virus* (GVLV)*
Viruses with a DNA genome		
Geminiviridae	*Grablovirus* Undetermined	*Grapevine red blotch virus* (GRBV)*; *Wild Vitis virus 1* (WVV1)*; *Grapevine geminivirus A* (GGVA)* *Temperate fruit decay-associated virus* (TFDaV)*
Caulimoviridae	*Badnavirus*	*Grapevine vein-clearing virus* (GVCV)*; *Grapevine Roditis leaf discoloration-associated virus* (GRLDaV)*; *Grapevine badnavirus 1* (GBV-1)*
Ill-defined, taxonomically unassigned viruses		Unnamed filamentous virus, *Grapevine Ajnashika virus* (GAgV), *Grapevine stunt virus* (GSV), *Grapevine labile rod-shaped virus* (GLRSV)

*Viruses marked by an asterisk were discovered by NGS

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