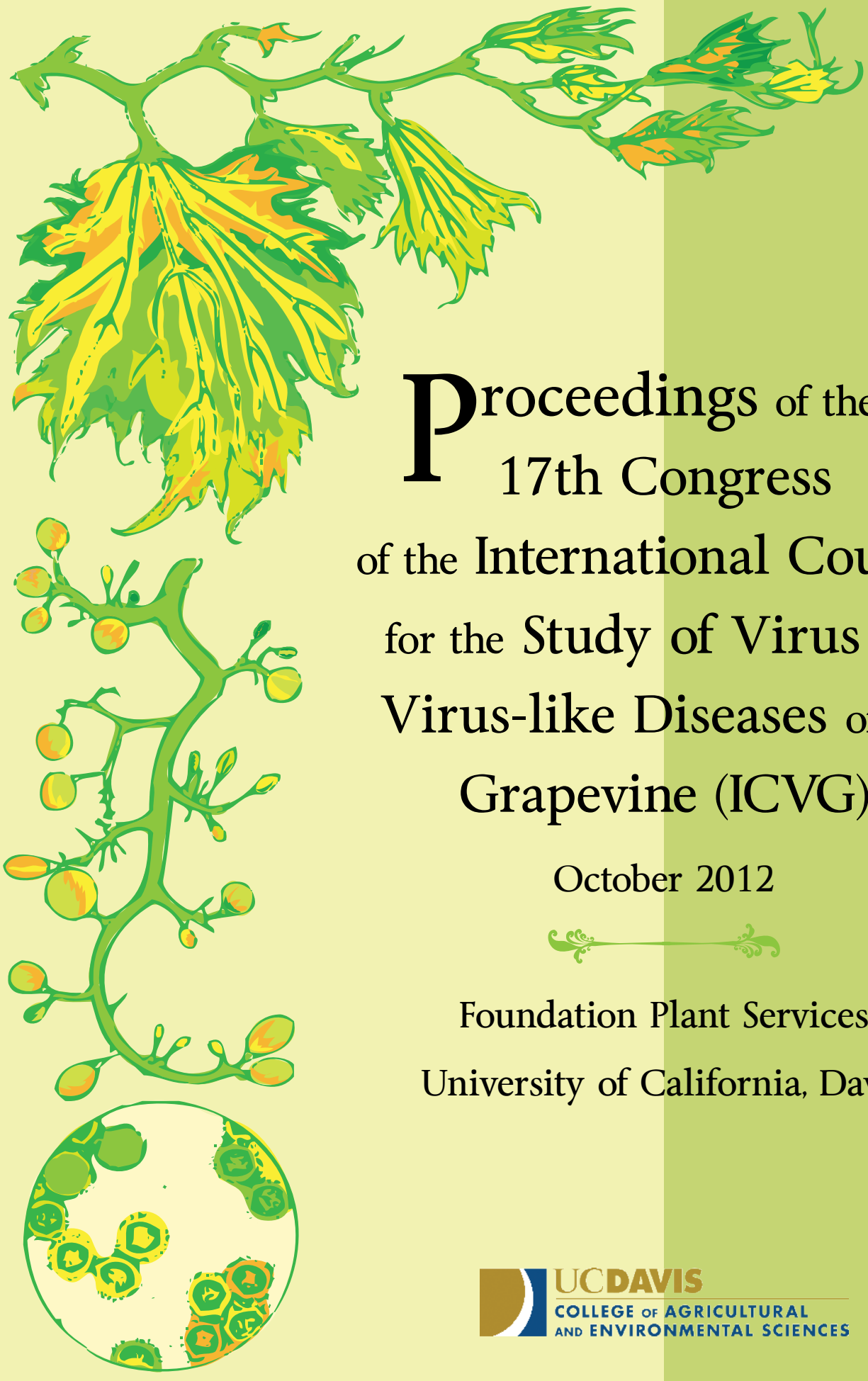

Proceedings of the 17th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG)

October 2012

Foundation Plant Services

University of California, Davis

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Our Thanks to our ICVG Supporters

We would like to acknowledge the special support of many who have made the 17th meeting of the ICVG possible. Because of their generosity, this meeting will be a memorable one.

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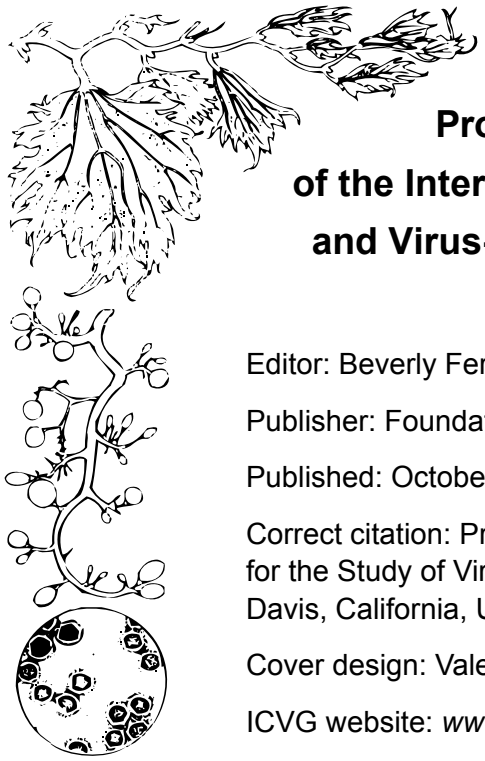
Welcome

It is a pleasure to welcome our colleagues and friends from around the world to the University of California at Davis for the 17th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG). ICVG has not met in Davis since 1965, during the early years of the organization. We feel honored to host this meeting celebrating 50 years of progress for ICVG, and hope that you enjoy the scientific presentations, opportunities to make new connections, and our beautiful State of California.

I would like to thank all of the attendees for coming, our sponsors for their financial support, and the staff of Foundation Plant Services for their many hours organizing the meeting and field trips.

Deborah Golino
Chair, 17th ICVG Organizing Committee



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A Short History of ICVG
Provided by R. Bovey and P. Gugerli

The idea of creating a scientific working group on virus diseases of grapevine came out during the third meeting on grapevine infectious degeneration organized by the *Office international de la Vigne et du Vin* (O.I.V.) in May 1962. The virologists present thought it would be useful to create an international study group independent from O.I.V., with the aim of providing an opportunity for grape virologists to discuss freely on their methods, their research and results.

Meetings
1. Changins, (Switzerland) 17-20 August 1964
2. Davis (California USA) 7-11 September 1965
3. Bernkastel-Kues (West Germany) September 1967
4. Colmar (France) 16-18 June 1970
5. Salice Terme (Italy) 16-19 September 1973
6. Cordoba and Madrid (Spain), 12-17 September 1976
7. Niagara Falls (Ontario, Canada) 7-12 September 1980
8. Bari (Italy) 2-7 September 1984
9. Kiryat Anavim (Israel) 6-11 September 1987
10. Volos (Greece) 3-7 September 1990
11. Montreux (Switzerland) 5-10 September 1993
12. Lisbon (Portugal) 28 September - 2 October 1997
13. Adelaide (South Australia) 12-17 March 2000
14. Locorotondo (Italy) 12-17 September 2003
15. Stellenbosch (South Africa) 3 – 7 April 2006
16. Dijon (France) 31 August - 4 September 2009
17. Davis (California, USA) 7-14 October 2012

17th Congress website: *ucanr.org/sites/ICVG*
Foundation Plant Services website: *fps.ucdavis.edu*
ICVG website: *www.icvg.ch*

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Session 3: Rugose wood and related viruses

Session 4: New viruses, disease of unknown etiology and viroids

Session 5: Diagnostics

Session 6: Virus effects

Session 7: Epidemiology

Session 8: Phytoplasmas

Dedication

These proceedings are dedicated to Mr. André Vuittenez. In 1962, André participated in the foundation of ICVG. He served for many years on our Steering Committee. It is fitting that we remember him with this dedication. We will all miss this very talented and active scientist in grapevine virology, who was also a founder of our organization and a perpetual Honorary Committee Member.

Grapevine Virology Highlights: 2010–2012

G. P. Martelli

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PREAMBLE

More than 300 papers dealing with various aspects of grapevine virology have been published since the Dijon Meeting in 2009, thus confirming, if there were the need of it, that infectious diseases of this crop still attract a lot of attention. Much goes to the merit of ICVG which, from its very foundation, has fostered the studies on these maladies and, thanks to the relentless activity of its members, has provided answers to some intricate problems such as, to quote a few, the aetiological nature of complex disorders like leafroll, rugose wood and graft-incompatibility. Much remains to be done, but with the help of increasingly powerful and sophisticated technologies, further advances can be expected in the years to come. As long as ICVG will remain the lively and enthusiastic group it proved to be for the last 50 years, hopes for further significant steps forward will be well founded.

SPECIALIZED MEETINGS AND REVIEWS

A meeting entitled 'Grapevine leafroll and vitivirus seminar - A continued and increasing problem for vineyards' was held in 2011 at Monterey (USA) in the framework of the 62nd National Conference of the American Society for Enology and Viticulture. The state of knowledge of both disorders and their agents was reviewed, with emphasis on the impact and incidence of the diseases, the molecular properties of the causal viruses, their epidemiology and the spatial pattern of spread.

The compilations analyzing in detail the properties of grapevine-infecting vitiviruses (35) and of *Grapevine leafroll-associated virus 3* (GLRaV-3) (88), are two of the endeavours aimed at summarizing the current information on specific grapevine-infecting viruses. Other reviews have adressed virus detection methods (48), grapevine virology at large (86) and the state-of-the-art of the knowledge on tolerance and resistance of *Vitis* to viruses and their vectors (102) and on leafrolll and vitivirus diseases (87).

SURVEYS AND NEW RECORDS OF KNOWN VIRUSES AND VIROIDS

First records of known viruses were: *Grapevine leafroll-associated virus 5* (GLRaV-5) in Turkey (25) and Spain (104); *Arabis mosaic virus (*ArMV) in Spain (2); GLRaV-4 and GLRaV-5 in China (108); GLRaV-5 in Portugal (43); GLRaV-4, -5, -7 and -9 and *Grapevine Syrah virus 1* (GSyV-1) in Chile (39,40,42); GSyV-1 in Washington state (94), Italy (51) and France (21), *Grapevine fleck virus* (GFkV) in Washington state (99), GLRaV-2 and GVB in Croatia (125, 126). *Vitis californica* and *Vitis californica* x *Vitis vinifera* hybrids proved to be natural hosts of *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll-associated virus 2* (GLRaV-2) and GLRaV-3 (74).

Citrus exocortis viroid (CEVd) was recorded in China (116), *Grapevine yellow speckle viroid 1* (GYSV-1) and *Hop stunt viroid* (HSVd) in New Zealand (130), *Grapevine yellow speckle viroid 2* (GYSVd-2) and *Grapevine Australina viroid* (GAVd) in Italy (F. Di Serio, personal communication).

A survey conducted in the Atacama region of Chile revealed a very high infection rate (*ca*. 70%) which was held responsible for a decrease in table grape production in the area (44). This situation pretty much resembles that observed in autochthonous wine grape cultivars in the island of Majorca (Spain), whose level of infection (60 to 78%) impaired also a proficient sanitary selection (33), and the condition of table grapes in Alicante (Spain) where infections of 96% (GFLV), 95% (GLRaV-3), 66% (GLRaV-1) and 65% (GfkV) were detected (19). The overall incidence of viral infections in Istriain (Croatia) vines, including autocthonous varieties, was over 82%, the most

This paper is dedicated to the memory of Dr. R.G. Bonfiglioli, in recognition of his academic skill, the clear vision in any aspect of research for the wine industry and his dedicated activity for the betterment of the New Zealand grapevine industry.

frequent virus being GLRaV-3 (69%), followed by GFLV (24%) and GLRaV-1 (17%) (109). No better was the sanitary conditions of two Croatian collections of native grapevine germplasm at Zagreb and in the island of Krk, in which very high infection levels by eight viruses (GFLV, ArMV, GFkV, GVA, GVB, GLRaV-1, -2, -3) were ascertained so that only 10.5% and 7.4% of the vines in the Zagreb and Krk collections, respectively, were free from these viruses (125). A survey conducted in the vineyards of the Argentinean province of Mendoza, specifically aimed at assessing the incidence of GLRaV-1, -2 and -3, disclosed that GLRaV-2 was the only virus present in all the clones analyzed with an incidence of 19%, whereas the infection rate by GLRaV-1 and -3 was negligeable (0.6 and 1.2%, respectively) (123). In the state of Virginia (USA) over 50% of the vines tested proved to be infected by distinct isolates of GLRaV-2, GLRaV-3 and GFkV (100), whereas the infection rate by GLRaV-1 and GLRaV-3 in the vineyards of the Finger lakes area (New York state), was estimated in the range of 60% (91).

NEW DISEASES AND VIRUSES

The increasingly popular “deep” or “high-throughoutput” or “next generation” sequencing allowed the discovery of three hitherto unknown viruses. Remarkably, one of these, denoted Grapevine vein clearing virus (GVCV), is the first with a DNA genome ever found in *Vitis*. It is associated with a disease called ‘Grapevine vein clearing and vine decline syndrome’ (133). GVCV is a putative member of the genus *Badnavirus,* has a circular double-stranded DNA genome 7,753 bp in size, comprising three ORFs, two of which (ORF1 and ORF2) code for proteins of 208 aa (24.2 kDa) and 127 aa (14,3 kDa) respectively, both with unknown function. ORF3 encodes a polyfunctional protein of 1,941 aa (219.5 kDa) containing the movement protein (MP), coat protein (CP), reverse transcriptase and RNase H signatures. GVCV was detected in six grapevine cultivars in three US states (Missouri, Indiana and Illinois), indicating that it may have a wide distribution in the country’s Midwest (128).

The second virus, which was given the provisional name of *Grapevine Pinot gris virus* (GPGV), was originally identified in a cv. Pinot gris plant showing a syndrome characterized by leaf mottling and stunting (51). This vine was also infected by two viroids (HSVd and GYSVd-1), and three additional viruses, i.e. *Grapevine rupestris stem pitting-associated virus* (GRSPaV), Grapevine rupestris vein feathering virus (GRVFV) and, as mentioned above, GSyV-1. GPGV has a linear, single-stranded, positive-sense RNA genome 5,744 nt in size, consisting of three slightly overlapping ORFs. These encode, in the order, the replication-associated proteins (ORF1, 1,865 aa, 214 kDa), a 30K-like MP (ORF2, 376 aa, 42 kDa) and the CP (195 aa, 22 kDa). This genome structure is typical of members of the genus *Trichovirus*, to which GPGV is being assigned. Interestingly, GPGV is phylogenetically closest to *Grapevine berry inner necrosis virus* (GBNV), a Japanese trichovirus transmitted by eriophyid mites. Although the first record of GPGV came from Trento and surrounding area (north-east Italy), the virus, which also infects cvs Traminer and Pinot noir, may have a wider distribution. Its epidemiology is now being investigated.

The third virus, a likely new member of the genus *Vitivirus* tentatively called Grapevine virus F (GVF), was found in accession AUD46129, a black-berried grapevine from California, which induces graft incompatibility in cv. Cabernet sauvignon grafted on different rootstocks. The virus has a single-stranded RNA genome 7,551 nt in size, comprising five ORFs with a vitivirus-like organization. These encode, in the order, the replication-associated proteins (ORF1), a 20 kDa protein with unknown function (ORF2), the MP (ORF3), the CP (ORF4) and a 12 kDa protein with putative RNA-binding properties (ORF5) (9a).

A fourth novel virus denoted Grapevine Cabernet franc-associated virus (GCFaV), reported from New York state (USA), has a circular single-stranded DNA genome with a structure comparable to that of members of the family *Geminiviridae*. The viral genome is 3,206 nt in size and contains six ORFs, three in the viral sense and three in the complementary sense orientation. In phylogenetic trees, constructed with the CP, polymerase, or the full-length sequence, GCFaV forms a distinct branch, separate from those comprising members of the four extant genera of the family *Geminiviridae* (75). This is the second geminivirus-like virus infecting a woody species, and the first ever found in grapevines.

With the addition of GCFaV, grapevine-infecting viruses grow to 63. This is a veritably unprecedented situation for, to the best of my knowledge, none of the woody crops hosts such a high and variegated number of viruses exhibiting the whole set of genome types: single-stranded DNA, double-stranded DNA, double-stranded RNA, single-stranded negative-sense RNA, single-stranded positive-sense RNA: a fascinating virus world (Table 1).

Deep sequencing has also been used to investigate the "virome" of a whole South African vineyard, revealing that the prevailing virus was GLRaV-3 (58.5%) followed by GRSPaV (3.8%), two vitiviruses [GVA (1.0%) and GVE (0.9%)] and representatives of different mycoviruses (31). The latter finding conforms to the data from a comparable analysis conducted in California, which disclosed in single vines a "virome" dominated by the presence of 26 putative fungal virus groups (9). Deep sequencing analysis of virus-derived short interfering RNAs (vsiRNAs) isolated from cv. Pinot noir clone ENTAV 115, one of the vines whose complete genome sequence

Table 1. The viral scenario of Vitis and Muscadinia: viruses and their taxonomic affiliation(a)

FAMILY	GENUS	SPECIES
A. Viruses belonging to genera included into families		
Viruses with a single-stranded DNA genome		
GEMINIVIRIDAE	Undetermined	Grapevine cabernet franc-associated virus (GCFaV)
Viruses with a double-stranded DNA genome		
CAULIMOVIRIDAE	*Badnavirus*	Grapevine vein clearing virus (GVCV)
Viruses with a double-stranded RNA genome		
REOVIRIDAE *ENDORNAVIRIDAE* *PARTITIVIRIDAE*	*Oryzavirus* *Endornavirus* *Alphacryptovirus*	Unnamed virus Two unnamed viruses Raphanus sativus cryptic virus 3 (RsCV-3) like Beet cryptic virus 3 (BCV-3) like
Viruses with a negative-sense single-stranded RNA genome		
BUNYAVIRIDAE	*Tospovirus*	*Tomato spotted wilt virus* (TSWV)
Viruses with a positive-sense single-stranded RNA genome (filamentous particles)		
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) GLRaV-4 strain 5 GLRaV-4 strain 6 GLRaV-4 strain 9 GLRaV-4 strain Car Grapevine leafroll-associated virus 7 (GLRaV-7)
ALPHAFLEXIVIRIDAE	*Potexvirus*	*Potato virus X (PVX)*
BETAFLEXIVIRIDAE	*Foveavirus* *Trichovirus* *Vitivirus*	*Grapevine rupestris stem pitting-associated virus* (GRSPaV) *Grapevine berry inner necrosis virus* (GINV) *Grapevine Pinot gris virus* (GPGV) *Grapevine virus A* (GVA) *Grapevine virus* B (GVB) *Grapevine virus D* (GVD) *Grapevine virus E* (GVE) Grapevine virus F (GVF)
POTYVIRIDAE	*Potyvirus*	Unidentified potyvirus-like virus isolated in Japan from a Russian cv *Bean common mosaic virus* (BCMV), peanut strain
Viruses with a positive-sense single-stranded RNA genome (rod-shaped particles)		
VIRGAVIRIDAE	*Tobamovirus*	*Tobacco mosaic virus* (TMV) *Tomato mosaic virus* (ToMV)

Table 1 continued

Viruses with a positive-sense single-stranded RNA genome (isometric particles)		
SECOVIRIDAE	*Fabavirus* *Nepovirus* *Sadwavirus*	*Broadbean wilt virus* (BBWV) *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 (*GDefV) *Grapevine chrome mosaic virus* (GCMV) *Grapevine fanleaf virus* (GFLV) *Grapevine Tunisian ringspot virus* (GTRV) *Peach rosette mosaic virus* (PRMV) *Raspberry ringspot virus* (RpRV) *Tobacco ringspot virus* (TRSV) *Tomato ringspot virus* (ToRSV) *Tomato blackring virus* (TBRV) *Strawberry latent ringspot virus* (SLRSV)
BROMOVIRIDAE	*Alfamovirus* *Cucumovirus* *Ilarvirus*	*Alfalfa mosaic virus* (AMV) *Cucumber mosaic virus* (CMV) Grapevine line pattern virus (GLPV) Grapevine angular mosaic virus (GAMoV)
TOMBUSVIRIDAE	*Carmovirus* *Necrovirus* *Tombusvirus*	*Carnation mottle virus* (CarMV) *Tobacco necrosis virus D* (TNV-D) *Grapevine Algerian latent virus* (GALV) *Petunia asteroid mosaic virus* (PAMV)
TYMOVIRIDAE	*Marafivirus* *Maculavirus*	*Grapevine asteroid mosaic-associated virus* (GAMaV) Grapevine redglobe virus (GRGV) Grapevine Syrah virus 1 (GSV-1) *Grapevine fleck virus* (GFkV) Grapevine rupestris vein feathering virus (GRVFV)
B. Viruses belonging to genera unassigned to families		
	Idaeovirus *Sobemovirus*	*Raspberry bushy dwarf virus* (RBDV) *Sowbane mosaic virus* (SoMV)
C. Taxonomically unassigned viruses		
		Unnamed filamentous virus Grapevine Ajinashika virus (GAgV) Grapevine stunt virus (GSV) Grapevine labile rod-shaped virus (GLRSV) Southern tomato virus (STV)

(a)Scientific names of definitive virus species are written in italics. The names of tentative species are written in Roman characters. The updated taxonomy of all classified grapevine viruses can be found in 73a. This table comprises also the new viruses reported from south-eastern USA (111) a detailed description of which has not yet been published.

has been published, cast light on the virome of this vine which, in addition to the GRSPaV and GFLV, appeared to be widely represented by the maculaviruses GFkV and Grapevine red globe virus (GRGV) and the marafiviruses Grapevine asteroid mosaic-associated virus (GAMaV) and Grapevine rupestris vein feathering virus (GRVFV), all of which are latent in *Vitis vinifera* (107).

In addition to the above-mentioned novel diseases (51, 128): (i) a very severe and fatal disorder of cv. Chardonnay, characterized by translucent vein clearing was observed in Missouri (USA) and attributed to a mixed infection by GFLV, GRSPaV and *Tomato ringspot virus* (ToRSV) (80a) and (ii) a new disease was described in California under the name of 'Grapevine necrotic union.' The symptoms (bright reddening of the canopy and pronounced swelling at the graft union) are those typically associated with graft incompatibility, but none of the viruses detected in affected vines, among which GRSPaV, could be identified as the causal agent of the disease, whose aetiology, therefore, remains undetermined (10).

NEW DEVELOPMENTS IN TAXONOMY: REVISION OF THE GENUS *AMPELOVIRUS*

It is common knowledge that grapevines host a high number of viruses belonging in the family *Closteroviridae,* all of which are associated with leafroll disease (GLRaVs). Currently, GLRaV-2 is assigned to the genus *Closterovirus,* comprising aphid-transmitted viruses, GLRaV-1, -3, -4, -5, -6, -8 and -9 are classified as approved or putative species in the genus *Ampelovirus,* comprising exclusively mealybug-transmitted viruses, whereas GLRaV-7 has the status of unassigned putative species to the family (90) (Table 2).

Table 2. Current classification and some properties of Grapevine leafroll-associated viruses (GLRaVs).

Virus	Genus	Coat protein (kDa)	Genome size (nts) (GenBank Access. No.)	ORFs (No.)	Vectors	First record *fide* 23, 90
GLRaV-1	*Ampelovirus*	34	18,659 (JQ023131)	9	Mealybugs, soft scale insects	59
GLRaV-2	*Closterovirus*	22	16,494 (AY88162)	8	Unknown	134
GLRaV-3	*Ampelovirus*	35	18,498 (EU259806)	12	Mealybugs, soft scale and scale insects	132
GLRaV-4	*Ampelovirus*	35	13,830 (FJ467503)	6	Mealybugs	65
GLRaV-5	*Ampelovirus*	35	13,384[a] (FR822696)	6	Mealybugs	127,134
GLRaV-6	*Ampelovirus*	35	13,807 (FJ467504)	6	Mealybugs	60,61
GLRaV-7	Unassigned in the family	37	16,496 (HE588185)	10	Unknown	28
GLRaV-8[b]	*Ampelovirus*	37	ND	ND	Unknown	96
GLRaV-9	*Ampelovirus*	35	12,588[a] (AY29781)	6	Mealybugs	8
GLRaV-Pr	*Ampelovirus*	30	13,696 (AM182328)	6	Mealybugs	83
GLRaV-Car	*Ampelovirus*	29	13,626 (FJ907331)	6	Unknown	4

[a]Nearly complete sequence; [b]Cancelled from the 9th ICTV Report (Martelli *et al*., 2012a); ND, not determined.

In the last four years or so, new ampelovirus isolates have been described, three of which have extensively or totally been sequenced and proposed as putative new species: Grapevine leafroll-associated virus Pr (GLRaV-Pr), Grapevine leafroll-associated virus De (GLRaV-De) (82, 83) and Grapevine leafroll associated-Carnelian virus (GLRaV-Car) (4). So, by 2011, the number of GLRaVs had grown to 12: one closterovirus (GLRaV-2), 10 ampeloviruses (GLRaV-1, -3, -4, -5, -6, -8, -9, GLRaV-Pr, GLRaV-De, GLRaV-Car) and one unassigned species (GLRaV-7) (Table 1). Such an unique situation -none of the known virus diseases of any woody crop has such a high number of agents of the same type implicated in its aetiology- called for its critical appraisal and, eventually, revision. This was made possible when: (i) the complete nucleotide sequence of GLRaV-4, -5 and -6, became available (5,11,71,120); (ii) a new sets of monoclonal antibodies to GLRaVs was produced (62); (iii) the discriminating species demarcation threshold for three taxonomically relevant genes (polymerase, HSP70h and CP) was raised from 10% to 25% (90); (iv) GLRaV-8 was removed from the membership of the genus *Ampelovirus* following the discovery that its sequence, rather than being of viral origin, is part of the grapevine genome (20).

A novel taxonomic scenario was therefore delineated whereby GLRaV-4 becomes a reference species comprising the formerly approved (GLRaV-5) and putative [GLRaV-6 (and its -De variant) and GLRaV-9] ampelovirus species and the unclassified GLRaV-Pr and GLRaV-Car (90).

The foundation on which this revision rests is the recognition that: (i) all viruses have the smallest (*ca.* 13,700 nts) and the simplest [six ORFs (seven genes) and the lack of a recognizable CPm] genome within the family *Closteroviridae;* (ii) the molecular divergence at the amino acid level of the polymerase, HSP70h and CP genes of none of the viruses exceeds 25%, with the exception of the 33% value shown by the GLRaV-Car HSP70h; (iii) all viruses have a similar biological behaviour, i.e. association with a symptomatology milder than that elicited by GLRaV-1 and GLRaV-3 and transmissibility by pseudococcid mealybugs, as experimentally ascertained for GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-9, and a Cypriot isolate of GLRaV-Pr (36a,78,117,121).

Thus, GLRaV-4 forms, together with *Plum bark necrosis stem pitting-associated virus* (PBNSPaV), *Pineapple mealybug wilt-associated virus 1* (PMWaV-1) and *Pineapple mealybug wilt-associated virus 3* (PMWaV-3), a phylogenetically coherent cluster of species comprised in a distinct clade denoted Subgroup II, which is significantly differentiated from Subgroup I (see also 24) (Table 3).

Table 3. The new configuration of the family *Closteroviridae*[(a)]

GENUS	**VECTOR**
Closterovirus	Aphids
Ampelovirus Subgroup I *Grapevine leafroll-associated virus 1* (GLRaV-1) *Grapevine leafroll-associated virus 3* (GLRaV-3) *Little cherry virus 2* (LChV-2) *Pineapple mealybug wilt-associated virus 2* (PMWaV-2) Subgroup II *Grapevine leafroll-associated virus 4* (GLRa-4) *Pineapple mealybug wilt-associated virus 1* (PMWaV-1) *Pineapple mealybug wilt-associated virus 3* (PMWaV-3) *Plum bark necrosis stem pitting-associated virus (*PBNSPaV)	Pseudococcid mealybugs, soft scale and scale insects
Crinivirus	Whiteflies
Velarivirus Grapevine leafroll-associated virus 7 (GLRaV-7) Little cherry virus 1 (LChV-1) Cordyline virus 1 (CoV-1)	Unknown
Viruses unassigned in the family	
Olive leaf yellowing-associated virus (OLYaV) Mint vein banding virus (MVBV)	Unknown Aphids

[a]Proposal submitted to the evaluation of the International Committee on Taxonomy of Viruses for formal approval.

NEW DEVELOPMENTS IN TAXONOMY: THE GENUS *VELARIVIRUS*

GLRaV-7, originally found in an unidentified grapevine cultivar from Albania (Choueiri *et al.,* 1996), has a rather wide geographical distribution comprising European (Albania, Armenia, Greece, Hungary, Italy, Switzerland), Near East (Egypt, Palestine, Turkey), North (USA) and South (Chile) American countries and China. Partial sequencing of its genome disclosed differences with members of the two monopartite closterovirus genera (*Closterovirus* and *Ampelovirus*) suggesting its classification as an unassigned putative species in the family *Closteroviridae*, a position that it shares with Little cherry virus 1 (LChV-1) (89).

Two GLRaV-7 isolates have now been sequenced (11,71). The viral genome consists of 16,496 nt arranged in 10 ORFs which encode in the order: (i) a polyprotein 267 kDa in size, comprising the protease, methyltransferase, and helicase domains (ORF1a) and the 60 kDa RNA-dependent RNA polymerase (ORF 1b); (ii) a 8 kDa putative transmembrane protein (ORF2) that overlaps ORF1b; (iii) a 4 kDa hydrophobic protein with a putative transmembrane domain (ORF3); (iv) the 62 kDa HSP70h protein (ORF4); (v) a 10 kDa protein showing homology with proteins p4 to p10 coded for by RNA-2 of some criniviruses (ORF5); (vi) a 61 kDa protein matching the comparable product, referred to as "p60", encoded by all members of the family *Closteroviridae* (ORF6); (vii) the coat protein (CP) 34 kDa in size (ORF6) and (viii) the minor coat protein (CPm) 69 kDa in size (ORF7). ORF9 and ORF10 putatively code for a 25 kDa and a 27 kDa protein, respectively, neither of which shares similarities with other viral proteins in the current database.

The genome structure of GLRaV-7 resembles that of LChV-1 and of Cordyline virus 1 (CoV-1), a novel closterovirus-like virus infecting ti plants (*Cordyline fruticosa*) in Hawaii (92). In phylogenetic trees constructed with the HSP70h sequences, these three viruses group together in a clade related to, but distinct from that comprising members of the genus *Crinivirus*. Differences of GLRaV-7, LChV-1 and CoV-1 with members of the three extant genera of the family *Closteroviridae* reside in:

Ampeloviruses: (i) genome size and structure [number of genes intermediate between that of the largest (Subgroup I) and the smallest (Subgroup II) members of the genus]; (ii) biological traits, i.e. lack of a recognized vector, transmission through dodder to herbaceous hosts [ascertained for GLRaV-7 and LChV-1 (70,95)]; (iii) distant phylogenetic relationships (RdRp, HSP70h and CP protein identity at the aa level always lower than 30% for any gene).

Closteroviruses: (i) genome size and structure (lower number of genes, CPm preceding CP); (ii) lack of a recognized vector; (iii) distant phylogenetic relationships, RdRp, HSP70h and CP protein identity at the aa level always lower than 30% for any gene.

Criniviruses: Genome structure (monopartite versus bipartite/tripartite, diverse gene arrangement); (ii) lack of a recognized vector; (iii) phylogenetic relationships closer than that with closteroviruses and ampeloviruses but still distant (RdRp, HSP70h and CP protein identity at the aa level slightly exceeding 50% only for the polymerase gene).

Overall, these differences seemed to be relevant enough to support the suggestion that a fourth genus with the provisional name of *Velarivirus* comprising GLRaV-7, LChV-1 and CoV-1 could be created within the family *Closteroviridae* (90). To become effective, the proposed taxonomic modifications must be examined by the various bodies of the ICTV and ratified by the ICTV Plenum. This procedure has just been initiated.

ADVANCES IN MOLECULAR BIOLOGY

The genome expression strategy of GLRaV-3 was found to conform largely to that of other closteroviriruses, encompassing: (i) direct translation and proteolitic processing of the polyprotein encoded by ORF1a; (ii) +1 ribosomal frameshift for the expression of the polymerase domain encoded by ORF1b; (iii) a set of eleven 3' co-terminal monocistronic subgenomic RNAs for the expression of proteins encoded by ORF2 through ORF12. Subgenomic RNAs for CP, p21, p20A and p20B genes of the US virus isolate WA-MR were the most abundant, being 4,699, 2,226, 1,744 and 1,234 nt in size, respectively (67). Eight subgenomic RNAs with a size ranging from 9,497 to 1,233 nts had also been identified in infections by the South African GLRaV-3 isolate GP18 (84). The difference in genome size between isolates NY-1/Cl-766 (17,919 nt) and five and more recently sequenced isolates (18,433-18,498 nts) depends on the length of the 5' non translated region (NTR), which was determined to be 158 nt long in NY-1/Cl-766, but 737 nt long in four of the other virus isolates. The latter figure (737 nt) is thought to be the correct one (67). The 5' NTR of isolate WA-MR folds into a complex secondary structure with

several substructural hairpins of variable length, differing from the less complex one reported for the South African isolates (67). By contrast, the 3' NTR of all isolates has the consistent length of 277 nts, is more conserved, and folds always into a secondary structure consisting of two principal hairpins, the 5' most of which contains four substructural hairpins (67).

The capsid protein of GRSPaV possesses a nuclear localization signal that actively targets it to the cell nucleus. The significance of this finding has not been determined. However, the similarity with the *Cucumber mosaic virus* (CMV) RNA silencing supressor protein 2b, which has a nuclear localisation, suggests that also GRSPaV CP may have a comparable role (93).

It was experimentally confirmed that the expression product of the ORF3 of GVA and GVB which, based on molecular evidence was identified as a movement protein (MP), has indeed this property. In fact, in *Nicotiana benthamiana* leaves agroinoculated with MP-green fluorescent protein constructs, the proteins of both viruses localize at plasmodemata and induce the formation of tubular structures at the periphery of *N. benthamiana* and *V. vinfera* protoplasts (64a).

Major advances are being made in the construction of infectious full-length clones of GLRaVs. Their availabilty will serve multiple purposes, among which the ultimate determination of the role of these viruses in the aetiology of leafroll disease. (i) An infectious full-length cDNA clone of GLRaV-3 was assembled through a multistep cloning strategy and, after insertion into a binary vector, was agro-infiltrated into *Nicotiana benthamiana* leaves. The contruct is infectious, as shown by the presence in *N. benthamiana* extracts of subgenomic RNAs with a profile similar to that obtained from naturally infected grapevines and of filamentous virus particles (69). (ii) As a follow up to the successful synthesis of an infectious cDNA clone of GLRaV-2 (80) a GLRaV-2-based vector which can accomodate large inserts (*ca.* 2kb) has been developed and successfully used for infecting *N. benthamiana* and *Vitis vinifera* plants by agroinoculation, micropropagated *V. vinifera* plantlets by vacuum infiltration, and a number of grapevine cultivars by grafting (77). This represents a veritable breakthrough in grapevine virology due to the multiple functions that such a powerful tool can perform. In fact, this vector appears to be extremely stable, hence liable to yield reproducible results. Furthermore, as it expresses recombinant proteins in phloem tissues throughout the plant, it can be used for introducing novel desired traits in the host without heritable modification to its genome, including resistance to pathogens, thus functioning as a substitute for the still widely opposed *Agrobacterium*-mediated plant transformation. As an additional desirable property, this vector is expected to be environmentally safe, since the virus it derives from is apparently not insect-transmitted (77). (iii) GFLV RNA-2 was modified by inserting a multiple cloning site, duplicating a protein cleavage site for the proteolitic realease of heterologus proteins, mutating the CP amino acid residues responsible for nematode transmission. The modified RNA-2 cDNA and the RNA-1 cDNA were cloned into a plant espression vector which, following agro-infiltration, proved very promising for it was able to silence *N. benthamiana* genes via virus-induced gene silencing and to overexpress heterologous proteins. Its behaviour in grapevines is being investigated (57, M. Fuchs, personal communication).

ADVANCES IN MOLECULAR BIOLOGY: NEW SEQUENCES OF KNOWN VIRUSES

A South African isolate of *Grapevine virus E* (GVE) has been completely sequenced (32). The genome is a single-stranded, positive-sense RNA 7,568 nt in size, that comprises 5 ORFs encoding in the order: ORF1 (5,100 nt, 1,699 aa), replication-associated proteins with an AlkB domain located within the helicase domain; ORF2 (576 nt) a protein with unknown function; ORF3 (807 nt), a 30K-like movement protein (MP); ORF4 (600 nt), the CP; ORF5 (351 nt), a nucleotide-binding protein. Such genomic organization conforms to that of known members the genus *Vitivirus.*

The newly sequenced larger segment (RNA-1) of the nepovirus *Grapevine deformation virus* (GDefV) consists of 7,836 nt and contains a single ORF coding for a polypeptide of 252 kDa. As with other nepoviruses, the GDefV RNA-1-encoded product is cleaved into 5 proteins of 45 kDa (putative proteinase co-factor), 88 kDa (helicase), 3 kDa (VPg), 25 kDa (proteinase), 91 kDa (polymerase). The unusual trait of this virus is that it is a recombinant between *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV). Recombination sites were identified in the previously sequenced RNA-2 (3) at the C-terminal part of the MP and CP cistrons, and in the 3' non coding region (38). This seems to be the first case of interspecies recombination in nepoviruses involving the CP domain that leads to the emergence of a new viral species. Interspecific GFLV/ArMV recombinants

are not rare. Similarly to past records, they were recently detected in the polymerase, homing protein (HP) and MP genes of a number of GFLV isolates from California but not in the CP gene, where, by contrast, intraspecies (GFLV/GFLV) recombinations prevail and do not modify the taxonomic status of the recombinant (101). This confirms earlier data showing the occurrence of intraspecies recombination in the MP and CP of GFLV field isolates between mild protective strains of GFLV and ArMV during a 12-year cross protection trial (122).

Grapevine Bulgarian latent virus (GBLV) is an additional nepovirus fully characterized molecularly (37). RNA-1 (7,452 nt in length) contains a single ORF encoding a polyprotein of 234 kDa with the conserved motifs proper of members of the order *Piconavirales.* RNA-2 (5,821 nt) has also a single ORF coding for a 167 kDa protein which is cleaved to yield the homing protein, MP and CP. The latter has the highest homology at the aa level with the CP of *Blueberry leaf mottle virus* (BLMV), which is in line with the notion that GBLV and BLMV are serologically related.

The genome of the molecular variant of GLRaV-2 from Italy denoted GLRaV-2 BD proved to consist of 16,535 nt organized in 8 ORFs like the other members of the genus *Closterovirus*. The molecular divergence with other recognized GLRaV-2 strains ranges from 21 to 28%, the closest being the more aggressive RG strain, which is involved in graft incompatibility (17).

ADVANCES IN MOLECULAR BIOLOGY: MOLECULAR VARIANTS OF KNOWN VIRUSES

Novel variants of GRSPaV have been identified. (i) A variant found in a vine of cv. Moscato giallo from southern Italy, called GRSPaV-MG, has a genome 8,725 nt in size, encompassing six ORFs with an arrangement typical of members of the genus *Foveavirus* and identical to that of virus isolates possessing a sixt putative ORF. ORF1 codes for a polypeptide 2,161 aa in size possessing the domains proper to the viral replication-associated proteins plus an AlkB and an OTU-like cystein protease domain; ORF2, 3 and 4 are the triple gene block whose expression products are involved in cell-to-cell virus movement; ORF5 is the CP cistron (28 kDa) and ORF6 encodes a 13 kDa protein with unknown function. Of the five already known isolates of this virus, denoted GRSPaV-SG1, GRSPaV-BS, GRSPaV-SY, GRSPaV-PN and GRSPaV-1, the latter is phylogenetically the closest to GRSPaV-MG. These two latter strains have a similar biological behaviour for neither of them induces pitting in *Vitis rupestris* but both cause vein necrosis in the indicator 110R (98). (ii) Two additional groups of distinct sequence variants, denoted GRSPaV-MT (from cv. Muller Thurgau) and ML (from cv. Merlot) were described following RT-PCR examination of collection of 101 grapevine accessions from Italy, Canada and the USA using a new set of degenerate primers and partial sequencing of viral genomes (118,119). The pathogenicity of these variants has not been investigated. None of the five “old” GRSPaV variants (see above) seems to be involved in the aetiology of Shiraz decline in South Africa (54), a finding confirmed by a more recent French study (21). A study of the GRSPaV population of the Pacific north-west of the USA, revealed virus segregation in four of the already known clusters of this virus. Interestingly, it was found that grafted cultivars contained more genetic variants than self-rooted vines and that the viral CP cistron was more variable than the helicase region (6). The collective recognition of nine divergent variants of GRSPaV makes this virus one of the most molecularly differentiated among the grapevine-infecting viruses.

In addition to GRSPaV, an increasing number of papers report the presence of molecular variants of different grapevine viruses from various countries. For example, comparison of the central part of the CP gene of 36 isolates of GFkV showed that this virus comprises two disticnt groups of molecular variants (52). Likewise, two distinct phylogenetic clusters were identified comparing the polymerase sequence of GFLV isolates from the USA, France, Italy, Czech Repubilc and New Zealand (36). Two clusters of molecular variants were identified also in *Tomato ringspot virus* (ToRSV) isolates from North America (79), and a Chinese study on the variability of GVA reported that the local isolates of group I cluster into two subgroups denoted IA and IB (129).

GLRaVs seem to be the preferred target for this type of exercises, which most frequently address the sequence of their HSP70h and CP genes. Thus: (i) 3 phylogenetic groups of GLRaV-1 were identified among isolates from three US states (California, Washington and New York) (7); (ii), 3 genetic variants of GLRaV-3 were found in South Africa (73), 5 in Portugal (58), 7 in California (115), 4 in 50 vines of cv. Merlot in California (128), and 2 in India (76); (iii) 6 lineages of GLRaV-2, were reported from the Pacific northwest of the USA (68), 5 from Italy (18); (iv) 8 lineages of GLRaV-5 were identified through the comparative analysis of the nucleotide sequences obtained from Portugese isolates and retrieved from database (43).

Undoubtedly, this type of information is useful as it assesses the intraspecies molecular variability of virus populations, that may originate from multiple introductions of inoculum in a given area by vectors or by infected propagative material, or from the differential transmission efficiency of vectors, or from man-made selection, but also from the pattern of evolution of the genes taken into account. Nevertheless, the usefulness of these data would be much higher if molecular differences were related to the biology of the diverging viral isolates. These are the cases of: (i) GLRaV-2, whose pathological behaviour was studied in Italy, except for lineage BD (18) determining that:

Lineage	Leafroll	Graft incompatibility
Pv20	+ (rarely)	-
RG	-	+
H4	+ (mild)	-
PN	+	+

and of (ii) GVA group II strains, some of which are involved in the aetiology of "Shiraz disease" in South Africa and have recently been found also in Australia and California (56); (iii) GRSPaV, whose variant GRSPaV-1, induces stem pitting in *Vitis rupestris* and vein necrosis in 110R, contrary to variants GRSPaV-SG1 and GRSPaV-MG which are latent in *V. rupestris* but strongly symptomatic in 110R (98); (iv) divergent variants of *Grapevine virus B* (GVB) from South Africa denoted GVB 935-1 and GVB-H1, which are consistently recovered from corky bark-affected vines and corky bark-negative plants, respectively (55).

ADVANCES IN DIAGNOSIS

That in a not far away future "next generation sequencing" will be used also as a primary diagnostic tool sems to be increasingly likely, as the platforms become more competitive and the custom-made runs cheaper. Till then, the traditional diagnostic approaches will continue to dominate the scene.

Serology. An antiserum raised the recombinant CP of GRSPaV was successfully used in ELISA for virus detection in infected grapevines (15). Decoration with a GRSPaV- antiserum revealed the presence in grapevines of filamentous particles *ca.* 800 nm in length (119).

Nucleic acid-based protocols. TaqMan one-step real-time qRT-PCR assays have been developed for determining the number of genome copies within vines infected by different viruses (GFLV, GFkV, GVA, GLRaV-1 and -3). Concentrations a low as 10 genome copies were estimated for GFLV (26), whereas Pacifico *et al.* (103), who referred viral quantity to the concentration of the grapevine glyceraldehyde-3P-dehydrogenase (GAPDH) gene, found that the mean load of each virus ranged between 3 (GLRaV-1 and GFLV) and 5,700 (GFkV) viral genomes per 100 GAPDH transcripts, with GLRaV-3 and GVA falling within this range. A 70-mer oligonucleotide microarray able to detect a broad spectrum of viruses has also been developed. It contais 570 unique probes designed against species-specific regions of 44 plant virus genomes and has successfuly been used for the detection of GLRaV-1, -2, 3-, 4-, 7-, -9, GFLV, GRSPaV, GVA and GVB (42). A single-colour microarray hybridization system which does not require sequence amplification of template RNA was designed and evaluated, with positive results, for the detection of 8 nepoviruses, two vitiviruses, and one each of closteroviruses, ampeloviruses, foveaviruses, maculaviruses and sadwaviruses (1). Primers designed within the highly conserved sequence of the 3' untranslated region of *Tomato ringspot virus* (ToRSV) proved to be more efficient and reliable for the detection of this virus, including a couple of grapevine isolates, than primers designed in other genomic regions (79). Finally, a panel was developed that includes a combination of RT-PCR and ELISA for the specific detection of GLRaV-1, -2, -3, -4, -5, -6., -7 and -9, plus GVA, GVB, GVD, GSyaV-1,GFkV and GRSPaV (97).

TRANSMISSION AND ECOLOGY

Nepoviruses. As a follow up to the discovery that viral CP was responsible for GFLV transmission by *Xiphinema index* (12) a new study based on the production of a series of mutants, ascertained that the sequence determining viral transmission consists of a stretch of 11 conserved amino acids located in an exposed region of the CP (112). The study of a poorly transmissible GFLV isolate showed that the transmission defect was due to a glycine/aspartate mutation in the CP (GFLV-TD). This mutation was localized on an exposed loop at the outer surface of the CP which did not affect the conformation of the capsid nor of individual CP subunits. This loop is

part of a positively charged pocket that includes the 11 amino acid transmission determininant. The suggestion is that perturbation of the electrostatic landscape of this pocket affects the interaction of the virus particles with specific receptors in the nematode's feeding apparatus thus decreasing transmission efficiency (113). As a follow up of this study, the poorly transmissible GFLV-TD and the efficiently transmissible GFLV-F13 were purified and crystallized and some characteristics of the crystals were determined (114). The essential role of CP in determining the specificity of transmission by nematodes was confirmed with a study in which the transmissibility by *X. index* and *X. diversicaudatum* of chimeric GLFV/ArMV viruses coding for the ArMV CP was tested. Results showed that transmission was operated only by *X. diversicaudatum* which was also the only species retaining the recombinant virus with ArMV-type CP (85). *Xiphinema index* populations from Cyprus, Israel, Italy, Spain, southern France, northern France and California showed remarkably different reproductive rates regardless of the grape genotypes (*Vitis rupestris* and *Vitis vinifera* cv. Cabernet sauvignon) on which they were reared. However, there was no differential vector competency among the seven nematode lines in the transmission of two distinct GFLV strains (F13 and GHu) (34). The suitability of 40 *Vitis* and *Vitis* x *Muscadinia* accessions for supporting the development a single line of *X. index* expressed as reproduction factor (RF) was investigated. The RF of some of the *Vitis* x *Muscadinia* hybrids was low indicating them as promising accessions worth of further evaluation with a wider number of nematode lines (41). An Austrian study found that ArMV-infected vines occur in patches, a behaviour explained with the high infectivity and longevity but the low mobility of the vector (50). The resistance to *X. index* derived from *Vitis arizonica* proved to be largely controlled by the quantitative trait locus XiR1 (*X. index* Resistance 1). The genetic map of this locus has now been reconstructed and markers have been developed that can expedite breeding of resistant grape rootstocks (66).

Closteroviruses. In New Zealand, GLRaV-3 is vectored by *Pseudococcus longispinus, Ps. calceolariae* and *Ps. viburni*. The latter two species thrive also on grapevine roots which constitute virus reservoirs after the uprooting of a vineyard, as shown by the long virus persistence (GLRaV-3, 12 months or more) in roots remnants and in mealybugs feeding on them. These findings establish a similarity in the epidemiology of nematode-borne nepoviruses and of mealybug-transmitted closteroviruses, both of which move from remnant roots to newly planted vines (16). This mechanism adds up to the 'classical' virus (GLRaV-3) dispersal methods operated by crowling mealybugs, that leads to within-row vine-to vine transmission, or by human-assisted movement of mealybug crawlers (e.g. with agricultural machinery), or by the aerial dispersal of viruliferus mealybugs which are responsible for random but localized infection on a between-block or within block scale (27). An epidemiological behaviour partly comparable with the above was ascertained for *Planococcus citri*/GLRaV-3 in an arbor-trained vineyard of north-west Spain were mealybug movement, thus virus spreading, was operated by passive aerial transport and moving of pruning remnants. In this case, there was no important downward movement of the mealybugs which were present on the leaves, branches and green canes but not on the vine roots (29). A different distribution pattern between GLRaV-1 and GLRaV-3 was detected in Austrian vineyards, in that vines affected by GLRaV-1 occurred in groups whereas those infected by GLRaV-3 were scattered, a surprising observation due to the fact that the vectors of both viruses are the same (50). Investigations on the feeding behaviour of *P. citri* showed that this mealybug ingests primarily from phloem cells but also from the xylem on which it spends long periods of time (up to *ca*. 9 h). However, the virus acquisition/transmision activity is associated with phloem feeding (30). A study of virus-vector specificity showed positive transmission of GLRaV-4 and GLRaV-9 by *Pl. ficus* and *Ps. longispinus* and confirmed that mealybug transmission of GLRaVs is non specific (121). The same conclusion was reached by Le Maguet *et al*. (78) who were able to transmit GLRaV-1, -3, -4, -5, -6, and -9, but not GLRaV-7 by a single mealybug species, *Phenacoccus aceris.*

TRANSGENIC RESISTANCE

This type of research, which was very active years ago, is progessively being abandoned primarily because the use of genetically modified plants is still strongly antagonized, especially in the European Union, regardless of the fact that their cultivatiom in the rest of the world has attained over 150 million hectares in 2011.

Eight grapevine lines transformed with the GFLV CP were analyzed to correlate transgene expression, small interfering RNAs (siRNA) production, and DNA methylation. No cytosine methylation was observed in challange-inoculated transgenic plants which, however, contained siRNAs 21-22 nt in size, indicating that they had responded to viral infection by activating post-transcriptional gene silencing (46). This finding contrasts with the results of a previous study reporting that efficient RNA interference in transgenic plants challange-inoculated with GFLV does not necessarily lead to detectable accumulation of siRNAs (131).

MicroRNAs (miRNAs) are post-transcriptional regulators of eukaryotic organisms that function through messenger RNA degradation, or suppression of translation, or gene silencing. Artificial microRNAs (amiRNA) operate in a similar way. Thus amiRNA technology has been used to protect plants from GVA and GFLV infection by contructing artificial amiRNA cassettes engineered with viral sequences targeting GVA ORF1 and ORF5 (110) or GFLV CP (72). *N. benthamiana* plants agro-infiltrated with GVA-derived amiRNAs showed varius levels of resistance. Likewise, GFLV-derived amiRNAs were transiently expressed in grapevine somatic embryos.

SANITATION

Heat therapy is recurrently accused of inducing modifications in treated vines. The analysis of the AFLP banding pattern of heat-treated or micropropagated vines showed that no significant variations occurred in the stressed and virus-infected vines, indicating that the level of genome uniformity was high (13), although heat therapy induced more DNA methylation changes than *in vitro* cultivation (14). Somatic embryogenesis was successfully used for the elimination of ArMV (22), and GFLV (45). This latter virus was abundantly present in some cell groups at the periphery of the embryogenic callus, whereas GVA and GLRaV-3 had a different topological localization and occurred in lower concentration, setting a difference in their ability to spread in callus tissues (Gambino *et al*., 2010b). Successful elimination of *Grapevine yellow speckle viroid 1* (GYSVd-1) and *Hop stunt viroid* (HSVd) was achieved through somatic embryogenesis but not with *in vitro* heat therapy. Interestingly, both viroids were experimentally identified within the nuclei of the host cells, proving true what had been just an assumption (49). Exposure to 37°C for 48 days of *in vitro*-grown explants of Kober 5BB singly infected by GVA, GFLV, GFkV, GLRaV-1 and GLRaV-3 resulted in the complete elimination of GFLV, no elimination of GFkV, and differential sanitation rates from the other viruses, i.e., 70% (GVA) and 25% for GLRaV-1 and GLRaV-3 (105). Electrotherapy, i.e. exposure of grapevines to a continuous electric field followed by *in vitro* growth of shoot apices was tested as a means for knocking out GLRaV-1 and -3. The treatment had apparently no detrimental effect on the stability of the host genome and yielded from 30 to 60% virus-free plantlets (63). A set of studies on chemotherapy have shown that : (i) ribavirin could eliminate only in part the GVA/GLRaV-1 complex (64); (ii) when thiopurine prodrugs such as 6-mercaptopurine (MP), 6-methylmecaptopurine riboside (MMPR), 6-thioguanine (6-TG), 1-amino-6-mercaptopurine (1A-MP) were tested against GLRaV-3 only 1A-MP and 6-TG, effectively eliminated the virus from grape explants (81); (iii) ribavirin and tiazofurin eradicated GLRaV-1 at a rate of 72 and 40%, respectively, whereas the most effective drugs against GLRaV-3 were a neuroaminidase inhibitor (78% sanitation) and a purine biosynthesis inhibitor (75% sanitation) (106).

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Grapevine Deformation Virus: Completion of its Sequence Reveals an Origin from Recombination Events Between *Grapevine Fanleaf Virus* and *Arabis Mosaic Virus*

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INTRODUCTION

Grapevine deformation virus (GDefV) is a nepovirus of the subgroup A (Çigsar et al., 2003) of which only the RNA-2 sequence is available (Abou Ghanem-Sabanadzovic et al., 2005). At the nucleotide level GDefV RNA-2 shared substantial identity with *Arabis mosaic virus* (ArMV) and *Grapevine fanleaf virus* (GFLV) (Abou Ghanem-Sabanadzovic et al., 2005), in particular with the $2C^{CP}$ domain of ArMV (72%). GDefV RNA-1 has now been sequenced and the complete genome compared with those of other subgroup A nepoviruses.

MATERIALS AND METHODS

GDefV was isolated from the infected Turkish grapevine accession N66 and purified as described by Çigsar et al. (2003). Viral RNAs were extracted from purified preparations. RNA-1 was excised from agarose gel using RNEasy Mini extraction kit (Qiagen, Italy) and reverse-transcribed using random hexamers and/or an Oligo(dT) primer. Internal sequence fragments were generated with primer DOP4 (Rott and Jelkmann, 2001) using the "DOP-PCR Master kit" (Roche, Switzerland). The 3' and 5´ends of RNA-1 were amplified using GDefV-specific primers designed on DOP-generated clones in conjunction with the oligo(dT) primer and the 5´ RACE-PCR System, respectively. Sets of specific sense and antisense primers were then designed for closing sequence gaps between all previously obtained clones. All amplicons were transformed in StrataCloneTM PCR Cloning vector pSC-A (Stratagene, USA), subcloned into *Escherichia coli* DH5α or SoloPACK cells and custom sequenced (Primm, Italy).

Nucleotide and protein sequences were analysed with the assistance of the DNA Strider 1.1 program. Multiple alignments of nucleotide and amino acid sequences were obtained using the default options of CLUSTALX 1.8 and search for homologies with proteins was done with the FASTA and BlastX and BlastP programs. Tentative phylogenetic trees were constructed using the NJPLOT package (Perrière and Gouy, 1996) with 1000 bootstrap replicates. The presence of possible recombination events was analyzed by RDP3 (Martin *et al*., 2009) using multiple alignments based on both the complete genome sequences and smaller genomic regions.

RESULTS AND DISCUSSION

The complete RNA1 sequence (acc. no. HE613269), excluding the poly(A) tail, consists of 7,386 nt in a single ORF. The coding region, 6,856 nt in size, potentially expresses a polypeptide (p1) of 2,284 amino acids (aa), The 287 nt 5´ noncoding region (NCR) is longer than that of GFLV (242 nt) and ArMV (229 nt). The 3′NCR is 244 nt in length. P1 contains the putative proteinase cofactor ($1A^{Pro\text{-}cof}$, 45 KDa), the NTB-binding protein ($1B^{Hel}$, 88 KDa), the viral protein genome-linked ($1C^{VPg}$, 3 KDa), the proteinase ($1D^{Prot}$, 25 KDa) and the RNA-dependent RNA polymerase ($1E^{Pol}$, 91 KDa) core domains of nepoviruses. Computer-assisted analysis for cleavage site prediction and pairwise alignment of deduced aa sequences of nepoviral p1 proteins from database identified in GDefV RNA-1 four dipeptides residues (C^{416}/A^{417}, C^{1217}/S^{1218}, G^{1241}/E^{1242} and R^{1460}/G^{1461}).

GDefV p1 shared the highest identity at aa level with subgroup A nepoviruses, in particular with GFLV (86-88%) and ArMV (73-74%) (Table 1), and to a lesser extent with the nepoviruses of subgroups B and C (data not shown). The 5´ and 3´NCR of GDefV RNA-1 showed the highest sequence identity with the comparable NCRs of GFLV (65-70% and 85%, respectively). The aa identity level between GDefV p1 domains and their GFLV orthologs was high, ranging from a maximum of 90-92% for $1B^{Hel}$ and $1D^{Prot}$ and a minimum of 83-84% for $1E^{Pol}$ (Table 1).

The comparative analysis of RNA-1 showed that GDefV is much closer to GFLV than to ArMV, so as to suggest that it could be regarded as a highly divergent strain of GFLV rather than a distinct nepoviral species. This,

however was not supported by a comparable analysis of RNA-2 (Abou Ghanem-Sabanadzovic et al., 2005). A more detailed study of RNA-2 showed that GDefV has a high genomic similarity with GFLV in the N-terminal part, comprising the 5'NCR and a large fraction of p2 that includes the homing protein ($2A^{HP}$) and the movement protein ($2B^{MP}$) domains, but this condition reverses at the coat protein ($2C^{CP}$) and 3'NCR levels, where GDefV shows a higher homology with ArMV than GFLV (Table 1).

The chimaeric nature of GDefV RNA-2 was confirmed by recombination analysis, using RDP3 program. Interspecies recombination events were predicted in the C-terminal portion of $2B^{MP}$ and $2C^{CP}$ domains and in the 3′NCR having ArMV isolates (But, NW, Lil) and GFLV (F13) as putative parents. At least six recombination events were identified as "significant" with crossover sites mapping to $2B^{MP}$, $2C^{CP}$ and 3'NCR domains (data not shown). Each of the aforementioned recombination sites was predicted by at least five different methods included in the RDP3 software package.

The occurrence of interspecific recombination events at the $2C^{cp}$ domain level of nepoviruses is deemed unlikely, since it concerns a gene encoding a structural protein involved also in determining virus-vector relationships (Vigne et al., 2008; Oliver et al., 2010). Therefore, to the best of our knowledge, this is the first case of interspecific recombination in nepoviruses involving the $2C^{CP}$ domain. Thus, the case of GDefV is unusual because recombination events gave birth to what has been recognized as a novel taxonomically valid viral species.

Table 1. Nucleotide and amino acid (shadowed boxes) identity matrix of RNA1- and RNA2-encoded polyproteins (p1: 1A–1E, and p2: 2A–2C) and of non-coding regions (NCR) at 5´ and 3´termini of *Grapevine deformation virus* (GDefV), with the corresponding sequences of *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV).

Virus	P1	5'NCR1	1A	1B	1C	1D	1E	3'NCR1	P2	5'NCR2	2A	2B	2C	3'NCR2
GDefV	86-88	65-70	84-86	90	87	90-92	83-84	85	73-75	65-79	71-84	90-92	61-62	63-72
vs.														
GFLV	80-81		84-85	80-81	75-77	80-82	77-78		71-73		74-85	81-82	61-62	
GDefV	73-74	44-46	60-61	77	75-79	78-80	75	74-78	71-73	46-53	47-55	86-87	70-72	79-87
vs.														
ArMV	67-69		65-66	70-71	61-69	73	67-69		66-69		51-64	75-77	66-67	
GFLV	74-75	52-55	59-61	80-82	70-83	80-81	74-76	74-79	71-77	42-55	44-73	84-87	66-69	61-71
vs.														
ArMV	68-70		65-66	72-73	65-79	72-74	68-69		64-73		49-76	74-77	63-65	
	92-93	83	89-92	97-98	95	96	87-91	87-91	89-99	72-99	74-99	95-99	93-99	80-98
GFLV	87-89		89-93	88-90	89-94	89-90	84-87		85-99		76-99	87-99	87-99	
	86	77	77	90	79	89	87	85	84-94	67-90	56-89	96-98	93-95	81-95
ArMV	81		80	81	80	80	80		79-90		57-86	84-91	83-96	

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A Novel Approach for Engineering Resistance to *Grapevine Fanleaf Virus*

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INTRODUCTION

Resistance to *Grapevine fanleaf virus* (GFLV) would be desirable for management purposes, but no source of resistance to GFLV has been identified in *Vitis* species (3). Transgenic resistance has proven to be an effective alternative means of obtaining GFLV-resistant plants (1, 2). Knowledge of the antiviral pathways of RNA silencing has led to the design of transgenes capable of stimulating RNA silencing more effectively and eventually conferring resistance that is more durable and broad-spectrum (4). However, the antiviral potential of a given transgene construct remains difficult to predict. In addition, the time and effort necessary to test numerous transgenic lines developed from each potential antiviral construct design can be prohibitive in perennial crops like grapevines. Therefore, more expedient and high-throughput methods are needed to streamline the testing of transgene constructs for their effectiveness at conferring virus resistance. In this study, we analyzed the genetic variability of GFLV and identified conserved nucleotide sequences within the two genomic RNA molecules. Conserved fragments were concatenated and cloned in a plant expression cassette for agroinfiltration and stable transformation experiments. Agroinfiltration was explored as a high-throughput and fast system for testing the capacity of antiviral constructs to interfere with GFLV multiplication following transient expression in the model host *Nicotiana benthamiana*. The robustness and versatility of the transient expression system was determined by comparing the performance of transgenic constructs in patch assays and stable transgenic *N. benthamiana* plants.

MATERIALS AND METHODS

GFLV RNA1 and RNA2 sequences available in GenBank were aligned using the algorithm Clustal W to identify regions of at least 25 nts in length where 85% of the nucleotide positions were conserved amongst 95% of the sequences. Subsequently, regions of at least 100 nts in length consisting of at least one or more of the regions identified were chosen for cloning.

N. benthamiana seedlings were used for agroinfiltration experiments and stable transformation. Agroinfiltration was carried out using a needleless syringe in two lower true leaves per plant, one of which received the antiviral construct of interest and the other of which received an eGFP control treatment. Five days after lower leaves were agroinfiltrated, upper leaves of *N. benthamiana* plants were mechanically inoculated with GFLV strains F13 or strain GHu using 1:50 dilutions of crude extracts of infected *N. benthamiana* leaves prepared in phosphate buffer. Agroinfiltration experiments were repeated at least three times. Stable transgenic *N. benthamiana* were developed using the antiviral constructs and tested for GFLV resistance following mechanical inoculation with different viral strains.

Transgene expression and accumulation was assayed by ELISA using antibodies to neomycin phosphotransferase II and by RT-PCR using appropriate primers. Similarly, the presence of GFLV was determined in plant tissue by ELISA with specific antibodies and semi-quantitative RT-PCR using GFLV RNA2 primers that did not bind to transgene sequences.

RESULTS AND DISCUSSION

Eight conserved genomic regions matching the selection criteria were identified by alignments of GFLV RNA1 and RNA2 nucleotide sequences. Three of these conserved regions were within the 5' half of the RNA1-encoded RNA-dependent RNA polymerase gene ($1E^{Pol}$) and five were within RNA2, including one within the homing protein gene ($2A^{HP}$), one consisting of a portion spanning the movement protein gene ($2B^{MP}$) and the coat protein gene ($2C^{CP}$), two entirely within gene $2C^{CP}$, and one including the 3' portion of gene $2C^{CP}$ with some of the 3' untranslated region. From the eight conserved genomic regions identified, concatenate constructs of three to six fragments were constructed by PCR and cloned into the binary plasmid pGA482G for *A. tumefaciens*-mediated plant transformation.

Following confirmation of transgene expression in agroinfiltrated tissue, reduced levels of GFLV accumulation was obtained in agroinfiltrated leaves receiving some of the anti-GFLV construct tested versus those from the same plant that were agroinfiltrated with *A. tumefaciens* containing an eGFP construct at six days post-inoculation (dpi), as shown by DAS-ELISA. A reduced GFLV RNA2 abundance was also confirmed by semi-quantitative RT-PCR using total RNA from leaf disks both inside and outside the agroinfiltrated areas (Fig. 1). As expected, reduced viral accumulation was more pronounced inside than outside the agroinfiltrated areas receiving an anti-GFLV construct. These results were consistent with the capacity of some constructs to significantly suppress virus accumulation in agroinfiltrated leaf patches.

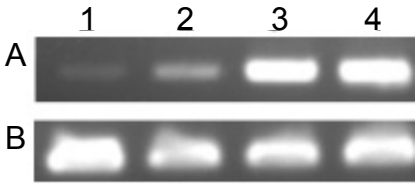

Figure 1. Effect of antiviral constructs on GFLV accumulation. (A) Comparative relative GFLV RNA2 abundance in leaf tissue agroinfiltrated with an antiviral (1, 2) or an eGFP (3, 4) construct both inside (1, 3) and outside (2, 4) the agroinfiltrated areas. (B) *Rcb1* RT-PCR normalized control.

In parallel, resistance to GFLV was tested in T_0, T_1 and T_2 transgenic *N. benthamiana* plants at 7, 14 and 25 dpi following mechanical inoculation. All constructs conferred some degree of resistance that ranged from immunity to delayed infection. Some constructs had a higher propensity than other constructs for resistance to multiple GFLV strains that had 20-33%, 14-17%, 12-26%, and 12-14% divergence at the nucleotide level in the RNA1-encoded $1E^{Pol}$ gene and RNA2-encoded $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$ genes, respectively.

These results indicate that (i) transient expression in *N. benthamiana* is robust for screening putative GFLV resistance constructs over a considerably shorter time frame than testing transgenic plants, (ii) most constructs reduced virus titers in agroinfiltrated plant tissues with differential levels of antiviral activity observed among constructs, and (iii) the transient expression system has the potential to predict the success of GFLV transgene constructs in stable transformants. Grapevine rootstocks were transformed with the most efficient antiviral construct. Their reaction to GFLV infection is being evaluated in a naturally infected vineyard.

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Molecular Characterization of South African Isolates of Grapevine Fanleaf Virus and an Associated New Satellite RNA

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is the causative agent of grapevine degeneration disease and infected grapevine (*Vitis vinifera*) display symptoms that include degeneration and malformation of berries, leaves and canes (Andret-Link *et al.*, 2004). The disease occurs worldwide where *V. vinifera* is cultivated and is considered the most important viral pathogen of grapevine in Europe. In South Africa, GFLV infections occur predominantly in the Breede River Valley in the Western Cape due to the prevalence of its nematode vector, *Xiphinema index,* in this region (Malan & Hugo, 2003). Grapevine fanleaf virus has a positive-sense, single-stranded bipartite RNA genome and is classified in the genus *Nepovirus*, family *Secoviridae*. Each RNA molecule contains a single open reading frame (ORF) encoding the polyproteins P1 and P2, which are proteolytically cleaved into functional proteins by the RNA1-encoded viral protease. To date, the full genome sequences of RNA1 and RNA2 are only available for three GFLV isolates; isolate GFLV-F13 from France, and GFLV-WAPN173 and GFLV-WAPN6132 from Washington, USA (Ritzenthaler *et al.*, 1991; Margis *et al.*, 1993; Mekuria *et al.*, 2009). Only partial GFLV movement protein and coat protein sequences are available from isolates found in African countries (Tunisia and South Africa) (Fattouch *et al.*, 2005; Liebenberg *et al.*, 2009). Here, we report the first full-length GFLV genome sequence from a South African isolate, GFLV-SAPCS3, and investigate putative recombination events in RNA1 and RNA2. We also report for the first time the presence of a satellite RNA (satRNA) that is associated with a South African GFLV isolate, GFLV-SACH44. The full-length nucleotide sequence of the satRNA was determined and was found to be more closely related to *Arabis mosaic virus* (ArMV) large satRNA than to the GFLV-F13 satRNA, the only other GFLV isolate known to be associated with a satRNA (Pinck *et al.*, 1988).

MATERIALS AND METHODS

The isolate GFLV-SAPCS3 was sampled from a grapevine plant (*V. vinifera* cv Cabernet Sauvignon) collected in the Paarl-Wellington wine growing region of South Africa. Virus was propagated and maintained in *Chenopodium quinoa*. Total RNA was extracted from *C. quinoa* leaves using a CTAB method (White *et al.*, 2008). Primers for cDNA synthesis and PCR were initially designed from GFLV-F13 sequences available on GenBank (RNA1 accession number NC003615 and RNA2 acc NC003623), as well as from newly generated GFLV-SAPCS3 sequences. High fidelity enzymes for cDNA synthesis and PCR were used throughout all amplifications. The 5' end sequence of GFLV-SAPCS3 was determined by 5' RACE (Invitrogen) and the 3' end sequence were determined by an oligo d(T) primer paired with a GFLV-F13 sequence-specific sense primer. Purified amplicons derived from total RNA were either sequenced directly, or sequenced bidirectionally after being cloned into a TA cloning vector (pGEM-T Easy, Promega). The sequences generated from the overlapping amplicons were used to build a contiguous sequence using Vector NTI Advance version 10 (Invitrogen). Multiple sequence alignments of RNA1 and RNA2 of GFLV and ArMV isolates were performed using ClustalW (Thompson *et al.*, 1994). The nucleotide and protein sequence identities, phylogenetic analyses and pairwise distance calculations were performed using the MEGA 5 analysis package (Tamura *et al.*, 2011). Putative recombination events were identified using Recombination Detection Program (RDP3 v. 3.44) (Martin *et al*., 2010) and Simplot (v. 3.2) (Lole *et al.*, 1999) with the full-length GFLV and ArMV sequences to find possible recombination events with GFLV-SAPCS3.

The satRNA was detected with diagnostic primers designed from the GFLV-F13 satRNA sequence (acc NC 003203) available on Genbank. Grapevine plants in the Robertson district (Western Cape, South Africa) were screened for the presence of satRNAs by RT-PCR. The purified amplicons were sequenced to confirm the presence of the satRNA. The full-length satRNA sequence of GFLV-SACH44 was determined by designing sequence-specific primers, cloning the resulting PCR amplicons into a TA cloning vector (pGEM-T-Easy, Promega) and sequencing the cDNA clones bidirectionally. The full-length nucleotide sequence of GFLV-SACH44 satRNA

was compared to the full-length satRNA sequences of GFLV-F13 and ArMV isolates by performing multiple sequence alignments and calculating pairwise distances.

RESULTS AND DISCUSSION

The complete sequences of RNA1 and RNA2 were determined for the South African isolate GFLV-SAPCS3. RNA1 and RNA2 are 7342 and 3817 nucleotides in length, respectively, excluding the poly(A) tails. Multiple sequence alignment of these sequences showed that GFLV-SAPCS3 RNA1 and RNA2 were the closest to the French isolate GFLV-F13, with a nucleotide identity of 86.5% and 90.4 %, respectively, and amino acid identities of 94% and 98%, respectively. The 5'UTRs of GFLV-SAPCS3 RNA1 and RNA2 are 243 and 272 nucleotides (nt) in length, respectively and the 3'UTRs are 244 and 212 nt in length, respectively. The GFLV-SAPCS3 RNA2 5' UTR is 32-53 nt longer and is also more closely related to GFLV-GHu and ArMV isolates when compared to other GFLV isolates. Putative intra- and interspecies recombination events between GFLV and ArMV isolates, involving GFLV-SAPCS3 RNA1 and RNA2, were investigated. Recombination analysis software have indicated that the GFLV-SAPCS3 RNA2 5'UTR might have evolved from a recombinational event between GFLV-F13-type and (ArMV) Ta-type isolate.

A satRNA was detected in field samples collected in the Robertson district (Western Cape, SA). The full-length sequence of GFLV-SACH44 satRNA is 1104 nt in length excluding the poly(A) tail. Interestingly, it is more similar to ArMV satRNA (86-88% nt identity) than to the satRNA of GFLV-F13 (82% nt identity), the only other GFLV isolate that a satRNA is associated with (Pinck *et al.*, 1988).

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Symptom Determinants of *Grapevine Fanleaf Virus* in *Nicotiana* Species

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INTRODUCTION

The mechanisms of symptom development following plant infection with viruses remain poorly understood although both, the crosstalk between the miRNA pathway and viral suppressors of RNA interference (VSR), and the induction of a hypersensitive response of the plant are involved. So far, no VRS encoded by a member of the genus *Nepovirus,* family *Secoviridae,* has been identified but RNAi and hypersensitive-like necrosis have been described for *Tomato ringspot vius* (ToRSV) in *Nicotiana benthamiana* (5). Also, little is known about viral sequences responsible for symptom development. Back in the 1970s, it was shown that RNA2 of *Raspberry ringspot virus* caused systemic yellowing in *Petunia hybrida* while RNA1 was responsible for the severity of systemic symptoms in *Chenopodium quinoa* and that both RNAs determined the lesion type in inoculated leaves of *C. quinoa* (2). More recently, the 5' untranslated region of *Grapevine chrome mosaic virus* was involved in the induction of necrotic symptoms in *Nicotiana* sp. when expressed from a viral vector, although typical symptoms were not reproduced, suggesting that this sequence may not act as a dominant determinant of symptomatology (1).

To get insight into nepoviral sequences involved in symptom development, we took advantage of two *Grapevine fanleaf virus* (GFLV) strains causing systemic infection in *Nicotiana* species but distinct symptomatology: strain GHu (4) induces a vein clearing on *N. benthamiana* and chlorotic spots on *N. clevelandii* while strain F13 (7) causes asymptomatic infections on both species. A reverse genetics approach was used to identify GFLV determinants of symptomatology by using infectious cDNA clones of strains F13 and GHu, which were available at the onset of this study (6) or developed over the course of this study, respectively. Here we describe how the use of assortants and chimeras of these clones were used in a gain-of-symptom approach to identify the determinants of symptomatology during systemic infection.

MATERIAL AND METHODS

GFLV strains F13 (7) and -GHu (4) were isolated from naturally infected grapevines. Full-length cDNA clones of GFLV-F13 RNA1 and RNA2 were used for *in vitro* synthesis of transcripts (6).

A full-length cDNA of RNA1 of GFLV-GHu was amplified by immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) using primers providing a T7 RNA polymerase promoter and a restriction linearization site. The full-length cDNA of RNA2 of GFLV-GHu was obtained by three RT-PCR steps using total RNA extracted from infected plants. The restricted PCR fragments were successively assembled in a plasmid carrying a T7 promoter and a unique linearizing site.

Chimeric cDNAs of RNA1 clones were constructed by introducing unique restriction sites by site-directed mutagenesis with overlap extension (3) in the parental infectious clones. Targeted sequences were then swapped using PCR amplification and/or restriction enzymes.

Transcripts of cDNAs were obtained by *in vitro* transcription with the mMESSAGE mMACHINE T7 kit (Ambion) according to the manufacturer's instructions. Size and integrity of transcripts were verified by electrophoresis on denaturing agarose gels prior to inoculation.

Four leave-stage *Chenopodium quinoa* and *N. benthamiana* plants were mechanically inoculated with purified transcripts. Symptoms were monitored and systemic infection was assessed by DAS-ELISA in apical leaves. Crude sap of infected *C. quinoa* or *N. benthamiana* was then used for passages in *C. quinoa, N. benthamiana* or *N. clevelandii*.

All the wild-type and chimeric cDNA clones were sequenced prior to transcription. The progeny viral RNAs were similarly checked for integrity by IC-RT-PCR followed by sequencing.

Coding cDNA sequences of interest were introduced in a binary vector for agroinfiltration assays in *N. benthamiana*. Electroporated *Agrobacterium tumefaciens* were grown at 28°C, centrifuged, resuspended in water and infiltrated using a needleless syringe.

RESULTS AND DISCUSSION

Infectious cDNA clones of GFLV-F13 RNA1 and RN2 (6) were re-sequenced for the purpose of this work. Although a few single nucleotide polymorphisms (SNPs) were detected compared to the published sequences, transcripts derived from the cDNA clones reproduced parental symptoms on *C. quinoa* plants for which a characteristic yellow mosaic developed on upper uninoculated leaves 6 to 8 days post-inoculation. On *N. benthamiana* and *N. clevelandii* the F13 cDNA derived transcripts also mimicked the parental virus, as these plants remained symptomless.

Infectious cDNA clones of GFLV-GHu RNA1 and RNA2 were developed and sequenced. A few SNPs were identified but, again, transcripts reproduced the parental symptoms on both *C. quinoa* and *Nicotiana* species.

We then assorted RNA1 from one strain with RNA2 from the other strain and could clearly conclude that the GFLV-GHu symptoms on *N. benthamiana* and *N. clevelandii* mapped to RNA1. Further experiments were undertaken to identify the coding region responsible for symptom development by swapping cDNA regions of RNA1 between the two strains. Eight chimeras were produced which allowed us to delineate a coding sequence responsible for typical GHu symptoms on *Nicotiana* species. This is the first identification of a nepoviral coding sequence eliciting symptoms in a plant host.

The RNA1 coding sequence that elicits symptoms on *Nicotiana* species was then tested in transient expression assays to determine whether it is able to suppress RNA silencing or induce a symptomatic response on its own. Neither silencing suppression nor symptoms were observed, suggesting that the mechanism underlying the induction of GHu symptoms on *N. benthamiana* and *N. clevelandii* is a complex phenomenon.

ACKNOWLEDGEMENTS

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Genetic Transformations of Grapevine Rootstocks by Different GFLV-Derived Constructs based on Local Isolates from the Czech Republic

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is the causal agent of highly damaging grapevine fanleaf disease that causes significant reduction in grapevine crop yield and a progressive decline of fruit quality. The fanleaf disease of grapevine is controlled by soil disinfection but this procedure is only partially efficient and in many countries forbidden due to high toxicity of nematocides. As an alternative, virus-resistant transgenic grapevine plants is possible to develop to control the disease. In the case of GFLV the grapevine rootstocks are the primary target of transformation experiments, because of its direct contact with soil nematodes and possibility to keep grapevine crop from grafted wine cultivars as GMO free product.

The objective of this study was to construct plant transformation vectors carrying different GFLV genes with sequences that were derived from local strains isolated in South-Moravia, Czech Republic. Subsequently genetic transformations of meristematic bulks derived from different grapevine rootstock cultivars were performed.

MATERIAL AND METHODS

Isolates from GFLV-positive plants found in the Czech vineyards were sequenced at different selected parts of GFLV genome. In addition to generally widely studied coding region of the coat protein, we decided to follow the variability within less frequently studied genes. In fact it was movement protein located on the RNA2 molecule and RNA-dependent RNA polymerase and RNA helicase located on the RNA1 molecule. Methodologies of sequencing experiments including description of using newly designed primers are summarized in these publications: Eichmeier et al., 2010, Eichmeier et al., 2011a, Eichmeier et al., 2011b.

Based on sequencing results the “average” sequences of Czech GLFV isolates were designed. The optimal sequence was designed to retain sequence motifs of at least some of the local isolates, allowing at the same time to avoid typical mRNA destabilizing sequences such as cryptic introns, internal ribosome binding sites and premature polyadenylation signals. Optimization procedure was performed with the help of commercial firm (GENEART, Regensburg, Germany) and the optimized genes were commercially synthesized. Resulting plant vector pCB3819 contains full length synthetic CP gene driven by tandemly arranged promoters pMan1´- p35S. Vectors pCB3820 with deleted C-terminal “standard sequence” part the gene and pCB3821 with N-terminal standard part deleted were also prepared. Similarly the plant vectors pCB3823 containing not reduced synthetic gene derived from RNA polymerase, pCB3825 with the gene for helicase in sense orientation and pCB3826 with the gene for helicase in antisense orientation were prepared too. For capacity reasons, the transformation experiments so far included only vectors carrying coat protein and RNA polymerase sequences. Finally six rootstock cultivars (SO4, Craciunel2, Teleki 5C, Kober 5BB, Kober 125AA and Amos) were transformed with *Agrobacterium tumefaciens* carrying pCB3819, pCB3820, pCB3821 and pCB3823 vectors, using the meristematic bulk tissue method (Mezzetti et al., 2002). To increase transformation performance, helpers LBA4404 and EHA105 were alternately used.

RESULTS AND DISCUSSION

It was performed 13 efforts for genetic transformation at all. Within these experiments 1207 cuttings originating form meristematic bulks were transformed, whereas 1072 of them regenerated on the selective medium with antibiotic kanamycine (25 $mg.l^{-1}$). Unfortunately a lot of them stopped growing and showed no regeneration of shoots. After subsequent selection on media with 50 $mg.l^{-1}$ and 75 $mg.l^{-1}$ of kanamycine, there remain 198 of regenerating shoots. Successful transformation by cultivar, vector and helper is summarized in Table 1.

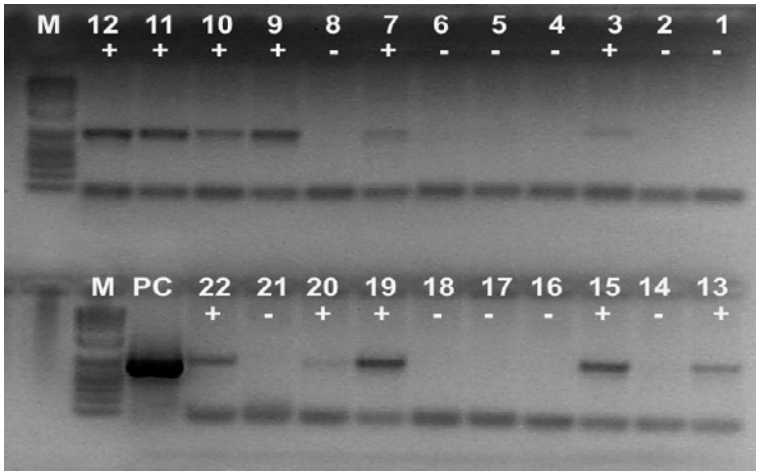
Table 1. Summary of obtained shoots after *A. tumefaciens* transformations of meristematic bulks derived from 6 rootstock cultivars.

	Rootstock cultivar						
Vector + helper	**T5C**	**SO4**	**CR2**	**5BB**	**125AA**	**Am**	**In sum**
3819 + LBA4404	10	3	0	19	6	1	39
3819 + EHA105	0	1	0	0	14	0	15
3820 + LBA4404	12	1	16	0	22	10	61
3820 + EHA105	5	0	0	0	5	0	10
3821 + LBA4404	12	3	2	7	14	0	38
3821 + EHA105	0	0	0	0	5	0	5
3823 + LBA4404	9	15	0	0	6	0	30
In sum	**48**	**23**	**18**	**26**	**72**	**11**	**198**

As visible, performance to obtain regenerated shoots was higher in variants where helper LBA4404 was used. From point of view of cultivar ability to regenerate shoots, the highest amount was obtained in the case of 125AA and T5C cultivars, lowest amount of regenerated shoots was obtained for Amos cultivar. Unfortunately, after longer period of selection the vast majority of the shoots subsequently died. One of the possible explanations is that the plants had chimerical character with majority of non transgenic tissues and that during further development of the plants the transgenic tissues were gradually silenced. But finally one of the transformation variant showed good long term regeneration ability and mainly, they showed positive results if presence of transgen vector was tested by PCR (see Fig.1). In fact, these are shoots derived from T5C cultivar, where helper plasmid LBA4404 was used and transformation was performed by pCB3820 vector carrying sequences of coat protein with C-terminal deleted. In the next period we suppose that positively PCR-tested transformants will be tested for the level of transgene expression and their resistance against GFLV.

Figure 1. Results of PCR test on the presence of transgen vector pCB3820 within 22 regenerating T5C-derived shoots.

M = 1 kb size standard; PC = positive control



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Preliminary Assessment of Grapevine Fanleaf Virus (GFLV) Isolate Variability in Portugal

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is a member of the genus Nepovirus, family Secoviridae and together with *Arabis mosaic virus* is responsible for infectious degeneration, the most severe viral disease of grapevines [1, 3]. Both viruses are naturally transmitted by soil borne nematodes, respectively *Xiphinema index* and *Xiphinema diversicaudatum*, with the coat protein determining transmission specificity in both cases [2, 6]. Exemption of both viruses is mandatory in certified propagation material within the EU countries. Symptoms of the disease include fanleaf, leaf mosaic, shortened internodes and in chromogenic strains severe leaf chlorosis.

Routine testing is based on DAS-ELISA for which commercial antibodies are available. RT-PCR based detection is also widely reported in the literature. Although infectious degeneration is studied in Portugal since 1959 [4], no contribution to international databases has been made available on the molecular variability of GFLV isolates in field grown plants of Portuguese origin. Within an ongoing survey of grapevine viruses in field grown *Vitis* species we have molecularly detected the presence of GFLV in *Vitis vinifera* and *Vitis rupestris*, in relation to an array of leaf symptoms. Preliminary molecular results obtained from different isolates are here reported.

MATERIALS AND METHODS

Infected material: plants showing symptoms (Table 1) were collected from the Coleção Ampelográfica Nacional (CAN-PRT051) situated at Dois Portos and from field grown plants in the Algarve. The CAN was established in 1982 in nematode free soil. The Algarve plants are not subjected to any level of sanitary control. Each isolate analyzed corresponds to an individual plant sample.

RNA extraction and amplification: for each isolate total plant RNA was extracted with the E.Z.N.A.™ *Plant RNA Kit* (Omega Bio-tek, USA). Synthesis of cDNA was done with *RevertAid*™ *First Strand cDNA Synthesis Kit* (Fermentas, Thermo Fisher Scientific, Inc.). PCR reactions were performed in a final volume of 50 µl with *Pfu* DNA Polymerase ™ DNA polymerase (Fermentas, Thermo Fisher Scientific, Inc.), using a primer pair designed in this work, based on GenBank accession NC_003623, and targeting the capsid protein (CP) gene.

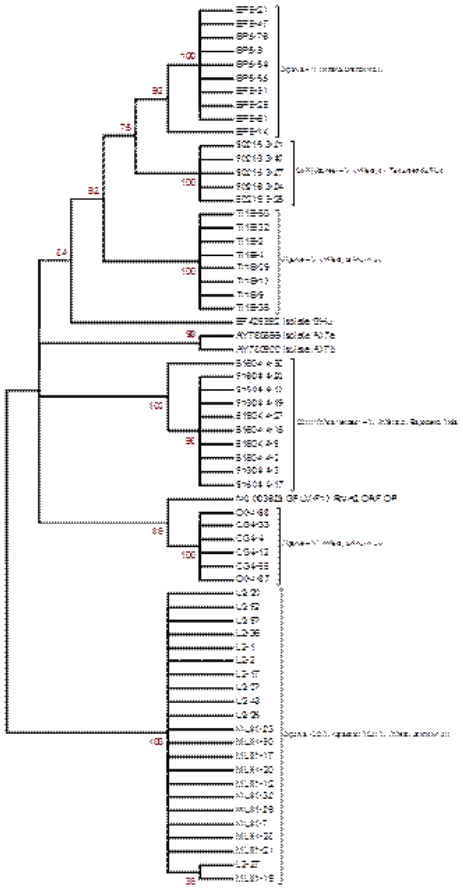
Cloning, SSCP analysis and sequencing: amplicons were ligated with the CloneJET™ PCR Cloning Kit (Fermentas, Thermo Fisher Scientific, Inc.) and used to transform *E. coli* XL1Blue (Agilent Technologies Inc., USA) competent cells. Recombinant clones (purified plasmid with insert) were obtained with the E.Z.N.A.® Plasmid Miniprep Kit II (Omega Bio-Tek, Inc.). Insert size was verified by PCR amplification and PCR products of at least 16 positive clones per isolate were subsequently analyzed by SSCP (Single Strand Conformation Polymorphism). For each SSCP pattern detected, at least two recombinant clones were selected for sequencing (CCMAR, UAlg, Portugal). The SSCP data were used to determine the heterozygosity level of the CP gene within each isolate, using Nei's *h* coefficient [7].

Sequence Data Analysis: the sequences obtained were initially aligned with BioEdit Sequence Alignment Editor (Bioedit) and visually screened, in order to exclude repeated sequences within isolate. Homologous complete sequences available at GenBank were included in the dataset. Phylogenetic trees were obtained in MEGA5.

Table 1. Identification of isolates used in this work.

Isolate ID	Species/Cultivar	Origin	Symptoms
BP5	*V. vinifera* unknown cv.	Algarve	Assymetric leaves; Mosaic
CG4	*V. vinifera* unknown cv.	Algarve	Assymetric leaves; Mosaic
L2	*V. rupestris* rootstock	Algarve	Assymetric leaves
MLII1	*V. vinifera* unknown cv.	Algarve	Assymetric leaves; Mild vein banding
TI1B	*V. vinifera* unknown cv.	Algarve	Assymetric leaves; Mosaic
50216	*V. vinifera* Terrantez do Pico	CAN - Azores	Assymetric leaves: Mosaic
51604	*V. vinifera* Espadeiro Mole	CAN – Minho	Assymetric leaves; Mosaic

Figure 1. Dendrogram based on the complete Capsid protein gene sequences. Bootstrap values (1000 replicates) are shown.



RESULTS AND DISCUSSION

All plants evidencing infectious degeneration symptoms tested positive in the molecular detection. The dendrogram obtained based on the alignment of the capsid protein (CP) gene with reference sequences available at GenBank (Fig. 1), conveyed no evidence of phylogroups with sequence variants from more than one isolate, with the exception of the cluster containing sequence variants from isolates MLII1 and L2. Also, in agreement with previous findings [6] no close relation between symptoms and genetic grouping was found. Dendrograms constructed including all complete CP gene sequences available at GenBank (data shown on poster) further supported the existence of isolate-specific phylogroups.

Intra-isolate genetic structure, based on Nei's heterozigocity index was further analyzed and compared in order to investigate the possible existence of quasi-species structure.

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Survey of the Presence of Nepoviruses in Montenegrin Vineyards

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INTRODUCTION

With around 4300 ha grapevine is the most important agricultural crop in Montenegro. Wine varieties are predominantly grown (90%). Autochthones varieties prevail. Nepoviruses can cause severe loses in grapevine; therefore a survey of the presence of 8 nepoviruses was done in 2006 and 2007 in Skadar Lake basin grapevine producing region.

MATERIALS AND METHODS

In 2006 and 2007, 165 samples were collected from four red (Vranac, Merlot, Kratošija, Cardinal), two white (Chardonnay and Rkacitel) and several unknown grapevine cultivars. 115 samples were taken in the two big commercial vineyards (location Lješkopolje and Ćemovsko polje) in Podgoričko sub-region of Skadar Lake basin where 75% of total grapevine production is concentrated. 50 samples were collected in 13 small vineyards around Godinje situated in Crmničko sub-region of Skadar Lake basin. Wines were sampled individually. Leaves with suspicious symptoms were taken. In the absence of visual symptoms several leaves were taken randomly throughout the canopy. All the samples were tested for the presence of *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV), *Cherry leaf roll virus* (CLRV), *Raspberry ringspot virus* (RpRSV), *Tomato ringspot virus* (ToRSV), *Tomato black ring virus* (TBRV), *Tobbaco ringspot virus (TRSV) and Strawberry latent ringspot virus* (SLRSV) using DAS-ELISA with antibodies and conjugated antibodies of Bioreba AG (Switzerland) for GFLV, ToRSV-Ch and ToRSV-PYBM, Loewe Biochemica GmbH (Germany) for RpRSV and SLRSV and Plant Research International (The Netherlands) for ArMV, CLRV, TBRV and TRSV. Total RNA was extracted with RNeasy Plant Mini KIT (Qiagen, Germany) from four GFLV infected samples of variety Vranac from Ćemovsko polje, one sample of the variety Vranac from Lješkopolje and one sample from about 200 years old wine of the variety Vranac from Godinje. DNA products obtained by IC RT-PCR with primers EV00N1 (5'- GACTATCTAGACACATATATACACTTGGGTCTTTTAA-3') and CPS (5'- TTGTGCGCCCAGATCTCTCTTTACCA-3') (Demangeat *et al,* 2004) from samples collected in Ćemovsko polje and Lješkopolje were sequenced directly (Macrogen, Korea). The amplicon obtained with the above mentioned primers from the total RNA of the sample from Godinje was successfully cloned into pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* JM109 competent cells (Promega USA). Six clones were sequenced (Macrogen, The Netherlands). Phylogenetic analysis was made using the MEGA version 5 program (Tamura *et al*., 2011). A phylogenetic tree was constructed using the neighbor-joining method, pairwise deletion option, and bootstrap analysis with 1000 replicates. Sequence identity matrices were generated with by BioEdit version 7.0.5.3 (Hall, 1999).

RESULTS AND DISCUSSION

The infection with GFLV was confirmed in 38 (23%) out of 165 samples analyzed. Other nepoviruses were not detected. On location Ćemovsko polje in the big commercial vineyard, GFLV was found only on variety Vranac. Nine out of 86 samples were infected. Varieties Merlot, Kardinal, Rkaciteli, Chardonnay and three samples of unknown variety were free from examined viruses. In location in Lješkopolje only one out of the nine assayed samples showed the presence of GFLV. Only variety Vranac was sampled on this location. A much higher incidence of GFLV was detected in Godinje, where numerous small private vineyards are present. 11 out of 13 examined vineyards proved to be infected. Variety Vranac was highly infected, since infection was confirmed in seven out of 13 samples. The infection was found also in the only sampled wine of variety Kratošija and in 20 (65,5%) out of 36 samples of unknown variety. Some of the vineyards in Godinje are very old, but many young vineyards are also present. A high proportion of grapevine planting material is imported to Montenegro and

imported planting material was the most probable source of infection of autochthonous varieties Vranac and Kartošija. These two red wine varieties are grown on 75% of Montenegrin total vineyard area and are thus very important. Grapevine planting material produced in Montenegro lacks adequate sanitary control. Main centre for production of grapevine plant material is situated at around 40 ha in the area of Ćemovsko field. In order to preserve autochthonous grape varieties, mainly production of plant material for varieties Vranac, Kratošija and Krstač is done. Our results show that the current practice, i.e. the use of scions from not tested wines grown in vineyards in the Ćemovsko polje, presents a danger for introduction of viruses into the new vineyards. Clonal selection and implementation of sanitation program according to the requirements of EU certification standards is therefore urgently needed.

GFLV is known to be very variable (Pompe-Novak *et al*., 2007). Although the number of sequences obtained in this study is very low, our results confirm the high variability of GFLV. Comparison of 507 nt long partial CP sequence from Montenegro and NCBI GenBank showed that Montenegrin sequences differ from other published sequences. High variability was observed also within Montenegrin sequences. The lowest identity determined between two Montenegrin sequences was only 0,889. Montenegrin sequences grouped in three separate clusters. Clones grouped in two of these clusters with four very similar to identical sequences in one cluster and two identical sequences in the other. Our results confirm the co-existence of different GFLV variants in the same plant and a quasispecies nature of this virus (Oivier *et al.*, 2010; Pompe-Novak *et al.,* 2007).

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New Progress in the Study of Grapevine Leafroll Disease in France

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INTRODUCTION

Grapevine leafroll disease is one of the most severe viral diseases of grapevine worldwide. It is associated to several virus species classified in the family *Closteroviridae*: *Grapevine leafroll-associated virus* (GLRaV) -1, -3 and some other species (genus *Ampelovirus*), GLRaV-2 (*Closterovirus*) and GLRaV-7 ('Velarivirus'). Leafroll ampeloviruses are disseminated by exchange of infected material, as well as by natural vectors, the scale insects (*Hemiptera Coccoidea*). Moreover, 'rugose wood'-associated *Grapevine virus A* (GVA) and *B* (GVB) (genus *Vitivirus*), often found in leafroll-affected vines, are also coccoid-transmissible. This raises questions about possible interactions between ampelo- and vitiviruses during transmission (Herrbach *et al.*, *in press*). Improving the protection of vineyards against leafroll requires further research in virology, entomology and epidemiology. The present communication aims at updating the results and prospects of the studies run in our institute.

BIOLOGY OF TRANSMISSION

Ampeloviruses are known to be transmitted by mealybugs (*Pseudococcidae*) and soft scales (*Coccidae*). In order to enrich our knowledge on virus–vector specificity, transmission experiments were performed with the mealybug species *Phenacoccus aceris* (Signoret) and the soft scale *Neopulvinaria innumerabilis* (Rathvon), both present in Burgundy and Beaujolais vineyards. Experiments were done using aviruliferous insects, given a 48 h-acquisition access period (AAP) on detached leaves of virus-infected grapevine followed by a 48 h-inoculation access period (IAP) on healthy vine cuttings.

The mealybug *P. aceris* was found to vector efficiently the ampeloviruses GLRaV-1, -3, -4, -5, -6, and -9, as well as GVA and GVB (Le Maguet *et al*., 2012a). It is the first vector species identified so far for GLRaV-6. Thus, *P. aceris* is shown to vector many grapevine viruses in both *Ampelovirus and Vitivirus* genera, which emphasizes the attention to be paid to this Holarctic widespread species. Moreover, the first instar larvae (L1) of *P. aceris* were shown to transmit more efficiently GLRaV-1 and -3 than do second instars (L2).

The soft scale *N. innumerabilis* was also shown to transmit GLRaV-1, -3 and GVA (Le Maguet, 2012). This species is increasingly present in France, causing direct damage where its population level is high. In regions where this species is present, its vector ability is also to be considered carefully in protection of grapevine against leafroll and rugose wood.

Moreover, the retention of particles in the viruliferous vector has been assessed. The RNA of GLRaV-1, -3 and GVA remained detectable up to 14 days in starving L2 of the mealybug *Heliococcus bohemicus*. Conversely, starving L2 of the coccid *Parthenolecanium corni* lost rapidly the detectability of GLRaV-1 and GVA by RT-PCR; however, this species retained the viral RNA up to 18 days when placed on a plant non-host of the viruses.

NATURAL SPREAD OF LEAFROLL VIRUSES

The spatio-temporal dispersal of leafroll in two Pinot noir vineyards in Burgundy has been surveyed over 8 years (Le Maguet, 2012; Le Maguet *et al*., *submitted*). On the first vineyard, planted with certified plant material, leafroll symptoms (mainly due to GLRaV-1) spread rapidly from the edges to virtually the entire plot. Biostatistical and molecular analyses showed that the ampelovirus and the vectors originated from the adjacent plots, planted several years earlier, heavily infested by *P. aceris* and highly infected by GLRaV-1. This highlights the risk generated by diseased neighboring plots to newly planted plots. The second vineyard displayed no or very little spread of leafroll. This vineyard was not surrounded by mealybug-infested and leafroll-infected plots.

NATURAL SPREAD OF SOFT SCALE CRAWLERS

Field experiments were set up to estimate the possible natural spread by the wind of *P. corni* L1 and L2 larvae from infested vineyards to a newly planted plot (Hommay *et al.*, 2012). Wind-borne nymphs were caught using glue-covered cylindrical traps, set up in the young plot during the crawler phase (L1) in late spring and during autumn migration of L2 nymphs down to the stock. Results reveal that crawlers were commonly trapped and that part of them were viruliferous. Very few L2 were caught in autumn.

PROSPECTS

Our studies shed new light on the vector biology of *P. aceris* and other species, and pave the way towards deciphering the virus–vector interactions. Pending questions are the relationships between vector activity and virus transmission, the possible interaction between ampelo- and vitiviruses during transmission (Hommay *et al.*, 2008), the localization of virus particles within the alimentary system of vectors, as well as the identification of viral determinants of the specific retention in the vector. A French transmissible isolate of GLRaV-1 is being fully sequenced for this purpose.

Finally, a more precise understanding of vector dispersal and virus spread in vineyard plots requires further experiments on vector biology, particularly with species such as *H. bohemicus* and *P. corni*, also able to vector viruses and abundant in many French vineyards. Data from such trials, together with in-depth knowledge of virus–vector interactions and improvement of virus detection methods, will provide new insights into leafroll epidemiology and help to improve the protection of grapevine against this major viral disease.

ACKNOWLEDGEMENTS

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Characterization of the Grapevine Leafroll-associated Virus 3 RNA Silencing Suppressor Encoded in the p19.7 Gene

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INTRODUCTION

Search for plant virus silencing suppressors has become an essential part of the functional characterization of viral genomes, not only for being the mediators of RNA silencing suppression but as they could cause changes in plant development through disruption of the miRNA pathway (Chapman *et al.*, 2004).

GLRaV-3, is the type member of the genus *Ampelovirus (*family *Closteroviridae)*. At least five phylogenetic groups have been reported for this virus based in the coat protein gene (Gouveia *et al.* 2011). Until now no RNA silencing suppressor (VSR) has been found among *Ampelovirus*, contrary to what happens with a large number of other viral genera (see review Li *et al.*, 2006) including the sister genus *Closterovirus*. By analogy with the genomic location and molecular signatures of the VSRs previously described for closteroviruses, we decided to screen the GLRaV-3 3'-terminal genes (p21, p19.6 and p19.7) for VSR activity. From these, only p19.7 revealed suppressing activity, demonstrated against diverse silencing inducing systems. This activity varies across the phylogenetic groups.

MATERIAL AND METHODS

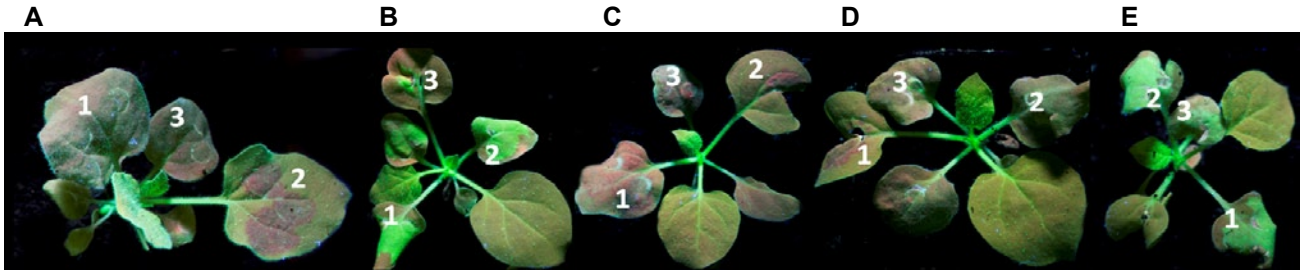
The p21, p19.6 and p19.7 genes were obtained from GLRaV-3 Portuguese isolates described previously by Gouveia *et al*. (2011). Each assayed gene was cloned under control of the CaMV 35S promoter in the binary plasmid pK7WG2, through two steps of Gateway recombination according to the manufacturer's manual (In-vitrogen). Assays were conducted in 16C *N. benthamiana* plants (constitutively expressing the GFP gene) and in wild type (WT) *N. benthamiana* plants. In 16C plants the silencing inducer was the mGFP5-ER, homologous to the GFP expressed in 16C plants. In WT plants the mGFP5-ER was used for transient expression of GFP and three silencing inducers were used: 1) long hairpin RNA of GFP (lhRNA-GFP); 2) an artificial miRNA (amiRNA-GFP) and 3) a double stranded RNA corresponding to the whole mGFP5-ER (dsRNA-GFP). Details for the construction of the silencing inducers are described in Gouveia *et al.* (2012a). The binary vectors were transferred into *Agrobacterium tumefaciens* strain C58C1. TAV-2b suppressor from Tomato aspermy virus was used as a VSR positive control. Co-infiltrations were done with equal volumes of each individual *Agrobacterium* culture, at an OD600 of 0.5 for each culture. Six plants were used for each modality. The GFP fluorescence was visualized by using a 100-W, hand-held, longwave UV lamp (Blak-Ray B-100AP, Ultraviolet Products) and plants were photographed with a Canon EOS 450D. Close up images were obtained with a stereo zoom microscope SZX16 (Olympus) under UV light. The fluorescent signal was also measured on ABI PRISM 7200 Sequence detector (Applied Biosystems). Isolation and northern blot analysis of the GFP-specific siRNAs were done as detailed by Gouveia *et al.* (2012a). The expression of GFP was quantified by qRT-PCR (iQ, Bio-Rad) using ubiquitin as a reference gene.

RESULTS AND DISCUSSION

Figure 1 shows 16C co-infiltrated with *Agrobacterium* cultures providing the GFP and the VSR gene candidates. Only plants that were co-inoculated with p19.7 or TAV-2b maintained a strong green fluorescence in each leaf's inoculation patch at plants 5 d.p.i. In the other modalities, the green fluorescence declined before 5 d.p.i. and was substituted by a reddish signal due to GFP silencing. The GLRaV-3 p19.7 construct behaved similarly to the TAV 2b construct which suggests that p19.7 has the ability to suppress RNA silencing. This data was corroborated by (results not shown): a significant difference of GFP fluorescence signal between the p19.7 construct and the other screened genes, measured at 475 nm; presence of GFP short-interfering RNAs (siRNAs) in northern blots from plants co-inoculated with GFP and p21 or p19.6 but not with p19.7; and a fourfold increase of GFP mRNA in plants co-inoculated with p19.7 relatively to plants singly inoculated with GFP.

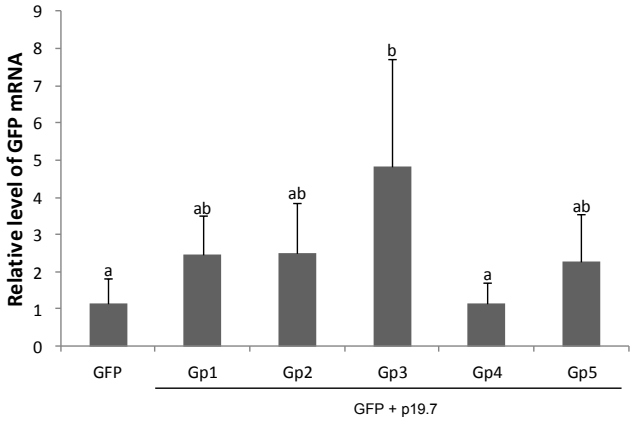
To test whether p19.7 could suppress silencing triggered by other inducers more likely to exist in a viral infection, i.e., long hairpin RNAs, micro RNAs and ds-RNA, a series of further assays were designed using WT plants. In all modalities, the presence of p19.7, originated a reduction in the level of GFP siRNAs. This data indicates that p19.7 is able to overcome strong silencing inducers.

Figure 1. 16C *N. benthamiana* leaves co-inoculated with *Agrobacterium* cultures containing GFP and the following constructs: A-none; B-TAV 2b; C-p21; D-p19.6; E-p19.7. Images were taken 5 d.p.i. under UV light. Each of the inoculated plant's leaves is numbered 1 to 3.



Five constructs of p19.7, belonging to different phylogenetic groups were compared for their suppressing activity (Gouveia et al., 2012b). For each p19.7 variant the accumulation level of GFP mRNA and specific siRNAs were determined using co-infiltration assays in 16C *N. benthamiana*. In agreement with the detection of siRNAs and mRNA northern blots (not shown), the lowest level of GFP expression was obtained in the presence of Gp4 variant of p19.7, while the highest corresponded to the Gp3 variant. The differences between of these two variants are statistically significant (Figure 2).

Figure 2. Relative GFP mRNA expression levels of co-infiltrated plant leaves at 5 d.p.i.. Letters indicate significantly different averages (P<0.05, Duncan's test)



The level expression of GFP for the other suppressors was in-between the Gp3 and Gp4 variants and their differences were not statistically significant. Systemic silencing was detected at 10 d.p.i and only in the presence of Gp4 variant. Some constructs originated virus-like mosaic symptoms which evolved into necrosis. The intensity of these symptoms appeared to be related to the strength of the suppressor activity. A comparison of the protein sequences suggested a few amino acid substitutions that could be associated with differences in the suppressing activity.

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Development of a LAMP Technique for Control of Grapevine Leafroll Associated Virus Type 3 (GLRaV-3) in Infected White Cultivar Vines by Roguing

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INTRODUCTION

Grapevine leafroll disease, associated primarily with grapevine leafroll associated virus type 3 (GLRaV-3) in South Africa (Pietersen, *unpublished results*) is a serious disease of grapevine worldwide (Bovey *et. al.,* 1980). In spite of successful virus-elimination within important clones by a certification scheme in South Africa, it spreads rapidly to new vineyards. Recent local epidemiological studies (Pietersen, 2006) have shown that new vines are often infected through secondary spread within vineyards. Roguing, within an integrated control strategy (Pietersen *et al.*, 2003), has been very effective at controlling the disease (Pietersen *et al*., 2009). In this study we report on a) successful control of leafroll in two phases at a commercial wine estate on red cultivars and b) development of a detection technique aimed at making control of leafroll by roguing in a third phase (on white cultivars) more feasible. Roguing of white cultivars is complicated by their lack of symptoms and hence virus detection relies on laboratory based detection before vine removal. Highly sensitive techniques, Nested-PCR and Real-time PCR, allow for the pooling of many samples but require expensive equipment and highly trained technicians. Currently the industry uses the less sensitive ELISA based detection systems, which is capable of processing high sample volumes. We propose the use of a sensitive, simple technique with the potential for high sample throughput, which does not require expensive equipment. Loop-Mediated amplification of Nucleic Acid (LAMP) is a isothermal means of amplifying a target sequence by a strand displacing DNA polymerase in conjunction with 4 primers which target 6 areas on target DNA (Notomi *et al.* 2000). The system can be used to detect DNA and RNA (with reverse transcriptase) (RT-LAMP) (Thai *et al*., 2004) and can be combined with a visual detection system for easily discernable positive results (Goto, et al., 2009). We have developed a GLRaV-3 specific RT-LAMP which is simple, rapid, and sensitive, which may make detection of infected white cultivar vines for roguing purposes more feasible.

MATERIAL AND METHODS

Control of LR on Vergelegen Wine Estate, near Somerset West, South Africa, was divided into three phases. In phase one, consisting of 34 vineyards (98200 vines), control was on five year-old vineyards established on ground not previously planted to *Vitis* with LR incidences below 2.5%, as well as new vineyards also on ground not previously planted to *Vitis.* In phase two, LR control was performed in 29 vineyards (111500 vines) where totally infected red cultivar vineyards were replaced with new red cultivar vineyards. Phase three, only recently initiated (not reported here) involves replacement of old LR infected white cultivar vineyards with new white cultivars vineyards. An integrated control strategy was applied (Spreeth *et al.*, 2006 ; Pietersen *et al.*, 2009) to control the spread of leafroll which includes annual roguing of infected plants. In the red cultivars annual visual detection of symptoms in autumn proved adequate for effective roguing. In phase three LR control in white cultivars required that, in addition to the strategies utilized in previous phases, LR infected plants be detected by ELISA as symptoms are too obscure or ambiguous to utilize for LR assessment for infected vine removal.

As an alternative to ELISA , RT-LAMP has been developed. GLRaV-3 specific primers were designed based on conserved regions of the genome. A 25ul RT-LAMP reaction consists of ; 0.2mM F3 and B3 primers, 1.6mM FIP and BIP primers, 8U Bst DNA polymerase, 1 x Bst buffer B (Lucigen) 1.4mM dNTPs, 6mM $MgCl_2$, 5M Betaine (Sigma) and 8U AMV reverse transcriptase (Roche) and 5U RNase inhibitor (Roche), to which 1ul of template was added. The reaction is incubated at 42°C for 10 min. then at 60°C for 1 hour. Initially amplification of DNA tested was with three recombinant plasmids containing GLRaV-3 specific sequence, this was followed by tests on total RNA extracts of GLRaV-3 infected vines in a one step RT-LAMP reaction. Visualization of the LAMP reaction was optimised for maximum turbidity with Hydroxy Napthol Blue (HNB) as an alternative means of

visualizing the amplification reaction. Results of RT-LAMP and nested PCR to GLRaV-3 were compared for 10 greenhouse-maintained grapevine samples of unknown GLRaV-3 status.

RESULTS AND DISCUSSION

In this study successful control of LR was demonstrated at a commercial wine estate. LR was reduced from essentially a 100% infection in 2002 on 41.26 ha (111431 vines) planted mainly from 1989 to 1992 (Phase 2 vineyards), to only 56 LR infected vines detected in 2012 on 77.84 ha (209626 vines) (Phase 1 and 2 new vineyards), an incidence of 0.026%. This was by replacement of fully infected vineyards and roguing 3105 infected vines within young and replaced new vineyards. Four vineyards are 13 years old in 2012 and have only four infected vines. This control was achieved in some instances where vineyards had significant numbers of LR infected vines on initiation of roguing (the highest being 12.2% or 548 vines).

A single tube, GLRaV-3 specific RT-LAMP method was developed to detect GLRaV-3 in grapevines from total RNA extracts . Turbidity and HNB were most promising amongst several visual detection systems analysed, and were optimised for use in the GLRaV-3 specific LAMP. Turbidity was optimised by increasing Mg^{2+} concentration until turbidity was observable to naked eye (8mM Mg^{2+}). Addition of HNB to the RT-LAMP resulted in clear colour changes from Violet to Sky blue for infected vines. In comparisons with a GLRaV-3 specific nested PCR it was shown that RT-LAMP and Nested PCR correlated well with all nested PCR positive samples (9 samples) also testing positive by RT-LAMP. Following improved template preparation methods LAMP has the potential to replace ELISA as a field diagnostic technique for GLRaV-3 in white vine cultivars.

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Grapevine Leafroll Associated Viruses Effects on Yield, Vine Performance and Grape Quality

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INTRODUCTION

Grapevine leafroll disease causes poor color development in red grapes and non-uniform maturation of fruit in *Vitis vinifera*. It is also reported to delay fruit maturation from three weeks to a month (Weber *et al*., 1993). Other symptoms include downward rolling of basal leaves followed by rolling of the leaves near the shoot tips, color change in the interveinal portions of the leaves, and phloem disruption. In most red varieties, leafroll disease causes reddening of the interveinal areas while the veins remain green. In white varieties, the interveinal area may become chlorotic and this symptom is often subtle and may not be recognized. The extent of leaf-rolling varies considerably among infected varieties; Chardonnay shows pronounced leaf-rolling by harvest while Thompson Seedless and Sauvignon Blanc show little or no leaf-rolling (Goheen and cook, 1959; Weber *et al*., 1993). In mixed infections, more severe symptoms occur including vine death (Golino, unpublished data). Grapevine leafroll disease has also been associated with yield losses as high as 20% to 40% (Goheen, 1988; Weber *et al*., 1993).

Our past research has shown that the effects of infection by the grapevine leafroll-associated viruses (GLRaVs) depend greatly on the virus as well as the grapevine variety. This information, along with new information on virus molecular biology and the increased ability to detect other GLRaVs is opening new doors of understanding. This, in turn, increases the potential to make practical predictions and recommendations when a certain GLRaV is detected in a vineyard. In this experiment, Cabernet Franc vines budded onto nine different rootstocks were inoculated with different GLRaVs and planted in the field to evaluate the symptoms, plant growth, yield, and berry qualities. The preliminary results are presented here.

MATERIALS AND METHODS

Reference sources of leafroll viruses were established in the Davis Grapevine Collection (Golino, 1992) and regularly updated with newly found viruses and virus strains. The GLRaVs used in this experiment were from this collection and included: GLRaV-1 (two isolates), GLRaV-2 (three isolates), GLRaV-3 (three isolates), GLRaV-4, GLRaV-4 strains 5 and 9 (Martelli *et al*., 2012) and GLRaV-7. Cabernet Franc plants were propagated on the following nine rootstocks: AXR #1, Mgt 101-14, 110R, 3309C, 5BB, 420A, Freedom, St. George 15 infected with grapevine rupestris stem pitting-associated virus (GRSPaV), and St. George 18 tissue cultured and free of GRSPaV . The rootstock portion of these vines was inoculated with two chip buds from different GLRaV-infected canes. The two chip buds were from either a single virus source (for single infections) or from two different virus sources (for mixed infections). Fifteen replicates per virus (or combination of two viruses) per rootstock in three different blocks (5 replicates per block) were used in the trial. The controls include vines chip budded from a healthy source and non-chip budded vines. The vines were planted in the field in a randomized complete block design with five vine plot replicates per rootstock treatment. The bud take was checked before planting them in the field.

RESULTS AND DISCUSSION

The data collected from the experiment in 2011 showed that the virus isolate LR132 had an unfavorable reaction on Cabernet Franc plants propagated on 420A, Freedom, 3309C, and 101-14 rootstocks, and many of these vines died within a few months after inoculation. The RT-PCR results showed that isolate LR132 is a mixed infection of GLRaV-1 and grapevine virus A (GVA). It is not clear whether a certain strain of GLRaV-1 is the cause of poor vine performance and vine death or if the presence of GVA creates a synergistic effect that kills the vines. It was also found that the presence of GLRaV-2 (isolates LR103 and LR119) had harmful reactions on vines propagated on Freedom and 5BB. These vines were extremely weak and exhibited red leaf symptoms

and short internodes. Furthermore, it was observed that different GLRaV types produced leaf symptoms of different severity. For example, leaf symptoms on vines infected with GLRaV-3 were more severe than on vines infected with GLRaV-4. None of the GLRaV-7 infected vines showed leaf symptoms.

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Nucleotide Sequence at the Beginning of the 5' Nontranslated Region is Critical for Replication of *Grapevine Leafroll-Associated Virus* 3

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3), the type member of the genus *Ampelovirus* in the family *Closteroviridae*, is the most predominant and widespread among several GLRaVs closely associated with grapevine leafroll disease (Rayapati *et al*., 2008). We recently determined the genome sequence of a Washington isolate of GLRaV-3 (WA-MR1, GU983863) to be 18,498 nucleotides (nt) long with a 737 nt long 5' nontranslated region (NTR) and 277 nt long 3' NTR (Jarugula *et al*., 2010). The unusually long 737 nt 5' NTR of GLRaV-3 showed non-uniform sequence identity distributed across the entire sequence when compared with a GLRaV-3 isolate from South Africa (Maree *et al*., 2008). Besides sequence divergence, a recent study showed differences in length of the 5'NTR of GLRaV-3 variants (Jooste *et al*., 2010). Since the 5′ NTR of most RNA viruses harbor RNA elements critical for genome replication (Gowda *et al*., 2003), we elucidated the role of different regions of the 5'NTR of GLRaV-3 in replication of a minireplicon of the virus.

MATERIALS AND METHODS

A full-length cDNA clone of GLRaV-3 isolate WA-MR1 (Jarugula *et al*., 2012) was used in this study to build a minireplicon cDNA clone. The clone contained the 5´NTR, the replicase module, a heterologous gene (green fluorescent protein gene under the control of GLRaV-3 coat protein promoter sequence), and the approximately 1.3 kilobase 3´ terminal sequences harboring p20B, p7 and p4 genes (Fig. 1a). The cDNA clone was sub-cloned into the T-DNA region of the binary vector pCAMBIA1380 in such a way that the CaMV 35S promoter with a double enhancer cassette is at the 5´ end and a hammerhead ribozyme sequence is at the 3´ end of the cDNA clone. (Fig. 1a). Different portions of the 5′ NTR were deleted by overlap extension PCR technique and used these truncated sequences to build a series of minireplicon cDNA clones with different lengths of 5'NTR (Fig. 1b). Additional minireplicon cDNA clones were built by replacing the complete 5'NTR sequence of WA-MR1 isolate with 5'NTR sequence of GP-18 (737 nt) and PL20 (672 nt) variants reported from South Africa (Maree *et al*., 2008; Jooste *et al*., 2010).

The plasmid constructs were mobilized into *Agrobacterium tumefaciens* strain EHA105 and individual colonies were checked for the presence of recombinant plasmids through colony PCR using virus-specific primers. Bacterial suspension derived from single, positive colony for each construct was adjusted to an OD_{600} of 1 and used for infiltration of *Nicotiana benthamiana* (3 to 4 weeks old) leaves. Infiltrations were done by gently pressing the blunt end of a 1-ml syringe filled with bacterial culture to the abaxial leaf surface and exerting gentle pressure to flood the interstitial areas within the leaf. The silencing suppressor gene HC-pro^{TuMV} of *Turnip mosaic virus* or p19^{TBSV} of *Tomato bushy stunt virus* were used in co-infiltration assays by mixing equal amount of bacterial cells (based on A_{600}=1) containing the suppressor construct and the minireplicon cDNA construct. After infiltrations, plants were maintained in a growth chamber at 25 ± 1°C with 14:10 hr photoperiod. The infiltrated leaves were monitored for GFP fluorescence under hand-held long wave UV-light and samples were harvested at 5-6 day post-infiltration. Total RNA isolated from these samples were analyzed by Northern blotting (Jarugula *et al*., 2010a) for the presence of genomic and sub-genomic(sg) RNAs specific to the virus, and the sg RNA of GFP.

RESULTS AND DISCUSSION

Agrobacterium-mediated delivery of the GLRaV-3 minireplicon into *N. benthamiana* leaves showed expression of GFP only in leaves co-infiltrated with a silencing suppressor (Fig. 1C-3 & 4). Detection of genomic and p20B sg RNA of GLRaV-3 and the GFP gene sgRNA in Northern blots further confirmed that the presence of the

ectopically expressed silencing suppressor is required for replication of the GLRaV-3 minireplicon (Fig. 1D, lane 3 & 4). Agrobacterium-mediated delivery of the GLRaV-3 minireplicon containing the 5'NTR sequence of GP-18 and PL20 from South Africa showed expression of GFP (Fig. 1C-5&6) and genomic and sgRNA of GLRaV-3 (Fig. 1D lanes 5&6) comparable to that of the minireplicon containing the 5'NTR of WA-MR1. This observation indicated compatibility of genetically diverse 5'NTRs with the replicase complex of GLRaV-3, thereby suggesting that cis-acting elements required for replication are conserved despite significant sequence and length variation in this region. To define further the RNA elements of the 5'NTR required for replication, different portions of the 5'NTR were deleted (Fig. 1B) and the resulting minireplicon constructs were tested for their replication competency by agroinfiltration of *N. benthamiana* leaves with silencing suppressors. Monitoring GFP expression in agroinfiltrated leaves and the presence of genomic and sgRNA of the virus in Northern blots demonstrated that deletion of the first 40 nt of the 5'NTR completely abolished replication (Fig. 1C-8 & 9; Fig. 1D lanes 8 & 9). Deletions between nt 41-737, either partial or complete, did not affect replication, although deletions between nt 41-120 resulted in slightly reduced level of replication (Fig. 1C 10-16; Fig. 1D, lanes 10-16). An identical sequence within the 40 nt at the beginning of the 5'NTRs of WA-MR1, GP18 and PL20 variants further support our observations that 40 nt at the beginning of the 5'NTR contains critical element(s) required for virus replication. Further studies are in progress to determine whether the primary structure or higher order structures of this sequence is involved in replication and virion formation.

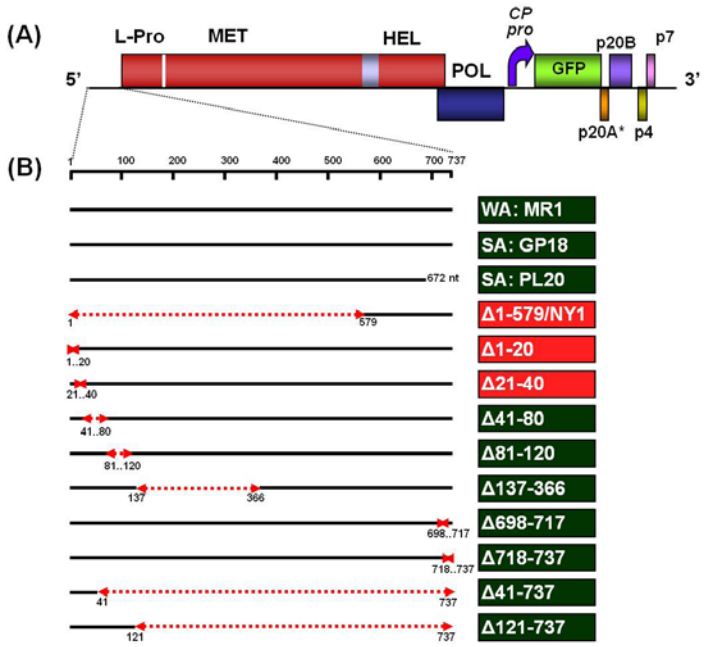
Fig.1: (A) Diagrammatic representation of GLRaV-3 mini-replicon harboring GFP gene.

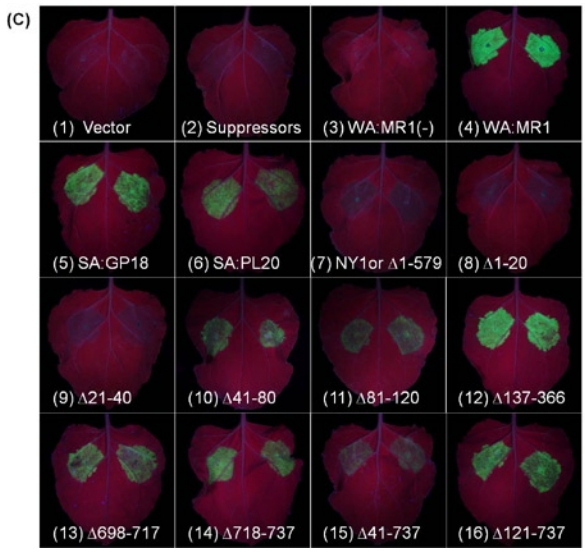
(B) Schematic representation of minireplicon constructs with the deletions in the 5′ NTR. The nucleotides that were deleted in the 5'NTR of each construct are shown by dots and designation of each construct is shown on the right.

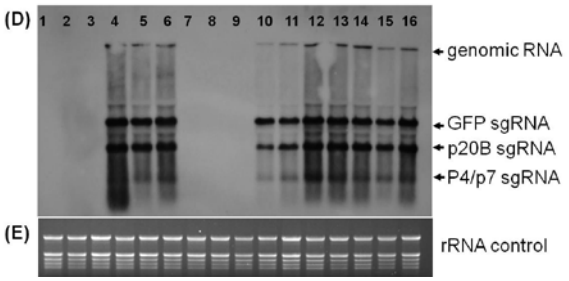
(C) Photographs taken under UV light of *N. benthamiana* leaves 6 days postinfiltration (dpi) with *A. tumefaciens* harbouring each construct in combination with silencing suppressor. The empty vector and minireplicon construct with no coinfiltration of a silencing suppressor were used as controls.

(D) Northern blot analysis of total RNA extracted from infiltrated leaves at 6 dpi. The detected bands indicated by an arrow on the right correspond to viral genomic RNA and sgRNAs of GFP and p20B.

(E) Ethidium bromide staining of rRNA used as loading control for mRNA.







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Economic Impact of Leafroll Disease in Vineyards of the Cultivar Albariño in Rías Baixas (Spain)

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INTRODUCTION

It is well documented that the physiological changes induced by the main viruses causing the leafroll disease (*Grapevine leafroll associated viruses*) give place to delays in the maturation of the grapes of the infected stocks (Charles *et al*., 2006). But the damages induced by the leafroll viruses are very variable according to factors as cultivars and clones, locations, age of the plants, rootstocks, crop management, virus or combination of viruses infecting the plants, and environmental conditions. But most grape growers do not regard leafroll to be economically important. Leafroll viruses are "quality" pathogens, and the losses are expected to be higher in cultivars and areas producing high quality wines, as it are the case of the Albariño white wines in Galicia. Since the ripening conditions may be quite variable, more problems are expected in cool climates than in warmer ones. In the last decade, several papers have been published regarding the economic impact of the leafroll disease in different grape growing regions in the world, mostly from cool climate areas as New Zealand, South Africa or northern USA (Walker *et al*., 2004; Nimmo-Bell, 2006; Freeboorough *et al.*, 2006; Shady *et al*., 2012) but not from Europe.

The cultivar Albariño is a good example of adaptation to the humid conditions of the vineyards close to the Atlantic Ocean. In the traditional vineyards, trained in a horizontal trellis ("parra"), some rainy summers Albariño may show delayed ripening and that is a great concern for growers. The Albariño is a traditional cultivar in the Rías Baixas region but its commercial cultivation did not start until the last quarter of the XXth century and it was necessary a certain period to adapt its vigor and agronomic performance to a modern viticulture. Most plant material used in the new vineyards until 1980 came from not-many, hundred-year plants, which were about 30% leafroll infected. The prevalence of GLRaV-3 is over 80% of the Albariño vineyards sampled between 1991 and 2003 in Rías Baixas and incidences up to 100%, with an average of 40%. Mealybugs are not common but in another vineyard they were found responsive for a 100% GRLaV-3 infection in a few years (Cabaleiro *et al.,* 2006).

The effects of *Grapevine leafroll associated virus 3* on the cultivar Albariño in the "Rías Baixas" have been evaluated since 1992 (Cabaleiro *et al*., 1999; García-Berrios *et al.*, 2007). The first data showing that on years with not favorable environmental conditions the damages induced by the virus were higher or at least with higher economic impact, prompted us to compare field data from several more years and from two locations in the region. The knowledge of the economic impact of the virus could help to stress the absolute need of using virus free plant material and prevent virus dispersal by mealybug vectors.

MATERIALS AND METHODS

The field studies were carried out in two vineyards included in the "Rías Baixas" DOC (Galicia, Spain), described in previous papers (Cabaleiro *et al.*, 1999; García-Berrios *et al*., 2007). Potential alcoholic degree (PAD) from 6 vintages from each vineyard is available: 1992-1994 and 2003-2005 for the vineyard in the North (N) and 2000-2005 for the vineyard in the South (S). The average reduction in PAD in the must from GLRaV-3 infected plants is 0.9º (1.5 ºBrix) in the N vineyard and 1.2º (2 ºBrix) in the S vineyard. The differences in the PAD among years are significant both for infected and virus free plants. The N vineyard has a 33% GLRaV-3 and the S about 23%.

Maximum kg·ha^{-1} allowed in the Rías Baixas is 12.000. There were no differences between the harvest weight of leafroll free and the infected plants. The base for the calculation of losses was PAD:

a) price scale according to PAD as in the Grape Purchase Agreements with value during 3 campaigns which assigns a base price per kg to each PAD from 11º to 13º;
b) price scale as in "Bodegas Martín Codax" with average PAD of each vintage; the price of the grapes over or under is increased or decreased using intervals of 0.5 º: the first interval of 0.5º gets a bonus or penalty of 0.02 € per each 0.1º, in the second interval (0.5-1º over or under) it is 0.04 € per each 0.1º and in the next 0.06 € per each 0.1º. Initially, when the grapes did not reach 11º there was an additional penalty of 0.30 €/kg per 0.5º interval but nowadays grapes under 11º are not allowed in the cellar and growers must delay harvest or sell the grapes out of the DOC for table wine at low prices.

The economic losses for increasingly infected vineyards are calculated as a percentage of the amount which could be obtained in a 100% leafroll free vineyard.

RESULTS AND DISCUSSION

With 40% of infected plants in the vineyards of Rías Baixas and taking into consideration the average of 6 harvests, the lower PAD of the GLRaV-3 infected musts could be responsive for 21-26% economic losses with a) option and 17-19% with b). In the conditions of the N model, losses could reach 33% some years or even more because 4 out of the 6 years the PAD of the infected plants was under 11º and that means that some years with only 10% leafroll incidence the PAD of the musts could not reach the minimum. The b) option is apparently less strict and gives lower losses but the prices paid by that winery are higher than in "Grape purchase agreements" and the real decrease in income could be very high. In the southern vineyard 3 years out of 6, the PADs of the leafroll infected plants were under the minimum but the good quality of the healthy compensates and the average PAD for the six years is always over 11º although losses could reach more than 32% when incidence is over 40%.

Data from Martín Codax winery show a trend to delay the harvest date probably because growers understood that they cannot afford their grapes being out of the DOC. A selective delayed harvest of symptomatic plants has been proposed in other regions, but in white cultivars with mild symptoms as in Albariño, that is not feasible because it is not so easy to identify the virus infected plants that are randomly spread all over the vineyards because come from initially infected plant material. A delayed harvest in the climatic conditions of Rías Baixas is dangerous because the probability of rain at the end of September is high and there will be a higher risk of attacks of *Botrytis cinnerea*. Other management strategies as partial defoliation could be used but at high cost especially in horizontal training systems (Pereira *et al*., 2012).

The greatest concern that show some grape growing regions in the world about leafroll spread and damages to the wine industry (Walker *et al*., 2004; Nimmo-Bell, 2006; Freeboorough *et al.*, 2006; Shady *et al*., 2012), do not exist in Europe; drastic measurements as roguing infected vines or vineyard removal are not even suggested. However, this preliminary data indicate that the current economic impact of the leafroll epidemics is not lower that anywhere else, at least in the cooler climate areas with premium quality wines.

ACKNOWLEDGEMENTS

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Novel Antibodies For ISIA Detection Of Grapevine Leafroll-associated Virus 1 (GLRaV-1) based on the Variability of the Capsid Protein Gene

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INTRODUCTION

Grapevine leafroll-associated virus 1 (GLRaV-1) is implicated in the grapevine leafroll disease (LRD), a syndrome of complex aetiology and one of the most important diseases of grapevine, reportedly capable of causing yield losses of up to 68% [5]. All serologically diverse viruses implicated in LRD have been assigned to the family Closteroviridae, the only virus family that encodes a 70-kDa heat-shock protein homolog (HSP70h). Based on its molecular and biological characteristics, the majority of the LRD implicated viruses are placed in the genus Ampelovirus (either as accepted or tentative members), with GLRaV-2 in genus Closterovirus and GLRaV-7 remaining unassigned [4]. Although serological methods target the capsid protein the fact remains that the variability of this gene has been described for only a few of the LRD related Ampeloviruses. Recent phylogenetic and evolutionary analysis within the genus, based on the HSP70 and the CP genes, has shown that GLRaV-1 is a member of a distinct lineage within Ampelovirus, which also includes Grapevine leafroll-associated virus 3 (GLRaV-3) [3]. Testing for GLRaV-1 and -3 is compulsory in certification programs in the EU. For this purpose several antisera are commercially available, raised against the capsid protein (CP). In spite of the relevance given to GLRaV-1 and the known molecular variability of the HSP70h gene [2], the variability of the CP coding gene has seldom been addressed [1]. Only one complete genome sequence (JQ023131) and four complete capsid protein (CP) sequences are available at GenBank. Since routine detection is based on the CP, clearly more information is needed in order to improve serological and molecular detection methods.

MATERIALS AND METHODS

Plant material and RNA extraction: the virus sources were selected from plants established in the field at the Portuguese national collection of grapevine varieties (CAN-PRT051, Dois Portos). Ten grapevine varieties were molecularly tested for the presence of GLRaV-1 after serological analysis with commercial antibodies. Viral RNA was extracted from phloem scrapings, with the E.Z.N.A.™ *Plant Kit* (Omega Bio-tek).

Amplification, cloning and sequencing: total RNA was used to synthesize cDNA with *iScript™ Select cDNA Synthesis Kit* (Biorad, USA). PCR reactions were performed with *Pfu* DNA Polymerase ™ DNA polymerase (Fermentas, Thermo Fisher Scientific, Inc.) using a primer pair designed in this work to amplify the capsid protein (CP) gene, based on the sequence AF195822: CPLR1F: 5'-tcaataatactgcgtgctt-3' (sense) and CPLR1R: 5'-ctaacgcagtcgccattgt-3' (antisense). Amplification of a 540 nt fragment of the HSP70h gene was obtained with primers previously published [2]. The amplified fragments were cloned into pGEM-TEasy vector (Promega Corporation) before sequencing (CCMAR, UAlg, Portugal).

Phylogenetic analysis: nucleotide and deduced amino acid sequences were edited with BioEdit, aligned with ClustalW, together with all homologous sequences available at GenBank. Phylogenetic analysis was conducted with MEGA5.

Monospecific IgG: the alignment of the deduced amino acid sequences was used to obtain the capsid protein hydrophilicity profile as well as to identify conserved residues across all molecular variants. Polyclonal antibodies were obtained commercially (Biogenes, Germany) in goat immunized with a selected 15 AA long synthetic peptide, encompassing AA residues conserved in all of the aligned sequences. Commercial purification of the monospecific IgG and respective conjugation with alkaline phosphatase was obtained (Biogenes, Germany).

In Situ Immuno Assay (ISIA): transversal sections of fresh tendrils and petioles (40 to 60 µm tick) were obtained in a cryostate (Leica CM1850, Leica Microsystems, Germany) and adhered to glass slides coated with Merkoglas (Merck, Germany). Sections were blocked and then incubated with monospecific IgG-AP conjugated, for one hour, followed by revelation of the CP clusters with NBT-BCIP.

RESULTS AND DISCUSSION

The primers designed in this work allowed the amplification of a fragment encompassing the 973 nt sequence corresponding to the ORF of the capsid protein of GLRaV-1. Two out of the ten accessions selected, had previously been found negative by DAS-ELISA in consecutive years with commercial antibodies. The dendrogram constructed based on the nucleotide sequences obtained from the ten isolates showed the existence of well-resolved clusters (data shown on poster). We did not detect more than one group of variants per isolate, nor per grapevine variety. We found however, clusters containing sequences of different varietal origin. Combined, these results suggested a degree of stability within each cluster that was further investigated by deducing the amino acid sequences and screening the length of the ORF for possible cluster related residue patterns. It became apparent that the first half of the ORF contains the molecular signature of each cluster, mostly concentrated between residue 10 and 100, of a total of 324. The dendrogram constructed on the basis of the deduced AA sequences is concurrent with the one obtained for the nucleotide sequences and better resolved (data shown on poster). Analysis of the mean hydrophilicity profile of the CP did not detect significant differences between the clusters.

The monospecific IgG-AP, designed on the basis of the information retrieved from the deduced AA sequences was able to detect all molecularly positive samples in the ISIA test (data shown on poster).

The above described evidence of i) a distinct AA profile for each group of variants identified, together with the fact that ii) serologically negative samples contained variants of the CP not found in positive ones, strongly suggests the existence of type-variants and respective putative antigen groups. Inclusion of more sequences into the analysis, preferably of diverse origin, can further attest the phylogenetic inference value of the CP.

The practical relevance of these results are also obvious for a virus of compulsory testing, since they strongly suggest the possibility of overlooking a high percentage of positive samples in routine screening by the available serological methods.

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Genome Organisation And Characterization of a Novel Variant of *Grapevine Leafroll-Associated Virus 3*

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INTRODUCTION

To date at least 11 species of grapevine leafroll-associated viruses have been identified in the grapevine (Martelli *et al*., 2012). In the late 2000s, we reported a novel isolate of *Grapevine leafroll-associated virus 3* (GLRaV-3) from *Vitis vinifera* cv. Crimson Seedless table grapes in Western Australia (Habili and Randles, 2008; Habili *et al*., 2009). Preliminary studies showed that this virus induced a mild leafroll symptom similar to that induced by members of the Subgroup II of Ampeloviruses (Martelli *et al.*, 2012). The other feature of this isolate was that it was not detectable by RT-PCR using our routine primers. However, it tested positive by ELISA using commercially available kits. We named this isolate as the mild isolate of GLRaV-3 (GLRaV-3m). In 2010, we detected another isolate of this virus in a symptomless Sauvignon Blanc variety in South Australia (unpublished). For its further characterization, we sent a sample of infected cuttings to the Canadian Food Inspection Agency. Here, we provide further information on its genomic structure based on the analysis of its complete sequence and provide evidence for the divergence of virus RNA.

MATERIALS AND METHODS

Dormant cane samples of leafroll infected (but symptomless) grapevine *cv.* Sauvignon Blanc (SB) were obtained from the Adelaide Hills, South Australia. This sample was sent with a dozen of other samples for a routine indexing by ELISA and RT-PCR. The SB sample tested negative by RT-PCR even when a number of different primer sets were examined. However, the GLRaV-3 specific polyclonal/monoclonal antibodies (either from Bioreba or Sanofi) reacted positively to the virus by ELISA.

To obtain the initial sequences of the virus, we isolated its dsRNA (Valverde *et al*., 1990) and synthesized cDNA using random hexamers. Total RNA was obtained using a QIAGEN total RNA kit (Chatsworth, CA). The ends of viral RNA were sequenced by the 5' and 3' RACE method. The PCR products were cloned in TOPO (Invitrogen), and sequenced.

The sequencing reactions were performed using a Quick Start Kit (Beckman), purified by Centri-Sep™ columns (Princeton Separations, Adelphia, NJ), and then run on CEQ™ 8000 Genetic Analysis System (Beckman, Fullerton, CA). Sequences were assembled and analyzed using Lasergene DNA software (DNASTAR Inc, Madison, WI). Multiple sequence alignment from different clones was carried out using the MegAlign program of the Lasergene Software package (DNASTAR Inc.) to produce the primer sets used in the PCR cloning of the fragments.

RESULTS AND DISCUSSION

The viral sequence showed a single stranded RNA genome with 18498 nuleotides (Figs 1, 2 and Tables 1-3). Based on the serology and the sequence analysis, the virus was found to have 10 open reading frames (ORF) (12 genes) namely methyl transferase with 6713 nt, RNA polymerase with 1742 nt, HSP70h with 1649 nt, HSP90-like with 1100 nt, and 6 ORFs with unknown functions ranging in size between 557 and 113 nts (Fig. 2, Table 3). Based on genome sequence analysis and the phylogenetic trees constructed from the amino acid (aa) sequences of CP and HSP70h, the virus was found to be closely related to GLRaV-3. It had a CP size of 35 kDa as judged by Western blotting. Both the genome size (18498 nts) and the CP size (35 kDa) matched exactly those for the typical GLRaV-3 (Martelli *et al.*, 2012). However, a higher divergence was observed at the aa level across the genome as compared to the severe strains of GLRaV-3.

The divergence in the aa sequence of the CP, HSP70h and CPd genes of GLRaV-3m **a**nd those of selected GLRaV-3 isolates averaged 15.8, 12.2 and 25.6%, respectively (Table 1). In Ampeloviruses (Table 2) the boundary

for the species demarcation has been set at an aa sequence divergence of 25% (Martelli *et al.*, 2012), Only the aa sequence of CPd of GLRaV-3m has surpassed this boundary. However, the divergent values for the CPd aa sequence have not been designated as an species demarcation for the Ampeloviruses by Martelli *et al.,* (2012). BLASTn showed that the HSP70h sequence of GLRaV-3m had the highest match at the nucleotide level with the two South African isolates, 623 (GQ352632) and GP18 (EU259816) (Jooste *et al.*, 2010) confirming a similar match at the aa level (not shown, see also Fig. 1). Anecdotal observations suggest that GLRaV-3m does not spread naturally in Australia.

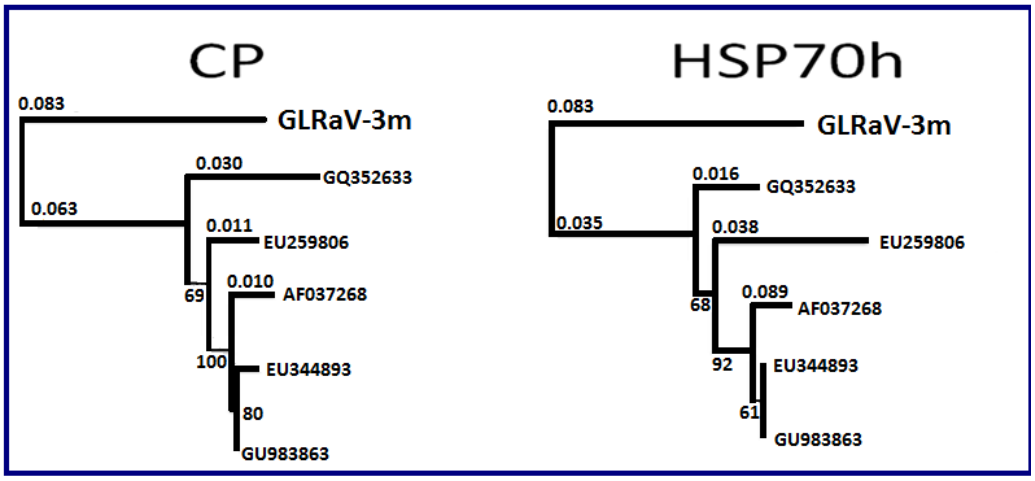

Fig.1. Phylogenetic tree constructed using CP and HSP70h of GLRaV-3m and other GLRaV-3 sequences in the Database. Bootstrap values (based on 100) are shown on nodes and branch lengths. Analysis is Distance Methods (BioNJ- Poisson- Squared).

Table 1. Comparison of amino acid sequences of GLRaV-3m to other GLRaV-3 isolates

Protein	Amino acid divergence (%)					
	AF037268 (NY1)	EU259806 (GP18)	EU344893 (CI-766)	GQ352633 (PL-20)	GU983863 (WA-MR)	Average
HSP70h	16	15	16	17	15	15.8
CP	12	14	12	11	12	12.2
CPd	26	25	25	26	26	25.6

Table 2. Comparison of amino acid sequences of GLRaV-3m to other Ampeloviruses

Protein	Amino acid identity (%)							
	Subgroup I				**Subgroup II**			
	GLRaV-3	**PMWaV-2[1]**	**GLRaV-1**	**LChV-2**	**PBNSPaV**	**GLRaV-4 strain 9[2]**	**GLRaV-4 strain Pr**	**PMWaV-1**
HSP70h	84	45	41	35	32	33	31	31
CP	88	39	31	19	20	22	22	21

[1]PMWaV, *Pineapple mealybug wilt associated virus*; LChV, *Little cherry virus*; PBNSPaV, Plum bark necrosis stem pitting-associated *virus.* [2] *see Martelli et al., 2012 for a new designation for the Subgroup II members.*

Fig. 2. Genome structure of GLRaV-3m (see Table 3).

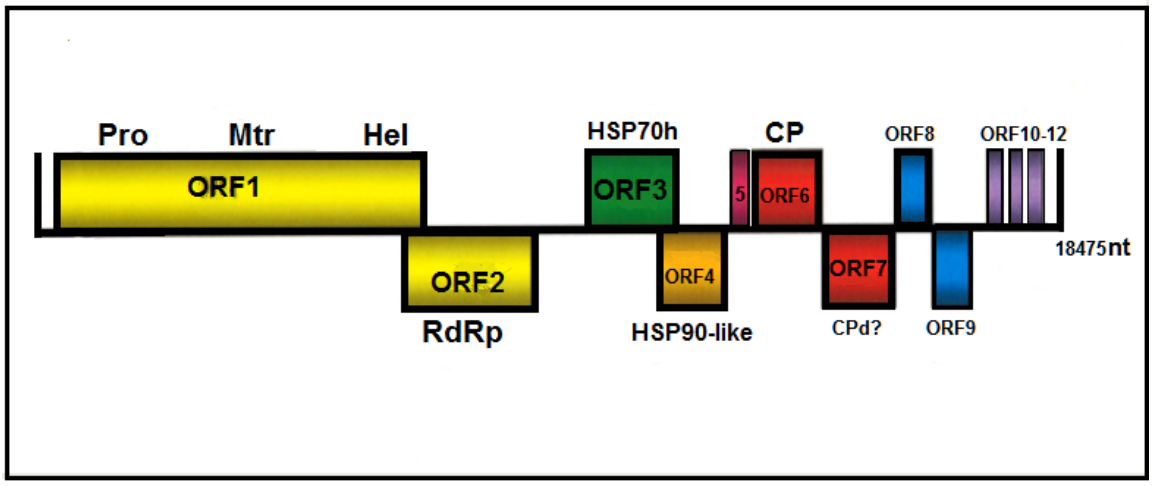

Table 3. Sequence annotation obtained from for 18498 nts of the GLRaV-3m genome.

ORF	Gene	Start	End	Size
1	Methyl Transferase	511	7224	6713
2	RNA Polymerase	7124	8866	1742
3	SHP 70h	10633	12282	1649
4	HSP 90-like	12275	13375	1100
5	unknown	13494	13727	233
6	Coat Protein (CP)	13817	14773	956
7	Divergent CP	14836	16269	1433
8	unknown	16280	16837	557
9	unknown	16834	17374	530
10	unknown	17374	17628	254
11	unknown	17631	17912	281
12	unknown	17912	18025	113

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Host Range and Biological Characterization of Argentinean Isolates of *Grapevine Leafroll Associated Virus* 2

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INTRODUCTION

Grapevine Leafroll associated Virus 2 (GLRaV-2), the only one member of the *Closterovirus* genus associated to Grapevine Leafroll disease, present a high genetic diversity. Recently up to six monophyletic groups were defined in function of coat protein (CP) sequence (Bertazzon *et al*., 2010; Jarugula *et al*., 2010). GLRaV-2 has been transmitted with difficulties to a restricted herbaceous host range. Using this approach, four isolates were biologically characterized, showing diverse symptomatology in *Nicotiana benthamiana, N.clevelandii* and *N.occidentalis*(Goszczynski *et al*., 1996; Abou Ghanem-Sabanadzovic et al. 2000). However there is not a comprehensive comparison of different isolates regarding the biological response of diverse host against the infection. In this work we attempt a biological characterization of eight Argentinean isolates of GLRaV-2 belonging to four different genetic groups in six different hosts belonging to the *Nicotiana* genus.

MATERIALS AND METHODS

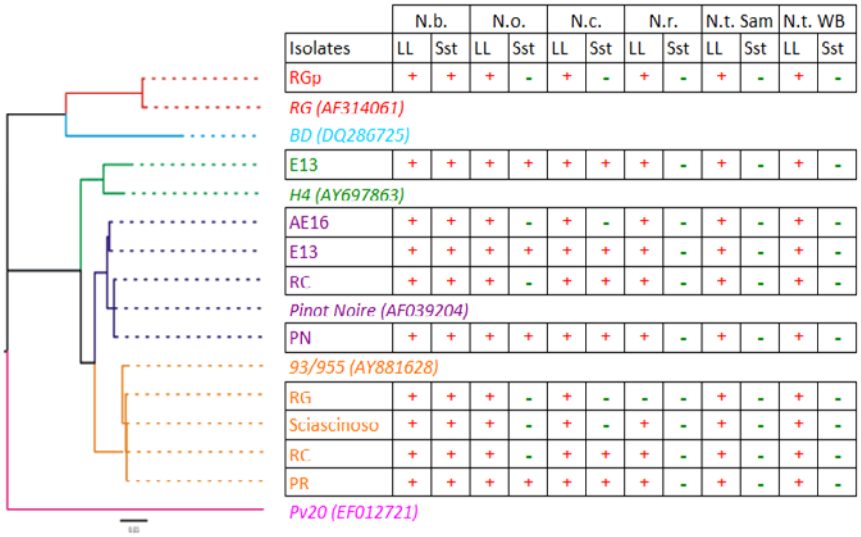
Over a previously established collection of field grown GLRaV-2-infected grapevine plants, eight accessions were selected (Lanza Volpe *et al*., 2009). dsRNA was extracted according to the method described by Zhang *et al*. (1998), retrotranscribed and subjected to PCR to amplify the entire CP ORF. The resulting PCR product was cloned and sequenced (at least three clones by product). The obtained sequences, together with six reference sequences belonging to each of the previously reported groups, were used for a phylogenetic reconstruction by Maximum Likelihood. Viral particles were purified from cortical scrapings of mature canes of the above mentioned plants, according to the method described by Gugerli *et al*.(1984). Carborundum-dusted leaves of *N.benthamiana* were rubbed with the viral extract, and after 30 days, apical leaves showing systemic symptoms were harvested, and stored at -85ºC until its inoculation over the selected herbaceous indicators. In order to establish the host range of the different isolates, batches of ten plants each of *N. benthamiana*, *N. occidentalis*, *N. clevelandii*, *N. rustica*, *N. tabacum* cv. Samsung and *N. tabacum* cv. White Burley were inoculated. The development of symptoms was recorded three times a week until 45 days after inoculation. Inoculated leaves were sampled 20 days after inoculation, and apical leaves 45 days after inoculation, in order to assess the presence of GLRaV-2 by DAS-ELISA.

RESULTS

The phylogenetic inference of the obtained sequences resulting from the infected plants used for transmission agree with the previous reports of clear group discrimination inside the viral specie. The eight isolates evaluated, belonging to four different genetic groups, were successfully transmitted from grapevine to *N.benthamiana*. In general terms, this initial efficiency of transmission, was quite low (approximately 20% of the inoculated plants developed systemic symptoms). However, the subsequent transmission from *N. benthamiana* to the other hosts was notably high. All the tested hosts developed at least local lesions against the infection with all the isolates. *N. benthamiana* was systemically infected with all the isolates, and *N. occidentalis* and *N. clevelandii* only were infected with some of the evaluated isolates. Neither *N. rustica*, *N. tabacum* cv Samsun or *N. tabacum* cv White Burley, developed systemic symptoms. In all the cases, the tissues showing symptoms of infection (local or systemic), gave a positive result in DAS ELISA. In same way, all the non-symptomatic tissues (even the apical leaves obtained from plants with local symptoms) gave a negative result in ELISA (Fig.1).

According to the results presented above, and using the terminology defined by Hull (2002), the six host evaluated are infectible by the eight strains of GLRaV-2 evaluated. However, differences in the interaction with the host appear to lead to a differential response of the last one. There is no clear association between the pertinence of a strain to a defined group, and the biological response from a host. The study of the host differential response

could allow a deep biological characterization of the virus-host interactions. Until our knowledge this is the first report of a compatible interaction between GLRaV-2 and *N. rustica* and *N. tabacum* cultivars. The clear behavioral differences among strains and host lead to infer that some of these biological systems could be very useful for the clarification of defense mechanism of the plants against GLRaV-2.



	N.b.		N.o.		N.c.		N.r.		N.t. Sam		N.t. WB	
Isolates	LL	Sst	LL	Sst	LL	Sst	LL	Sst	LL	Sst	LL	Sst
RGp	+	+	+	-	+	-	+	-	+	-	+	-
RG (AF314061)												
BD (DQ286725)												
E13	+	+	+	+	+	+	+	-	+	-	+	-
H4 (AY697863)												
AE16	+	+	+	-	+	-	+	-	+	-	+	-
E13	+	+	+	+	+	+	+	-	+	-	+	-
RC	+	+	+	-	+	+	+	-	+	-	+	-
Pinot Noire (AF039204)												
PN	+	+	+	+	+	+	+	-	+	-	+	-
93/955 (AY881628)												
RG	+	+	+	-	+	-	-	-	+	-	+	-
Sciascinoso	+	+	+	-	+	-	+	-	+	-	+	-
RC	+	+	+	-	+	+	+	-	+	-	+	-
PR	+	+	+	+	+	+	+	-	+	-	+	-
Pv20 (EF012721)												

Figure 1. Phylogenetic inference and presence of sysmptoms in the evaluated combinations of GLRaV-2 isolate-*Nicotiana* specie. **N.b.:** *N. benthamiana*; **N.o.:** *N. occidentalis*; **N.c.:***N. clevelandii*; **N.r.:** *N. rustica*; **N.t. Sam:** *N. tabacum* cv Samsum, **N.t. WB:** *N. tabacum* cv White Burley; **LL**: Local lesions and **Sst**: systemic symptoms. References sequences and their corresponding GenBank accession numbers are shown in italics.

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Genetic Diversity of the Silencing Suppressor of Grapevine Leafroll-associated Virus 2 and its Inhibition

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INTRODUCTION

GLRaV-2 is the only member of the genus *Closterovirus* (family *Closteroviridae)* that has been associated to the leafroll disease of the grapevine (Martelli *et al.*, 2002). Graft incompatibility and other symptoms have also been associated with GLRaV-2 (Bertazzon *et al.* 2010). A study on the genomic variability of HSP70h and CP nucleotide sequences revealed the existence of six phylogenetic groups (Jarugula *et al.* 2010). However very little is known regarding the genetic variability of the 3' – end of the genome, namely of the p24 gene. As many other viruses, GLRaV-2 encodes in its genome an RNA silencing suppressor protein (p24) that enables to overcome host antiviral defence (Chiba *et al.*, 2006). A logical strategy for obtaining resistance against GLRaV-2 is to target its VSR. *A priori*, this might be difficult to attain as p24 has been considered a strong silencing suppressor (Chiba *et al.*, 2006). In this paper we studied the genetic diversity of the whole p24 gene obtained from isolates taken of infected *V. vinifera* Portuguese varieties. Using a p24 clone from the most common phylogenetic group we designed different experiments to characterize the RNA silencing suppression triggered by GLRaV-2 p24 in *agrobacterium*-mediated transient expression assays.

MATERIAL AND METHODS

Total RNA was extracted from bark shavings with the aid of a magnetic particle processor KingFisher™ mL (Thermo Scientific) using the reagents from the MagMAX™-96 Total RNA Isolation Kit (Ambion), as described by Gouveia *et al*. (2011). The cDNA obtained was amplified by PCR using the primers LR2uP24_1 (5'-TCGTTAAGATGARGGTKATAGT-3') and LR2dP24_2 (5'-AAGTTGATACGTCAGGTAGAT-3') which flanked the p24 gene. The amplified cDNA fragments were TA cloned and a SSCP analysis was performed prior to sequencing in order to ensure that the clones representative of the most common patterns were be sequenced. A sequence database was constructed and subjected to phylogenetic analysis. The p24 gene from clone p2207-1 was inserted under control of the CaMV 35S promoter in the binary plasmid pK7WG2 through Gateway® Technology (Invitrogen). A long hairpin RNA of p24 (lhRNA-p24) was constructed by cloning p24 gene in the destination vector pK7GWIWG2(I), using a similar approach. The resulting lhRNA-p24 is constituted by the p24 gene in an antisense orientation, an intron that is part of the vector backbone and the p24 gene in sense orientation. The binary vectors were transferred into *Agrobacterium tumefaciens* strain C58C1.

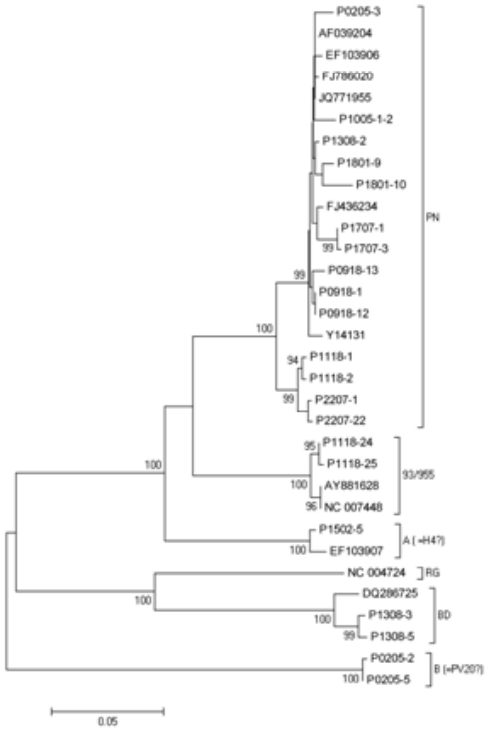
The *Agrobacterium*-mediated transient expression assays were done in WT *Nicothiana benthamiana.* GFP expression was obtained by infiltration with pK7WG2-mGFP5-ER. Silencing of GFP was obtained with a GFP specific long-hairpin (lhRNA-GFP) or an artificial micro RNA (amiRNA-GFP) as described by Gouveia *et al.* (2012). The silencing was suppressed by co-inoculation with pK7WG2-p24. Inhibition of suppression was studied in fourfold inoculations with the previous constructs plus the lhRNA-p24. Fluorescence analysis, northern blot assays and real-time quantification was done as previously described by Gouveia *et al.* (2012).

RESULTS AND DISCUSSION

Figure 1 shows the phylogenetic tree gathering the new and the Genbank available complete sequences of the p24 gene. The mean diversity for the entire population is 13.8%, a value similar to the genetic diversity of the CP gene (13.0%, data not shown). The same pattern of clustering found previously for the CP and HSP70h genes (Jarugula *et al.*, 2010) was obtained for the p24 gene, with correspondence for the lineages PN, 93/955, RG and BD. Due to the absence of reference sequences for this region, the correspondence of groups A and B cannot be ascertained. However, taking into account the topology for the HSP70 and CP, groups A and B corresponds probably to the lineages H4 and PV20, respectively.

The ability of p24 to suppress silencing was tested against a GFP specific artificial micro RNA or a long hairpin in *N. benthamiana* WT plants. In both cases the p24 suppressed the GFP silencing, as verified through real-time quantitation of GFP mRNA (Fig. 2), GFP specific siRNAs and visual observations (results not shown). An almost total restoring of silencing was obtained when the lhRNA-p24 construct was jointly inoculated.
These results demonstrate that although being a strong suppressor, the p24 can by its turn be silenced by an homologous long-hairpin construct. Nevertheless, in view of the diversity of p24 it remains to be verified whether a construct from the PN group is able to silence the p24 gene from a distant group (e.g. BD or RG)

Figure 1. Phylogenetic tree (Neighbour-joining, K2P) of the p24 gene from Portuguese isolates and Genbank sequences (identified through the accession number). Only bootstrap values above 90% are shown.



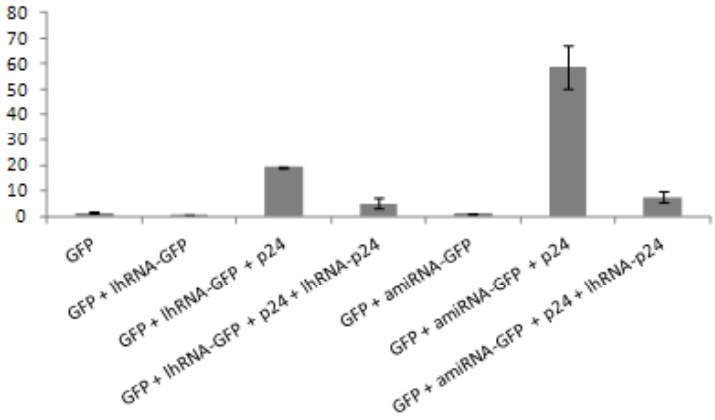


Figure 2. Relative GFP mRNA expression levels of WT co-infiltrated plant leaves at 5 d.p.i. Mean GFP expression of plants singly infiltrated with GFP construct was used as reference. Error bars represent SD of three independent determinations.

ACKNOWLEDGEMENTS

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Genome Sequence Analysis of *Grapevine Leafroll-associated Virus 2* Isolate from Own-Rooted, Asymptomatic Wine Grape Cultivar

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INTRODUCTION

Grapevine leafroll disease (GLRD) is one of the most economically important virus diseases affecting wine grapes (*Vitis vinifera* L.) in many grape-growing regions. GLRD produces distinct symptoms in red- and white-fruited cultivars (Rayapati et al. 2008). Among the currently recognized virus species associated with leafroll disease, only *Grapevine leafroll-associated virus 2* (GLRaV-2) belongs to the genus *Closterovirus*, while other GLRaVs belong to the genus *Ampelovirus* in the family *Closteroviridae* (Al Rwahnih et al., 2012). In addition to being distinct molecularly from grapevine-infecting closteroviruses, GLRaV-2 has been reported to occur as distinct biological and molecular variants in different grape-growing regions (Jarugula et al., 2010). Some of these variants have been documented in grapevines showing GLRD symptoms and others were implicated in several disease syndromes that are distinct from GLRD, such as graft-incompatibility (Alkowni et al., 2011). In a recent study, molecular diversity of field isolates of GLRaV-2 has been reported in the Pacific Northwest region of the U.S.A (Jarugula et al., 2010). Although most of these isolates were collected from wine grape cultivars showing GLRD symptoms, some isolates were obtained from asymptomatic own-rooted vines of cv. Sangiovese in a commercial vineyard. Since GLRaV-2 isolates from asymptomatic Sangiovese vines segregated into a separate sub-lineage within the H4 lineage (Jarugula et al., 2010), we determined the complete genome sequence of GLRaV-2 isolate from cv. Sangiovese (designated as GLRaV-2-Sg) and compared with full-length genome sequence of other GLRaV-2 isolates.

MATERIALS AND METHODS

Cambial scrapings of canes from a single, asymptomatic Sangiovese grapevine, tested positive only for GLRaV-2, were used for isolating genomic-length replicative-form double-stranded (ds) RNA. The same dsRNA-enriched preparation was used as a template for amplification of different portions of the virus genome. Primers were designed based on nucleotide sequence of GLRaV-2 isolates available in GenBank and used in reverse transcription-PCR to amplify overlapping portions spanning the entire genome. Sequences obtained from two independent clones specific to each amplicon were sequenced in both orientations to obtain a consensus sequence. Wherever necessary, additional clones were sequenced to resolve nucleotide sequence ambiguities. The 3' and 5' terminal sequences were confirmed using FirstChoice® RLM-RACE Kit (Ambion) following manufacturer's instructions. Sequences were analyzed using Vector NTI Advance11 software (Invitrogen Corporation, Carlsbad, CA, USA). Nucleotide and predicted amino acid sequences were aligned and edited, and sequence identities were determined using Simplot (Lole et al., 1999).

RESULTS AND DISCUSSION

The genome of GLRaV-2-Sg isolate was determined to be 16,461 nucleotide (nt) long. Similar to other GLRaV-2 isolates, the genome of GLRaV-2-Sg contains nine putative open reading frames (ORFs) with 106 nt long 5′non-translated region (NTR) and 244 nt long 3'NTR (Fig. 1). A pairwise comparison of GLRaV-2 genome sequences showed that GLRaV-2-Sg isolate is more closely related to the OR1 isolate (FJ436234) from Oregon, USA (84% identity) and the 93/955 isolate (AY881628) from South Africa (86% identity), and distantly related to the BD isolate (DQ286725) from Italy (73% identity) and the RG isolate (NC004724) from USA (73% identity). Pairwise comparison of individual ORFs of GLRaV-2-Sg with corresponding sequences from other isolates showed greater than 90% identities at nt and amino acid (aa) level among all ORFs, except p19, with GLRaV-2 strains, except BD and RG strains. The p19 ORF showed 84% aa identity with corresponding sequence of all GLRaV-2 isolates and 71% identity with RG and BD isolates. Since the nucleotide identity values of the coat protein and heat-shock protein-70 homolog (HSP70h) genes fall within the 10% identity range considered for

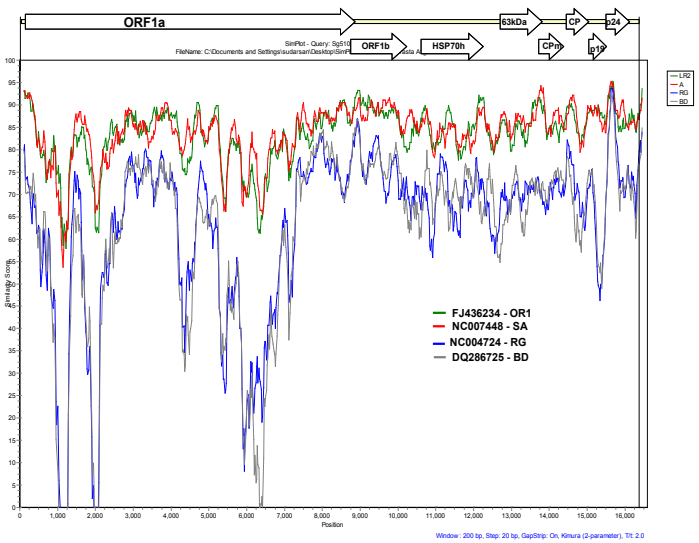

Figure 1. Schematic diagram of the genome organization of GLRaV-2-Sg (top, drawn to scale) depicting the positions of 5' and 3' nontranslated regions and open reading frames. The nucleotide sequence was compared with the genome of GLRaV-2 isolates OR1 (FJ436234), SA (NC007448), RG (NC004724) and BD (DQ286725). Sliding-window simplot graph was generated using the multiple alignment of all isolates using a window size of 200 nt and a step size of 20 nt. Lowest sequence identities (<50%) were observed in four different regions (1000 to 1300 nt, 1700 to 2100 nt, 4250 to 4650, and 5200 to 7200 nt) of the ORF1.

strain demarcation (Martelli et al., 2005), it can be concluded from our data that the Sg isolate is a distinct strain of GLRaV-2 causing asymptomatic infection in own-rooted wine grape cv.Sangiovese. Further studies are in progress to determine if the GLRaV-2-Sg isolate can produce symptoms or remain asymptomatic in other wine grape cultivars planted as own-rooted or grafted vines. Nevertheless, from a practical point of view, the presence of asymptomatic strains of GLRaV-2 have implications in symptom-based surveys for the diagnosis of GLRD and underscores the importance of rigorous testing of planting materials in clean plant programs to ensure that virus-tested planting materials are provided to nurseries and grape growers.

ACKNOWLEDGEMENTS

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Development of Full Length Infectious cDNA Clone of *Grapevine Leafroll-associated Virus* 3

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INTRODUCTION

In recent years, the generation of infectious virions from full-length genomic cDNA clones has been achieved for many positive-sense RNA viruses infecting plants. These advances provided possibilities to use reverse genetics system as a powerful research tool for studying the etiology of virus diseases in plants and for directed genetic manipulation of virus genomes to understand their molecular biology and various aspects of virus-host interactions at the molecular level. In these regards, the development of infectious cDNA clones for viruses in the genera *Closterovirus* and *Crinivirus* of the family *Closteroviridae* has greatly advanced our understanding of the molecular biology of closteroviruses and offered an opportunity to manipulate their genomes for both fundamental and practical applications (Dawson, 2010). An extension of these studies to members of the genus *Ampelovirus* that cause serious diseases in grapevines would provide basic knowledge that has potential practical value and offer avenues for a better understanding of fundamental differences between closteroviruses and ampeloviruses infecting perennial crops. In this study, we report the development of a full-length infectious cDNA clone for *Grapevine leafroll-associated virus 3* (GLRaV-3), the type species of the genus *Ampelovirus*, and the most complex among the different GLRaVs.

MATERIALS AND METHODS

The cDNA clones of GLRaV-3 generated from our previous study (Jarugula et al., 2010) were used to assemble full-length clones of the virus in a pUC119-based vector using a multi-step cloning strategy. We also built less-than full-length cDNA clones of GLRaV-3 by deleting varying portions of the viral sequence between Open reading frame (ORF) 1a/b and 3'nontranslated region (NTR). After verifying their integrity by sequencing, each of the full-length and less-than full-length cDNA clones were used for generating *in vitro* RNA transcripts and tested their replication using mesophyll protoplasts isolated from *Nicotiana benthamiana* leaves (Satyanarayana et al., 1999). Subsequently, candidate cDNA clones were sub-cloned into the T-DNA region of the binary vector pCAMBIA1380 in such a way that the CaMV 35S promoter with a double enhancer cassette is at the 5´ end and a hammerhead ribozyme sequence is at the 3´ end of each cDNA clone (Fig. 1A). The plasmid constructs were mobilized into *Agrobacterium tumefaciens* strain EHA105 and individual colonies were checked for the presence of recombinant plasmids through colony PCR using virus-specific primers. Bacterial suspension derived from single, positive colony for each construct was adjusted to an OD_{600} of 1 and used for infiltration of *Nicotiana benthamiana* (3 to 4 weeks old) leaves. Infiltrations were done by gently pressing the blunt end of a 1-ml syringe filled with bacterial culture to the abaxial leaf surface and exerting gentle pressure to flood the interstitial areas within the leaf. The silencing suppressor gene $HC\text{-}pro^{TuMV}$ of *Turnip mosaic virus* or $p19^{TBSV}$ of *Tomato bushy stunt virus* were used in co-infiltration assays by mixing equal amount of bacterial cells (based on A_{600}=1) containing the suppressor construct and the candidate cDNA construct. Plants were maintained in a growth chamber at 25 ± 1°C with 14:10 hr photoperiod. The infiltrated leaves were harvested at daily intervals until 8 days post-infiltration and analyzed for GLRaV-3 genomic and sub-genomic(sg) RNAs by Northern blotting (Jarugula et al., 2010). Virion formation was ascertained by enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM) using polyclonal antibodies for the coat protein of GLRaV-3 (Bioreba AG, Switzerland).

RESULTS AND DISCUSSION

Using a multi-step cloning strategy, we have assembled the 18,498 base pair full-length genomic cDNA clone of GLRaV-3 in a modified pUC-119 vector. The capped RNA transcripts generated *in vitro* from full-length cDNA clones were used for inoculating protoplasts isolated from *N. benthamiana* leaves. Total RNA was isolated from protoplasts 4-5 days post-inoculation and analyzed by Northern blot hybridization for the presence of 3'-co-terminal sgRNAs (Jarugula et al., 2010). Since the presence of sgRNAs in Northern blots is indicative of virus replication, the results gave a definitive measurement of infectivity of *in vitro* transcripts generated from cDNA clones. Similarly, RNA transcripts generated from smaller size subset of cDNA clones of GLRaV-3, with sequences of varying length deleted between ORF 1b and 3'NTR, were able to replicate in *N. benthamiana* protoplasts. To negate some of the disadvantages associated with protoplast systems, we pursued *A. tumefaciens*-assisted inoculation of *N. benthamiana* leaves to test replication competency of individual recombinant binary vectors containing full-length or less-than full-length cDNA clones. We co-infiltrated with heterologous viral-encoded

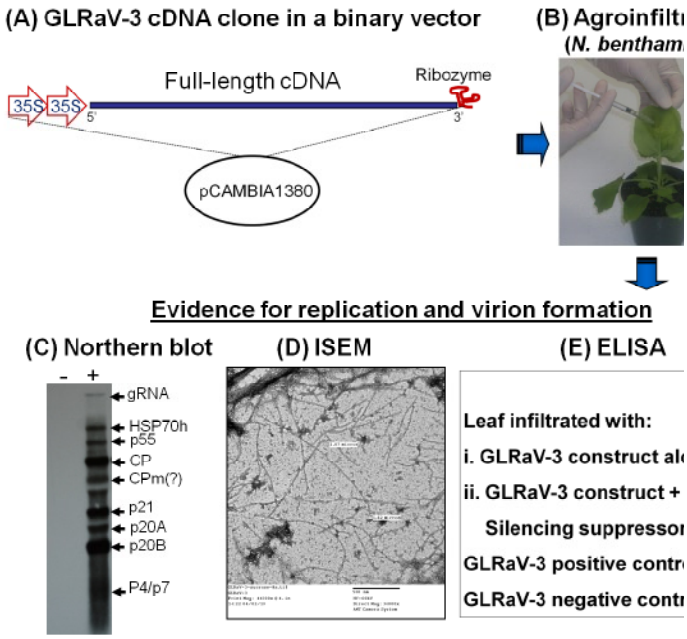

Fig. 1. (A) Full-length cDNA clone of GLRaV-3 in a binary vector between enhanced double 35S promoter and a hammerhead ribozyme sequence. (B) Full-length cDNA clone introduced into *N. benthamiana* leaves via infiltration using *A. tumefaciens* strain EHA105. (C) Northern blot showing the presence of 3' co-terminal sgRNAs in leaves co-infiltrated with a heterologous viral-encoded gene silencing suppressor (right lane) and no sgRNAs in leaves infiltrated with cDNA construct alone (left lane). (D) Flexuous filamentous particles observed under electron microscope and (E) A405 values in DAS-ELISA with samples from agroinfiltrated and immunoelectron microscopy with antibodies to the wild type virus confirmed virion formation.

suppressor of gene silencing (HC-pro^{TuMV} or p19^{TBSV}) with viral cDNA construct to alleviate host silencing response. Northern blot analysis of total RNA from agro-infiltrated leaves showed that in leaves co-infiltrated with full-length GLRaV-3 and a binary vector containing a heterologous silencing suppressor, sgRNA for p20B was the first progeny sgRNAs detected at 6 days post-infiltration (dpi). Accumulation of sgRNAs specific to p4/p7, p20A, p21 and CP were readily observed on 7 dpi and all these sgRNAs increased substantially by 8 dpi as deduced from the intensity of the hybridization signal in Northern blots. The presence of these sgRNAs in Northern blots was comparable with the six sgRNAs detected in grapevine (*Vitis vinifera*) naturally infected with GLRaV-3 (Jarugula *et al*., 2010). These results provided a definitive measurement of replication of full-length cDNA of GLRaV-3. Filamentous virion particles ranging in length from 1000-2200 nm were observed in agroinfiltrated leaves and their reaction specificity in ISEM and ELISA to polyclonal antibodies to the CP of wild-type GLRaV-3 further confirmed that the full-length cDNA clone of GLRaV-3 can replicate and form authentic virion particles in agroinfiltrated leaves. These results represent the establishment of a full-length infectious cDNA clone for GLRaV-3. Since less-than full-length cDNA constructs showed replication in protoplasts, we tested these constructs for their ability to replicate in agroinfiltrated *N. benthamiana* leaves. Northern blot analyses of total RNA extracted from agroinfiltrated leaves showed sgRNAs corresponding to the ORFs present in each of these constructs. Replication of GLRaV-3 cDNA construct consisting only the 5'NTR, ORF1a/b and 3'NTR indicated that genes between ORF1a/b and 3'NTR are not essential for virus replication, as reported for other closteroviruses like Citrus tristeza virus and Beet yellows virus. The availability of infectious cDNA clones of GLRaV-3, together with assays available for infecting grapevine with a cDNA clone of GLRaV-2 (Kurth et al., 2012), provides the critical reagents needed to apply reverse genetics for understanding the role of different GLRaVs in the etiology of GLRD and to study the molecular biology of ampeloviruses in comparison with other monopartite closteroviruses.

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Partial Characterization of a New Divergent Variant of GLRaV-4

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INTRODUCTION

Grapevine leafroll disease (GLD) is one of the most economically important viral diseases. GLD is associated with a complex of filamentous viruses referred to as Grapevine leafroll-associated viruses (GLRaVs). All GLRaVs identified so far belong to the family *Closteroviridae*. Up to now, 11 different GLRaVs have been identified: one in the genus *Closterovirus* (GLRaV-2), nine in the genus *Ampelovirus* and one in the new-defined genus *Velarivirus* (GLRaV-7) (Al Rwahnih *et al.* 2012). The genus *Ampelovirus* is further divided into subgroup I (GLRaV-1 and GLRaV-3) and subgroup II containing the short ampeloviruses GLRaV-4, -5, -6, -9, GLRaV-De, GLRaV-Pr and GLRaV-Car. A recent taxonomic revision of the genus *Ampelovirus* proposed that GLRaV-5, -6, -9, GLRaV-De, GLRaV-Pr and GLRaV-Car are all molecular variants of a single species, GLRaV-4 and not, as has been assumed previously, distinct species in the genus *Ampelovirus* (Martelli *et al.* 2012). Here we report the detection of a new variant of the GLRaV-4 and examine the relationship with other members of the genus *Ampelovirus*. For convenience, all variants belonging to the reference species GLRaV-4 will be referred in this work as to “GLRaV-4 group”.

MATERIALS AND METHODS

During field inspection of the grapevine collection at Agroscope ACW in Nyon, a vine (No 11970) was detected with clostero-like particles but it was not infected by one or more of the known grapevine leafroll-associated viruses. Nested RT-PCR was used with a set of degenerate primers designed on conserved regions of the heat shock protein 70 homologue gene to identify Grapevine leafroll viruses (Dovas and Katis, 2003). The CODEHOP (Consensus Degenerate Hybrid Oligonucleotide Primers) approach has been used to characterize further parts of the viral genome (Boyce *et al.* 2009). Amplification products were subsequently cloned into pGEM-T Easy plasmid (Promega Corporation, USA) and Sanger DNA sequencing was performed by FASTERIS SA (Geneva, Switzerland). Open reading frames were identified using the NCBI ORF Finder. Multiple alignments with published sequences were carried out and the percent amino acid sequence identity was calculated using the program Clustal W. The phylogenetic relationships were determined with the maximum likelihood algorithm of the MEGA 5 package.

RESULTS AND DISCUSSION

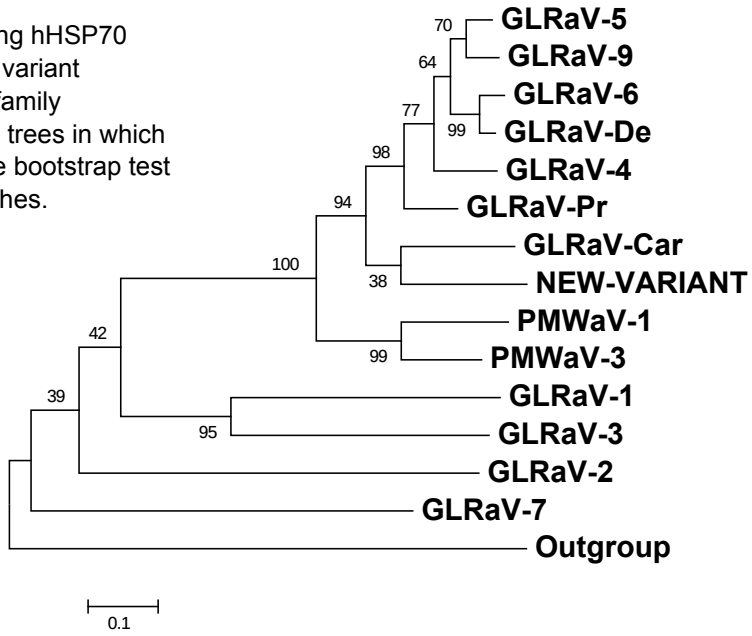
Initial molecular characterization of this virus was carried out. An hHSP 70 amplicon of 502 bp was obtained from the nested RT-PCR with degenerate primers. Using TBLASTX algorithm, the obtained sequence showed a limited amino acid identity with the hHSP70 genes of P*ineapple mealybug wilt-associated virus* 1 and 3 and of viruses belonging to “GLRaV-4 group”. Further sequence data were obtained using a CODEHOP approach based on the sequences of “GLRaV-4 group” already available in GenBank. The generated sequence data consisted of 5500 nucleotides and putatively encodes 5 complete ORFs: p5, hHSP70, p60, CP and p23. The genomic arrangement was typical of members of the “GLRaV-4 group” (Abou Ghanem-Sabanadzovic *et al.* 2012). However, the sequence data showed that this virus was fairly different from other ampeloviruses of subgroup II. For example, amino acid identity in the gene of hHSP70 between the new variant detected in this study and other viruses of “GLRaV-4 group” vary from 63-66% (Table 1).

In phylogenetics analyses, performed on amino acid sequences of hHSP 70 and CP, the new variant always clustered with viruses of the subgroup II in the genus *Ampelovirus* (Figure 1). GLRaV-Car (Abou Ghanem-Sabanadzovic *et al.* 2010) and the new variant described here appeared to be the most distinct member of the GLRaV-4 cluster. Those two viruses were consistently placed slightly apart from the rest of the “GLRaV-4 group” (Figure 1). Based on our results, the virus identified during the inspection of our grapevine collection appears to be a new divergent strain of the “GRLaV-4 group”. The description of this new divergent variant confirmed the high genetic diversity within the species GLRaV-4.

	GLRaV-9	GLRaV-5	GLRaV-4	GLRaV-6	GLRaV-Car	GLRaV-De	GLRaV-Pr	**New variant**	***Mean Divergence (%)***
GLRaV-9	100	89	81	84	67	84	79	65	22
GLRaV-5		100	84	84	67	85	80	65	19
GLRaV-4			100	78	66	81	77	66	24
GLRaV-6				100	66	92	78	65	22
GLRaV-Car					100	66	69	63	34
GLRaV-De						100	78	66	21
GLRaV-Pr							100	64	25
New variant								100	35

Table 1. Heat shock protein 70 homologue amino acid sequence identity (%) of members of the "GLRaV-4 group".

Figure 1. Phylogenetic tree constructed using hHSP70 complete amino acid sequences of the new variant of GLRaV-4 and some other species in the family *Closteroviridae*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.



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Current Status of Major Grapevine Viruses in La Côte Vineyards of Switzerland

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INTRODUCTION

Viral diseases are reported to cause several detrimental effects on grape and wine production. However, except for Valais vineyards (Besse and Gugerli, 2009), the sanitary status elsewhere in Switzerland has not been studied by modern diagnostic tools. Therefore, we have undertaken a survey of viruses occurring in the vineyards of La Côte in the canton of Vaud. The vineyards of the La Côte region cover a surface of ca. 2000 ha and are located on the edge of Lake Geneva between Lausanne and Geneva. Chasselas is the predominant cultivar (65 % of the surface). The aim of the present work was to evaluate in commercial vineyards the occurrence of viruses and their rate of incidence. The results will help to evaluate the economic impact of viral diseases and to define an efficient control strategy to mitigate their negative impact.

MATERIALS AND METHODS

Sixty commercial vineyards were selected on a random basis in the La Côte region. Vineyards are at least ten years old. Within a given vineyard, a plot (500m^2) was defined and 30 individual grapevines were sampled at random. Samples were collected in January 2012 and consisted of dormant canes. To account for the possible uneven distribution of the virus within a vine, three dormant canes per plant were collected and bulked for virus testing. Cane samples were tested by double-antibody-sandwich ELISA (DAS-ELISA) with reference monoclonal antibodies from commercial kits from BIOREBA AG (Reinach, Switzerland). The following viruses were assessed by DAS-ELISA: *Grapevine leafroll-associated virus (GLRaV) 1, -2, -3, -4*, *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV) and *Grapevine fleck virus* (GFkV). The following viruses were assessed by RT-PCR on pooled samples (20 individual grapevines per vineyard): *grapevine virus A* (GVA), *grapevine virus B* (GVB), *grapevine rupestris stem pitting-associated virus* (GRSPaV) and GLRaV-7. As viruses (GLRaV-4, -5, -6, -9) belonging to subgroup I of ampeloviruses are divergent variants of a single species, GLRaV-4 (Martelli *et al.*, 2012), they were grouped together under the name GLRaV-4.

RESULTS AND DISCUSSION

Samples from 1800 individual grapevines were collected from 60 vineyards (locations). All samples tested negative for GVA and GLRaV-7 (Table 1). The viruses GRSPaV, GFkV and GLRaV-2 were predominant in the La Côte vineyard. All tested locations were infected by GRSPaV. GFkV infections were detected in nearly all tested locations and its incidence was high (Table 2). GLRaV-2 is the third most frequent virus which was detected in 72 % of all locations. As reported by various authors (Rayapati *et al.*, 2009, Gangl *et al.*, 2011), the mild viruses GLRaV-2 and GFkV are widely distributed in certain wine regions.

Virus	Tested locations	Virus infected locations (%)
GLRaV-1	60	37
GLRaV-2	60	72
GLRaV-3	60	67
GLRaV-4	60	20
GLRaV-7	47	0
GFLV	60	57
ArMV	60	13
GFkV	29	97
GVA	47	0
GVB	47	36
GRSPaV	47	100

Table 1. Viral infection frequency in commercial vineyards in the La Côte region.

Virus incidence (%)	Percentage of locations						
	ArMV	GFLV	GLRaV-1	GLRaV-2	GLRaV-3	GLRaV-4	GFkV
0 (none)	87%	43%	63%	28%	33%	80%	3%
1-10 (low)	8%	33%	29%	13%	31%	18%	10%
11-20 (moderate)	3%	10%	5%	19%	33%	2%	4%
21-50 (high)	2%	8%	0%	13%	3%	0%	59%
51-90 (very high)	0%	3%	3%	25%	0%	0%	10%
91-100 (extremely high)	0%	3%	0%	2%	0%	0%	14%

Table 2. Incidence of ArMV, GFLV, GLRaV-1, -2, -3, -4, and GFkV in La Côte vineyards.

GFLV infection was present in 57 % of the tested locations. After GLRaV-2 and GFkV, GFLV was the virus with the highest incidence. The other nepovirus tested, ArMV, was detected at a lower level and always on locations already infected by GFLV.

After GLRaV-2, GLRaV-3 was the most common virus associated to leafroll in the region. GLRaV-3 infection was detected in 2/3 of the tested locations and in higher percentage than GLRaV-1. The incidence of these two viruses was moderate, nearly all infected locations showed less than 20 % of infected samples (Table 2). GLRaV-4 was only occasionally detected. Grapevine leafroll disease appeared to be widespread in La Côte vineyard as 3/4 of the locations were infected by GLRaV-1 or GLRaV-3. The spread of leafroll disease in La Côte is similar to what has been reported for Valais area, another Swiss wine region (Besse and Gugerli 2009). However, contrary to the latter region, La Côte vineyard is characterized by a high percentage of GLRaV-2 infection, a result inherent to the prevailing cultivar Chasselas.

As shown by Spring *et al.* (2012), although GFkV infection is apparently latent in *Vitis vinifera*, the presence of GFkV in mixed infections with GLRaV can contribute to increase the negative effects associated with leafroll disease. Here the reported high rate of GFkV and GLRaV infection found in the La Côte vineyard might have detrimental effects on grape and wine production. Considering those results, there is little doubt that it is important to use virus free plant from certificated material and that grapevine certification program is of paramount importance for a sustainable wine production.

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Influence of the Grapevine Leafroll Associated Virus (GLRaV-1) and Grapevine Fleck Virus (GFkV) on the Grape and Wine Production of cv. Gamay

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INTRODUCTION

Viral disease are reported to cause several detrimental effects on grapevine. Grapevine leafroll disease, due to single or mixed infections of different Ampelovirus and Closterovirus, was shown to affect grape production both quantitatively and qualitatively. Grapevine fleck virus (GFkV), one of the most spread virus in grapevine, is latent in European cultivars. As reported by Besse and Gugerli (2009), GFkV can co-infect GLRaVs-infected vines at a relatively high rate. However, few scientific reports have been published on the effect of mixed viral infections on grape and wine production. Therefore, the aim of the present work was to evaluate the effects of GFkV co-infection with GLRaV-1 on physiological, agronomic and oenological characteristics of Gamay.

MATERIALS AND METHODS

Gamay Rouge de la Loire (indicator used at ACW for indexing for leafroll) was grafted on healthy 3309C. Virus inoculation was done by triple grafting according to the following variants:

A: Healthy control (triple grafting with two healthy interscions and one healthy Gamay scion)
B: GLRaV-1 (triple grafting with one healthy interscion, one GLRaV-1infected interscion and one healty Gamay scion)
C: GFkV (triple grafting with one healthy interscion, one GFkV infected interscion and one healthy Gamay scion)
D: GLRaV-1 + GFkV (triple grafting with one GLRaV-1infected interscion , one GFkV infected interscion and one healthy Gamay scion)

Plots were planted in 1998 at the Agroscope research station in Nyon (Switzerland) in four randomized blocks with 10 vines per replicate. Vine yield components (bud fertility, cluster weight, and berry weight), fruit composition at harvest (sugar content °Oe, pH, titratable acidity expressed as tartaric acid equivalents, tartaric and malic acid and yeast assimilable nitrogen) and pruning weight was measured from 2001 to 2010. Trimming parts of the canopy were weighted during summer of 2001 to 2004. From 2001-2005, foliar analysis was performed to evaluate vine mineral status (N, P, K, Ca and Mg). Chlorophyll index was measured from 2001 to 2003. From 2001 to 2004 grapes from the four variants were collected for identical winemaking procedures.

RESULTS AND DISCUSSION

Yield components. Figure 1 illustrates the yield at harvest of the four variants from 2001 to 2010. Two groups can be distinguished: the healthy control together with the variant with only GFkV which showed a significantly higher yield compare to the two variants infected by GLRaV-1 with or without GFkV. Significant differences were also observed between those two groups concerning bud fertility, cluster weight and berry weight.

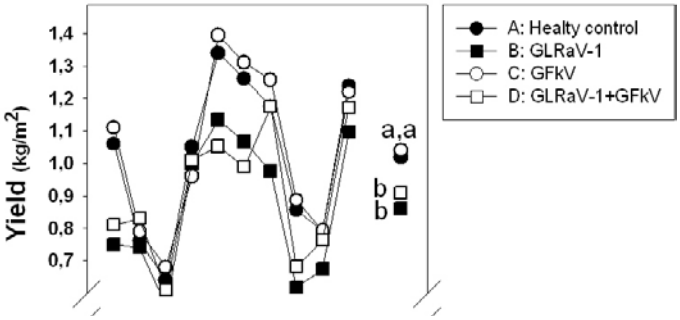

Figure 1. Effects of GLRaV-1 and GFkV infection on Gamay. Yield at harvest, 2001-2010. Means with different letters are significantly different at P ≤ 0.05.

Vegetative growth. Vine growth, indicated by pruning weight and trimming weight, was significantly reduced by GLRaV-1 infection (average reduction for pruning weight: -20% and -50% for trimming weight). The negative impact on vegetative growth of a mixed infection by GLRaV-1 and GFkV did not differ compare to an infection by GLRaV-1 alone.

Mineral nutrition. The effects of GLRaV-1 with or without GFkV co-infection were the following on vine mineral status: significantly decrease of the leaf nitrogen, calcium and magnesium content and a significant increase

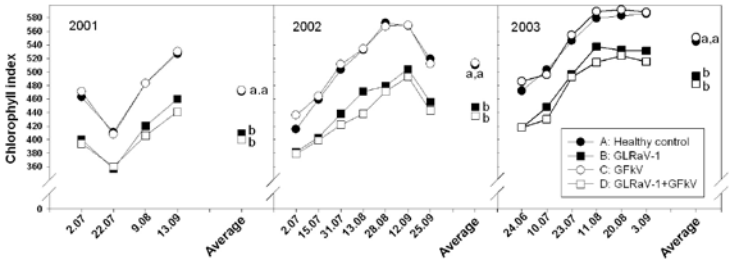

Figure 2. Effects of GLRaV-1 and GFkV infection on Gamay. Chlorophyll index, 2001-2003. Means with different letters are significantly different at P ≤ 0.05.

of the leaf potassium content. Chlorophyll index is a measure of the density of the green color in the leaves, its evolution for the four variants is depicted in Figure 2. The infection by GLRaV-1 had a clear effect on leaf colour. The mixed infection by GLRaV-1 and GFkV tend to aggravate symptoms compared to infection by GLRaV-1 alone, although this relation was not statistically significant.

Must composition. The infection by GLRaV-1 in combination or not with GFkV was associated with modifications of must composition (Table 1). The variant infected by GLRaV-1 alone presented a higher fruit sugar content (°Oe), due probably to lower yield (-20%). On the other hand, the variant co-infected by GLRaV-1 and GFkV had the lowest value for fruit sugar content. The presence of GFkV in mixed infection with GLRaV-1 contributed to increase the negative effects associated with GLRaV-1 infection. Similar results were observed for must nitrogen content : mixed infection by GLRaV-1 and GFkV had a negative impact on must nitrogen content, but infection by GLRaV-1 alone did not have any impact.

Wine analysis. Concerning wine composition parameters, none of the observed differences between the four variants were significant. The results of the sensory analysis showed a significant decrease of tannin intensity for the variant with co-infection by GLRaV-1 and GFkV. The overall quality of wine tannin was evaluated generally lower in the co-infected variant compared to the others variants.

Variant	Soluble solids content (°Oe)	pH	Titratable acidity (g/l)	Tartaric acid (g/l)	Malic acid (g/l)	Yeast assimilable nitrogen (g/l)
Healthy control	90,6 b	3,09 a	10,8 a	7,2 a	5,4 b	171 b
GLRaV-1	92,9 a	3,10 a	10,7 a	6,9 b	5,4 b	174 b
GFkV	89,5 c	3,07 b	10,8 a	7,1 a	5,6 a	176 a
GLRaV-1 + GFkV	88,6 c	3,08 a	10,8 a	7,3 a	5,1 c	150 c

Table 1. Effects of GLRaV-1 and GFkV infection on Gamay. Must composition, average over years 2001-2010. Means with different letters are significantly different at P ≤ 0.05.

Discussion. In this study, yield parameters and vine vigor were significantly reduced by GLRaV-1 infection, which Mannini *et al.* (1997) and Bertamini *et al.* (2005) noted as well. Co-infection by GLRaV-1 and GFkV did not have a significant effect on those parameters compared to infection by GLRaV-1 alone. We observed a synergistic effect between GLRaV-1 and GFkV on fruit composition (reduction of sugar and nitrogen content) and wine quality. Other authors have reported the negative effect of leafroll on grape and wine production (Jungmin and Martin, 2009, Besse *et al.,* 2009), here we showed that GFkV, when present in co-infection with GLRaV-1, had the potential to increase the negative impacts of GLRaV-1 (Spring *et al.,* 2012).

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Rugose Wood – associated Viruses and *Similia*: 2010-2012

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Rugose wood-associated agents comprise viruses belonging in the genera *Vitivirus* and *Foveavirus*, family *Betaflexiviridae*, order *Tymovirales.* A brief review of the recent information on grapevine-infecting flexivirids is provided, together with a description of a new disease caused by a betaflexivirus belonging in the genus *Trichovirus*.

FOVEAVIRUS: *Grapevine rupestris stem pitting-associated virus*

Updates from the Dijon Meeting shows that, among viruses associated with Rugose wood many research groups payed much attention to *Grapevine rupestris stem-pitting associated virus* (GRSPaV). A perusal of the recent literature shows that six full papers dealing with this virus have been pulbished (1, 30,31, 13, 20, 23), addressing primarily: (i) the extreme molecular diversity of this virus; (ii) its multi-faceted involvement in three diverse diseases: Vein Necrosis on 110 Richter (6); Rupestris Stem Pitting (32) and 'Syrah Decline' (18).

According to the existing literature (21, 24) several authors (1, 23) found that GRSPaV segregates in four different lineages comprising six fully [GRSPaV-1, -SG1, -BS, -SY, -PG, -MG (1, 12, 23)] and one partially sequenced (31) genomes, regardless of the gene (coat protein) or domain (Helicase or RdRp) used for phylogenetic studies. Further studies (31) identified two new virus variants, tentatively proposed as lineages VI (GRSPaV-MT) and VII (GRSPaV-ML) from cvs Muller Thurgau and Merlot, respectively. These last sequences are limited to short regions in the Helicase and the RdRp domains.

Because of the co-infection of GRSPaV variants in the same plant, recombination events were identified when the CP and Hel genes are compared (1). According to (1) these two genes are subjected to a different purifying selection since the lack of vector transmission makes the CP more tolerant to mutations, whereas constraints imposed by replication makes the Hel domain less prone to variability.

The number of abstracts in the RW session of this Meeting devoted to this virus, which are five out of nine, also follows this trend. Confirming previous findings (21) Sevin et al. (29) analyzed several American and European grapevine accessions and concluded that an ancestor of GRSPaV infected the *Vitis* species long time ago since it is found in cultivated (*Vitis vinifera* ssp. *vinifera*) and wild (*V. vinifera ssp. sylvestris*) grapevines. A couple of abstracts address GRSPaV involvement in "vein necrosis" of 110 Richter (VN) . Whereas Della Bartola *et al*. (7) substantiated the association of GRSPaV variants belonging to groups 2a and 2b with VN, Alliaume *et al.* (4) found this relationship less consistent. The widespread presence of this virus prompted Gambino *et al.* (10) to explore GRSPaV effects on cultivated grapes. In a field study which combines physiological observations and global gene expression analysis by microarray, these authors demonstrated that this virus establishes a unique interaction with the plants as a result of a long co-existence with the host.

A significant promise for future studies on the pathogenicity of GRSPaV and development of a system for reverse genetic and RNA silencing in grapevine, comes from the assembly of a second generation cDNA clones of this virus (22). These clones were infectious in both *Nicotiana benthamiana* and grapevine. Interestingly, the virus did move systemically only in its natural host although at a very slow rate of replication and movement.

Progresses in understanding the cellular life cycle of GRSPaV are reported in an elegant work by Meng (20) showing that the CP of this virus localizes into the nucleus of transfected BY-2 cells when the protein is transiently expressed. The protein is also able to interact with itself as supposed from its role in the assemblage of the virus protein shell. Speculations on the effects of the nuclear localizations range from a modulation of gene expression to suppression of RNA silencing.

VITIVIRUSES

The state-of-the-art on the knowledge of grapevine vitiviruses with particular emphasis on *Grapevine virus A* (GVA), was the object of a recent review by du Preez *et al*. (8). The paper besides revising the recent achievements in the molecular biology of GVA, mainly originating from the development and use of an infectious clone

underlines the fact that the exact role of vitiviruses in grapevine diseases is largely unknown. Involvement of *Grapevine virus B* (GVB) in the etiology of Corky bark found confirmation in the work of Goszczynski (14) who also highlighted the high variability of this virus which explains the existence of strains differing in their respective pathogenicity tp grapes. The same author provided further data (15) on the etiology of Shiraz Disease (SD) and its relationship with Australian Shiraz Disease (ASD). Some GVA variants of group II were strongly associated with both SD and ASD. However since this virus was always present in multiple infections with *Grapevine leafroll-associated virus 3* (GLRaV-3), this author speculates that suppression of plant defense system induced by this latter virus, could help these GVA variants to express their pathogenicity. Likely, other closteroviruses (GLRaV-1 and/or GLRaV-9) could exert the same suppressive role in the expression of ASD. In recent studies, presented in this Meeting, the involvment of GVA in ASD found further confirmation (16). Shiraz vines top-grafted with GVA-infected Chardonnay rootstocks developed severe disease in a vineyard of South Australia.

Regarding Syrah decline, a different disease affecting the same cultivar with which a robust association with GRSPaV was previously found (19), a study of Goszczynski (13) reported that neither this foveavirus, nor GVA or GVB are involved in etiology of the disease.

GVA variability was also reported by Alabi *et al.* (2) who analyzed the CP and RdRp sequences of this virus from 27 isolates from California and Washington states. The study highlights the high genetic diversity of this virus determined by mutations and recombinations events.

Expansion of the genus *Vitivirus* is represented by the recently discovered Grapevine virus E (GVE), a South African variant of which was fully sequenced showing the feature, unique among plant viruses, to possess the AlkB domain within the helicase domain. Interestingly the virus was isolated from a SD plants co-infected by GLRaV-3, GVA and GRSPaV. A further variant of this virus originating from a Cabernet Sauvignon grape in the Washington state, was completely sequenced by Alabi *et al.* (3) showing a high homology with the South African isolate. GVE was also detected in several vineyards of the Pacific Northwest of the USA but its role in disease expression is still undetermined. Plasticity of the genus *Vitivirus* was further stressed by the discovery of a new member in California (5), tentatively named Grapevine virus F (GVF). This virus, identified in a Cabernet Sauvignon accession causing graft incompatibility of Freedom, 420A, 3309C, and 101-14 rootstocks, was completely sequenced disclosing a typical vitiviral genome organization.

Insights into the cellular life cycle of GVA and GVB came from the study of Haviv *et al.* (17) which follow the distribution of the GFP- or RFP-tagged movement proteins (MP) of these viruses in infected *N. benthamiana* cells. Both MPs are routed to plasmodesmata from where they assemble tubule-like structures extending from the surface of *N. benthamiana* protoplasts. The same distribution patterns is observed in transfected *V. vinifera* protoplasts thus showing that the proteins behave similarly in both hosts. The study also demonstrate that the CP is necessary for the virus systemic movement and that both viruses do not exploit the cytoskeleton for intra-cellular routing to the cell wall and plasmodesmata. These data fully confirms previous studies of immunodetection of both MPs (28) in plasmodesmata. The same authors (28) had shown also GVA MP localizes to cytoplasmic aggregates of viral particles. Combining both studies a MP-mediated routing of GVA particles to plasmodesmata could be supposed.

NEW DISEASES AND VIRUSES

A new disease was described in the Trentino region (northern Italy) affecting the cv Pinot Gris. Symptoms, observed in the field from 2003 consist in chlorotic mottling of the leaves, delayed bud break and shoot necrosis. Yield of symptomatic plants was also affected leading to a 47% reduction because of a lower number (-22%) and weight (-37%) of the clusters (M. Varner, personal communication). A three-year survey in the originally affected Pinot Gris vineyard showed an increase of the number of symptomatic plants from 11.8% to 21.1% and, concurrently, an increase of dead vines from 0.05% to 0.34%. The disease was also observed in cvs Pinot noir and Traminer the latter variety being more severly affected After repeated laboratory assays aiming at exploring the “virome” of the affected vines, a new virus was identified by high throughput sequencing (12). This virus, provisionally named *Grapevine Pinot gris virus*, showed striking homologies with the trichovirus *Grapevine berry inner necrosis virus* (GINV) with which it shares also similar symptoms (S. Terai, personal communications). The virus genome organization in three ORFs expressing proteins of 214 kDa (replication-associated), 42 kDa (MP) and 22 kDa (CP), clearly classifies GPGV in the genus *Trichovirus* in which it was recently accepted as a new

member. The involvement of GPGV in the disease shown by infected vines is now under investigation since it was also detected in symptomless plants. Further analogies with the Japanese GINV lie on the typical reaction on the indicator *Vitis riparia*. How and when GPGV popped up in Italy is unknown, but the recent report of its discovery in cv. Tamnara in Korea (GenBank accessions numbers AB731567.1 and AB731566.1) indicates that this virus is not confined to Italy.

ADVANCES IN DIAGNOSIS OF RW-ASSOCIATED VIRUSES

In the frame of a national project (ARNADIA) of validation and standardization of serological and molecular diagnostic protocols of economically important grapevine viruses (9), ELISA was shown to be as efficient as RT-PCR for the detection of GVA and GVB providing that accurate extraction methods are used. Similarly, comparison among tissue disruption methods and RNA extraction protocols for the detection of grapevine viruses, showed that the use of Tissue Lyser (Qiagen) and a bead-based protocol for RNA purification, significatively improve the detection (25). Pacifico et al. (26) quantified GVA viral load in grapevine by a TaqMan one-step qRT-PCR assays in cv. Nebbiolo. Relative quantitation was determined with respect to the housekeeping GAPDH mRNA and consisted on 116 viral genome copies for 100 GAPDH mRNAs.

CONTROL MEASURES: TRANSGENIC RESISTANCE AND SANITATION

A minimally invasive strategy for inducing transgenic resistance to GVA was followed by developing an artificial microRNA (amiRNA) system based on the scaffold of *Vitis vinifera* pre-miR166f (27). Two constructs were engineered to express 21nt RNA sequences targeted to GVA ORF1 and ORF5. Transient expression of GVA amiRNAs induced promising levels of resistance in *N. benthamiana* co-inoculated plants. Gambino *et al.,* (11) investigated on the cellular localization of GVA in callus tissues. These authors found that regeneration of healthy plantlets by somatic embryogenesis depends on virus concentration and the ability of spreading, which was lower for GVA as compared to GLRaV-3 and GFLV.

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Infectivity Assays of Second-Generation cDNA Clones of *Grapevine Rupestris Stem Pitting Associated Virus*

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) is a single-stranded, positive sense RNA virus in the genus *Foveavirus,* family *Betaflexiviriae* (Meng *et al*., 1998; Zhang *et al*., 1998; King *et al*., 2011). GRSPaV is perhaps the most prevalent virus of grapevines and has a worldwide distribution. Extensive evidence suggests that GRSPaV is composed of a multitude of molecular variants (for a review, see Meng & Gonsalves, 2007). GRSPaV is reportedly associated with three distinct diseases: 'Rupestris Stem Pitting', 'Vein Necrosis' (Bouyahia et al., 2005) and 'Syrah Decline' (Lima *et al*., 2006). However, direct evidence for the causative role of GRSPaV in any of these diseases is still lacking. It is possible that different strains of GRSPaV may be responsible for the different diseases. The ultimate resolution to this enigma depends on the availability of infectious viral clones for each of these strains, followed by inoculation of these viral clones into different grapevines that are free of the virus. At the molecular and cellular level, mechanisms that govern different aspects of the replication cycle have not been explored for GRSPaV or members of the *Betaflexiviridae* as a whole. To enable these studies, one would also need to first establish infectious cDNA clones and systems for infectivity assays. Previously, we made full-length GRSPaV clones and its GFP-tagged variant using $pHST_{40}$, a pUC18 derivative, as the base vector. Rub-inoculation into numerous herbaceous plant species that are commonly used as alternative hosts for many RNA viruses failed to show infectivity.

This further study was carried out with the following objectives: (1) to subclone these viral constructs into a binary vector for the purpose of the highly effective agrobacterium-based inoculation; (2) to demonstrate that these viral clones are infectious in the natural host grapevine and in an experimental host; and (3) to set up an effective system to deliver these viral clones into grapevine and grapevine protoplasts for downstream investigations.

MATERIALS AND METHODS

Subcloning of viral constructs into a binary vector and agro-inoculation. The cDNA fragment corresponding to the 35S promoter and the first 1,420 nts of the viral genome was amplified from pRSP28 with primers 35S-Xba and Bam1420. The PCR product was digested and cloned into pCambia-1390, resulting in $pRSP5'\text{-}3_{(Cam)}$. SmaI site was then inserted into $pRSP5'\text{-}3_{(Cam)}$ via site-directed mutagenesis, producing $pRSP5'Sm\text{-}3_{(Cam)}$. Subsequently, the cDNA fragment flanked by SpeI and SmaI was released from pRSP28 and pRSP-GFP1 by restriction digestion and sub-cloned into $pRSP5'Sm\text{-}3_{(Cam)}$, producing full-length clones $pRSP28\text{-}2_{(Cam)}$ and $pRSP\text{-}GFP2_{(Cam)}$, respectively. Agro-infiltration of *N. benthamiana* plants was performed using *A. tumefaciens* strain EHA105 carrying either $pRSP28\text{-}2_{(Cam)}$ or $pRSP\text{-}GFP2_{(Cam)}$. After induction with acetosyringone, agrobacterial suspensions were infiltrated into fully or nearly fully expanded leaves of *N. benthamiana* plants at the 4 -6 leaf stage. Agrobacterial cultures containing pPVX-GFP were also used in infiltration experiments as positive control. Agro-inoculation of grapevine plantlets was carried out following the protocol developed by Muruganantham et al. (2009).

Electron microscopy and immuno gold labelling. Infiltrated *N. benthamiana* leaves were collected at 5 dpi, ground in 50 mM Tris buffer and used to prepare EM grids. Virus particles were decorated by GRSPaV-specific antiserum, followed by immuno-gold labeling. The grids were stained with uranyl acetate and viewed under a Philips CM-10 transmission electron microscope.

Western blotting. Tissue from infiltrated *N. benthamiana* leaves was collected at different times post-infiltration, ground in liquid nitrogen and extracted in 2.5 volumes of extraction buffer. Resulting crude extracts were electrophoresed on 6-20% gradient polyacrylamide gels. Extracts obtained from plants infiltrated with pPVX-GFP were included as positive control. Proteins were transferred to a PVDF membrane and GFP expressed from viral clones was detected using anti-GFP antibody.

RESULTS AND DISCUSSION

Infectivity of GRSPaV clones in N. benthamiana. Filamentous virions were observed in *N. benthamiana* leaves infiltrated with pRSP28-$2_{(Cam)}$ as well as in leaves infiltrated with pRSP-GFP$2_{(Cam)}$ at 5 dpi. It is worth noting that the number of virions produced by pRSP-GFP$2_{(Cam)}$ was much lower than that for pRSP28-$2_{(Cam)}$. Immunogold labeling with virus-specific antiserum confirmed that these virions were indeed of GRSPaV (Fig. 1). The infectivity of pRSP-GFP$2_{(Cam)}$ was confirmed by fluorescence microscopy of infiltrated leaves. Green fluorescence due to GFP was observed in epidermal cells starting at 1 dpi and peaking at 7 dpi. The green fluorescence was distributed along the cell periphery and also as globular bodies. GFP expression was further verified by a time course western blot analysis, which showed that the intensity of GFP steadily increased with time and reached a maximum by 7 dpi (Fig. 2). It is evident that the GFP levels from pRSP-GFP$2_{(Cam)}$ were much lower than those of the positive control, pPVX-GFP (Fig. 2). Surprisingly, both GRSPaV clones could not initiate systemic infection in *N. benthamiana* or three other *Nicotiana* species as judged by absence of the virus or viral proteins in non-inoculated upper leaves. This suggests that GRSPaV can infect these herbaceous plants locally but not systemically.

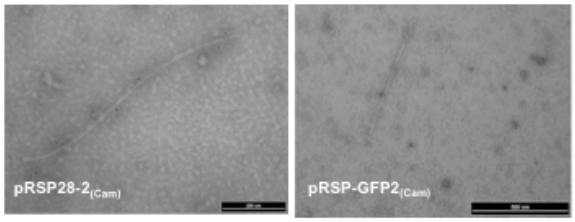

Figure 1. Detection of GRSPaV virions in infiltrated leaves of N. benthamiana with immuno electron microscopy.

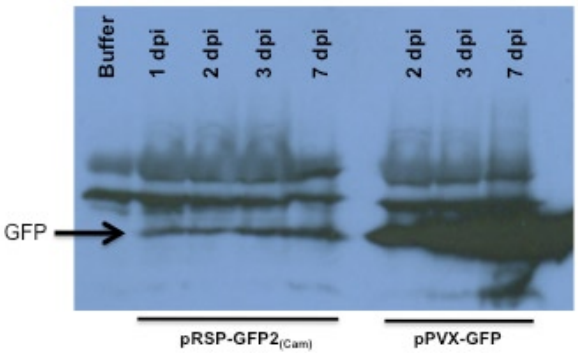

Figure 2. Time course Western blot analysis of GFP expression from infectious viral clones in infiltrated leaves of *N. benthamiana*.

Infectivity of GRSPaV clones in grapevine. To test if these viral clones were infectious in grapevine, we inoculated tissue-cultured plantlets of Thompson using *Agrobacteria* containing pRSP-GFP$2_{(Cam)}$. Inoculated plantlets were monitored by fluorescence microscopy and RT-PCR. One month post agro-inoculation, GFP was clearly detected in roots from 8 of the 10 plantlets by both methods. To test for systemic infection, nested RT-PCR was conducted using total RNAs isolated from these inoculated plants six months after inoculation. One of these plants was positive for GRSPaV. These data demonstrated that pRSP-GFP$2_{(Cam)}$ was infectious in the natural grapevine host. However, these viral clones seem to have a slow rate of replication and movement. To set up an alternative system for infectivity assays and for further downstream studies, we have recently established an effective system for isolation and transfection of grapevine protoplasts. We successfully isolated viable protoplasts, which were able to express a nucleus-targeting GFP marker. We are currently testing the feasibility of using these grapevine protoplasts for further studies concerning GRSPaV replication.

CONCLUSION

After years of exploratory work, we are pleased that we have finally won the battle of making infectious clones for GRSPaV. We have demonstrated, with multiple lines of evidence, that these viral clones are infectious in both the natural host grapevine and *Nicotiana* species. This is the first infectious clone for the genus *Foveavirus* and one of the first for the family *Betaflexiviridae*. The potential of developing GRSPaV as a model system for the study of other members of the *Betaflexiviridae* family and as a silencing vector for grapevine functional genomics awaits realization.

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Grapevine Vein Necrosis is not Exclusively Associated to GRSPaV Group 2 Molecular Variants

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV), member of the genus *Foveavirus,* is widely detected in grapevine. Six GRSPaV variants have been fully sequenced: GRSPaV-1, -SG1, -BS, -SY, -PN, -MG (Morelli *et al*., 2011; Terlizzi *et al*., 2011). They are divided into three main clusters defined as: cluster **1** (SY), cluster **2** split in sub-cluster **2a** (SG1, MG) and sub-cluster **2b** (-1) and cluster **3** (BS, PN). Some of them are supposed to be involved in diverse syndromes such as vein necrosis (VN) (Bouyahia *et al.,* 2005; 2009; Borgo *et al*, 2009; Morelli *et al*., 2011).In the present study we analyzed the genetic diversity of GRSPaV in fifteen grapevine accessions showing different behaviors towards VN.

MATERIALS AND METHODS

Grapevine material: Biological indexing was realized by green grafting onto 110R as *Grapevine Vein Necrosis* (GVN) indicator (accession known to be GRSPaV free). Ten rootstock and five scion varieties collected from IFV experimental station (Le Grau du Roi, France) were tested.

Total RNA extraction: RNA extraction was performed on 200 mg of cambial tissues from wood mature canes taken from the same plants according to the "RNeasy Plant Mini Kit" protocol (Qiagen, France).

RT-PCR amplifications: Two-step RT-PCR was performed to generate GRSPaV amplicons within ORF5 encoding the protein capsid for subsequent sequencing (HotGoldStar Taq polymerase, Eurogentec) using the universal primers GRSPaV-CPF1/CPR2 (Beuve *et al*., 2012).

Cloning and sequence analyses: The GRSPaV specific 423 bp PCR products were cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen, France). Eight to fifteen recombinant clones per plant were sequenced. The 231 sequences were aligned using CLUSTAL W. A phylogenetic tree was constructed with Mega 4 software and clustering of the GRSPaV variant was defined according to the bootstrap values above 70% using the neighbor-joining algorithm (Tamura *et al*., 2007).

RESULTS AND DISCUSSION

GRSPaV variability associated to vein necrosis symptomatology

As shown in table 1, biological indexing revealed that 7 plants were symptomless while 8 expressed symptoms of the disease.

Table 1. Distribution of molecular variants of GRSPaV in the two symptomatic categories.

			GRSPaV Variants					
	Variety	Biological Indexing	SY (1)	BS (3)	PN (3)	SG1 (2a)	MG (2a)	1 (2b)
Rootstock varieties	5 BB (E27)	VN-	15[a]	-	-	-	-	-
	Fercal (E2)		15	-	-	-	-	-
	Riparia (E21)		14	-	-	-	-	-
	4010 C (E1)		-	15	-	-	-	-
	161-49 C (E22)	VN+	-	-	-	-	-	15
	Riparia (E23)		-	-	-	-	-	15
	101.14 Mgt (E73)		10	-	-	-	-	5
	5 BB (E12)		8	-	-	-	-	6
	Rupestris (E1)		-	6	-	-	9	-
	SO4 (E24)		-	5	3	-	-	-
Scion varieties	Corrin SDL B (E1)	VN-	-	15	-	-	-	-
	Chasselas B (E221)		13	-	-	-	-	2
	Merlot N (E132)		2	-	-	13	-	-
	Cardinal Rg (E16)	VN+	13	-	-	-	-	2
	Aligoté B (E56)		9	-	-	-	-	6

[a] number of sequences belonging to the phylogenetic group

VN symptomless (VN-): 5 accessions out of 7 contained a homogeneous population of a single molecular variant belonging to either the group 1 (SY) or the group 3 (BS). The two other accessions harbored a mix of two groups: group 1 (SY) and 2b (-1) or group 2a (SG1) and 1 (SY). Interestingly, the VN- plants were essentially infected with viral variant belonging to group 1 (SY) or group 3 (BS). **Neverthless, the group 2 was identified in 2 accessions without developing VN symptoms** (Chasselas and Merlot); moreover it appeared to be the main variant in Merlot.
VN symptomatic (VN+): 2 accessions out of 8 presented a single variant belonging to group 2b (-1). 5 accessions contained heterogeneous GRSPaV populations including the group 2. Thus, 7 out of 8 accessions exhibiting a VN positive phenotype, were infected with a variant belonging to group 2 (2a or 2b). We can notice that the variant 2a (SG1) was never detected here.

Table 2. Number of accessions infected/free from variants belonging to group 2 in both symptomatic categories.

	VN+	VN-
Presence of viral variants belonging to group 2	7	2
Absence of viral variants belonging to group 2	1	5

A good correlation (80%) between vein necrosis disease and the presence of viral variants belonging to group 2 (-SG1, -1, -MG) is obtained (Table 2). **Nevertheless, some exceptions can be noticed with, first, the absence of group 2 in one accession VN+ (SO4) and secondly, its identification in 2 accessions VN- (Chasselas and Merlot).**

Viral repartition in rootstock varieties
Interestingly, three rootstock varieties presented an atypical viral diversity with the presence of mixtures of divergent sequence variants as previously found in scion varieties but not in rootstock varieties (Meng *et al*., 2006). This is contradictory with the hypothesis of the adaptation between GRSPaV variants and *Vitis* species.

Our results lead to moderate the restricted relationship previously proposed between vein necrosis syndrome and molecular variants from GRSPaV group 2. Vein necrosis symptoms can develop in the absence of group 2 or when this group 2 remains in minority. By contrast, some plants remain free of symptoms even with the presence of variants from group 2 as the major group. This study sheds light on the pathogenicity of GRSPaV found almost ubiquitously in grapevine genotypes.

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Sequencing and Prevalence of Grapevine Virus E in South African Vineyards

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INTRODUCTION

Grapevine virus E (GVE) was first identified in 2008, in the Japanese table grape, *Vitis labrusca* cv. Aki Queen and Pione. Two sequence variants of GVE were identified, TvAQ7 and TvP15 (Nakaune *et al*., 2008). This virus was classified as a new member of the genus *Vitivirus*, family *Betaflexiviridae*. In 2010, a metagenomic sequencing study in a severely diseased vineyard reported a partial GVE sequence (Coetzee *et al*., 2010a). The metagenomic data generated two GVE scaffolds with high sequence homology to the partial sequence available for GVE-TvP15. This was the first report of GVE in South Africa.

In this study we determined the first complete genome sequence of a GVE isolate. We studied the seasonal titre in grapevine with quantitative reverse transcription-PCR (qRT-PCR) and determined the prevalence of GVE in South African vineyards.

MATERIALS AND METHODS

Based on available sequence information (Nakaune *et al*., 2008 and Coetzee *et al*., 2010a) diagnostic primers were designed. A grapevine, SA94 (*V. vinifera* cv. Shiraz) affected by Shiraz disease and infected with GVE, was identified. Total RNA was extracted with a modified CTAB method (White *et al*., 2008) and used to determine the complete nucleotide sequence. Nine primer pairs were designed to amplify overlapping regions spanning the GVE genome. The 3'-terminal nucleotide sequence of the genome was determined by cDNA synthesis from total RNA with an oligo(dT) primer. RNA-ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was used to determine the 5'-terminal end.

Primers were designed for detection and quantification with qRT-PCR of all known isolates of GVE. Petioles of five GVE infected grapevine plants were randomly collected every second week during the growing season of 2010/2011 (15 time points) and total RNA extracted. Actin was used as the reference gene to normalize the data (Reid et al., 2006). Standard curves were constructed for GVE and actin, using a dilution series of total RNA from a GVE positive sample. The relative titre of GVE was calculated using reaction efficiencies from the standard curves and ct values for the five GVE positive samples according to the standard curve method for relative quantification. A standard concentration of 50 ng total RNA was used and reactions performed in duplicate.

The incidence of GVE was determined by conducting a survey in the grapevine growing areas of the Western Cape, South Africa. The vineyards used in this survey were previously used as mother blocks, written off in the 2009/2010 growing season and selected to obtain potential newly-infected plants. Plants displaying typical leafroll disease symptoms were collected. In total, 130 plants from 10 different regions were screened for GVE infection with qRT-PCR high resolution melting analysis.

RESULTS AND DISCUSSION

The GVE SA94 genome sequence is 7568 nucleotides in length and has a genome organization typical of vitiviruses, consisting of five open reading frames (ORFs), and polyadenylated at the 3' end (Figure 1). The 5' terminus most likely contains a methylated cap structure due to the presence of the methyl-transferase domain in the first ORF. In contrast to other vitiviruses, the AlkB domain is located within the helicase domain of ORF 1. Grapevine virus E SA94 shares 98.1% nucleotide identity with the Japanese TvP15 isolate, indicating that it belongs to the same putative strain (Coetzee *et al*., 2010b).

When plotting the relative virus titre against the week of sample collection no significant fluctuation in GVE titre was observed, indicating a constant virus titre throughout the season. The relative titre for one of the samples,

Plant 3, was marginally higher than that of the other samples. These experimental procedures were performed for samples from the same vineyard for only one growing season as an initial indication of GVE virus titre. To confirm these results, more data from additional growing seasons are required.

The survey results indicated a GVE infection occurrence of ~20% in leafroll diseased vines. No clear clustering of infection was observed, as the GVE infected plants were found throughout the Western Cape, South Africa (Figure 2). This is the first survey of GVE conducted in South Africa after it was discovered here in 2010.

Figure 1: a Schematic diagram of the genome organization of grapevine virus E (SA94). **Mtr** methyltransferase, **Hel** helicase, **Alk B** AlkB conserved domain, **RdRp** RNA-dependent RNA polymerase, **MP** movement protein, **CP** coat protein, **NB** nucleic-acid-binding protein, **?** protein with unknown function (Coetzee *et al*., 2010b).

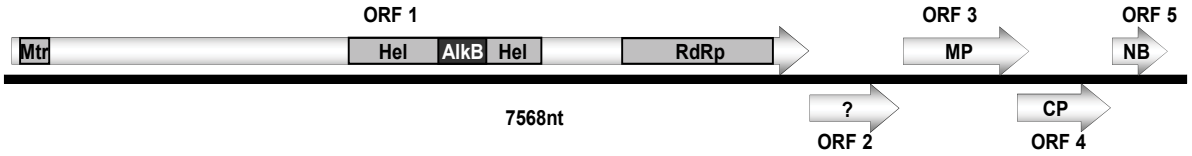
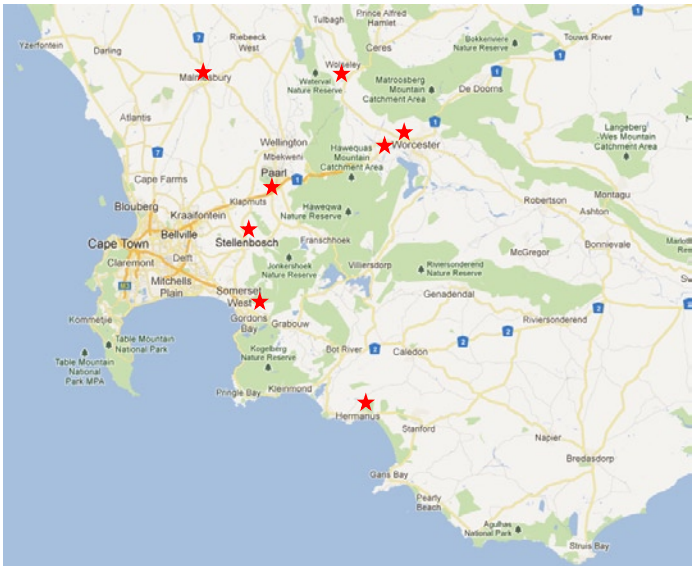


Figure 2: A Google map image of the Western Cape region, South Africa where the survey was conducted; "★" indicates the areas where GVE positive grapevines were identified.



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Grapevine Rupestris Stem Pitting-associated Virus and Vein Necrosis Disease: Further Data on the Molecular Characterization of Biologically Divergent GRSPaV Isolates

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) is a member of the genus *Foveavirus* in the newly established family *Betaflexiviridae* (Martelli *et al*., 2007). GRSPaV has been reported from almost all vine growing areas in the world, where it seems to have a high incidence. This virus was found to be consistently present in Vein Necrosis (VN) infected vines, suggesting its involvement in the determinism of VN disease (Bouyahia *et al*., 2005). The genome of several GRSPaV isolates has been sequenced and like other flexiviruses, it has been shown to be extremely variable. According to the CP sequence analysis, GRSPaV isolates clustered in four groups (Nolasco *et al*., 2006). Moreover, successive investigations on the aetiology of VN disease indicated that such variants might have a diverse pathological role and expression of VN symptoms is likely to be restricted to grapevine accessions infected with GRSPaV variants belonging to groups 2a and 2b, while other GRSPaV molecular groups seem to be latent (Bouyahia *et al.*, 2009).

In the present work, we provide further data on the molecular characterization of biologically divergent GRSPaV isolates, by RT-PCR amplification and sequencing of a fragment of ORF1.

MATERIALS AND METHODS

Object of this study were 7 grapevine putative clones from Tuscany. Accessions were previously indexed for VN on 110 Richter and tested for the presence of GRSPaV by RT-PCR. Each accession was infected by a single group of GRSPaV variants: 2 VN-affected accessions infected by group 2a variants (CC8 and MLOC2), 1 VN-affected accession infected with group 2b variants (CC17), 2 VN-free accessions infected by group 1 variants (5/1 and SMH22) and 2 VN-free accessions infected with isolates belonging to group 3 (MSAS1 and MSAS3).

Total RNA was extracted from cortical scrapings of mature canes and a new set of degenerate primers, RSP D1f/D1r, was designed to amplify a 656 bp fragment in the helicase domain of ORF1. RT-PCR using primers RSP 13/14 (Meng *et al.*, 1999) and RSP D1f/D1r was performed in order to compare the performance of both primer pairs. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sequenced. Sequence and phylogenetic analyses were conducted using Mega 5 (Tamura *et al.*, 2011). Tree was constructed according to the neighbour-joining method.

RESULTS AND DISCUSSION

RT-PCR amplification: The amplification results varied depending on the primers used. Only GRSPaV isolates belonging to groups 2a and 2b were detected by primers RSP 13/14. In contrast, when primers RSP D1f/D1r were used, all of the 7 GRSPaV isolates produced the expected 656 bp amplicon.

Sequence analysis: A 299 bp sequence internal to the 656 bp amplicons obtained with primers RSP D1f/D1r was used for sequence analysis, in order to compare our GRSPaV isolates with the ones available in Genbank.

Analysis confirmed the existence of the four groups, but the isolates MSAS1 and MSAS3 clustered outside of these groups (Fig. 1). We designated each molecular group with a full-sequenced reference isolate. To be consistent with the existing nomenclature based on the CP-sequence (Nolasco *et al.*, 2006), RSPaV-1, GRSPaV-SG1, GRSPaV-BS and GRSPaV-SY lineages correspond to groups 2b, 2a, 3 and 1, respectively.

GRSPaV isolates from VN-affected accession clustered in groups 2a and 2b, which share 88% nt. similarity. Group 2a includes isolates from CC8 and MLOC2 VN-affected accessions, which shared 98% nt. identity between them and respectively 93% and 92% with GRSPaV-SG1. Group 2b includes GRSPaV isolate from VN-affected accession CC17, which share 99% nt. identity with RSPaV1. Isolates from VN-free accessions 5/1 and SMH22 shared 99% nt identity between them and respectively 94% and 95% with GRSPaV-Sy. These isolates

clustered in group 1, which share less than 82% nt. similarity with groups 2a and 2b. None of our isolates clustered in group 3, with GRSPaV-BS as reference isolate. GRSPaV isolates from VN-free accessions MSAS1 and MSAS3 grouped separately from the aforementioned four groups. They share 99% nt. identity between them and 84% with GRSPaV-PN. The ORF1-based clustering of MSAS1 and MSAS3 isolates is not consistent with the CP-based one, where they were grouped in group 3, together with GRSPaV-BS (Bouyahia *et al*., 2009).

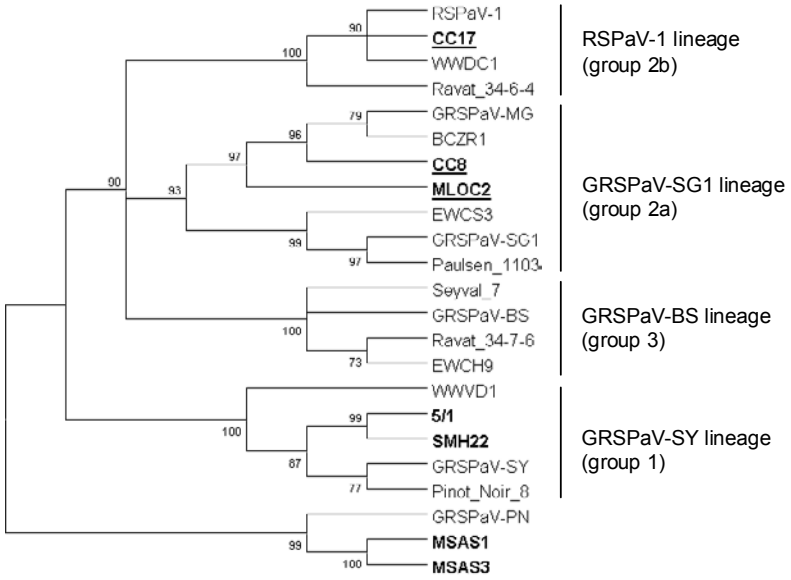

Figure 1. Phylogenetic relationship among GRSPaV isolates. Multiple alignment included 299 bp sequences from VN-affected (emboldened, underlined),VN-free (emboldened) isolates and sequences available in GenBank: RSPaV-1 (AF057136); GRSPaV-SG1 (AY881626); GRSPaV-BS (AY881627); GRSPaV-SY (AY368590); GRSPaV-PN (AY368172); WWDC1 (FJ943372); Ravat_34-6-4 (DQ278641); GRSPaV-MG (FR691076); BCZR1 (FJ943358); EWCS3 (FJ943407); Paulsen_1103 (DQ278620); Seyval_7 (DQ278646) Ravat_34-7-6 (DQ278643); EWCH9 (FJ943371); WWVD1 (FJ943404); Pinot_Noir_8 (DQ278636). Phylogenetic tree was constructed with neighbour-joining method, evolutionary distances were computed using the Kimura 2-parameter method. Only bootstrap values higher than 70% are shown.

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Effects of *Grapevine Rupestris Stem Pitting-Associated Virus* on *Vitis vinifera* L.

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) is a member of the genus *Foveavirus*, family Betaflexiviridae, associated with rupestris stem pitting (RSP), a disorder of the rugose wood complex (Martelli, 1993). RSP is characterised by pitting symptoms on the woody cylinder below the graft union on the *Vitis rupestris* cv. St George indicator. GRSPaV is the most prevalent among grapevine viruses, and it is usually found in *Vitis vinifera* L. cultivars in a latent state without the development of phenotypic alterations. To date, the molecular interactions between GRSPaV and grapevine are largely unknown and little has been reported on the agronomic effects of this virus. In the present work, we discuss the results obtained by applying global gene expression analyses combined with physiological and agronomic studies in grapevine affected by GRSPaV.

MATERIALS AND METHODS

The study was carried out in a vineyard planted in 2002 in Albenga (Liguria), North-West Italy, where a row was established with the white grape cultivar 'Bosco' (*V. vinifera* L.) grafted onto 1103 P rootstock. All 'Bosco' plants derived by vegetative propagation from a single mother plant originally infected by several viruses and further subjected to sanitation. The sanitation techniques did not provide satisfactory results in the eradication of GRSPaV, leaving some lines still infected by the virus, as results by multiplex RT-PCR (Gambino *et al.*, 2006). Twelve vines along the row, six GRSPaV-free and six GRSPaV-infected (two replicates of three plants each), were analysed during the growing season in 2010. Eco-physiological parameters were registered at berry pea size (E-L31) and véraison (E-L35) (Coombe, 1995). Some relevant agronomic parameters, and the main ripening parameters were determined for each vine (Table 1).

Microarray analysis was carried out on RNA extracted from three biological replicates of leaves at véraison. Hybridisation was carried out on a NimbleGen microarray 090818 Vitis exp HX12 (Roche, NimbleGen Inc., Madison, WI), representing 29,549 predicted genes on the basis of the 12X grapevine V1 gene prediction version (http://srs.ebi.ac.uk/). A Significance Analysis of Microarray (SAM) was implemented with a false discovery rate (FDR) of 1%. Validation of microarray data was carried out by real-time RT-PCR.

RESULTS AND DISCUSSION

In terms of vine physiological behaviour, the chlorophyll content (Fig. 1a) and photosynthetic rate (P_n) (Fig. 1b) were significantly lower in GRSPaV-infected plants at the end of the season. Yield and sugar content were in favour of uninfected plants. It is interesting to note that, in control vines, higher yields were mainly due to bigger bunches with bigger berries and not to higher fertility (Table 1). These findings suggest that GRSPaV eradication resulted in a moderate overall improvement of field performance and photosynthetic efficiency.

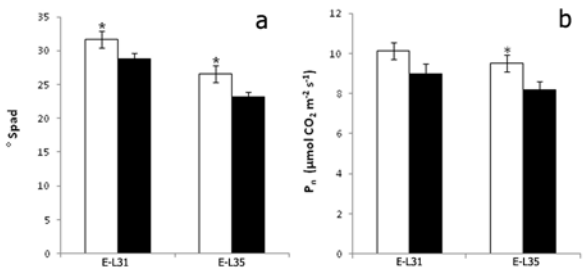

Figure 1. a) Chlorophyll content (Spad), and b) net photosynthesis (P_n), in GRSPaV-free (white columns) and GRSPaV-infected (black columns) grapevine leaves. Measurements (n = 36) were taken during two phenological periods: berry pea size (E-L31) and véraison (E-L35). Bars are standard error of the mean. Asterisks show significant differences between infected and GRSPaV-free leaves ($p < 0.05$).

The decrease in physiological performance induced by GRSPaV infection was less relevant than what observed in other grapevine-virus combinations. For instance, in GRSPaV-infected plants, Pn decreased more than 15%, while it is reduced up to 60% in the 'Nebbiolo' variety infected by GLRaV-3 and GVA (Guidoni *et al*., 1997).

Table 1. Field performances of GRSPaV-infected and uninfected grapevines of 'Bosco'. All data are expressed as average values ± standard errors. Significance: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, ns = not significant.

Data	GRSPaV-free	GRSPaV-infected	Sign
Fertility (n° inf/shoot)	0.49 ± 0.10	0.62 ± 0.13	ns
Yield (kg/vine)	2,80 ± 0.34	1.92 ± 0.13	*
Bunch/vine (n°)	12 ± 0.7	10 ± 0. 7	ns
Bunch weight (g)	250 ± 24.3	191 ± 15.0	ns
Berry weight (g)	2.64 ± 0.41	2.34 ± 0.5	***
Total soluble solids (°Brix)	22.22 ± 0.26	21.10 ± 0.42	*
Titratable acidity (g/l)	2.89 ± 0.08	3.4 ± 0.15	*
pH	3.27 ± 0.03	3.38 ± 0.03	ns

The transcriptomic changes that take place in grapevine leaves in response to GRSPaV were analysed through microarray analysis. We found 877 genes showing significant expression changes in GRSPaV-infected grapevines compared to their own control. P_n rates were significantly lower in infected plants, whereas, surprisingly, several genes involved in photosynthesis and CO_2 uptake were upregulated (Fig. 2). GRSPaV caused a reduction in P_n in the absence of environmental limitation, thus, the infected grapevine attempted to increase P_n rates by inducing genes involved in photosynthesis and CO_2 fixation. The downregulation of many genes involved in the defence response (e.g. thaumatins, chitinases, pathogenesis related proteins, NBS-LRR class, β 1-3 glucanases) was another surprising result observed in grapevines affected by GRSPaV (Fig. 2). Generally, in other plant-virus interactions, this functional category is the most induced (Whitham et al., 2003). Signal transduction was strongly downregulated by GRSPaV, about 50 genes encoding for protein kinases were repressed. This virus caused a deep alteration in the expression of genes involved in hormone metabolism. Transcripts tied to ethylene, cytokinins, gibberellins, ABA, and jasmonate biosynthetic pathways were activated, while other genes linked to auxin signalling were repressed. In addition, we have shown a significant overlap in cellular responses between GRSPaV infection and abiotic stresses, such as water deficiency and salinity.

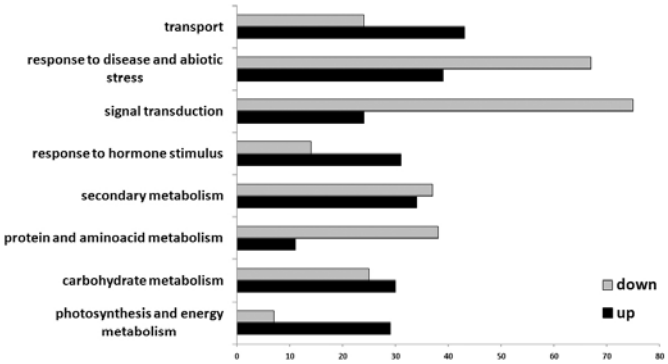

Figure 2. Functional distribution of grapevine transcripts significantly induced and repressed in GRSPaV-infected leaf at véraison. Bars represent the number of transcripts downregulated (grey) or upregulated (black) in each functional category.

In grapevines infected by GRSPaV, global gene expression analysis combined with physiological and agronomical studies showed some unique responses, never before reported for other plant-virus interactions. We hypothesise that the long co-existence between grape and GRSPaV has resulted in the evolution of a form of mutual adaptation between this virus and *V. vinifera*, which has resulted in the specific modulation of several transcripts and in the absence of visible symptoms.

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Grapevine Virus A Occurs as Genetically Diverse Variants in California and Washington States

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INTRODUCTION

Grapevine virus A (GVA), the type member of the genus *Vitivirus* (family *Betaflexiviridae*) is widespread in many grape-growing regions of the world (Du Preev et al., 2011). GVA has been consistently found closely associated with Kober stem grooving disorder of the Rugose Wood complex (Minafra, 2000) and with Shiraz disease in South Africa and Australia (Goszczynski and Habili, 2012).. A few studies have investigated the genetic diversity of GVA isolates using variant-specific reverse transcription polymerase chain reaction (RT-PCR; Goszczynski and Jooste, 2003), single-strand conformation polymorphism (Goszczynski, 2007) and RT-PCR-restriction fragment length polymorphism (Murolo et al, 2008). Using nucleotide sequences of the coat protein (CP) and the RNA-dependent RNA polymerase (RdRp) genes of GVA, we showed in this study genetic diversity among natural populations of GVA isolates collected from California and Washington, the two leading grapevine-producing States in the U.S.

MATERIALS AND METHODS

GVA isolates analyzed in this study were collected from a variety of red- and white-berried table and wine grapevine cultivars during 2005 to 2010 from commercial vineyards in the Yakima Valley of Washington State and grapevine collections maintained by the Foundation Plant Services (FPS), University of California, Davis, CA. Isolates from Washington were derived from own-rooted vines whereas source grapevines of isolates from California came originally from grafted vines from different sources worldwide prior to being planted as own-rooted vines at the FPS vineyard blocks. For each isolate, full-length CP gene was amplified using primers GVA-CPF6356 (5'-GATACYCTAGTTATGCCAGA-3') and GVA-CPR7096 (5'-GCACCACACTTACACACATTC-3') to yield approximately 741 base pair (bp) fragment. A portion of the RNA-dependent RNA polymerase (RdRp) was amplified from each isolate using primers GVA-RdRp3987F (5'-ACMTCWGAYGAYACDGCHAC-3') and GVA-RdRp4894R (5'-CTCATYCKCCANCCRCAGAA-3') to yield approximately 908 bp fragment. The amplicons specific to CP and RdRp were cloned and at least two independent clones per amplicon sequenced in both orientations. Sequence analysis and phylogenetic assessment were performed as described previously (Alabi et al., 2011).

RESULTS AND DISCUSSION

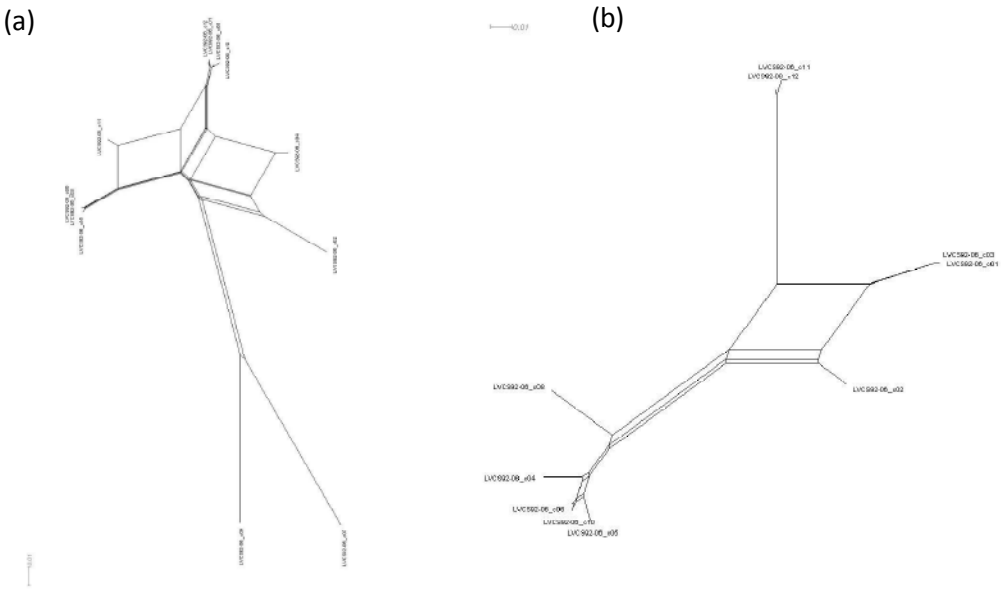
In pairwise comparisons, the CP and RdRp sequences obtained from 27 isolates showed nucleotide identities ranging from 74-100% and 72-100%, respectively, among themselves (Table 1) and 74-98% and 73-89%, respectively with corresponding sequences of GVA isolates available in GenBank. These identity values are within the limits of species demarcation in the family *Betaflexiviridae*, where isolates sharing greater than 72 % nt or 80 % aa sequence identities between their CP or polymerase genes are considered as one species (Adams *et al.*, 2005). Further analyses showed the presence of distinct haplotypes and considerable haplotype diversity among clones obtained from individual grapevines. Phylogenetic analysis of CP and RdRp sequences of GVA isolates from CA and WA showed random distribution in among different phylogroups. Phylogenetic network analysis revealed non-treelike phylogenetic networks among the GVA sequences (Fig. 1), suggesting that a complex evolutionary scenario such as mutations and recombination events could be contributing to genetic diversity among natural populations of GVA. Such networks also indicate that mixed infections of distinct virus variants in individual grapevines could be contributing to quasi-species nature of the virus.

In summary, our study revealed the presence of genetically diverse populations of GVA in CA and WA vineyards. This knowledge will help grape clean plant programs across the country in improving the sanitary status of planting materials provided to nurseries and grape growers.

Table 1. Range of nucleotide (nt) sequence identities among GVA isolates from California and Washington States.

Gene	Source	Number of isolates	% nt identity
CP	California	16	74.5-100.0
	Washington	11	74.3-100.0
RdRP	California	7	72.5-100.0
	Washington	9	72.1-100.0

Figure 1. Non-treelike phylogenetic networks depicting relationships among distinct haplotypes of Grapevine virus A present in an individual isolate of the virus from California. Such networks were observed among haplotypes obtained from individual vines based on the (a) RdRP and (b) CP gene sequences.



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This work was supported, in part, by Washington State University's Agricultural Research Center in the College of Agricultural, Human, and Natural Resource Sciences, Wine Advisory Committee of the Washington Wine Commission, USDA-ARS-Northwest Center for Small Fruits Research, and USDA-NIFA-Specialty Crop Research Initiative (Award Number 2009-51181-06027).

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Occurrence of *Grapevine virus E* in the Pacific Northwest Vineyards

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INTRODUCTION

Grapevine virus A (GVA), *Grapevine virus B* (GVB), *Grapevine virus D* (GVD)and *Grapevine virus E* (GVE) are the four definitive species in the genus *Vitivirus* (family *Betaflexiviridae*) reported from grapevine (*Vitis* spp.) across many grape-growing regions (Du Preev et al., 2011). Among them, two isolates of GVE - one (isolate TvAQ7) from table grape (*V. labrusca* cv. Aki Queen and another (isolate TvP15) from a wine grape (V. vinifera cv. Pione – were reported from Japan (Nakaune et al., 2008). Subsequently, a variant of GVE (isolate SA94) was sequenced from wine grape (*V. vinifera* cv. Merlot) from South Africa (Coetzee et al., 2010). A pairwise comparison of genomic sequence showed 69.6% nucleotide (nt) identity between SA94 (GU903012) and TvAQ7 (AB432910) isolates indicating that they are distinct strains of GVE. A partial genome sequence of TvP15 isolate (AB432911) shared 98.1% nt identity with SA94 isolate, suggesting that both TvP15 and SA94 are closely related isolates of GVE. In this study, we have determined the complete genome of an isolate of GVE from a wine grape (cv. Cabernet Sauvignon) and a comparison with corresponding sequences of SA94, TvP15 and TvAQ7 isolates indicated that the Washington isolate is highly similar to SA94 and TvP15 than TvAQ7.

MATERIALS AND METHODS

The GVE isolate (WAHH2) was collected from a single, own-rooted grapevine (cv. Cabernet Sauvignon) planted in a commercial vineyard block in Yakima Valley of Washington State. Initially, primers GVE Rbp F2 (5'-GCCAAGGSAGTATTTGATG) and GVE Rbp R2: 3 (5'-AWGGGTACTCAGACTTCC) were designed, based on consensus sequence specific to open reading frame 5 of GVE isolates available in GenBank, to amplify a 327 bp fragment from petiole extracts in one step-single tube reverse transcription polymerase chain reaction (RT-PCR) assay. Sequences obtained from cloned amplicons were compared with corresponding sequences of GVE isolates available in GenBank. Additional primers were designed, based on consensus sequence of TvAQ7 and SA94, to obtain overlapping amplicons spanning the GVE genome. The 3'-terminal sequence was determined using FirstChoice RLM-RACE Kit (Ambion) following manufacturer's instructions. Sequences from the overlapping fragments were assembled and pairwise comparisons done using Vector NTI Advance 11 (Life Technologies Corp., CA, USA). In addition, a limited number of grapevine samples were tested using primers specific ORF5 described above to obtain a snapshot of the distribution of GVE in Washington (WA) and Oregon (OR) vineyards.

RESULTS AND DISCUSSION

The complete genome of WAHH2 isolate from Washington (GU000000) is 7,568 nt long, excluding the poly(A) tail, and similar in size to GVE SA94 (7,568 nt) than to GVE TvAQ7 (7,576 nt). In pairwise comparisons, WAHH2 genome showed 98% and 70% identity with genome sequences of SA94 and TvAQ7 isolates, respectively, and 45% and 46% identity with GVA (DQ855087) and GVB (EF583906), respectively. These results suggest that WAHH2 is more closely related to SA94 than to TvAQ7 of GVE and other vitiviruses. GVE WAHH2 encodes five ORFs and their arrangement (Fig. 1) was identical to those described previously for SA94 and TvAQ7 isolates (Coetzee et al., 2010; Nakaune et al., 2008). Similar to SA94 and unlike TvAQ7, the ORF1 of WAHH2 does not overlap with ORF2.

In pairwise comparison of different ORFs, WAHH2 isolate shared a higher sequence identity at both nt and amino acid (aa) levels with SA94 (97-99% at nt level and 98-100% at aa level) and TvP15 (95-99% at nt level and 91-99% at aa level) isolates than with TvAQ7 (53-78% at nt level and 45-87% at the aa level). These values, except for ORF2 of TvAQ7, are within the limits of species demarcation proposed for the family *Flexiviridae*, where

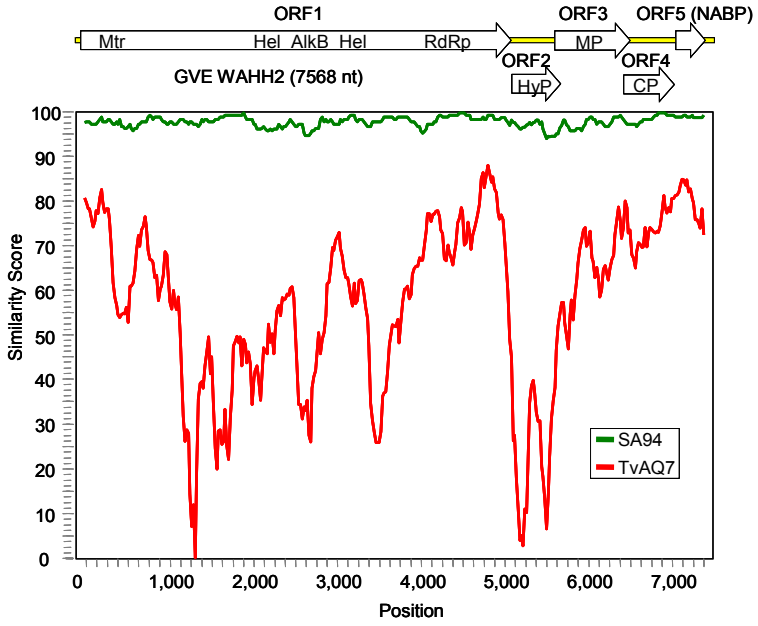

Figure 1. Complete genome organization of a Washington isolate (WAHH2) of *Grapevine virus E* derived from cv. Cabernet Sauvignon and its comparison with complete genomes of GVE isolates SA94 (GU903012) and TvAQ7 (AB432910). Mtr, methyltransferase; Hel, helicase; AlkB, AlkB conserved domain; RdRp, RNA-dependent RNA polymerase; HyP, hypothetic protein – a protein with unknown function; MP, movement protein; CP, coat protein; NABP, nucleic-acid-binding protein.

isolates sharing greater than 72% nt or 80% aa sequence identities between their CP or polymerase genes are considered one species (Adams et al., 2005). In this regard, it can be concluded that WAHH2, SA94 and TvP15 are closely related sequence variants and TvAQ7 is a distant variant of GVE. Thus, it is likely that divergent variants of GVE are present in different grape-growing regions. Our limited survey of WA and OR vineyards indicated the presence of GVE in different wine grape cultivars. Further studies are in progress to determine genetic relationships and biological properties of GVE isolates from WA and OR vineyards

In summary, our study documented the presence of GVE in the Pacific Northwest vineyards. Sequence analysis of GVE isolate from Washington indicated that it is closely related to SA94 and TvP15 isolate from South Africa and Japan, respectively, and distantly related to TvAQ7 from Japan. Although the economic impact of GVE is yet to be realized, information on GVE variability will help in the development of better diagnostic tools to detect all strains of the virus to in clean plant programs to provide virus-tested planting materials for wine grape growers in the Pacific Northwest region.

ACKNOWLEDGEMENTS

This work was supported, in part, by Washington State University's Agricultural Research Center in the College of Agricultural, Human, and Natural Resource Sciences, Members of the Wine Advisory Committee of the Washington Wine Commission, USDA-ARS-Northwest Center for Small Fruits Research, and USDA-NIFA-Specialty Crop Research Initiative (Award Number 2009-51181-06027).

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Preliminary Study on Artificial MicroRNA Induced Resistance Against GVA in *Nicotiana benthamiana*

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INTRODUCTION

Grapevine virus A (GVA), the type member of the genus *Vitivirus* (King et al. 2012), is one of most widespread grapevine viruses. GVA is associated with the rugose wood complex (Martelli and Boudon Padieu, 2006) and Shiraz disease of grapevines (Goszczynski *et al*. 2008). Since no useful natural resistance to viruses has been identified in grapevines (Martelli and Boudon-Padieu, 2006), genetic engineering is of importance to develop virus-resistant grapevines. In this study, we explored efficiency of artificial microRNA resistance (amiRNA) technology to target GVA in the herbaceous host, *Nicotiana benthamiana.*

MATERIALS AND METHODS

V. vinifera cv**.** Pinot Noir clone ENTAV 115 was used as source plant for amplification of *Vitis vinifera miR166f (*vvi-miR166f) pre-miRNA which was used as a backbone to express amiRNA sequences. We chose miR166f precursor because of its simple 2D structure, high level of expression and effective processing in various grapevine tissues including leaves, tendrils, inflorescence and berries (Pantaleo *et al.* 2010).

Construction of vector expressing miR166f

The vvi-miR166f pre-miRNA plus 50 bp flanking regions was amplified and cloned in the vector pSCA (Stratagene, USA), sequenced and sub-cloned into the binary vector pBIN61. The resulting recombinant plasmid (pBinMir166f), was transferred into *Agrobacterium tumefaciens* strain C58C1 and infiltrated into *Nicotiana benthamiana* as described by Voinnet *et al.* (2003). Its transcription and processing was assessed using semi-quantitative RT-PCR (Liu *et al.* 2002), Northern blot (Canizares *et al.* 2004) and stem loop realtime RT-PCR (Gasic *et al.* 2007).

Construction of amiRNAs

GVA amiRNA candidate sequences were generated using WMD3 web microRNA designer (Ossowski *et al.* 2010). Two amiRNAs, amiRNA-1 (5´-TTTAGAAAAATAGTTCGGCGC-3´) and amiRNA-5 (5´-TACTTACACACATTCATGCGC -3´), which target GVA ORF1 and ORF5, respectively, were selected. AmiRNA-1 and amiRNA-5 were generated by PCR-based mutagenesis according to Warthmann *et al.* (2008) using the pBinMiR166f as template. The obtained PCR fragments were cloned into pSCA, sequenced and sub-cloned into pBin61, resulting in pBin61-amiRNA-1 and pBin61-amiRNA-5. Binary vectors were transferred into *A. tumefaciens* strain C58C1. Transcription and processing of the amiRNAs were investigated through stem loop realtime RT-PCR using transient expression assay in *N. benthamiana* plants.

Challenge inoculation and GVA accumulation

N. benthamiana plants were infiltrated using the above mentioned constructs. Three days post infiltration (dpi), plants were inoculated by GVA PA3 (Galiakparov *et al*. 1999). Tissues from non-inoculated systemic leaves were sampled at 0, 7, 14 and 21 dpi and used for monitoring GVA accumulation through DAS-ELISA using a GVA commercial kit (Agritest, Italy).

RESULTS AND DISCUSSION

Transcription and processing of pBinMir166f

Because of the existence of endogenous transcription of miR166f in *N. benthamiana*, we used semi-quantitative RT-PCR to detect overexpression of the agroinfiltrated vvi-pre-miR166f. This was demonstrated by the earlier appearance of the amplicons in the infiltrated tissues. No PCR products were detected in the control treatment, containing no reverse transcription, indicating the lack of *Agrobacterium* DNA in the samples after DNAse treatment. Accordingly, Northern blot analysis also confirmed the expression of pre-miR166f RNA in

pBinMiR166f-infiltrated leaves of *N. benthamiana*. Moreover, stem-loop real-time RT-PCR confirmed the specific processing of the construct and miRNA166f expression in *N. benthamiana* agroinfiltrated plants, in which the expression of miR166f was increased by more than five times compared with wild type plants.

Construction of amiRNAs

The pSCA cloning vector containing vvi-pre-miR166f was used as template in fusion-PCR reactions to produce mutagenized 215 bp fragments containing GVA amiRNA-1 and amiRNA-5 sequences. Correctness of both amiRNA-ORF1 and amiRNA-ORF5 constructs was confirmed by sequencing.

Functionality of the amiRNAs

Correct expression of amiRNA-1 and amiRNA-5 in tissues infiltrated with pBin61-amiRNA-1 and pBin61-amiRNA-5 was assessed by stem-loop RT-PCR assay. The expected amplicons were selectively obtained from the corresponding infiltrated tissues and their sequences were confirmed. This is taken as an indication that the amiRNAs expressed from the pMiR166f-215 vector are properly processed by the DICER.

Evaluation of resistance

GVA symptoms were delayed in 50% of pBin61-amiRNA-1-infiltrated plants and 20% of pBin61-amiRNA-5-infiltrated plants. At 15dpi, GVA was undetectable in all mock inoculated plants and in 30% and 40% of pBin61-amiRNA-1 and pBin61-amiRNA-5 plants, respectively. In inoculated symptomless plants GVA was undetectable, indicating resistance induction. By contrast, GVA accumulated at high levels in the inoculated controls and in infiltrated symptomatic plants. The transient expression of amiRNAs containing sequences complementary to either ORF1 or ORF5 induced promising levels of resistance to GVA in *N. benthamiana*. However, the real effectiveness of the system needs to be evaluated first in transgenic *N. benthamiana*, then in grapevines.

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GRSPaV, a Prevalent Virus in Most of the *Vitis* species

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV), is closely related to the disease Rupestris Stem Pitting, a component of the the rugose wood disease (Zhang *et al.,* 1998, Meng *et al.* 1998). GRSPaV is commonly detected in cultivated grapevine worldwide. The hypothesis of co evolution between the ancestor of GRSPaV and *Vitis* species associated to its diffusion in scion varieties via the grafting was previously proposed (Meng *et al.,* 2006). To test this hypothesis we investigated the GRSPaV infection of numerous grapevine accessions never grafted.

MATERIALS AND METHODS

26 rootstocks varieties (1 to 10 clones per variety), 34 *V. vinifera ssp. vinifera* own-rooted and never grafted and 6 *V. vinifera ssp. sylvestris* from collections maintained in Languedoc-Roussillon (Espiguette and Vassal repositories) were used for this study.

Total nucleic acid was extracted from 200 mg of cambium scrapings dormant cane, using the Mac Kenzie extraction buffer modified (Mac Kenzie *et al.*, 1997) and the NucleoSpin® RNA II kit (Macherey-Nagel). RNA were amplified with QuantiTect® SYBR® Green RT-PCR Kit (QIAGEN®, France) with primers 18 S as internal control (Gambino *et al.,* 2006) and RSP F1-R1 for GRSPaV detection (Beuve *et al.,* in press).

RESULTS AND DISCUSSION

Rootstocks

Except 3 (34 EM, 110 R and 99 R), the whole varieties of rootstocks analyzed were infected by GRSPaV independently of their genetic background (Table 1). Among the 26 rootstocks analyzed, 19 rootstocks had a homogeneous status. 8 presented a heterogeneous status with identification of positive and negative clones in the same variety even if each of these varieties came from a unique seed.

Table 1: Detection of GRSPaV in rootstocks accessions with various parentages.
Number of positive versus number of tested samples. **: a variety with at least one clone positive is considered positive. (1) plus *V. longii* for Fercal ; *: variety found free of GRSPaV; Variety with positive and negative clones.

Genetic origin	Rootstock varieties	positive varieties **	positive clones
V. berlandieri - V. vinifera (1)	333EM, 41B, Fercal	3/3	4/8
V. riparia Michaux	Riparia Gloire de Montpellier	1/1	4/5
V. riparia - V. berlandieri	125 AA, 161-49 C, 34 EM*, 420 A, 5 BB, 5 C, RSB 1, SO4	7/8	21/41
V. riparia - V. labrusca	Vialla	1/1	2/2
V. riparia - V. longii	1616 C	1/1	1/1
V. riparia - V. longii - V. rupestris	216-3 Cl	1/1	1/1
V. riparia - V. rupestris	101-14 Mgt, 3309 C	2/2	7/7
V. riparia - V. rupestris - V. berlandieri	Gravesac	1/1	1/1
V. riparia - V. rupestris - V. cordifolia	44-53 Ma	1/1	2/3
V. riparia - V. rupestris - V. vinifera	4010 Cl, 196-17 Cl	2/2	3/3
V. rupestris Scheele	Rupestris du lot	1/1	2/6
V. rupestris - V. berlandieri	99 R*, 110 R*, 140 Ru, 1103 Pa	2/4	2/12

To study the differences in a variety, the 8 rootstocks varieties presenting heterogeneous results (positive and negative clones) were more precisely described (Table 2).

Table 2: Number of GRSPaV positive clones in rootstock varieties with heterogeneous results.
Sex (M= Male; F= Female) and sanitized status of the tested clones are given.

Variety	Riparia Gloire de Montpellier	Rupestris du Lot	44-53 Ma	140 Ru	1103 Pa	SO4	161-49 C	41 B	Total
Sex	M	M	M	M	M	M	F	F	6 M / 2 F
Not sanitized	3/3	2/4	2/3	1/2	0/1	3/5	2/4	0/1	**13/23**
Sanitized	1/2	0/2	0	0	1/1	4/5	2/10	1/4	**9/24**

Some tests were realized to verify that the absence of GRSPaV detection is not due to a sampling heterogeneity (results not shown). 56% of the not sanitized clones and 37% of the sanitized ones found positive. More interestingly, some of the clones found free of GRSPaV become infected after sanitation without any grafting (canes taken from the mother plants). This suggests the possibility of a contamination in the vineyard by an unknown vector. These heterogeneous results are observed in female as in male varieties (Table 2). The presence of positive in male varieties does not match with the possibility of a contamination by pollen as previously suggested (Rowhani *et al.,* 2000).

Table 3: Detection of GRSPaV in different accessions of *Vitis vinifera* never grafted from diverse geographical origins.

Vitis subsp.	Geographical origin	GRSPaV positive/ total tested
V. vinifera ssp. sylvestris	France-Tarn	0/2
	France-Hérault	2/2
	Tunisia	1/2
	Total	**3/6**
V. vinifera ssp. vinifera	Afghanistan	1/1
	Australia	3/3
	Chile	5/7
	Greece (Santorin)	0/6
	Peru	1/1
	Tunisia	2/2
	Yemen	2/2
	Total	**14/22**
V. vinifera x V. vinifera		**0/12**

Vitis vinifera own-rooted never grafted

GRSPaV is detected in the two sub species of *Vitis vinifera* and in some seedlings (Table 3). No clear connection can be established between the GRSPaV presence and geographical origin of these accessions. However, it can be noticed that the greek varieties taken from Santorin island were all free of GRSPaV. We observe a fewer proportion of positives in the *V. vinifera ssp. vinifera* accessions never grafted than generally observed in *V. vinifera ssp. vinifera* grafted. Indeed, 98% on the 147 grafted *V. vinifera* previously analyzed were positive (results unshown) against 64% of 22 *V. vinifera* own-rooted. Interestingly the half of the *V. vinifera ssp. sylvestris* accessions analyzed appeared infected by GRSPaV. By contrast, *V. vinifera* seedlings tested are all negative (Table 3). The GRSPaV appeared to be a virus prevalent in the American and the European species of *Vitis* even in the wild ones *(Vitis vinifera ssp. sylvestris*) as previously reported by Nolasco *et al.* (2006). The GRSPaV identification in *Vitis vinifera* never grafted, and in all the families of rootstocks may indicate it would have been introduced in *Vitis* species a long time ago. However, the different sanitary status of different clones, inside a same variety, suggests the existence of a vector able to transmit this virus. The study of diversity in GRSPaV in the wild species and some varieties of rootstocks would allow providing clues important in answering this question.

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A Study of Shiraz Disease Etiology Using Next-Generation Sequencing Technology

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INTRODUCTION

The emergence of a new disease in South African *Vitis vinifera* cv Shiraz, vineyards have been reported and described as Shiraz disease (SD) (Engelbrecht and Kasdorf, 1990). Since then, SD has also been observed in other *V. vinifera* cultivars (Gamay, Malbec, Merlot, Shiraz, Viognier, Chardonnay, Semillon and Tempranillo). The vines of affected plants remain green and immature for extended periods in the growing season (Figure 1). Cross sections of vines show excessive phloem and cambium growth and underdeveloped non-lignified xylem causing the shoots to have a rubbery consistency (Goussard and Bakker, 2006). Affected vines show postponed budding and fruit production is diminished. Shiraz diseased vines never recuperate and die within five years of first symptoms observed. The disease is dormant in non-susceptible grapevine cultivars, from which it can be transmitted by grafting and the mealybug *Planococcus ficus* to SD susceptible grapevine cultivars (Goszczynski & Jooste, 2003). Grapevine leafroll-associated virus 3 and grapevine virus A (GVA) have been found to be consistently associated with SD (Goszczynski and Jooste 2003; Goszczynski, 2007). Grapevine virus A, has also been shown to be involved in other grapevine disease complexes, and although it is an abundant virus in SA vineyards, it does not cause disease by itself or in complexes within any cultivars (Boscia *et al*., 1997). It was shown that genetic variants of molecular group II are closely associated with SD, and variants of molecular group III are present in GVA-infected SD susceptible grapevines that do not show symptoms of the disease (Goszczynski, 2007). Although, GVA was shown to be associated with this disease (Goszczynski & Jooste 2003), the role of the virus and possible other viruses in the disease complex is still unknown. In order to progress in the understanding of grapevine disease complexes, the host plant and all viruses involved need to be extensively studied. Next-generation sequencing (NGS) technologies have the potential to produce large amounts of sequence information. This technology has the advantage that disease complexes, such as SD, can be screened for all possible viruses without the need for prior knowledge of the pathogens involved. It can thus provide an unbiased picture of the etiology of the SD viral complex and generate sequence information for previously unknown viruses and their variants or viruses previously unknown to infect grapevine.

The aim of this project is to elucidate the complex viral disease etiology associated with SD with a metagenomic sequencing approach using the latest technology in NGS. The data generated will not only indicate which viruses are the causative agents of this destructive disease, but also which genetic variants or strains of these viruses are involved.

MATERIALS AND METHODS

Shiraz diseased and leafroll diseased (control) vines were sampled from the same vineyards in late autumn when symptom expression is most distinct. Double-stranded RNA was extracted using an adapted dsRNA protocol (Valverde *et al*., 1990). Sequencing libraries were prepared using the ScriptSeq™ v2 RNA-Seq Library preparation kit (Epicentre) and sequenced on an Illumina HiScanSQ. Reads were filtered and trimmed for quality using Fastx toolkit and used in *de novo* assemblies and read-mapping analysis mainly using CLC genomic workbench. BLAST homology searches were performed in batches using Blast2Go.

Figure 1: Typical SD symptoms as described by Goussard and Bakker (2006) in late autum. Left: Vine with droopy appearance and delayed senescence and leaf drop. Right: Non-lignified canes with senescent leaves.

RESULTS AND DISCUSSION

Table 1 summarizes the preliminary results from this study (five vines sequenced). BLAST analysis of de novo assembled contigs revealed the presence of several viruses known to infect grapevine and commonly found in South African vineyards. Virus variants were identified by read-mapping using the full genomes of variants identified with the BLAST homology searches as the reference. Fungal viruses were only classified to family level by amino acid similarity due to the lack of sufficient information in sequence databases. Fourty-six different mycoviruses were identified belonging to at least 6 families. Even though the complexity of virus infection was greater in SD plants than in the control plants thus far, no association with specific viruses can be confirmed. Additionally, four different viroids (GYSVd-1, GYSVd-2, AGVd, and HSVd) were identified in South African vineyards for the first time. GLRaV-3 was identified in all samples and GVA in four of the samples. The hypothesis that GVA group II variants are associated with SD holds true, so far. More sequencing is planned and additional results will be presented.

Table 1: Table of virus and virus variants identified in each sample.

Family	***Closteroviridae***				***Betaflexiviridae***			
Genus	***Ampelovirus***				***Vitivirus***			
Species	Grapevine leafroll-associated virus 3				Grapevine virus A			Grapevine virus E
Reference genome [b]	Group I (621)	Group II (623)	Group III (PL-20)	Group VI (GH11)	Group I (GTG11-1)	Group II (BMo32-1)	Group III (GTR1-1)	(SA94)
Sample								
SD 3 - Control	□	-	-	-	-	-	-	-
SD 11 - Control	□	-	-	□	-	-	□	-
SD 4	□	□	-	□	-	□	-	□
SD 8	-	□	-	-	-	□	□	-
SD12	-	□	-	-	-	□	□	-

a Plant virus variants identified by read-mapping to complete genomes of known variants identified from *de novo* results.
b Genetic variant group indicated with isolate code in brackets

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Electronic resources

Fastx toolkit: http://hannonlab.cshl.edu/fastx_toolkit/
FastQC: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Blast2GO: http://www.blast2go.com/b2ghome

Complete Genome Sequence of a New Circular DNA Virus from Grapevine

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INTRODUCTION

Geminiviruses are plant viruses with geminate icosahedral particles and a circular single-stranded DNA genome. Their recent emergence is notable based on a rapidly expanding geographic distribution and host range, as well as recombination propensity that can cause new diseases and new epidemics (4, 6). We provide here the first description of a gemini-like virus sequence from grapevine (*Vitis vinifera*) for which the corresponding virus is provisionally named grapevine cabernet franc-associated virus (GCFaV).

MATERIAL AND METHODS

Circular DNA was amplified from grapevine total nucleic acid extracts by RCA and resolved by RFLP (2). RCA product was cloned and sequenced by standard laboratory techniques. Sequences were assembled with VectorNTI program and analyzed with BLAST (1).

RESULTS AND DISCUSSION

RCA products were detected from four different *Vitis vinifera* 'Cabernet franc' vines, all originating from the same declining vineyard in New York. No comparable DNA was detected in 18 other grapevine samples collected from different sites, including two ‚Cabernet Franc' accessions from independent sources. Cloning and Sanger sequencing (4x coverage, both strands) revealed a single DNA circle of 3,206 nucleotides. This genome size is larger than the largest previously reported geminivirus genomic DNA of 3,080 nucleotides (3). The GenBank accession number for the sequence of GCFaV is JQ901105.

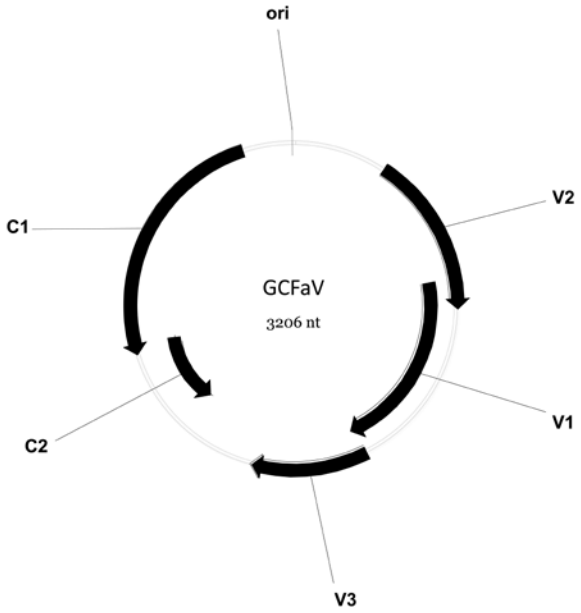

Figure 1. Genome organization of grapevine cabernet franc-associated virus (GCFaV). Arrows indicate open reading frames (C1:C2, replication-associated protein; V1, coat protein; V2, unknown; V3, unkown; ori, origin of replication with nonanucleotide sequence).

Consistent with other monopartite members of the family *Geminiviridae* the orientation of the predicted GCFaV ORFs is bidirectional with three ORFs in the viral-sense (V) and three in the complementary orientation (C). Importantly, the nonanucleotide signature for the geminivirus origin of replication, 'TAATATT|AC', was present in an intergenic region as observed in all members of the family *Geminiviridae* (5). BLASTN analysis (1) showed the closest related sequence to be that of a dicot infecting mastrevirus, *Chickpea chlorotic dwarf Syria virus*, the genome of which is 634 nt smaller and shares only 50% identity. The GCFaV ORF V1 (coat protein, CP) showed a maximum amino acid sequence identity of 26% with *Mesta yellow vein mosaic virus* (genus *Begomovirus*). Remarkably, the V2 and the V3 ORFs had no apparent sequence similarity with other geminiviral sequences at the nucleotide and amino acid levels. ORFs C1 and C2 showed a subgenomic organization strikingly similar to those of mastreviruses (including a spliced transcript; (7)) and a maximum identity of 33% to 52%, respectively, with 74% and 79% coverage (BLASTX analysis) of the respective ORFs of *Bean yellow dwarf virus*. In phylogenetic analyses, maximum likelihood and neighbor-joining trees for the CP gene, replicase gene, and full length sequences, GCFaV formed a distinct branch (bootstrap >70%) apart from all members of four genera within the family *Geminiviridae*.

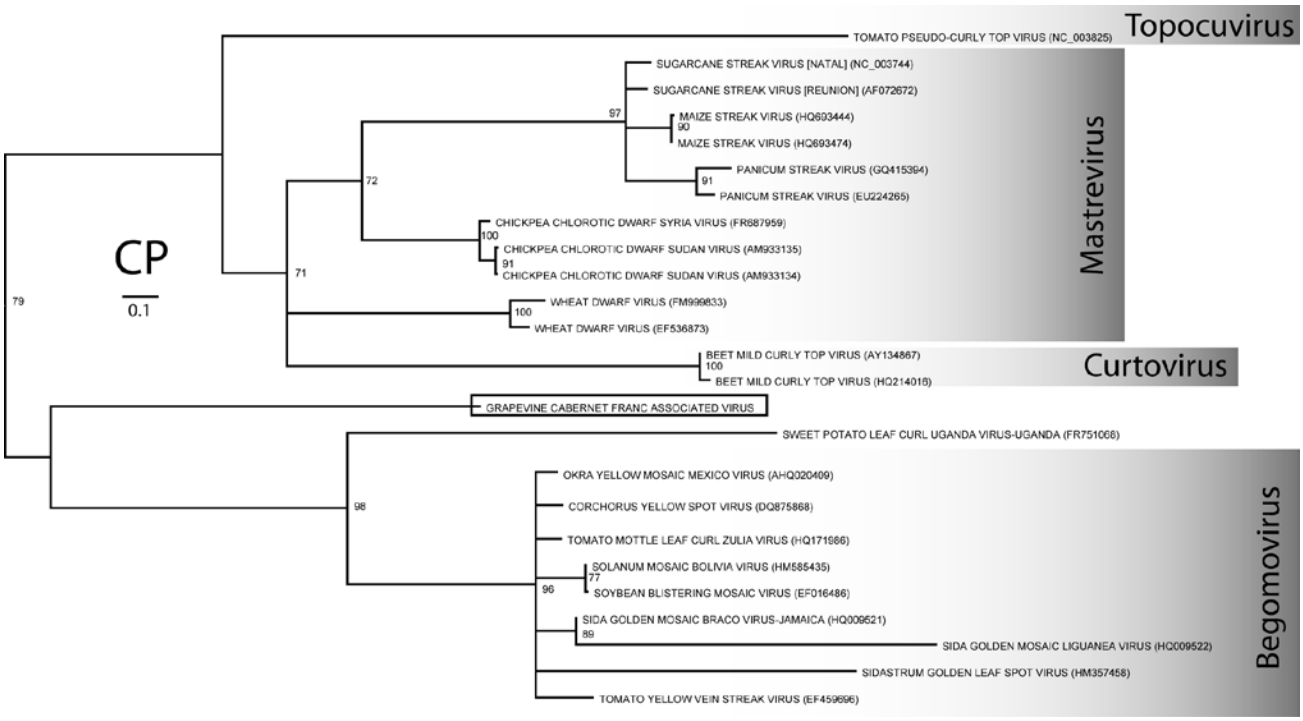

Figure 2. Maximum likelihood unrooted trees of the coat proteins (CP) of selected members of the family *Geminiviridae*, including GCFaV (boxed). Genera groupings are indicated in bold on the right. Sequence accession numbers are in parentheses. Percent bootstrap values are shown at each node (all branches with less than 70% support were collapsed).

A DNA virus belonging to the genus *Badnavirus,* family *Caulimoviridae* was recently detected in grapevine by deep sequencing (8) but this is the first report of a geminivirus sequence in grapevine. Further studies are needed to determine the prevalence of GCFaV and its impact.

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Association of a Circular DNA Virus in Grapevines Affected by Red Blotch Disease in California

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INTRODUCTION

In 2008, a new disease consisting of patches of red blotches along leaf margin, and red veins under the leaf surface were observed in red grape varieties in a few vineyards in Napa Valley, CA. Brix units of fruit juice of symptomatic, but not asymptomatic grapevines, were reduced (Calvi 2011). Anecdotal observations suggested that the disease was spreading in the vineyards. The name 'grapevine red blotch (GRB)' was proposed to distinguish this disease from leafroll disease. Absence of signs and symptoms associated with bacterial and fungal pathogens prompted investigations to determine the causal agent of GRB. RNA extracts derived from petioles obtained from symptomatic grapevines tested negative for known grapevine viruses in RT-PCR assays.

Recently, metagenomic analysis using next generation sequencing (NGS) has successfully revealed the presence of previously uncharacterized viruses (Al Rwahnih et al., 2009; Kreuze et al., 2009; Zhang et al., 2011). Herein we report on the identification of a new DNA virus in nucleic acid extracts obtained from grapevines showing GRB symptoms using NGS.

MATERIALS AND METHODS

Dormant canes were collected in fall 2010 from three symptomatic grapevines, one from each of the three commercial vineyards, and bark scrapings were obtained. Double stranded RNA was extracted without DNAase treatment, cDNA prepared and the library was amplified as described by Al Rwahnih et al. (2009). Sequence reads were generated by Eureka genomics (Hercules, CA, USA) using an Illumina Genome Analyzer IIx. After sequencing, the contigs were assembled and BLASTN and TBLASTN analysis were performed at the NCBI web site. Primers were designed to detect a new DNA virus identified in the TBLASTN analysis and PCR assays followed by agarose gel electrophoresis were conducted to detect the new virus in DNA extracts obtained from source vines and several symptomatic grapevines in fall 2011. Complete sequence of the new virus was obtained by sequencing amplified products obtained using Illustra™ TempliPhi kit (GE Healthcare Biosciences, Philadelphia, PA, USA).

RESULTS AND DISCUSSION

The TBLASTN analysis using the nucleotide sequence of contigs obtained from each of the three source vines indicated a distant homology at the amino acid level with geminiviruses. The complete sequence determined by sequencing the products obtained by rolling circle amplification of DNA from symptomatic grapevines indicated the presence of a new circular DNA virus.

PCR assays using primers specific to the new virus were able to amplify a product from DNA extracts obtained from petioles of grapevines showing red blotch symptoms. Similar results were also obtained from DNA obtained from bark scrapings of dormant canes. The new virus has been named 'Grapevine red blotch-associated virus'. We are currently investigating the biological properties of the virus to ascertain its role in red blotch disease.

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Grapevine Vein Clearing Virus Exists as Genetically Diverse Populations in Seven Grape Varieties in Three Midwestern States

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INTRODUCTION

Grapevine vein clearing virus (GVCV) is the first DNA virus discovered in grapevine that contains a circular DNA genome of 7,753 base pairs (bp) (Zhang, *et al.*, 2011). GVCV has three open reading frames (ORFs), among which ORF 3 encodes for a polyprotein including Zinc finger (ZF), Reverse transcriptase (RT) and RNase H domains. The symptoms closely associated with GVCV are translucent vein-clearing on young leaves, short internodes and the decline of vine vigor. The syndrome has been found on grape varieties Cabernet Sauvignon, Chardonnay, Chardonel, Cabernet Franc, Riesling, Vidal Blanc, and Corot noir in Missouri, Illinois and Indiana (Zhang, *et al.*, 2011). GVCV-associated diseases have caused significant yield lost and are becoming a serious threat to the production of grapes in vineyards in Midwest region of America. To have a better understanding of GVCV population structures on grapevine varieties grown at different geographical locations, a 2,580 bp fragment of GVCV was amplified and cloned from thirteen single vines. Two separate regions (ZF and RT region) were sequenced and analyzed for phylogenic relationship and pressure under selection.

MATERIALS AND METHODS

Thirteen isolates were collected from grapevines in commercial vineyards in Missouri, Illinois and Indiana in 2009 and 2010. Total DNA was extracted from each isolate using DNeasy ® Plant Mini Kit (Qiagen). A 2,580 bp fragment (From 4,142 to 6,721 in the genome) was amplified by polymerase chain reaction (PCR) using primer set DBV3956F and GVCV-R1, and cloned into pCR8/GW/TOPO vector which was transformed into *E. coli*. Three colonies for each isolate were selected randomly and cultured. Plasmids were purified by QIAprep Spin Miniprep Kit (Qiagen). ZF and RT regions were sequenced (Nevada Genomic Center, Reno, Nevada). The sequences were aligned in the CodonCode Aligner software. Phylogenic analysis was performed for these sequences by MEGA5 program. The selection pressure on ZF and RT regions were analyzed by DataMonkey program.

RESULTS AND DISCUSSION

As a result, 78 sequences for 13 isolates were obtained, 6 for each isolate (3 sequences of ZF region and 3 sequences of RT region). Based on the sequences of ZF and RT regions, GVCV clustered into three and two subgroups, respectively (Figure 1), suggesting the presence of genetically diverse isolates in vineyards. Moreover, purifying (negative) selection pressure was acting on both ZF and RT regions, implying that there was a restrain on nucleotide changes in RT and ZF domains to retain their functionality. In conclusion, GVCV occurs as genetically diverse populations that are subject to the purifying selection pressure in ZF and RT regions.

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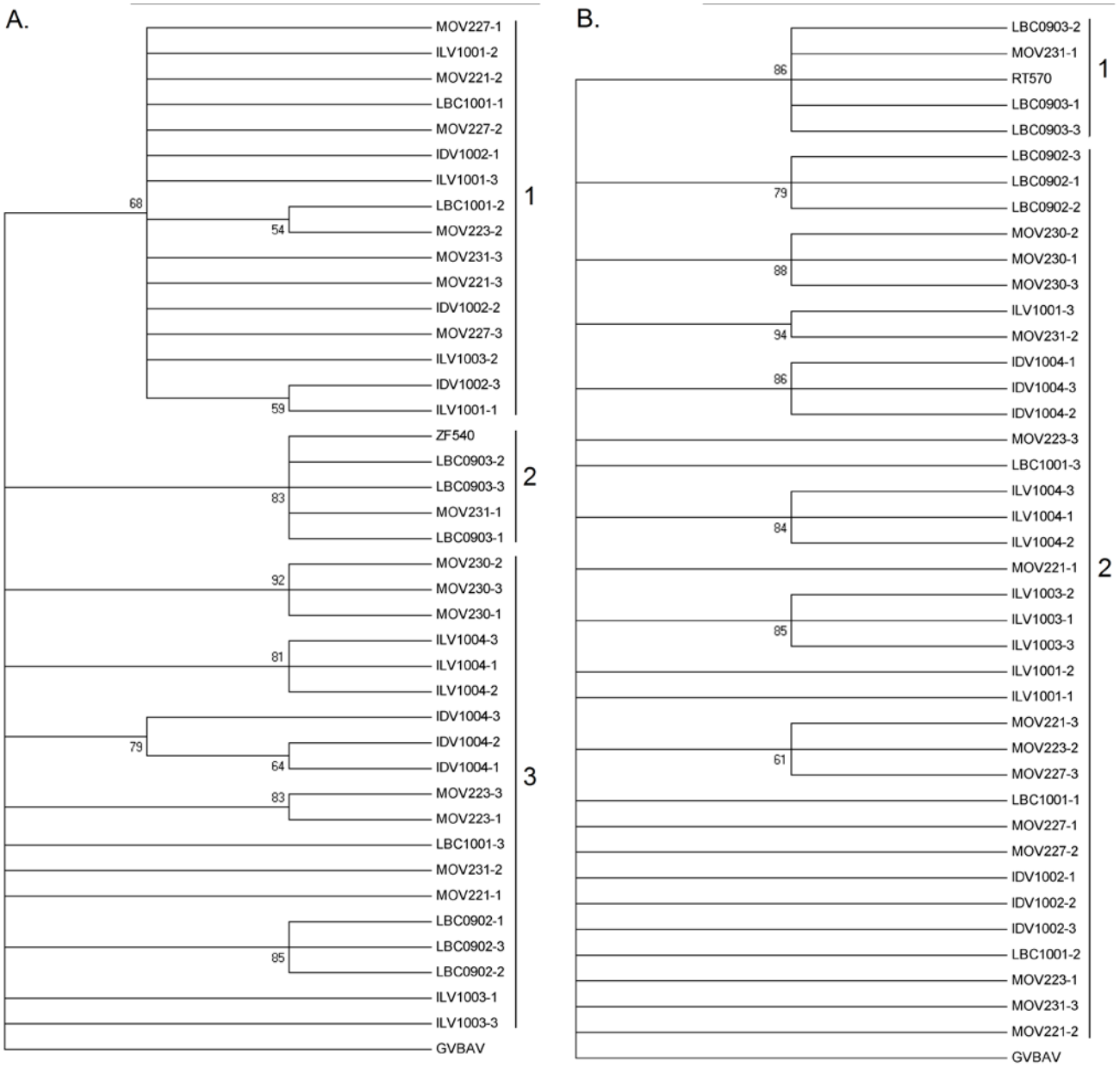

Figure 1. Phylogenic analysis of thirteen *Grapevine vein clearing virus* isolates. A. Phylogenic analysis of ZF region of 39 clones; B. Phylogenic analysis of RT region of 39 clones. The trees were generated using maximum likelihood (ML) method in the MEGA5 software with bootstrap replicates at 1,000. Branches reproduced in <50% of bootstrap replicates were collapsed. ZF540 and RT570 (GenBank accession: JF301669) were reference sequences of ZF and RT regions of GVCV from NCBI. *Gooseberry vein banding associated virus* (GVBAV) was used as outgroup (Xu, *et al*., 2011).

Grapevine Yellow Speckle-1 Type 4: A New Proposed Type of *Grapevine Yellow Speckle*-1

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INTRODUCTION

Five viroids are reported to infect grapevines (Flores *et al*., 2005) Which include Hop stunt viroid (HSVd), Citrus exocortis viroid (CEVd) two Grapevine yellow speckle viroids 1 and 2 (GYSVd-I and GYSVd-2) and the Australian grapevine viroid (AGVd). Heterogeneity in natural GYSVd-1 populations was demonstrated (Polivka *et al*., 1996). Based on sequence variations and possible symptom-inducing abilities, the GYSVd-1 populations are classified as types 1, 2 and 3 (Redgin and Reziaian 1993; Szychowski *et al*., 1998).

We already reported that vineyards in Northwest Iran are infected by multiple viroids (Hajizadeh *et al*., 2010). But, there is no study on molecular characterization of grapevine viroids in this part of Iran. This paper reports a new proposed type of GYSVd-1-type 4 in Iran based on sequence variations.

MATERIALS AND METHODS

Grapevine leaves were collected from Iranian vineyards (East and West Azerbaijan and Ardabil provinces) during summer 2010 and the silica-capture extraction method (Foissac *et al*., 2000) was used with minor modifications for preparing total nucleic acid (TNA) extracts. First strand cDNA was synthesized using the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, CA, USA) following manufacturer's instructions and PCR was done by specific GYSVd-1 primers (GY2P: TAAGAGGTCTCCGGATCTTCTTGC and GY1M: GCGGGGGTTCCGGGGATTGC) to amplify full length of GYSVd-1. PCR products and DNA marker (marker VI, Fermants, Lithuania) were analyzed by electrophoresis through 1.4% agarose gels in the presence of 1µg mL^{-1} ethidium bromide using 1X Tris-Acetic EDTA buffer (Sambrook *et al*., 1989). Gels were visualized and photographed with a UV-illuminator.

PCR products were purified from the agarose gel using the "Quantum PrepTM Freeze ′N Squeeze DNA Gel Extraction Spin Columns" (Bio Rad), and ligated into the pGEM-T Easy Vector (Promega, USA) and transformed into chemically competent *Escherichia coli* strain DH5α cells. Sequences of recombinant plasmid were obtained by automatic sequencing at MWG (Biotech, Ebersberg, Germany) using M13 universal forward and reverse primers.

Phylogenetic tree was constructed with MEGA 5 program (Tamura *et al*., 2011) using UPGMA method with bootstrap values (in %) calculated for 1000 replications.

RESULTS AND DISCUSSIONS

GYSVd-1 was positive in 91% of 137 samples examined (Hajizadeh *et al*., 2010). An isolate was chosen randomly from each distinct geographical region (Mahabad, Urmia, Maraghe and Fakhrabad) totaling 8 independent cDNA clones (2 clones for each isolate) which were sequenced. The sequences obtained in this experiment were edited, joined and aligned with those of the three types of GYSVd-1 sequences (type 1 (accession number: X87905), type 2 (accession number: Z17225) and type 3 (accession number: AF059712) and two GYSVd-1 sequences (accession numbers: FJ940920.1 and DQ408542.1) reported from the Southern part of Iran (Zaki-Aghl and Izadpanah, 2009) using the Mega5 program.

Molecular analysis showed some differences between Iranian isolates and their non-Iranian counterparts. Our sequences had 80-99% homologies with the sequences deposited in the GenBank. Also, our sequences had 5, 6 and 16% differences with GYSVd-1 type 1, type 2 and type 3 in nucleic acids (NA) level respectively. Phylogenetic relationships were determined from the aligned sequences by using the neighbor- Joining method inplemented in MEGA 5. As shown in Fig. 2, the Fakhrabad isolate is identical to the type 1 of

GYSVd-1 whereas Mahabad, Maragheh and Urmia isolates together with an isolate from Southern Iran formed a distinct clade. So, we here propose a new type 4 variant of GYSVd-1 from Iran. According to the typing, the Fakhrabad isolate is included in type 1 variant of GYSVd-1 (a symptomless variant), whereas the other isolates seem to have formed a distinct clade based on sequence variations and geographic region (including the symptomless variants Mahabad and Urmia isolates) and yellow speckle-inducing variant (Maragheh isolate).

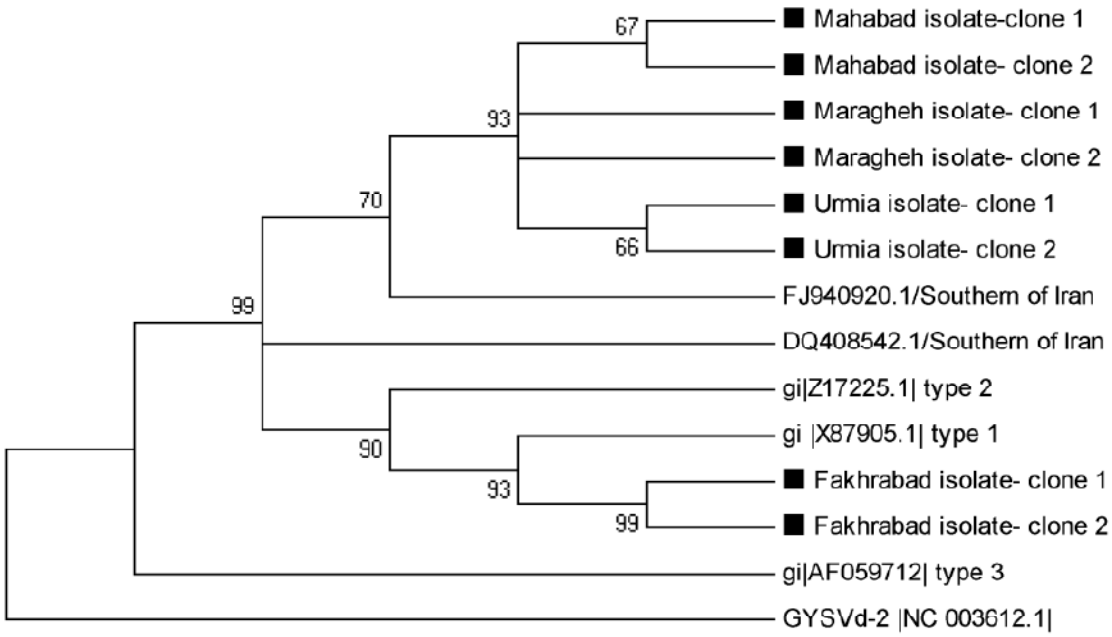

Fig.2. Phylogenetic relationships among GYSVd-1 variants and GYSVd-2 (outgroup). The percentages on the branches are show bootstrap values. Nucleotide sequences of GYSVd-1 types, two isolates from Southern of Iran and GYSVd-2 were obtained from GenBank database. The numbers starting with a letter are database accession numbers. The sequences generated in this study (■) will appear in the GenBank database.

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Molecular Characterization of Two dsRNA Viruses in Native *Vitis* spp

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INTRODUCTION

Vitis vinifera and related species are susceptible to more than 60 different viruses (4), all of them containing single stranded RNA (ssRNA) genomes. However, two new viruses with DNA genomes have been recently reported from *Vitis vinifera* and hybrid grapevines in the Midwest US (9) and State of New York (3).

During the study on viruses infecting plants in Great Smoky Mountains National Park (GSMNP) we identified and molecularly characterized a spectrum of viruses with dsRNA genomes (authors, unpublished). Here we report results on molecular characterization of two new dsRNA viruses infecting native *Vitis* spp. and belonging to the families *Partitiviridae* and *Reoviridae*.

MATERIALS AND METHODS

Virus sources. Plants used in this work belonged to several species of native *Vitis* spp collected from natural ecosystems in Great Smoky Mountains National Park and in Mississippi.

Molecular analyses. Double stranded RNAs (dsRNAs) extracted from phloem tissues were selectively treated with DNAse and RNAse and used as a template for random-primer generated cDNAs according to previously published protocol (2,7). PCR-enriched complementary DNAs were cloned into proper vectors and sequenced. Sequences were mapped/assembled with Lasergene-DNAStar package. Analyses were performed with a number of "stand-alone" or on-line resources (MEGA, BLAST; CD, Pfam, etc).

RESULTS AND DISCUSSION

Cryptovirus. A doublet of dsRNA molecules was frequently found in extracts from samples collected in GSMNP and MS. Complete sequencing showed that they represent a genome of a novel cryptic virus, provisionally named Grapevine cryptic virus 1 (GCV-1). Larger molecule (1,588 bp) coded for viral RNA-dependent RNA polymerase, closely related to orthologs of Pepper cryptic viruses 1 and 2 (PCVs -1 and -2), *Raphanus sativus cryptic virus 3* (RsCV-3) and Persimmon cryptic virus (PsCV). Putative coat protein, encoded by smaller genomic molecule, shares ca 35-40% aa identities with counterparts in PCV-1, PCV-2 and PsCV. Pairwise comparisons and phylogenetic analyses indicate that GCV-1 represents a novel species in the genus *Alphacryptovirus* (fam. *Partitiviridae*).

Reovirus. A novel reovirus was originally identified in a symptomless sample of summer grape (*Vitis aestivalis*) during a virus survey in GSMNP (7). The genome of this virus, referred to as Summer grape latent virus (SGLV) comprises 10 molecules of dsRNAs, ranging from 3.5 kbp (Segment 1) to 1.1 kbp (Segment 10). Complete genome of SGLV have been determined and analyzed. All segments are monocistronic, except one coding for putative viral RdRp and the smallest segment (S10), which are both bicistronic. All SGLV segments contain conserved terminal nucleotide sequences which are identical to those reported for Raspberry latent virus (RpLV), recently sequenced aphid-transmitted reovirus reported from raspberries in the Pacific Northwest of the US. As suggested by phylogentic analyses, these two viruses belong to the same evolutionary lineage within the subfamily *Spinareovirinae*. Taking into account several peculiar features shared by SGLV and RpLV (i.e. dicot host, nucleotide termini, phylogeny, etc), that distinguish them from the rest of the extant taxa in the subfamily *Spinareovirinae* (fam. *Reoviridae*)(1) strongly support the proposal for the establishment of a novel genus in this subfamily (5,6). SGLV was found in few additional samples of native grapes in southeastern US. However, it has not been detected in any of 25 samples of *Vitis vinifera* tested in our lab and its potential importance, if any, to cultivated grapevines is yet to be understood.

ACKNOWLEDGEMENTS

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Complete Genome Sequence of a Novel *Vitivirus* Isolated from Grapevine

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INTRODUCTION

To date four different viruses, all members of the genus *Vitivirus* (family *Betaflexiviridae*) are reported to be associated with diseases in grapevine. These viruses have been referred to as *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), Grapevine virus D (GVD) and Grapevine virus E (GVE) (Adams et al., 2011). This group of viruses are reported to be associated with the rugose wood complex, which includes several important diseases in grapevines and produce woody cylinder modifications. We have encountered a novel *Vitivirus* during the characterization of black grape accession AUD46129. Bioassay of that accession resulted in death within 1-2 years of Cabernet Sauvignon plants propagated on Freedom, 420A, 3309C and 101-14 rootstocks.

MATERIALS AND METHODS

In order to understand the pathological reaction, we extracted double stranded RNA (dsRNA) from the accession AUD46129 source and used the dsRNA as a template for DNA library construction (Al Rwahnih *et al*., 2009). The library was subjected to high throughput sequencing using the Illumina platform (Eureka Genomics, Hercules, CA). BLAST analysis of the assembled reads against the GenBank database (Altschu *et al*., 1997) revealed fifteen contigs that ranged in size between 114 to 988nt, and showed a distant relationship with grapevine vitiviruses. Using total RNA from the original vine as template, Polymerase Chain Reaction was used to re-confirm the presence of the novel sequences, and to generate sequence information to fill in the gaps between the fifteen contigs. The exact 5` and 3` end sequences of the putative new virus were obtained using the FirstChoice® RLM-RACE Kit (Life Technologies, Grand Island, NY, USA).

RESULTS AND DISCUSSION

A novel virus-like sequence was identified from grapevine by Illumina sequencing. The complete genome was 7539 nucleotides in length, plus a polyadenylate 3' tail. The genome structure revealed five open reading frames (ORFs) organized similarly to *Grapevine virus A* (Fig 1). ORF 1 encoded a polypeptide of 1727aa with a calculated molecular mass of 196.9 kDa, and contained conserved domains characteristic of methyltransferase, RNA helicase and an RNA-dependent RNA polymerase. ORF2 encoded a 17.9 kDa hydrophobic protein of unknown function. ORF3 encoded a 30.4 kDa movement protein homolog. ORF4 encoded a 21.7 kDa capsid protein-like gene. ORF5 encoded a 12.3 kDa protein similar to an RNA binding protein. ORFs -1 through -5, respectively, shared 31-49%, 8-26%, 28-47%, 40-70% and 19-51% homology with other previously known grapevine vitiviruses.

We have tentatively named the novel virus described here Grapevine virus F (GVF). The genbank accession number for the sequence of this virus is JX105428. We are currently pursuing field surveys and biological studies to show the possible involvement of this novel virus in graft incompatibility reactions.

ACKNOWLEDGEMENTS

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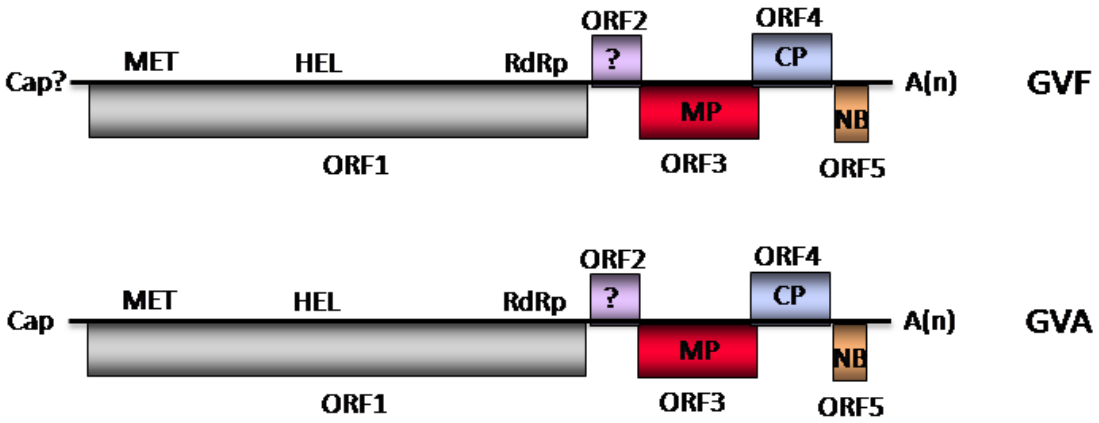

Fig. 1. Genome organizations of GVF in comparison to GVA approximately to scale. The different segments represent open reading frames; their vertical heights represent the different frame registers Mtrmethyltransferase, Hel helicase, Pol RNAdependent RNA polymerase, MP putative movement protein, CP coat protein, NB nucleic-acid-binding protein, ? protein of unknown function.

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The Use of Next-Generation Sequencing to Identify Novel Mycoviruses in Individual Grapevine Plants

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INTRODUCTION

Next-generation sequencing (NGS) has become a powerful tool for detecting viral populations in samples as it is more cost-effective and less time-consuming than traditional detection techniques (Beerenwinkel & Zagordi, 2011). One of its biggest advantages is however the ability to detect new variants of viruses as well as completely novel viruses.

Mycovirology is a relatively new field of study when compared to plant and animal virology (Pearson *et al*., 2009) and it is proposed that a large number of mycoviruses remain undiscovered (Ghabrial & Suzuki, 2008). This is because mycoviral infections are frequently symptomless and usually go undetected. The majority of mycoviral research is aimed at economically important or pathogenic fungi (Pearson *et al*., 2009). Although dsRNA profiles from fungi have shown that mixed mycoviral infections occur (Ghabrial & Suzuki, 2008), little research has been done on mycoviral populations in environmental samples. Next-generation sequencing is therefore a valuable approach to detect mycoviral populations in samples, especially in samples such as grapevine, which is known to be susceptible to a large number of viruses and fungal pathogens (Martelli, 2009).

Mycoviral sequences have recently been found to be strongly represented in grapevine viromes (Al Rwahnih *et al*., 2011; Coetzee *et al*., 2010). The mycoviral species identified, differed between the two studies and most were novel, uncharacterised mycoviruses. This project therefore aimed to further investigate the presence and diversity of mycoviral sequences in grapevine using NGS.

MATERIALS AND METHODS

Double-stranded RNA was extracted from phloem tissue of five *Vitis vinifera* (cv Shiraz) plants (Valverde et al., 1990). The dsRNA from individual samples were used to construct RNA sequencing libraries (ScriptSeq™ v2 RNA-Seq Library preparation kit, Epicentre) and sequenced as single reads using an Illumina HiScanSQ platform. Two sequencing runs were performed: a small run with ~2.5 million reads and a large run with ~6 million reads per sample. The pooled dsRNA from two plants were also sequenced on a 5500xL SOLiD System. The sequence data from the small Illumina sequencing run was trimmed and filtered for quality and *de novo* assemblies were performed using Velvet (Zerbino & Birney, 2008) and CLC Genomics Workbench. The resulting scaffolds were subjected to nucleotide and protein BLAST searches against the NCBI non-redundant database using Blast2GO (Conesa et al., 2005) and mycoviral diagnostic primer sets were designed to target the most prominent mycoviral hits. Read-mapping assemblies were performed on the scaffolds used for primer design to ensure that primers were designed in areas with adequately deep coverage. The primer sets were used to screen the original plant dsRNA that was used for sequencing, as well as total RNA and DNA extracted from the same plants using RT-PCR and PCR. Double-stranded RNA, total RNA and DNA extracted from fungal cultures that were isolated from the plant material were also screened. The read data from the large Illumina sequencing run as well as from the SOLiD run were assembled using Velvet and the identity of the resulting scaffolds were determined using Blast2GO.

RESULTS AND DISCUSSION

The Illumina HiScanSQ sequencer generated 2 353 938, 3 113 510, 2 728 458, 1 981 508 and 2 626 429 reads respectively for the five samples during the first run. The reads were assembled separately for each sample using the Velvet *de novo* assembler as well as with CLC Genomics Workbench and the resulting scaffolds were subjected to nucleotide and protein BLAST searches. Twenty-eight different mycoviruses were identified

belonging to the families *Chrysoviridae, Endornaviridae, Narnaviridae, Partitiviridae* and *Totiviridae*. Four of the identified mycoviruses could not be assigned to known viral families. Multiple mycoviral sequences were detected in each of the five samples, with as many as 11 species in a single sample. The majority of the BLAST hits to mycoviruses were however only on protein level and the scaffolds had low similarities to the mycoviral sequences on the GenBank database. This indicates that the mycoviruses present are probably novel, uncharacterised viruses. The large diversity of mycoviruses detected is consistent with previous research where fungal dsRNA profiles differed between trees within the same nectarine orchard and even between fruit from a single tree (Tsai *et al*., 2004).

Primer sets were designed to target 13 of the most prominent mycoviral hits. A second Illumina sequencing run on the same cDNA library was used to validate the scaffolds used for primer design. This was a larger run with 5 863 651, 4 255 719, 7 943 190, 3 681 062 and 7 879 193 reads respectively for the five samples. The mycoviral primer sets were used to screen the plant dsRNA, total RNA and DNA as well as fungal dsRNA, total RNA and DNA from isolates that were cultured from the plants. Only two mycoviruses were successfully amplified from both the plant material and fungal isolates. The targeted mycoviruses were related to Sclerotinia sclerotiorum partitivirus and to Chalara endornavirus 1.

The SOLiD sequence data was used as further verification for the mycoviral sequences. New dsRNA extractions were performed on two of the samples, the dsRNA was pooled and a new RNA sequencing library was constructed and sequenced. A Velvet *de novo* assembly was performed on the 142 609 606 reads and the resulting scaffolds were identified through Blast2GO analysis. As with the Illumina run, the identified mycoviruses belonged to the families *Chrysoviridae, Endornaviridae, Narnaviridae, Partitiviridae* and *Totiviridae*. Although the presence of mycoviruses could not be adequately confirmed by RT-PCR or PCR screening, similar mycoviral profiles were produced by the three different sequencing runs.

ACKNOWLEDGEMENTS

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF. The authors also thank Dr. Lizel Mostert for her assistance with the fungal work and Beatrix Coetzee and Anelda van der Walt for help with the bioinformatic analyses.

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Approaches of Next Generation Sequencing to Investigate Grapevine Diseases of Unknown Aetiology

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INTRODUCTION

Analysis of small RNAs (sRNAs) and /or double-stranded RNAs (dsRNA) libraries by next generation sequencing (NGS) is a novel technology that is proving increasingly useful for the discovery of previously unknown viruses associated with diseases of undetermined aetiology (e.g. Loconsole *et al.,* 2012). Among virus-like diseases of the grapevine, enations, an erratic disorder whose appearance may depend on seasonal conditions (Martelli and Boudon Padieu, 2006), is characterized by morphological modifications of the leaves that recall the consequences of hormonal unbalance. Even though graft transmissibility of enation disease supports its viral etiology, the putative agent has not yet been identified. NGS technology was used to investigate the "virome" of enation-infected grapevines and of an accession of cv. Moscato giallo showing an unusual interveinal spring chlorosis, that tends to turn whitish-yellow as the season advances.

MATERIALS AND METHODS

Grapevine sources: Leaves collected in late spring from eight plants of cv. Panse precoce with enations in a 10-year-old commercial vineyard from Trani (Apulia, Southern Italy) and symptomatic leaves of cv. Moscato giallo from a vineyard at Locorotondo (Apulia, Southern Italy) were pooled for NSG.

Libraries preparation and analysis: Purified dsRNAs and small sRNAs from leaf tissues were used to synthesize cDNA libraries according to the Illumina protocol (Giampetruzzi *et al.*, 2012). A 50 base-single read run was done on a HiScan SQ apparatus. Short sequences were processed with Fastx toolkit, *de novo* assembled with Velvet (Zerbino and Birney, 2008) and searched for homologies with viral sequences with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) tools. Guided assembly of viral genome sequences was with SOAP (http://soap.genomics.org.cn/) using reference sequences of GFLV, *Grapevine leafroll-associated virus 3* (GLRaV-3) and *Grapevine virus A* (GVA), retrieved from the RefSeq database (ftp://ftp.ncbi.nih.gov/refseq/release/viral/).

RESULTS AND DISCUSSION

Enations: Assembly of sequences obtained from the dsRNA library produced 2,040 contigs, whose BLASTN analysis revealed homologies with 10 different viruses: *Grapevine leafroll-associated virus 1, 2, 3, 5 and 9* (GLRaV-1,-2,-3,-5,-9), GVA, *Grapevine virus B* (GVB) *Grapevine rupestris stem-pitting associated virus* (GRSPaV), GFLV and *Grapevine fleck virus* (GFkV). From the small RNAs library 355 contigs were obtained that showed homologies to six viruses: GLRaV-1, GLRaV-2, GVA, GFLV, and GFkV; and two viroids: *Grapevine yellow speckle viroid-1* (GYSVd-1) and *Hop stunt viroid* (HSVd). Comparative analysis of both libraries disclosed the prevailing presence of GLRaVs sequences in the dsRNA library, presumably due to the propensity of these viruses to accumulate RNA replicative intermediates. Conversely, the small RNA library allowed the assembly of a larger number of GFLV contigs probably consequent to its active replication during spring.

Routine virus detection by RT-PCR assays (Table 1) confirmed the NGS-detected viruses and confirmed the occurrence of GLRaV-9 in Italy (Giampetruzzi *et al.*, 2011). A comparative analysis of a group of plants from the same vineyard subjected to sanitation by thermotherapy, which did not show symptoms of enations for over three years (Table 1), showed a less compromised sanitary status although being still infected by GFLV. A similar situation had been encountered in enation-affected vines of cv. Michele Palieri (unpublished data). This data confirms the alleged lack of relationship between GFLV and enation disease but does not clarify the nature of the disease whose aetiology still remains undetermined.

	Healthy	E1	E2	E3	E4	E5	E7	E8	T1	T2	$T2_1$	$T2_2$	$T2_3$
GRSPaV	-	+	+	+	+	+	+	+	+	+	+	+	+
GFLV	-	+	+	+	+	+	+	+	+	+	-	+	+
GLRaV-1	-	+	+	+	+	+	+	+	-	-	-	-	-
GLRaV-2	-	+	+	+	+	+	+	+	-	-	-	-	-
GLRaV-3	-	+	+	+	+	+	+	+	-	-	-	-	-
GLRaV-5	-	-	-	-	-	+	-	-	-	-	-	-	-
GLRaV-9	-	+	+	-	+	+	+	-	+	-	-	-	-
GFkV	-	+	+	+	+	+	+	+	+	+	+	+	+
GVA	-	+	+	+	+	+	+	+	+	-	-	-	-
GVB	-	-	-	-	+	-	-	-	-	-	-	-	-

Table 1. Sanitary status of grapevine plants with enations (E series) assessed by NGS and RT-PCR. A group of partially sanitized plants (T series), originating from the same vineyard and not showing enation symptoms was tested for comparison.

Spring chlorosis: assembly short sequences from the Moscato giallo vine produced 10,198 and 1,708 contigs respectively from the dsRNA and sRNA libraries. Six different viruses and one viroid were identified in the library from dsRNA: GLRaV-2*,* GLRaV-3*,* GVA*,* GRSPaV*,* GFkV and GFLV and GYSVd1 whereas the analysis of the sRNA library showed the presence of GFLV*, Grapevine Red globe virus* (GRGV)*, Grapevine rupestris vein feathering virus* (GRVFV)*,* and two viroids: GSYVd1 and HSVd. GLRaVs , GVA and GRSPaV were not found, the same as in the libraries from enation disease. Also with Moscato giallo, the presence of the detected viruses was confirmed by RT-PCR. The complete RNA sequences of the GFLV isolate from these plants was reassembled and showed a significant amino acid identity of both polypropteins P1 (98,1%) and P2 (97,6%).

The conclusion is that NGS technology gives a very wide if not complete picture of the virome of a given vine (Al Rwahnih *et al*., 2009; Coetzee *et al*., 2010), an that the use of dsRNAs or sRNAs as template for library preparation and the run of samples in a multiplex format is a better option for the identification of the viruses present in the plant under study.

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Detection of New Strains of GLRaV-3 in New Zealand Using ELISA and RT-PCR

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is the most damaging virus in New Zealand (NZ) vineyards, resulting in major reductions in grape quality, particularly for red grape varieties. NZ Winegrowers obtained funding from the Ministry of Primary Industries for project 09/144 ('Leafroll virus control in New Zealand vineyards'). This project uses visual surveys supplemented by ELISA detection to identify and rogue infected vines annually, combined with monitoring and control of mealybugs. We have modified ELISA protocols to increase their sensitivity in order to provide a robust and cost-effective method to screen vineyards using composite samples from 20 vines. In addition, an investigation of the diversity of GLRaV-3 strains in NZ (Chooi *et al*., 2009, 2011) has been undertaken using a combination of ELISA, RT-PCR and sequencing.

MATERIALS AND METHODS

Grapevine samples were collected from commercial vineyards in Marlborough, Wairarapa and several blocks in Hawke's Bay that are part of the MPI project. Vein samples from grape leaves, or phloem scrapings from grape canes or roots were macerated in extraction buffer (GEB, 200 mM Tris buffer (pH 8.2) containing 137 mM NaCl, 2 % PVP 40kD, 1% PEG 8 kD, 2% Tween® 20 and 0.02% NaN_3). Extracts were tested using a DAS-ELISA protocol with Bioreba reagents. Samples were also tested in a modified TAS-ELISA protocol in which plates were coated with goat polyclonal anti-GLRaV-3 antiserum, (G5/1, obtained from Darius Goszczynski, ARC, South Africa), and GLRaV-3 and diluted rabbit polyclonal anti-GLRaV-3 antibodies (163-15 provided by Mark Fuchs, Cornell University) mixed with anti-rabbit-AP (Sigma A3687) were used for detection and incubated in the same way as a conjugated antibody in DAS-ELISA. All ELISA protocols were run as kinetic assays, with plates read several times over 2-4 hours and reaction rates (mOD/min) calculated between two selected readings. The ratio of reaction rates from the two protocols (polyclonal/Bioreba) indicates variations in reactivity to the Bioreba-conjugated antibody. Samples were also tested by RT-PCR using the primers and protocols developed by Chooi *et al*., (2011 and these proceedings).

RESULTS AND DISCUSSION

In commercial vineyard leaf samples, some blocks gave low reaction rate ratios using Bioreba reagents, whilst, in other blocks the ratio varied widely. Samples from the low ratio samples were often difficult to detect by RT-PCR using MacKenzie primers (MacKenzie *et al*., 1997). Primers have been designed that amplify GLRaV-3 from these samples (Chooi et al. 2011) and sequencing showed the presence of novel NZ strains of GLRaV-3 (NZ1 and/or NZ2) in these samples. With phloem scrapings from roots infected with only a mixture of NZ1 and NZ2, GLRaV-3 was detected in all samples using the polyclonal antibodies, whereas the Bioreba reagents did not detect GLRaV-3 in many samples. High-throughput screening for GLRaV-3 requires a robust, cost-effective and sensitive test that is able to detect all strains of GLRaV-3. Although the Bioreba reagents fulfilled most of these requirements for cane and leaf samples, sensitivity was an issue with root samples.

We have increased the sensitivity of ELISA in order to be able to detect a single infected vine in a composite of tissue from 20 vines. Key factors in this development have been the source of tissue for the tests, the extraction protocol and extended substrate reaction time. Leaf petioles are commonly used for leaf tests but we have shown comparable reaction rates per cm of leaf veins, despite the petioles weighing ~4 times more per cm. Leaf veins are easily sampled using an 8-mm Harris Uni-core™ punch and are more readily crushed using a mechanical ball-bearing macerator. To enhance release of virus, samples are frozen to disrupt membranes before addition of GEB. Increasing the substrate reaction time to 3 or 4 hours aids the distinction between low reaction rate samples and uninfected controls.

As a trial for our ELISA protocols, leaf and cane samples were collected in 2011 from 232 symptomatic and 11 asymptomatic vines in a Hawke's Bay vineyard that had been planted in 2006 and rogued annually of symptomatic vines since 2008. Extracts from all leaves and canes were screened by ELISA using both Bioreba and polyclonal reagents. GLRaV-3 was detected in 229 samples including several from asymptomatic vines. The reaction rate ratios were calculated and 159 cane samples were tested by RT-PCR using strain-specific primers. GLRaV-3 was detected in 152 of the 159 samples, with 98% of these vines being infected with a single strain. When the ELISA results are sorted with increasing reaction rate ratios (Fig. 1), all samples that were infected with either NZ1 or NZ2 had a reaction ratio above 2 and all except one sample infected with NY1 had a reaction ratio below 2. These results demonstrate the complementarities of ELISA and RT-PCR in the detection and analysis of virus variants.

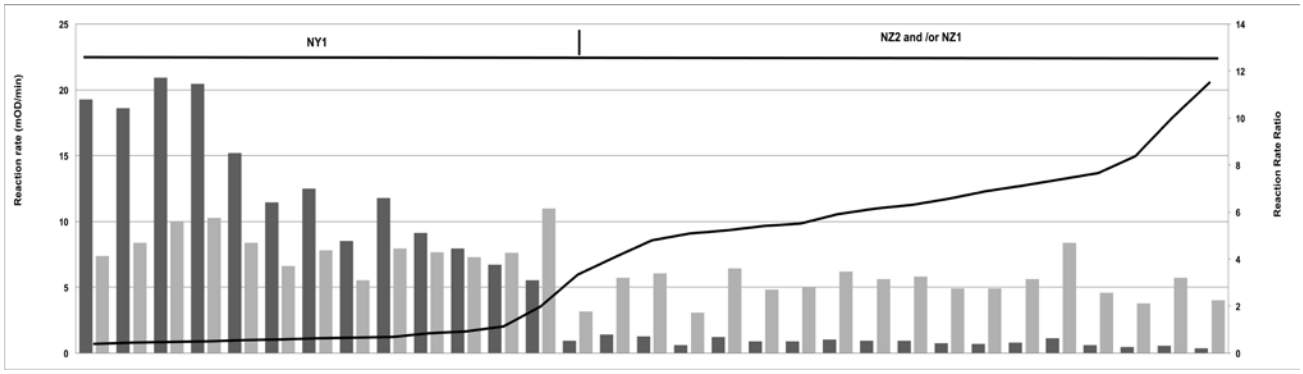

Figure 1. ELISA reaction rate ratios of GLRaV-3 samples tested using two antibody combinations. Samples were tested using Bioreba reagents (dark gray bars) or only polyclonal antibodies (pale gray bars) and the reaction rate ratio was calculated (black line). Representative samples are ordered by ascending reaction rate ratio.

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First Report on the Detection of Grapevine Virus and Viroid RNA in Bottled Wines

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INTRODUCTION

Plant viruses can withstand adverse environmental conditions such as rivers and sewage (Koenig, 1986). *Pepper mild mosaic virus*, a *Tobamovirus*, is resistant to physical and chemical agents and is still viable after passing through the digestive tract of humans (Colson et al., 2010). Infectious *Tobacco mosaic virus* can be recovered from cigarettes (Habili, 1985). Grapevine viruses can survive as RNA for years in dried leaves stored at ambient temperature (Habili and Randles, 2010).

A number of virus and viroid species are widespread in the grapevine. Of the five viroids isolated from the grapevine, the most prevalent is *Grapevine yellow speckle viroid 1* (GYSVd 1) (Little and Rezaian, 2003). Of the viruses, *Grapevine rupestris stem pitting-associated virus* (GRSPaV) is present in over 90% of the grapevine samples tested by us (unpublished). We selected these pathogens as candidates to see if they are present in bottled wines. There have been no reports on the detection of virus or viroid RNA sequences in wine, although the detection of DNA as a tool in the authentication of wine grape varieties has been described (Nakamura et al., 2007). One possible reason is that RNA is prone to ribonuclease attack. RNAse is abundant in various stages of winemaking and hence the survival of intact RNA during this process is assumed to be low. Here we report the detection of sequences of GRSPaV RNA and viroids from wine. A sequence matching that of *Apple scar skin viroid* (ASSVd) is the first report, albeit indirect, that this viroid infects grapevine.

MATERIALS AND METHODS

Various wine samples were obtained from the Australian Wine Research Institute. Grapevine wood samples from cultivar Cabernet Sauvignon as well as its associated wine made in 2006 were received from McLaren Vale (South Australia). Total nucleic acids (TNA) were extracted from vine and wine samples by the guanidine hydrochloride method of McKenzie et al (1997) using a SiO2 mix for the absorption, washing and elution of nucleic acids. Five ml wine batches were dialysed to dryness against solid PVP 40 and resuspended in 4 M guanidine hydrochloride for the TNA extraction. Virus and viroid amplicons obtained from single tube RT-PCR were cloned using the Invitrogen TOPO cloning system and sequenced by AGRF (Adelaide). For the detection of viroids by RT-PCR the following Apscaviroid generic primers designed by Sano et al (2000) were used: PBCVd100C Primer 5'-AGACCCTTCGTCGACGAC and PBCVd94H Primer 5-TGTCCCGCTAGTCGAGCGG. This pair targets a 220 bp segment of the central conserved region of Apscaviroids. For GRSPaV, the primer pair RSP48 (AGCTGGGATTATAAGGGAGGT) and RSP49 (CCAGCCGTTCCACCACTAAT) targeting a 329 bp segment on the virus coat protein gene was used in a single tube RT-PCR.

RESULTS AND DISCUSSION

PCR amplicons of expected size using primer pairs specific either for GRSPaV (Fig. 1) or Apscaviroids (Fig. 2) were detected in wine TNA. The amplicons from vines and wines were cloned and sequenced and their specificity was confirmed. The BLASTn analysis showed that the partial CP sequence of GRSPaV was 99% identical with the vein necrosis variant of GRSPaV reported from Italy. Sequences of GYSVd-1, GYSVd-2 and ASSVd were also detected in wine. The Apscaviroid generic primers amplified a product in a 2006 Cabernet Sauvignon wine from McLaren Vale which had 90% similarity with an isolate of ASSVd from apples in India. ASSVd was present in three cDNA clones from this wine. In contrast, in the Cabernet mother vine and in a Cabernet wine sample of the 2004 vintage from Yarra Valley (Victoria, Australia) no ASSVd was found. However, GYSVd-1 and GYSVd-2 were detected, and the GYSVd-1 sequence had highest identity with an isolate of the viroid reported from vineyards in Washington State, USA. GYSVd-1 has 37% sequence homology with ASSVd (Little and Rezaian, 2003) and chimeric recombinants of multiple viroid species to produce *Australian grapevine viroid* have been described in grapevine (Little & Rezaian, 2003). Finding ASSVd in wines may help elucidate the molecular evolution of Apscaviroids in grapevines. So far no ASSVd has been detected in the limited grapevine samples tested by us.

To detect other nucleic acids in wine a RAPD analysis was carried out. DNAs of up to 5 kb were detected. No significant matching was observed when the RAPD profiles of the cv. Cabernet Sauvignon DNA were compared with its own wine DNA (not shown). This may suggest the presence of various DNA molecules in wine. Deep sequencing of the wine nucleic acids is in progress.

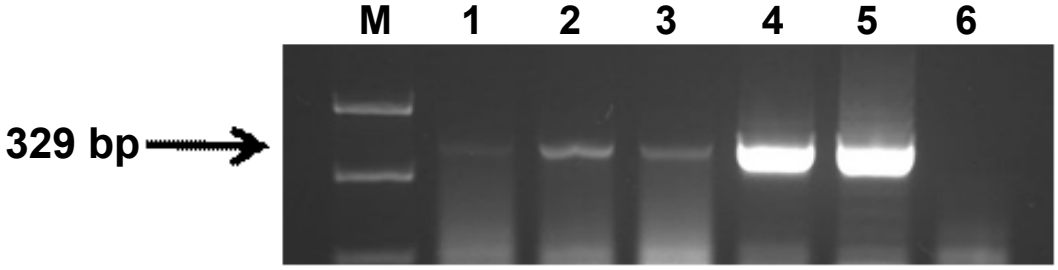

Fig.1. Single tube RT-PCR analysis of wine and vine samples using GRSPaV specific primers generating an amplicon size of 329 bp. 1, Chardonnay 2011 wine, 2, Cabernet Sauvignon 2006 wine, 3, Cabernet Sauvignon 2011 wine, 4, Cabernet Sauvignon grapevine, 5, Chardonnay grapevine, 6, H2O. M, DNA markers

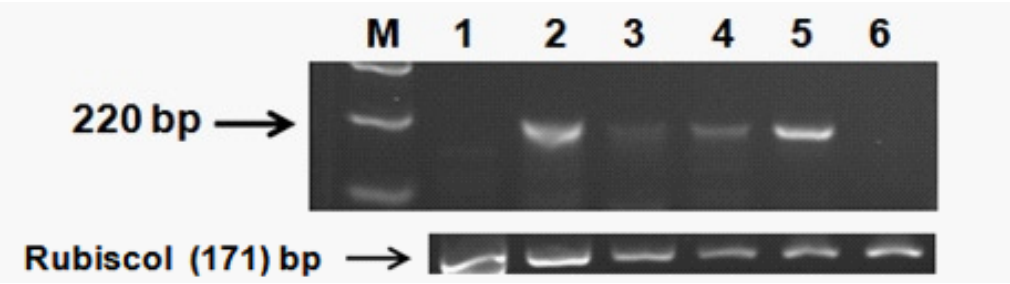

Fig.2. Single tube RT-PCR analysis of wine and vine samples using Apscaviroid group specific primers generating an amplicon size of 220 bp. 1, Shiraz 2011 wine, 2, Cabernet Sauvignon 2006 wine, 3, Cabernet Sauvignon 2004 wine, 4, Chardonnay 2011 wine, 5, Cabernet Sauvignon grapevine and 6, H2O. M, DNA markers. RubiscoL (Malk et al., this proceedings) was used as internal control.

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A Survey of Red and White Cultivars to Test an Improved Detection Technique for Grapevine Leafroll Associated Virus 3 (GLRaV-3) Variants Identified in South African Vineyards

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INTRODUCTION

The *Ampelovirus*, grapevine leafroll associated virus 3 (GLRaV-3), is the most widespread virus in South African vineyards. The molecular variability of the virus was studied in more detail during the past three years from different regions, especially in South Africa, Portugal and the USA (Fuchs *et al*., 2009, Jooste *et al*., 2010, Gouveia *et al*., 2010, Sharma *et al*., 2011, Bester *et al*., 2012b). The availability of more full-length sequences is significantly accelerating the genetic study of GLRaV-3 variants.

To date, six genetic variant groups of GLRaV-3 were identified world-wide (Maree *et al*., 2008., Joosteet *al*., 2010, Gouveia *et al*., 2010., Sharma *et al*., 2011, Bester *et al*., 2012b). Six full genome sequences, representing four of the genetic variant groups of GLRaV-3, were published from South African studies namely, group I (represented by isolate, 621), group II (represented by isolates GP18, 623), group III (represented by isolate PL-20), group VI (represented by isolates GH11, GH30) (Maree *et al*., 2008, Jooste *et al*., 2010., Bester *et al*., 2012b).

In previous studies GLRaV-3 variants were identified based on single-strand conformation polymorphism (SSCP) of a genomic region in ORF5 (Jooste *et al*., 2010, 2011). This technique was able to identify variants from groups I, II and III, but was unable to detect variants from group VI.

In this study an improved detection method, a one-step real-time reverse transcriptase polymerase chain reaction (qRT-PCR) followed by high-resolution melting (HRM) curve analysis, was optimized and used in a survey of field collected plants in the Western Cape Province of South Africa (SA). For a detailed description of this technique, refer to Bester *et al*. (2012a). The prevalence of the newly described group VI variants in South African vineyards was also determined, and possibility of additional GLRaV-3 variants evaluated.

MATERIALS AND METHODS

Petioles from 130 plants were collected during a survey of vineyards in the Western Cape in the winter of 2012. The vineyards selected for the study were all previously used as mother blocks, but lost their status in the 2009/2010 growing season due to GLRaV-3 infection. These vineyards were chosen in order to collect newly infected plants and to determine if new GLRaV-3 variants occur. Four infected plants, showing typical leafroll symptoms, were collected randomly per block. In total 30 blocks from twelve different farms were sampled, 16 blocks of white cultivars and 14 blocks of red cultivars, that represented nine white and eight red cultivars.

Total RNA was extracted from 0.2g petiole tissue using an adapted CTAB method (2% CTAB, 2.5% PVP-40, 100mM Tris-HCL pH8, 2M NaCl, 25mM EDTA pH8 and 3% β-mercaptoethanol) (White et al., 2008).

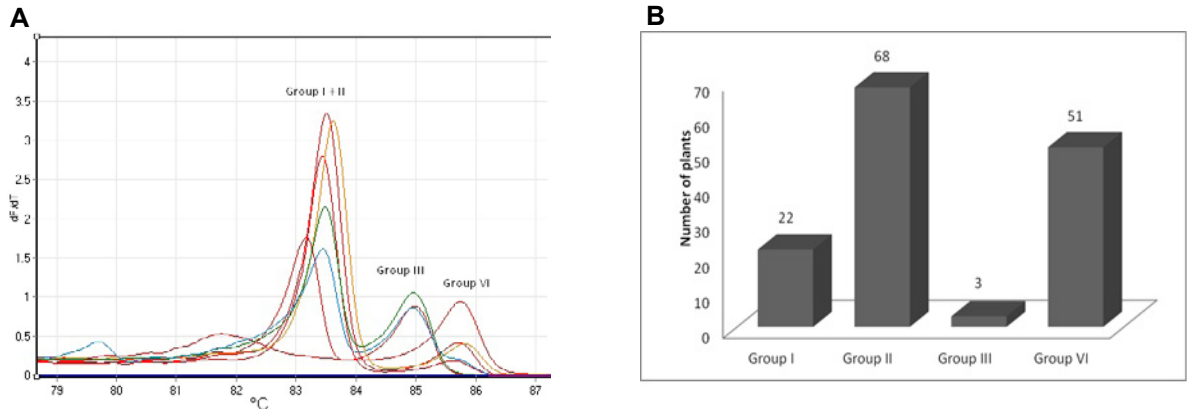
Multiple alignments of sequences of isolates of four GLRaV-3 variant groups (I, II, III, VI) were used to identify regions of high homology for universal detection of these isolates. The primer pair situated in ORF4, LR3. HRM4F and LR3.HRM4R which could most effectively differentiate between the variant groups based on the HRM curves, was used in the one-step real-time RT-PCR. The one step real-time RT-PCR HRM assay was performed on a Qiagen Rotor-Gene Q. See detail of the reaction mixture and cycling conditions in poster abstract by Bester et al. (2012a) To differentiate between group I and II variants an additional real-time RT-PCR using primer pair LR3.HRM6F and LR3.HRM6R positioned in the coat protein (CP) region, was done. Plants with known variant status were used as positive controls.

RESULTS AND DISCUSSION

Real-time RT-PCR followed by HRM curve analysis proved to be a very accurate and sensitive technique to determine GLRaV-3 variant status in plants. Most of the sampled plants tested positive for GLRaV-3. Of the 130

plants tested, only 23 tested negative. Negative plants were expected since the white cultivars do not clearly display leafroll symptoms and plants were collected randomly. Results of real-time RT-PCR HRM analysis of GLRaV-3 variant groups are shown in Figure 1.

Figure 1. An example of the real-time RT-PCR HRM analysis showing three melting curves (A), representing group I+II, group III and group VI variants. The number of plants infected with a specific GLRaV-3 variant is indicated in the graph (B).



From Figure 1B it is clear that the group II variants occured in the highest number followed by the group VI variants. Single infections with group II variants were also the most dominant and mixed variant infections were detected in 26 plants, where group II and VI variants occurred in combination.

In this study we applied a newly developed detection technique for GLRaV-3 variants successfully. We confirmed the existence of four genetic groups of GLRaV-3 in South African vineyards, compared to the six genetic variant groups described world-wide. With the use of this sensitive technique we could also confirm that group II variants occur predominantly in Western Cape vineyards, as reported previously (Jooste et al., 2011). Even though white cultivars were included in the study no association of a specific genetic variant to cultivar was observed. No additional genetic variants of GLRaV-3 were detected.

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Diagnostic Performance of Foundation Plant Service Assays

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SUMMARY

The diagnostic performance of three assays currently used at Foundation Plant Services (FPS) was evaluated using three years of test data from the FPS Importation program. The three assays evaluated were ELISA, RT-PCR, and RT-qPCR. The viruses included in the evaluation were *Grapevine leafroll-associated virus* 2 (GLRaV-2), *Grapevine leafroll-associated virus* 3 (GLRaV-3), *Grapevine fleck virus* (GFkV), and Grapevine virus A (GVA). The two parameters estimated were diagnostic sensitivity and diagnostic specificity. The results indicated that in general, RT-qPCR had the highest sensitivity, followed by RT-PCR. ELISA had the lowest sensitivity, especially for GLRaV-2. In contrast, RT-qPCR had the lowest specificity of all the assays.

INTRODUCTION

Lab assays for grapevine viruses provide useful information for diagnosing vineyard problems and preventing disease spread. Commonly used assays include serological methods such as ELISA, and nucleic acid-based methods such as conventional and RT-qPCR. When determining which of the available assays to use, a diagnostic lab typically goes through a validation process that includes optimizing the analytical sensitivity and specificity of an assay and then determining its diagnostic performance using a reference population that is more diverse than the experimentally-derived controls used for analytical validation.

We introduced RT-qPCR to our panel of assays in 2005 and determined that its analytical sensitivity and specificity were sufficiently high (Osman *et al*., 2007; Osman *et al*., 2008) to expand our validation of the assay as a diagnostic tool. Therefore, from 2005 through 2010, we assayed grapevine samples received in the FPS Importation Program with RT-qPCR in addition to ELISA, RT-PCR, and the field indexes. We analyzed the data from 2007-2010 and determined each assay's diagnostic sensitivity and specificity for GLRaV-2 and -3, GFkV, and GVA.

MATERIALS AND METHODS

Test results for 449 samples received in the FPS Importation Program from 2007-2010 were included in the analyses. We did not include the data from 2005 and 2006 because of the low number of infected samples and because we did not consider the RT-qPCR assay to be analytically optimized during those first two years. Values for the field indexes, ELISA and RT-PCR were entered as positive or negative based on previous review of the data. Threshold values (Cq) from RT-qPCR assays were entered without making positive or negative decisions unless the test result was required to determine whether the sample was infected or uninfected (see below).

A sample was considered infected if the field index was positive or if at least two lab tests were positive; otherwise it was considered uninfected. The field indexes included Cabernet Franc for Leafroll Disease, St. George for Grapevine Fleck, and Kober 5BB for Grapevine Kober Stem Grooving. Since the Cabernet Franc field index is not specific for individual leafroll-associated viruses, a positive Cabernet Franc index was assigned to a sample only if that sample also had at least one positive lab test. If RT-qPCR test results were required to classify samples as infected or uninfected, Cq values below 25.0 were considered positive.

After classifying samples as infected or uninfected, test results were marked as true positives (TP), false negatives (FN), true negatives (TN), or false positives (FP). Results from each year were compiled so that the sample size for the infected group was at least 30. If the number of infected samples for a given virus was less than 30, those assays were not analyzed further. Diagnostic sensitivity (D-Se) and diagnostic specificity (D-Sp) were calculated according to the following formulas:

$$\text{D-Sn} = \text{TP}/(\text{TP}+\text{FN})$$

$$\text{D-Sp} = \text{TN}/(\text{TN}+\text{FP})$$

RESULTS AND DISCUSSION

Estimates of D-Sn and D-Sp are listed in Table 1. The grapevine viruses included in our analyses were limited to GLRaV-2 and -3, GFkV, and GVA due to the small number of infected samples for other viruses.

Virus	GLRaV-2			GLRaV-3			GFkV			GVA	
Assay	RT-qPCR	RT-PCR	ELISA	RT-qPCR	RT-PCR	ELISA	RT-qPCR	RT-PCR	ELISA	RT-qPCR	RT-PCR
D-Sn	0.98[a]	0.89[b]	0.21[c]	0.91[a]	0.91[a]	0.72[b]	0.93[a]	0.81[b]	0.85[b]	0.91[a]	0.68[b]
D-Sp	0.97[a]	1.00[b]	1.00[b]	0.96[a]	0.99[b]	1.00[b]	0.88[a]	0.98[b]	1.00[c]	0.96[a]	0.99[b]

Table 1. Mean values for diagnostic sensitivity (D-Sn) and diagnostic specificity (D-Sp) for RT-qPCR and conventional RT-PCR and ELISA. Values with different letters are significantly different at the 95% confidence level.

In general, RT-qPCR had a higher probability of detecting these four viruses if they were present in a vine than the other two lab assays. The only case in which RT-PCR sensitivity was similar to RT-qPCR was in GLRaV-3 infected vines. ELISA sensitivity was highly variable, ranging from 0.21 for GLRaV-2 to 0.85 for GFkV. These lower sensitivity values for ELISA are probably explained by both the lower analytical sensitivity of this assay and the lower quality of our GLRaV-2 antiserum. Despite this lower sensitivity however, three of the 33 samples that were positive by the Cabernet Franc index had GLRaV-3 positive test results for only ELISA. These three samples were received in the same year and are from CA. It is possible they are genetically diverse GLRaV-3 isolates that are not detected by the more specific nucleic acid-based assays.

RT-qPCR diagnostic specificity, in contrast, was always lower than the specificity of the other assays. This was due to a number of samples that were negative by their respective field index and other lab assays but positive by RT-qPCR. The threshold (Cq) values for these samples were between 27.0 and 39.9, indicating that very little viral RNA was present. Since the field index for the respective virus was negative, we classified these test results as false positives. This classification is supported by our recent experience that low levels of cross-contamination can occur when samples are collected, processed and ground in the presence of strong positives.

Given that no assay is 100% correct, estimates of the rate at which false positives and false negatives occur provide a measure of how much confidence to place in test results. This information enables labs to make informed decisions on which assay or assays to use, given the perceived cost of false test results and the cost of individual assays.

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Comparative Study of TaqMan® and RT-PCR combined with ELISA for the Detection of Grapevine Viruses

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SUMMARY

Eurofins STA has developed test panels (HealthCheck™) that include a combination of reverse transcription (RT) PCR and ELISA for the specific detection of important *Grapevine viruses*. Known infected samples (positive and negative controls) and field samples of unknown virus status were subjected to HealthCheck™ Panel and TaqMan® RT-PCR specific for the detection of the following viruses: *Grapevine leafroll associated virus* -1,,-2, GLRaV-2 *Red Globe*, -3, -4, -5, -7, -9, *Grapevine virus A*, *Grapevine virus B*, *Grapevine virus D, Grapevine fleck virus, and Rupestris stem pitting associated virus*. Our work shows that both the TaqMan® and in house RT- PCR had the same sensitivity as measured by the limit of detection using serial dilutions of known infected grapevine template RNA. However, TaqMan® RT-PCR failed to detect virus infection in a number of the field samples and positive controls tested. The lack of detection can be explained by the diverse genetic variant populations present in virus infected grapevines. We conclude that more than one complementary detection method should be used for accurate and sensitive pathogen detection.

INTRODUCTION

The objective of the study was to compare the sensitivity and specificity of Eurofins STA HealthCheck™ Panel A + GFLV against TaqMan® to determine if recent scientific reports on the use of TaqMan® could be applied in a commercial lab setting for the detection of important grapevine viruses. HealthCheck™ Panel A + GFLV includes the following assays for the detection of *Grapevine leafroll associated virus* (GLRaV -1, -2, -3, -4, -5, -6, -7, -9): Grapevine virus A (GVA), *Grapevine virus B*, *Grapevine virus D*, *Grapevine* Syrah *virus (GSyV-1), Grapevine fleck virus, Rupestris stem pitting associated virus (including the* Syrah strain) and Grapevine fanleaf virus (GFLV). Our panels are routinely updated (after careful validation) when new ELISA reagents or sequences for new viruses or strains become available. TaqMan® RT-PCR is available for the detection of the following viruses: *Grapevine leafroll associated virus* -1, 2, GLRaV-2 Red Globe, -3, -4, -5, -7, -9, *Grapevine virus A*, *Grapevine virus B*, *Grapevine virus D, Grapevine fleck virus, and Rupestris stem pitting associated virus* (Osman *et al.*, 2008 and Klaassen *et al.*, 2010).

The key to any diagnostic technique is that it must be tested and validated with many field samples. Therefore the work here compares the results using both methodologies testing samples collected in vineyards and nursery rows as well as our in-house sets of positive and negative controls.

MATERIALS AND METHODS

Initially our experiments focused on comparing different sample grinding methods using the Homex-6 (Bioreba AG) and Geno/Grinder (SPEX CertiPrep) and extraction methods using the RNAeasy Plant Kit (Qiagen) and MagMax (Life Technologies). A limited number of samples (nine positive and one negative control) were processed in all possible combinations to determine if grinding or extraction methods had an effect on the specificity of detection. Subsequently, a series of dilutions of positive and negative controls ground with a Homex-6 and extracted with RNAeasy Plant Kit combined treatment were run in our lab to determine the sensitivity of each technique. Finally, field samples provided by Eurofins STA clients consisting of dormant plant material collected in the 2011-2012 fall/winter season were tested using TaqMan® and our in -house testing methods. When samples are tested with our standard protocol, each sample is processed as two sub-samples, one of the sub-samples is subjected to ELISA for the detection of the viruses described above and the other sub-sample is

subjected to RT-PCR. TaqMan® was run following methods previously described by Osman *et al.,* (2008) and Klaassen *et al.*, (2010) using the Viia7 (Life Technologies) and CFX (BioRad) real time detection systems.

RESULTS AND DISCUSSION

There was no significant difference in virus detection between the different grinding and extracting methods. However, HealthCheck Panel A detected GFkV and GLRaV-4-9 in a couple of the samples that remained undetected by TaqMan®.. To further address the sensitivity question we ran side by side TaqMan® and RT-PCR with serial dilutions of template of known infected samples. Our work showed that both TaqMan® and RT-PCR had the same sensitivity as measured by the limit of detection using serial dilutions of RNA of known infected grapevine samples (representative data will be presented). Once the detection sensitivity was confirmed as equivalent, we proceeded to the main goal of this work: the comparison of TaqMan and RT-PCR capabilities using field samples. Within the group of 251 client samples tested, TaqMan® failed to detect virus infection in many of the samples compared to the combined RT-PCR and ELISA panel. Remarkably, the TaqMan® probes missed the detection of samples infected GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV -5, GFKV, and GVB (Table1). No difference in detection between the methods was found when testing for GLRaV-2RG, GLRaV-7, GLRaV-9, GVA, and GVD.

Virus Tested	TaqMan® Detected	HealthCheck™ Detected	Percentage TaqMan® Missed
GLRaV-1	12	14	14%
GLRaV-2	23	25	8%
GLRaV-3	20	30	33%
GLRaV-4	3	6	50%
GLRaV-5	4	6	33%
GVB	15	19	21%
GFkV	16	24	33%

Table 1. Detection of virus infected samples by HealthCheck™ and TaqMan®

The decrease in detection capability of viruses by TaqMan® relative to our HealthCheck panel can be explained by the variability of virus populations present in vineyards. HealthCheck Panel uses two complementary detection techniques: ELISA with broad spectrum detection and RT-PCR with specific and sensitive detection capabilities. We recommend that more than one complementary detection method, namely ELISA and one of the PCR methods described here should be used for accurate and sensitive pathogen detection.

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Development and Validation of a Multiplex Quantitative PCR Assay for the Rapid Detection of Grapevine Vitivirus A, B and D

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SUMMARY

A single quantitative multiplex real-time PCR assay was developed to detect Grapevine vitiviruses A, B and D (GVA, GVB and GVD). This multiplex qPCR assay was validated against 48 different grapevine varieties of GVA, GVB and GVD obtained from a wide range of cultivars and locations. The specificity and robustness of the multiplex qPCR assay has been examined by parallel testing of the same 48 samples with GVA, GVB and GVD singleplex qPCR assays. It was shown that the multiplex qPCR assay was as robust as the other singleplex qPCR assays it replaces, with comparable specificity. None of the isolates were missed by the new multiplex qPCR assay. The multiplex qPCR assay developed in this work streamlines the testing of grapevine vitiviruses, replacing three separate qPCR assays for vitiviruses with a single multiplex qPCR assay, thus reducing time and labor, while retaining the same sensitivity and specificity. This is the first report of a multiplex qPCR assay designed to detect grapevine viruses.

INTRODUCTION

Several strategies have been successfully employed that allow the simultaneous detection and (or) identification of several plant pathogens in a single multiplex qPCR. The two main approaches for quantitative pathogen detection using real time PCR are using non-specific probes such as SYBR green and using specific probes such as TaqMan® probes or molecular beacons. However, recently, when detecting pathogens, there is a tendency to prefer specific probes, such as dual-labeled probes or MGB-TaqMan® probes due to their elevated specificity, thus are considered the preferred candidates for multiplex qPCR assays.

Singleplex real time PCR assays for the detection of GVA (Pacifico *et al*., 2011; Osman *et al*., 2008), GVB and GVD (Osman *et al*., 2008) have been reported. The aim of this study was to develop a multiplex qPCR assay for the simultaneous detection of GVA, GVB and GVD. This new multiplex qPCR was compared to singleplex qPCR assays detection of each of the individual viruses tested. In addition, an internal control (IC) based on the 18 S rRNA specific for Grapevine was included to verify the integrity of the RNA.

MATERIALS AND METHODS

Sample preparation*:* Grapevine samples were collected from cambial scraping from six different dormant wood branches and homogenized using the 2010 Geno/Grinder (SPEX SamplePrep, Metuchen, NJ). RNA extraction was performed using the MagMax™ Express-96 (Applied Biosystems) as described by Osman *et al*., 2012. Total RNA was eluted in a final volume of 100 µl, converted to cDNA for further testing of qPCR experiments.

Singleplex and Multiplex qPCR*:* The primers and probes for GVA, GVB and GVD used for singleplex qPCR were described in Osman *et al*., 2008, while those for the multiplex assay were newly designed incorporating 3 distinct 5' terminal fluorophore on the probes of each vitivirus; for GVA 6-carboxyfluorescein (FAM), for GVB TET and for GVD VIC. In all of the three assays the 3' quencher dye was a non fluorescent dye.

Testing the Viral load in singleplex and Multiplex qPCR*:* Standard curves for qPCR assays for GVA, GVB and GVD were performed. Amplicons for GVA, GVB and GVD were obtained for each primer set individually. Synthetic genes were ordered through Eurofins MWG Operon (Huntsville, AL) by submitting the amplicon sequences for every virus in different primer probes combination, cloned into pCR2.1 plasmids (Eurofins MWG Operon (Huntsville, AL) and the final product confirmed via sequencing. Plasmids were then linearized using HindIII enzyme, to increase the efficiency of dilutions. Serial tenfold dilution of plasmids carrying the GVA, GVB and GVD inserts were done to construct the DNA standard curves. Reactions were performed in triplicates to establish the linear response between the Cq values and the log of known copy numbers. The copy numbers for each samples were calculated using the equation $y = mx + b$.

***Assay Validation, Intra-assay and Inter-assay variation**:* The intra-assay variation was calculated, by determining the Intra assay coefficient of variation (CV), whereby total RNAs from three different GVA, GVB and GVD infected grapevine were extracted, 10 fold serial dilutions were prepared for each and tested by qPCR in triplicate and their Cq values were calculated. The intra-assay variation has been performed for both singleplex and multiplex qPCR. To confirm the accuracy and reproducibility of qPCR within the experimental plates, the inter assay variation was also determined within three separate plates as per Pfaffl, 2001.

***Evaluation of singleplex and Multiplex qPCR assays in detection**:* Forty eight randomly selected grapevine samples with broad geographical origin of known disease infection status of GVA, GVB and GVD were used to evaluate and compare singleplex and multiplex qPCR assays. The data were analyzed quantitatively by measuring the Cq values.

RESULTS AND DISCUSSION

In order to assess if the multiplex assay could detect all three viruses in mixed infections across a range of concentrations, dilution limits were established whereby series of cDNA from samples infected with each of the viruses were tested in singleplex and multiplex formats. In this way, assessments could be made to see if low titers of one virus could be detected with high titers of the other, and vice versa. Detection of the three viruses in a multiplex qPCR format was found to be possible across a range of dilutions of the other virus

In order to obtain accurate and reproducible results, all assays were determined to have an efficiency of >93.8% with the exception of GVB3 with an efficiency of 88.7%. Based on the amplification efficiencies detection limits were approximately 10 copies of cDNA per reaction.

For singleplex qPCR, for GVA infected sample the CV% was in the range of 0.23 % to 1.612%, for GVB infected sample was in the range of 0.63% to 1.494% and for GVD infected sample was in the range of 0.413% to 1.002%. For multiplex qPCR; GVA, the intra-assay variations were found to be 0.86% to 3.71% and the inter-assay variations of the same sample in 2 separate runs were 1.13% to 3%. For GVB, the intra-assay variations were found to be 0.38% to 1.36% and the inter-assay variations of the same sample in 2 separate runs were 0.65% to 1.27%. For GVD, the intra-assay variations were found to be 0.63% to 4.75% and the inter-assay variations of the same sample in 2 separate runs were 1.39% to 3.09%.

This paper describes the development of a multiplex qPCR assay for the detection of GVA, GVB and GVD using differently labeled MGB qPCR probes for each virus and comparing its performance with singleplex qPCR assays. The new vitivirus multiplex qPCR assay allows the replacement of the three separate testing protocols (a conventional PCR, using primers specific for each virus, a multiplex PCR using degenerate primers designed to detect all viruses and a singleplex qPCR of each virus).

Multiplex qPCR proved to be as robust as the three individual singleplex qPCR assays it replaced, with comparable specificity where none of the isolates were missed by the new multiplex qPCR assay. The test reliably detected 45 different grapevine varieties mixed infected with GVA, GVB and GVD obtained from a wide range of different cultivars and geographical locations, including some samples in which existing tests failed to detect virus, where conventional multiplex PCR using degenerate primers detected 77.08% of the isolates while multiplex qPCR detected 93.75% of the same isolates. This demonstrates that this multiplex qPCR assay has a broad specificity.

One of the main advantages of this vitivirus multiplex qPCR assay when used in routine diagnosis is that it offers the possibility of replacing three tests with a single multiplex assay, thus reducing time and labor, while maintaining a high degree of specificity.

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Detection and Discrimination of Grapevine Leafroll-Associated Viruses by Real-Time PCR and Amplicon Melting Curve Analysis

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INTRODUCTION

Several strategies have been successfully employed for accurate identification of plant viruses (James *et al*., 2006). Conventionally, serological methods such as enzyme-linked immunosorbent assay (ELISA) are used for the detection of viruses in grapevine (*Vitis* spp.). However, accurate detection of viruses by ELISA is hampered in many situations due to lack of virus-specific antibodies, low concentration of viruses in grapevines and their uneven distribution. To circumvent these limitations, molecular techniques such as reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) followed by agarose gel electrophoresis are employed for the detection of viruses. In recent years, quantitative real-time PCR (qPCR) and DNA-microarray technologies have become the preferred methods for rapid and specific detection of viruses in plants (Osman *et al*., 2012; Thomson *et al*., 2012). With an increasing demand for high sample throughput and multiplexed assays in virus diagnosis and research programs, we developed RT-qPCR coupled with SYBR Green (SG)-based amplicon melting curve analysis (MCA) for reliable and sensitive detection of grapevine leafroll-associated viruses (GLRaVs) in grapevines and insect vectors.

MATERIALS AND METHODS

Petiole samples from grapevines (*V. vinifera*) previously tested positive for GLRaV-1, -2, -3, -4, and -9 were collected from red- and white-fruited wine grape cultivars planted in commercial vineyards. Total RNA was isolated from petiole samples using Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA), and the quality and quantity of RNA was determined using a spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). One microgram (µg) of total RNA was used for cDNA synthesis using random hexamers and reverse transcription was carried out using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Species-specific primers were designed targeting either helicase (GLRaV-2 and -3) or RdRp (GLRaV-4 and -9) domains of the replication gene block to estimate virus load as number of virus genomic copies in a given sample. PCR was carried out using the following conditions: denaturation for 5 min at 95°C; 35 consecutive cycles of 10 s at 95°C, 10 s at 58°C and 30 s at 72°C. Amplicons were cloned and sequenced to confirm their specificity to the respective viruses. RT-qPCR was carried out in 12 µL reaction mixture containing 2 µl of cDNA, 0.5 µM each of virus specific forward and reverse primers and 6µl of 2X SG Master Mix using the LightCycler 480 (Roche Diagnostics, Mannheim, Germany). Appropriate controls and replicates were included in compliance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al*., 2009). At the end of RT-qPCR, amplicons were subjected to MCA to determine the T_m of virus-specific products. The standard curve, range of detection, correlation coefficient (R^2) and the amplification efficiency ($E=10^{(-1/slope)}-1$) were determined as previously described (Gutha *et al*. 2010).

RESULTS AND DISCUSSION

In initial experiments, primers targeting the helicase domain of the replication gene block of GLRaV-2 and -3 were designed and tested for their specificity in detecting individual viruses by RT-PCR. For GLRaV-4 and -9, conserved sequences in the RdRP domain were used to amplify sequences specific to GLRaV-4 and -9. The specificity of amplification obtained for each virus (241 base pair [bp] for GLRaV-2, 170 bp for GLRaV-3, 457 bp for GLRaV-4 and -9) was further ascertained by sequencing cloned PCR products and comparing them with sequences of GLRaVs available in GenBank. Subsequently, the same primer sets were tested for detection and quantification of each virus by RT-qPCR using SG. Melting curve analysis of amplicons showed a single melting

peak for each set of primers (Fig. 1) confirming the homogeneity and specificity of reaction products with distinct T_m for GLRaV-2 (80^{0}C ±0.20), -3 (84^{0}C±0.20), -4 (83^0±0.20C), and -9 (81^0±0.20C). Since the primers were designed to target a genomic region spanning the 'replication gene block' of GLRaVs, this method is highly reliable in quantifying virus genomic RNA copies in a given sample with concomitant elimination of the risk of overestimation of copy numbers by co-amplification of virus subgenomic RNAs (Pacifico et al., 2011). The standard curve derived from tenfold serial dilution series of known concentration of virus-specific recombinant plasmid DNA showed R^2 value of 0.987for GLRaV-2 (-2.96 slope, PCR efficiency 1.176), 0.99 for GLRaV-3 (slope -3.33, PCR efficiency 0.996) and 0.988 for GLRaV-4 (slope -3.37, PCR efficiency 0.97).

Our data also revealed that the RT-qPCR is approximately 10^3-fold more sensitive than conventional RT-PCR in the detection of these viruses. For instance, ~4x10^{-4} molecules/µL of GLRaV-3 could be detected using RT-qPCR suggesting that the sensitivity of this method is comparable to the existing TaqMan-based RT-qPCR diagnostic methods described previously for grapevine viruses (Osman *et al*., 2012). The increased sensitivity will help in the timely detection of very low virus titers in plants and vectors. In addition, the SG-based RT-qPCR developed in this study provides a rapid and cost-effective alternative to other detection chemistries with similar efficiency and accuracy (such as the TaqMan-based RT-qPCR), particularly when large numbers of samples are to be analyzed. Also, the SG-based RT-qPCR combined with MCA allows rapid optimization of assays and confirmation of amplicons by producing a characteristic T_m for each amplicon analogous to the detection of a specific sized fragment by agarose gel electrophoresis. Since this is a closed-tube method, it replaces time-consuming procedures such as agarose gel electrophoresis after PCR assays. Further studies are in progress to validate RT-qPCR in combination with MCA in virus diagnostics and epidemiological studies.

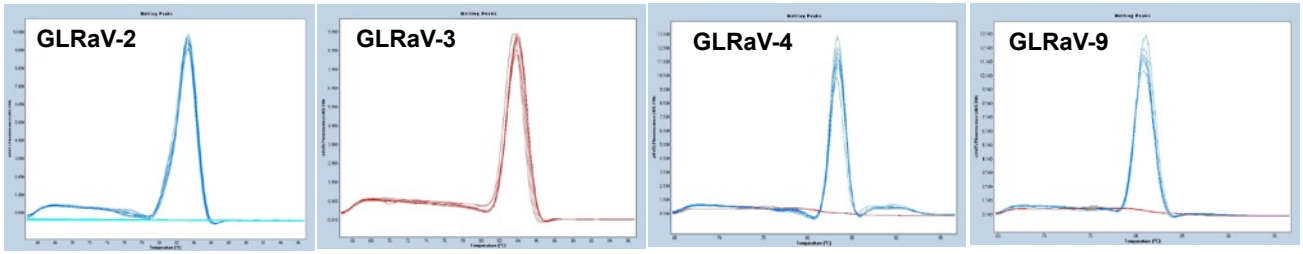

Figure 1. Real-time RT-qPCR with melting curve analysis for the separate detection of GLRaVs. Separate peaks of T_m for GLRaV-2 (80^{0}C±0.20), -3 (84^{0}C±0.20), -4 (83^0±0.20C), and -9 (81^0±0.20C) enable discrimination of GLRaVs. Multiple curves for each virus represent the replicates of different isolates.

ACKNOWLEDGEMENTS

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Multiplex Detection of Grapevine Viruses using a Randomly Primed RT-PCR/Macroarray Platform

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INTRODUCTION

Currently, the two most commonly used laboratory methods used in certification schemes are based on either PCR or ELISA technologies. An important consideration with future diagnostic methods will be their ability to be multiplexed thus enabling the simultaneous detection of a finite number of target molecules. In plants, the initial adoption of microarrays for virus detection began with a limited number of virus targets (Boonham *et al*., 2003; Wei *et al*., 2009), although recent reports demonstrate a much broader applicability with probes designed to detect up to 52 virus species (Nicolaisen, 2011). The real potential of macroarrays was first exploited in the detection of fungal, bacterial, and oomycete plant pathogens (Levesque *et al*., 1998; Lievens *et al*., 2003). More recently macroarray detection methods have been developed for potato (Agindotan and Perry, 2008; Maoka *et al*., 2010), solanaceous crops (Perry and Lu, 2010), and grapevine (Thompson *et al*., 2012) thus demonstrating the practicability of crop-specific assays. In this study we report the development of a macroarray assay for the detection of the majority of viruses found in grapevine by using, as substrates for labeling, random sequence-nonspecific amplified complementary DNAs (cDNAs) derived from plant total RNA extracts. This demonstrates a proof-of-principal, for the unbiased multiplex detection of all grapevine viruses using a robust and transferrable platform.

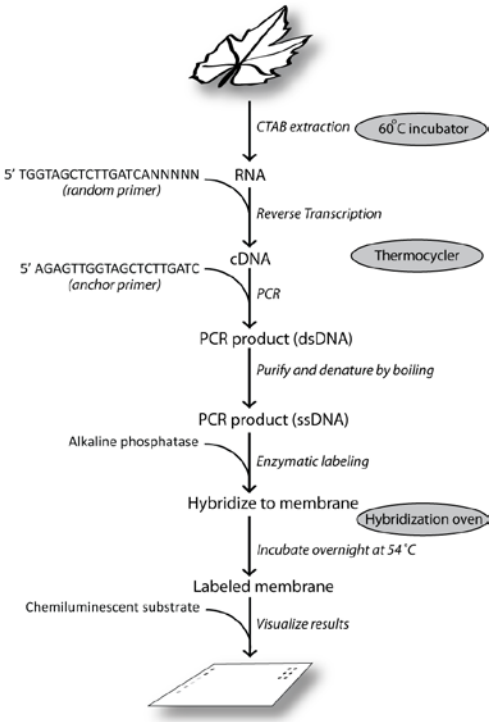

Figure 1. Flow chart showing steps in the processing of grapevine material for macroarray detection.

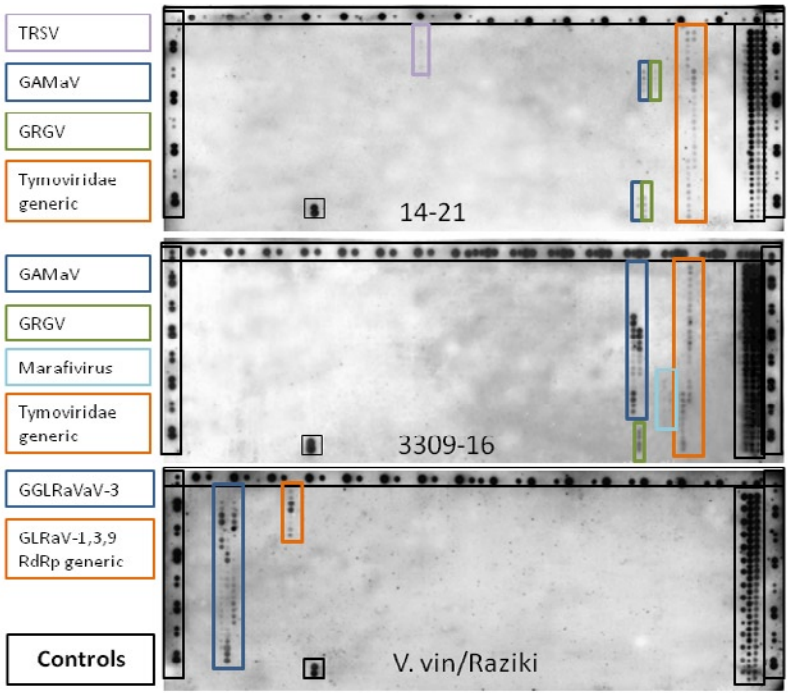

Figure 2. Examples of array results for multiple infections in three individual grapevine samples. Detected viruses are indicated as acronyms with color coded boxes outlining the hybridized probes.

MATERIALS AND METHODS

Total RNA was extracted from 100mg of leaf, petiole or bark scrapings using the method previously described (Gambino *et al*., 2008). Reverse transcription, cDNA PCR amplification and enzyme labeling was carried out as described previously (Agindotan and Perry, 2008) with minor modifications. Viral probes were designed both in the plus and minus viral sense and were derived from three sources; 1) in-house 70-mers, 2) 70-mer oligonucleotides designed for a microarray (Engel *et al*., 2010), and 3) 60-mer oligonucleotides designed for a microarray (Bagewadi *et al*., 2010). The printing procedure and hybridization steps were carried out as described previously (Agindotan and Perry, 2007) with minor modifications (Fig. 1).

RESULTS AND DISCUSSION

Here we report the development of a macroarray assay for the detection of most all recognized grapevine infecting viruses. The first array, GrapeArray1 with 314 virus-specific probes, was able to detect all described grapevine leafroll-associated viruses, nine in total representing members of the family *Closteroviridae* (Thompson *et al*., 2012). The second, GrapeArray2 (Fig. 2) contained 1308 probes specific for the detection of 35 viruses, including members of the families *Betaflexaviridae*, *Bromoviridae, Bunyaviridae, Closteroviridae, Secoviridae, Tombusviridae and Tymoviridae*, and the genus *Ideaovirus*. Plant-specific and other oligonucleotide internal controls (e.g. for rubisco, ubiquitin, ETFs, and NADH dehydrogenase genes) allow for monitoring each step in the amplification/hybridization protocol. The most recent GrapeArray4 includes probes for nine additional grape viruses for which sequence information is now available, and a total of 1600 virus specific probes will be incorporated. Infections by single and multiple viruses were observed, including members of the families that include betaflexivirids, closterovirids, secovirids and tymovirids. Results were largely consistent with those obtained by ELISA and PCR, and in some cases detected viruses not usually screened for.

ACKNOWLEDGEMENTS

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Study on the Time and Tissue for Reliable Detection of *Grapevine Leafroll-associated Virus* 3 in Cabernet Gernischt

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INTRODUCTION

Grapevine is a host to more than 60 viruses that result in constant adverse impact on the grape yield and the berry quality (Martelli and Boudon-Padieu, 2006). Currently more than ten *Grapevine leafroll-associated viruses* (GLRaVs) are associated with grapevine leafroll disease (GLRD). GLRaVs have been found in major grape-growing regions in China. For instance, GLRaVs were detected in 77% of samples of 39 grape varieties that are grown in the repository of grape germplasm at the State Fruit Tree Germplasm at Zhengzhou, China (He *et al*., 2001). A survey of GLRD showed that 12% to 29% of red grape varieties were affected with GLRD by visual inspection in three grape-growing areas in Ningxia Hui Autonomous Region, China (Wang *et al*., 2002).

Ningxia is a new grape-growing region where Cabernet Sauvignon, Cabernet Franc and Cabernet Gernischt (a.k.a Carmenère) are three main red grape varieties. Propagation and planting of virus-tested grape varieties is the most effective scheme of preventing the spread of GLRD and the most significant strategy of sustaining the healthy growth of the new grape and wine industry in Ningxia. The sensitive and timely detection of GLRaVs is one of the key components in the establishment of foundation vineyards of virus-tested grape varieties in Ningxia. In this study, we employed the reverse-transcription polymerase chain reaction (RT-PCR) to investigate the sampling time and the type of tissue for most reliable detection of GLRaV-3 in Cabernet Gernischt.

MATERIALS AND METHODS

Grapevines: Cabernet Gernischt vines were collected from Luhuatai vineyard, Ningxia and planted in the vineyard at the State Key Laboratory of Seedling Bioengineering, Yinchuan, Ningxia, China. The vines showed reddish tissues between major veins and rolling backward of leaves in the fall. The vines with no visible symptoms were used as negative control.

Extraction of RNA: Full-expanded leaves at the lower part of the vines, petioles, and phloem scrapings were collected for extracting RNA. A total of 400mg tissues were frozen and grounded to fine powder after addition of 0.05g PVPP in pre-cooled mortar under liquid nitrogen. The subsequent procedures of extracting RNA and DNase-treatment followed the protocol as described previously (Fung *et al*., 2008). Further purification was conducted by using the RNA kit (TianGen Biocompany, Beijing). At final step, RNA was eluted in 20 µL RNase-free water.

cDNA synthesis and PCR: cDNA was synthesized as described (Lunden *et al*., 2009). Primers for detecting GLRaV-3, GLRaV-3F1 5′-TACGTTAAGGACGGGACACAGC-3′ and GLRaV-3R1 5′-TGCGGCATTAATCTTCAT-TG-3′ were adopted from a previous study (Gambino and Gribaudo, 2006). A set of primers for 18S rRNA are 18SrRNAf1 5’-CGCATCATTCAAATTTCTGC and 18SrRNAR1 5’-CAGCCCTTGCGACCATACT. Thermal cycling conditions are: initial denature at 94°C, 3 min; 35 cycles of 94°C, 30 sec; 54°C, 30 sec; 72°C, 1 min; and final extension at 72°C for 5 min.

RESULTS AND DISCUSSION

Intensity of 18S rRNA RT-PCR indicates the comparable quantity and quality of RNA among samples

RT-PCR results showed that the 844bp fragment of 18S rRNA was amplified from all samples and their intensity was relatively similar across samples, which verified the comparable concentration and quality of all samples that were subjected to the subsequent analysis (Figure 1).

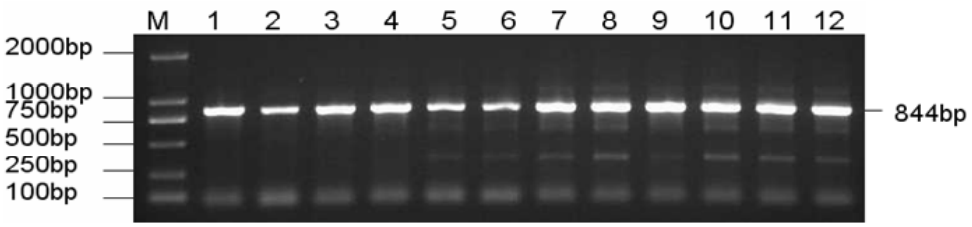

Figure 1. Agarose gel image showing the relative intensity of 18S rRNA DNA fragments as amplified by RT-PCR. Lane 1 to 4 RNA from leaves; Lane 5 to 8 petioles; Lane 9 to 12 phloem. Lane 1, 5, 9 leaf, petiole, phloem collected in June; Lane 2, 6, 10 in July; 3, 7, 11 in August; Lane 4, 8, 12 in September.

The time and tissue specificity of detecting GLRaV-3

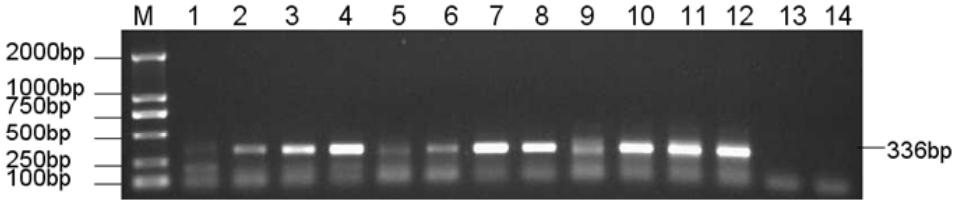

Figure 2. Agarose gel image showing the relative intensity of the GLRaV-3 specific 336bp fragment as amplified by RT-PCR. Lane 1 to 4 RNA from leaves; Lane 5 to 8 petioles; Lane 9 to 12 phloem. Lane 1, 5, 9 leaf, petiole, phloem collected in June; Lane 2, 6, 10 in July; 3, 7, 11 in August; Lane 4, 8, 12 in September. Lane 13 negative control without addition of cDNA; Lane 14 a grapevine without visible leafrolling symptom. The PCR was performed in 35 cycles.

Leaf, petiole and phloem tissue were chosen to test the type of tissue in which GLRaV-3 can be detected. The results showed that GLRaV-3 was detected in phloem as early as in June, but can be detected in all three tissues in July, and reached the highest level in September (Figure 2). It is clear that GLRaV-3 accumulates gradually over time in all three tissues. The intensity of GLRaV-3 DNA fragment was much brighter in phloem tissue than in leaf and petiole tissues under the same conditions, suggesting that GLRaV-3 accumulates most abundantly in the phloem tissue of Cabernet Gernischt. It is possible that the most abundant accumulation in the phloem provides advantages for GLRaV-3 to be transferred to leaves quickly and to be transmitted to other vines easily via the insect vector.

From these results, it is concluded that the most reliable time for detecting GLRaV-3 by the RT-PCR is in September and GLRaV-3 can be detected in all three tissues. Since it is more convenient to extract RNA from leaves than from petiole and phloem, leaf tissues shall be collected for detecting GLRaV-3 in September. If the purpose of a project is to detect GLRaV-3 as early as possible, phloem tissue shall be collected in June for extracting RNA and for detecting GRLaV-3, which gives sufficient time to replace the GRLaV-3 infected vines with new vines. This is particularly important for Ningxia region of China where temperature and moisture drops sharply in winter time.

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Development of a Reliable Diagnostic Test for 10 Viruses and Evaluation of its Potential Use in Different Organs of Grapevine

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INTRODUCTION

The grapevine industry of many countries is expanding and will continue to do so in the years to come. The necessity then arises to distribute large amounts of planting material virus-free. Sensitive, rapid and reliable laboratory diagnosis is so desirable for certification, exportation and quarantine purposes. If virus detection is efficient on woody canes, fewer things are known on its efficiency on other organs. Some studies were done on leaves throughout the year (Fiore *et al*., 2009; Stewart and Nassuth, 2001). Nevertheless, little is known about viruses' detection in roots on grafted vines. The purpose of this study was to test the method of detection developed to detect 10 viruses on woody canes, onto leaves and roots to determine if these organs would be usable for routine detection.

MATERIALS AND METHODS

Grapevine Material: A list of positive controls identified to be mono or multi-infected for a range of 10 viruses (analysis realized on lignified canes) were selected. Leaves were collected from 21 accessions from IFV virus collection (Le Grau du Roi, France) in fall. Leaves were sampled at the bottom and in the middle of two canes per vine and analyzed separately to test the potential uneven distribution of these viruses. Woody canes selected from the infected control vines were prepared for grafting on SO4 (controlled free of viruses) in April 2011. The grafted vines were planted in a greenhouse. Tests on roots were done on three individual roots per plant collected 6, 8 and 11 months after grafting. Leaves or wood of the scions and the rootstocks were used as controls. In October, one plant was selected and tested per batch whereas two plants were separately analyzed in December and in March.

Total RNA extraction: RNA extraction was performed on 200 mg of roots, leaf petiols and on cambial tissues from wood mature canes according to the "NucleoSpin RNA II" kit protocol (Masherey Nagel, France).

RT-PCR amplifications: One step RT-PCR was performed with Quantitect SYBR Green RT-PCR kit (Qiagen, France) in conventional RT-PCR and with Ready-To-Go™ RT-PCR Beads (GE-Healthcare, France) for the GLRaV-6 and GLRaV-7, according to the manufacturer protocol. LR1-H70F1/R1, P19qtF/P24qtR, LR3-POLF1/R1, LR4F2/R2, LR5-F4/R3, LR6-SN1F/SN10R, LR7-F4/R1, LR9-FV/RC, GVA-CPF1/CPR1 and GVB H28/C410 were used as specifics primers (Beuve *et al*., 2007, 2012; Rowhani, pers. comm.; Alkowni *et al*., 2004; Minafra and Hadidi., 1994). The primers 18 S were used as internal control (Gambino and Gribaudo., 2006).

RESULTS AND DISCUSSION

RT-PCR detection on fall leaves. As shown in table 1, results revealed a high rate of detection as 96% of the samples tested were detected positive in accordance with the sanitary status of the plants tested. Only one plant was found negative for GLRaV-6 in each of the 4 samples. RNA extraction and RT-PCR conditions usually applied on woody canes can be used on leaves during fall. It can be a suitable season for routine tests at least for the viruses involved in leafroll and rugose wood.

Table 1. RT-PCR detection of ten viruses on fall leaves.

C1B (bottom of the cane 1); C1M (middle of the cane 1); C2B (bottom of the cane 2); C2M (middle of the cane 2).

Positives samples / total number of samples tested.

Virus	C1B	C1B	C2B	C2M
GLRaV-1	3/3	3/3	3/3	3/3
GLRaV-2	3/3	3/3	3/3	3/3
GLRaV-3	3/3	3/3	3/3	3/3
GLRaV-4	3/3	3/3	3/3	3/3
GLRaV-5	4/4	4/4	4/4	4/4
GLRaV-6	1/2	1/2	1/2	1/2
GLRaV-7	1/1	1/1	1/1	1/1
GLRaV-9	1/1	1/1	1/1	1/1
GVA	3/3	3/3	3/3	3/3
GVB	3/3	3/3	3/3	3/3
Total	**24/25**	**24/25**	**24/25**	**24/25**

Table 2. RT-PCR detection of six viruses in roots of grafted vines during fall and winter.

nt: not tested samples

		October 2011			December 2011		March 2012		
		Roots	Rootstock wood	Scion	Roots	Rootstock wood	Roots	Rootstock wood	Scion
GVA	C1	3/3	1/1	1/1	6/6	2/2	6/6	2/2	2/2
	C2	0/3	1/1	1/1	5/6	2/2	4/6	2/2	2/2
	C3	3/3	1/1	1/1	0/6	2/2	6/6	2/2	2/2
	C4	3/3	1/1	1/1	1/6	2/2	2/3	1/1	1/1
	C5	nt	nt	nt	0/6	2/2	3/6	2/2	2/2
	C6	nt	nt	nt	5/6	2/2	5/6	2/2	2/2
	C7	nt	nt	nt	3/6	1/2	1/6	2/2	2/2
GLRaV-1	C8	2/3	1/1	1/1	0/6	0/2	6/6	2/2	2/2
GLRaV-3	C1	3/3	1/1	1/1	6/6	2/2	6/6	2/2	2/2
GLRaV-4	C3	0/3	1/1	1/1	0/6	2/2	6/6	2/2	2/2
	C5	nt	nt	nt	0/6	2/2	3/6	2/2	2/2
	C9	nt	nt	nt	0/6	0/2	0/6	0/2	2/2
GLRaV-5	C2	3/3	1/1	1/1	1/6	2/2	3/6	2/2	2/2
	C6	nt	nt	nt	3/6	2/2	6/6	2/2	2/2
GLRaV-7	C4	0/3	0/1	1/1	0/6	0/2	0/3	1/1	1/1
		63%	**89%**	**100%**	**33%**	**77%**	**68%**	**93%**	**100%**

RT-PCR detection on roots

The rootstocks were globally found positive, with some few exceptions, whereas the scions were always found positive (Table 2). Concerning the roots, the results were more variable as viruses were only detected in 30% to 63% of the samples tested. Only 3 viruses (GVA, GLRaV-3 and GLRaV-5) on the 6 tested were detected at the three dates. Some of these results can be related to the absence of detection in the rootstocks that may indicate that the viruses had not migrated to the rootstock yet. Nevertheless, in many cases, the viruses were not detected in roots whereas they were found in the rootstock wood. This does not appear to be related to the method used as the internal control was systematically found positive.

The differences between the plants infected with the same viruses are important. Thus the viruses seem to infect the roots in a heterogeneous way that make their systematic detection difficult, especially in December. The reduced number of the samples analyzed here does not allow extrapolating our results to a general behavior to any particular virus. Nevertheless, our results clearly indicate that detection on root was less efficient than on wood or leaf. Complementary analyses have to be realized to improve the sampling technique for viruses detection on grafted vines.

ACKNOWLEDGMENTS

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Phytoplasma Detection and Identification in Grapevine by Deep Amplicon Sequencing

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INTRODUCTION

Grapevine yellows is one of the major diseases affecting the vineyards in Europe as well as in the majority of grapevine cultivation areas worldwide. Phytoplasma presence seriously affect quality and quantity of production therefore accurate and sensitive pathogen detection and identification are relevant for the disease management. Phytoplasmas within single plants are populations of individuals; routine techniques such as PCR followed by RFLP or sequencing of uncloned or cloned products do not show the diversity of these populations. These techniques only allow identification of the most frequent genotypes in the samples and not mixed phytoplasma or pathogen infection. A number of grapevine samples in which single or mixed phytoplasma infection was detected by routine methods were employed for deep amplicon sequencing on the Roche Genome Sequencer FLX system to compare the two detection systems.

MATERIALS AND METHODS

Samples employed were selected from those that are routinely processed during surveys for phytoplasma detection and identification in Northern Italy where both 'flavescence dorée' (FD) and 'bois noir' (BN) diseases are widespread or epidemic (Botti and Bertaccini, 2007). Total DNA was extracted from 1 g of mid-vein leaf tissue following the procedure of Angelini *et al*., (2001). Phytoplasma detection was carried out by direct PCR on ribosomal gene and spacer region (Martini *et al*., 2002) followed by nested amplification with R16(I)F1/R1 (Lee *et al*., 1994) and 16R758F/V1730 (Martini *et al*., 1999) primer pairs. RFLP analyses with *Tru*I on the first amplicons and *Taq*I on the second one allow identification of BN and FD phytoplasmas. Samples showing single and mixed phytoplasma infection were selected for deep amplicon sequencing. Tagged primers were used for generating pyrosequencing samples following described PCR protocol (Nicolaisen *et al*., 2011). PCR products were pooled in equimolar amounts, run on an agarose gel and a band of the correct size was excised from the gel and purified using QIAquick gel extraction kit from QIAGEN. Two pools of 13 and 11 samples respectively were sequenced on a GS FLX plate at Eurofins MWG. Tag-sorted sequences were quality filtered using CLOTU software at the Bioportal webportal (http://www.bioportal.uio.no/). To minimize sequencing errors, only the first ~200 nucleotides of each sequence were used. Accepted sequences were clustered using CD-HIT with a 99% similarity threshold, and singleton sequences were discarded. To identify sequences, these were aligned together with reference sequences from GenBank using MEGA and phylogenetic trees were constructed.

RESULTS AND DISCUSSION

A total of 35521 sequences were generated from the first pool of 13 field collected samples of phytoplasma infected grapevine in which single infection was detected by routine procedures. Four of these samples were tested twice to verify result consistency (table 1). These data overall confirmed RFLP results.

Table 1. Number of sequences determined in each sample with single phytoplasma infection by routine methods.

	FD-C 46	FD-C 51	FD-D 53	FD-D 64	FD-D 66	FD-D 54	BN 33	BN 43	BN 59	FD-C 56	FD-C 63	FD-C 68	FD-C 71	FD-C 46	FD-D 53	BN 33	FD-C 56
16SrV-C/D	2109	1709	3032	885	1559	42	19	15	10	2040	1876	1520	1356	2126	2240	0	2540
16SrXII-A	0	0	0	0	24	0	2213	2730	4716	3	1	0	0	0	0	2756	0

Table 2. Number of sequences determined in each sample with mixed phytoplasma infection determined by routine methods.

	FD+BN 63	FD+BN 71	FD+BN 73	FD+BN 60	FD+BN 72	BN 67	FD+BN 76	FD TV1	FD+BN 112	FD+BN 120	FD+BN 80
16SrV-C/D	3009	414	54	612	359	2158	130	201	914	3846	33
16SrXII-A	361	5447	4442	1424	3964	1019	9727	4574	698	0	6823
16SrX-B	189	0	0	0	0	109	0	0	278	0	0
Chimeras	39	17	16	8	26	0	11	0	24	0	0
Total	3598	5878	4512	2044	4349	3286	9868	4775	1914	3846	6856

A total of 50,926 sequences were generated from the pool of 11 field collected samples in which mixed BN and FD phytoplasma infection was determined by nested PCR with group specific primers or RFLP analyses (table 2). After clustering at 99% similarity threshold, sequences were aligned to a reference set of sequences of known identity. This showed that 11,730 sequences belonged to phytoplasma 16SrV group, 38,456 sequences were belonging to 16SrXII group, 576 sequences were of '*Candidatus* Phytoplasma prunorum' (16SrX-B) origin whereas the last 164 sequences could not be assigned to one single group. There was a large variation in the number of sequences within each sample: 16SrV 33 – 3,009 sequences; 16SrXII 0 – 6823 sequences; '*Ca*. P. prunorum' 0 – 278 sequences.

Interestingly, the samples showing single infection in routing testing were shown by pyrosequencing to be infected by both 16SrV and 16SrXII phytoplasmas which is highly congruent with the fact that samples were collected in the same viticultural area having long time described BN and FD epidemic. The presence of a low copy number of 16SrX-B phytoplasmas is in agreement with previous finding of these phytoplasmas in grapevine in Italy and other European viticultural areas. Deep amplicon sequencing could be therefore employed in the future especially for testing propagation material for quarantine phytoplasma detection.

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Real-Time RT-PCR High Resolution Melting Curve Analysis and Multiplex RT-PCR to Detect and Differentiate Between Grapevine Leafroll Associated Virus 3 Variant Groups

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is a positive-sense single-stranded RNA virus that is the type member of the genus *Ampelovirus* in the family *Closteroviridae* (Martelli et al., 2002). This phloem-limited virus is considered the main contributing agent of leafroll disease worldwide with detrimental effects on both wine and table grapes. Six variant groups of GLRaV-3 have been identified of which four are known to be present in South Africa (Jooste et al., 2010, Jarugula et al., 2010, Ling et al., 2004, Engel et al., 2008, Maree et al., 2008, Bester et al., 2012, Gouveia et al., 2010). These variants commonly occur as mixed infections. However, no specific disease symptoms or geographic distribution could so far be assigned to a specific variant group. It is therefore necessary to develop an effective method to detect and differentiate between GLRaV-3 variants.

The aim of this study was to develop a simple and reliable one-step real-time RT-PCR assay with high-resolution melting (HRM) curve analysis (RT-PCR HRM) for the simultaneous detection and identification of GLRaV-3 variants of groups I, II, III and VI. A universal primer set for GLRaV-3, targeting the heat shock protein 70 homologue (Hsp70h) gene of GLRaV-3, and that is able to detect these GLRaV-3 variant groups, was designed. A multiplex RT-PCR was also designed to validate the RT-PCR HRM results. The application of these protocols will aid in the understanding of the molecular epidemiology of GLRaV-3 variants and leafroll disease.

MATERIALS AND METHODS

Total RNA was extracted from 173 grapevine samples using an adapted Cetyltrimethylammonium bromide (CTAB) method (White et al., 2008). Six primer pairs were evaluated for their ability to detect and differentiate between GLRaV-3 variant groups I, II, III and VI, utilizing the RT-PCR HRM analysis. The primer pair that could most effectively detect and differentiate between GLRaV-3 variant groups I, II, III and VI was used to screen the 173 samples to optimize the assay. Each reaction was performed in duplicate on a Qiagen Rotor-Gene Q thermal cycler.

Real-time RT-PCR amplicons of GLRaV-3 variant groups I, II, III and VI were cloned into a pGEM-T-easy vector and sequenced to obtain variant-specific plasmid DNA. The plasmid DNA was use to determine whether the chosen primer pair could differentiate between variants if mixed infections were present in field plants. Artificial *in vitro* duplex infections were made between the variant-specific plasmid DNA in a 1:3, 1:1 and 3:1 ratio for each combination of two variant groups. In order to use the RT-PCR HRM analysis to differentiate between variants, a melting point confidence interval was determined for each variant group to include at least 90% of all melting points observed. Variant-specific end-point reverse primers targeting the 5' UTR of the GLRaV-3 variant groups I, II, III and VI were designed to be used in a single reaction with one forward primer. This multiplex RT-PCR was designed to validate the HRM analysis and assign each sample to a specific variant group.

RESULTS AND DISCUSSION

In this study a real time RT-PCR assay was designed that can detect all GLRaV-3 variant groups described in South Africa thus far. The PCR targeted a conserved region in the Hsp70h gene of GLRaV-3. Primer pair LR3.HRM4 could most effectively detect GLRaV-3 variant groups and when HRM curve analysis was added to the real-time RT-PCR, distinct melting profiles were observed for each variant group (Figure 1).

In order to differentiate between variant groups based on HRM curve analysis, a melting point interval for each variant group was determined by calculating the largest interval with the highest confidence without overlap with the adjacent interval. No discriminatory difference could be found between the intervals for groups I and II, therefore an additional primer pair, LR3.HRM6, was designed for this purpose (Figure 1). One hundred and sixty nine grapevine samples were screened with the LR3.HRM4 primer pair, of which 48 samples tested negative for GLRaV-3. Of the 121 GLRaV-3 positive samples, 73 samples had multiple infections.

The multiplex RT-PCR was optimized to detect GLRaV-3 variant groups I, II, III and VI in a single reaction. The multiplex RT-PCR protocol validated 94% of the infections detected with the RT-PCR HRM analysis. The 12 infections that were not detected, indicates that the RT-PCR HRM analysis is more sensitive than the multiplex RT-PCR protocol. The RT-PCR HRM analysis provides a more sensitive, automated and rapid tool to detect and differentiate between different GLRaV-3 variant groups. The multiplex RT-PCR protocol offers an end-point PCR alternative to differentiate between the variant groups present in South Africa, or to be used as a validation method for the RT-PCR HRM analysis. The abovementioned tools will contribute to the understanding of the pathogenesis of leafroll disease and aid epidemiology studies to investigate how these different GLRaV-3 variant groups are spreading.

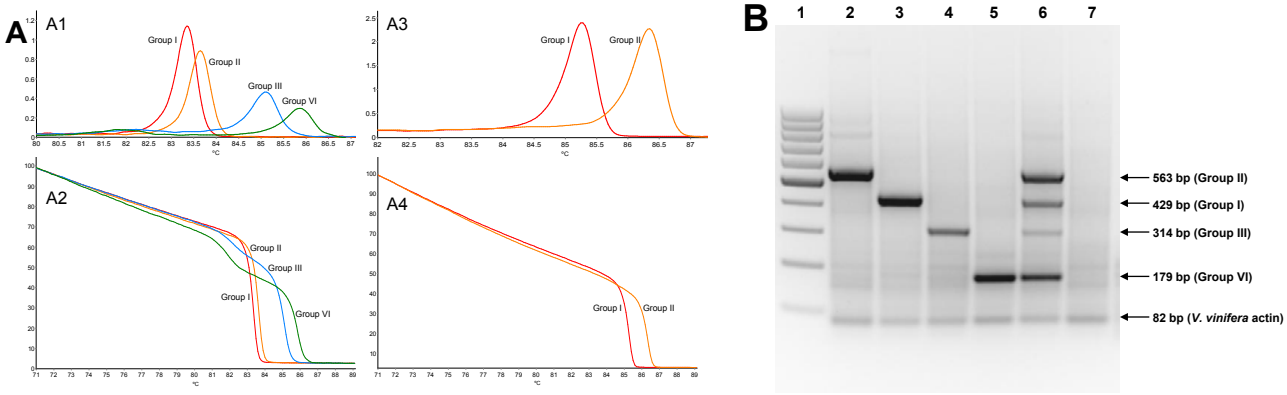

Figure 1: Example of the derivative HRM curves (dF/dT) (A1,A3) and normalized HRM curves (A2,A4) obtained using primer pair LR3.HRM4 (A1,A2) and primer pair LR3.HRM6 (A3,A4) in the RT-PCR HRM. Figure 1B represents the multiplex PCR. Lane 1: 100bp ladder, Lane 2: Group II, Lane 3: Group I, Lane 4: Group III, Lane 5: Group VI, Lane 6: All 4 variant groups, Lane 7: RNA negative control.

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Development of Generic and Variant-specific Molecular Assays for the Detection of the Highly Variable *Grapevine Leafroll-associated Virus 3*

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is an economically important virus that is found in all grapevine growing regions worldwide. A reliable and cost-effective GLRaV-3 detection method to test nursery and field grapevine plants is a critical component of any disease management programme. However, genetic variability within the virus population can compromise detection. Recent studies have shown high genetic variability in GLRaV-3 populations from different countries (Gouveia et al., 2011; Jooste et al., 2010; Sharma et al., 2011; Wang et al., 2011). Therefore, understanding sequence variability is essential to ensure that RT-PCR protocols detect all variants. It is also important to determine the biological significance of these variants, where differences in vector transmission, graft transmissibility, and severity of symptom expression need to be considered. Consequently tests that can detect and identify multiple variants/genotypes/strains economically and with high sensitivity are advantageous. The objectives of this study were to (i) investigate the extent of sequence variation in New Zealand (NZ) GLRaV-3 isolates, (ii) determine whether variation affects detection using molecular-based methods, and (iii) develop molecular assays to improve the detection of GLRaV-3 and aid biological studies of individual GLRaV-3 variants.

MATERIALS AND METHODS

Cane and leaf material were sourced from a varietal collection and several commercial vineyards in NZ and screened for GLRaV-3 by DAS-ELISA using Bioreba reagents (Bioreba AG, Switzerland) and RT-PCR using primers targeting ORF4 (608bp) and 6 (527bp). All GLRaV-3 positives were confirmed by sequencing and further analysed by single-stranded conformational polymorphism analysis. Isolates of interest were further sequenced. Phylogenetic analysis was conducted using Geneious v5.5, ClustalX v2.0, and MEGA5. Sequences from this study and GenBank, representing various phylogenetic groups, were used to design generic and variantspecific primer sets for conventional RT-PCR, multiplex RT-PCR (mRT-PCR), and real-time RT-PCR diagnostic assays. Generic primers that target ORF4 and generate a 120bp amplicon were used for conventional and real-time RT-PCR. The mRT-PCR assay uses six primer sets, five targeting virus variants and one internal plant control (*nad5* gene). Strain specific primer sets, for mRT-PCR, targeted ORFs 4 to 7 with expected amplicon sizes of 94 to 681bp. Four of the sets were designed specifically to detect variants from group 1, group 2, NZ1, and NZ2, while the fifth set was generic, detecting all variants from groups 1 to 5. The specificity of the primers was assessed against a range of sequence variants and the sensitivity tested using ten-fold serial-dilutions of *in vitro* RNA transcripts ranging from 10^1 to 10^8 amplicon copies/µL, diluted with healthy Cabernet Sauvignon RNA.

RESULTS AND DISCUSSION

The NZ GLRaV-3 population showed significant genetic variation, with isolates clustering with phylogenetic groups 1, 2, 3 and 5 (based on Gouveia et al., 2011), plus isolates that did not fit the current groupings, based on 488 bp of the ORF6. In particular, isolates NZ1 and NZ2 showed considerable genetic variation, differing from NY1 (group1) by 19.5% and 20.2% respectively. The high genetic variability of NZ1 and NZ2 isolates was further confirmed over 1418 nucleotides of the ORF4 region. The genetic variability within the NZ GLRaV-3 population affected GLRaV-3 detection. Outlier isolates, NZ1 and NZ2, have low immunological reactivity with Bioreba reagents, resulting in the occasional false negative. False negatives were also encountered when primer sets H330/C629 (MacKenzie et al., 1997) and LC1/LC2 (Turturo et al., 2005) were used to test NZ1 and/or NZ2 positive samples. To accommodate for the increased GLRaV-3 genetic variability, a new set of **generic** primers was designed and optimised for use with conventional and real-time SYBR Green RT-PCR protocols. For the detection of mixed GLRaV-3 infections, **variant-specific** primer sets were designed for mRT-PCR. Within a single hexaplex RT-PCR reaction, isolates from all phylogenetic groups could be detected. The generic and sequence-

specific assays detected GLRaV-3 readily and reproducibly regardless of the plant tissue type (Figure 1) or variety of grapevine (over 20 different varieties). The assays have high specificity, with no non-specific amplification of healthy or no template controls, and high sensitivity, detecting as few as 10 transcribed RNA copies per µl.

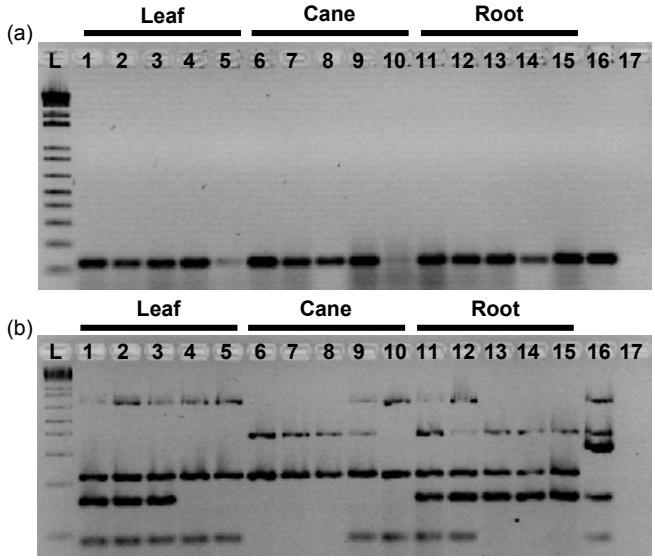

Figure 1. Detection of GLRaV-3 from leaf (lanes 1–5), cane (lanes 6–10), and root samples (lanes 11–15), using (a) the generic RT-PCR protocol and (b) the variant-specific mRT-PCR protocol. Lane L, 1 Kb plus DNA (Invitrogen, Carlsbad, CA, USA); lane 16, positive control using RNA transcripts diluted in water to $1x10^6$ amplicon copies per µl; lane 17, no template control.

The hexaplex RT-PCR assay was successfully used to screen 316 field samples from an historic grapevine collection and a commercial Hawke's Bay vineyard. Although little is known about the specific biological properties of the NZ1 and NZ2 variants, both were common within the NZ GLRaV-3 population at both sites. In particular, NZ2 was present in more than half the GLRaV-3 positive samples from both collections, with 64.6% and 53.3% respectively. Our results show NZ's GLRaV-3 population has high genetic variability, with the identification of at least six different variants, which can reduce the reliability of current diagnostic protocols. To improve GLRaV-3 detection, particularly of NZ1 and NZ2 variants, new assays for (i) the generic detection of all known GLRaV-3 variants in NZ and (ii) specific detection of variants from groups 1 to 5, and outlier variants NZ1 and NZ2, were developed and optimised. The generic assay is useful for certification schemes as it provides automated identification of GLRaV-3, and the field survey results from over 300 samples demonstrate the reliability and robustness of the mRT-PCR assay, providing researchers with a simple and cost-effective test to identify different GLRaV-3 variants in singular and mixed infections, which will facilitate biological and spatial distribution studies.

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Multiplex ELISA for the Simultaneous Detection of Grapevine Viruses

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INTRODUCTION

In this study, the possibility was investigated to reduce costs and time of ELISA (Clark and Adams, 1977) by mixing specific antibodies for the simultaneous detection of different viruses in a single test (Multiplex ELISA), and by analysing the effects of this combination on the sensitivity and specificity of diagnosis. To this aim four viruses were analysed, i.e. *Gr. fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Gr. leafroll-associated virus 1* (GLRaV-1) and *Gr. leafroll-associated virus 3* (GLRaV-3).

MATERIALS AND METHODS

Commercial kits for GFLV, ArMV, GLRaV-1 and GLRaV-3 detection (Agritest, Italy) were used. According to the manufacturer, the best antibody combinations for trapping and detection were: GFLV (1:1000/1:500), ArMV (1:1000/1:500), GLRaV-1 (1:500/1:1000), GLRaV-3 (1:1000/1:1000). Multiplex ELISA was first used for detecting viruses of the same genera (GFLV/ArMV nepoviruses; GLRaV-1/GLRaV-3 ampeloviruses), then it was extended to combination of viruses belonging to different genera (e.g. GFLV+GLRaV-1+GLRaV-3).

RESULTS AND DISCUSSION

After comparative testing of different antibody combinations, the antibody ratios adopted for simultaneous detection were: GFLV+ArMV (1:1/1:1,5), GLRaV-1+GLRaV-3 (2:1/1,5:1), GFLV+GLRaV-1+GLRaV-3 (1:2:1/1,5:1,5:1).

Simultaneous detection of GFLV and ArMV (Fig. 1). The sensitivity and the specificity of mixed antibodies in a single test resulted as effective as those of single antibodies, since all GFLV- and ArMV-infected vines were clearly detected by both procedures. Mixed antibodies gave a significant better response (*ca*. 30% increased absorbance) than single antibodies for GFLV detection (Fig. 1a), possibly because antibodies to ArMV (a virus serologically related to GFLV) in the mixture enhanced the activity of GFLV antibodies. Conversely, a slight reduction in sensitivity, but not in specificity, was observed in the detection of ArMV in the multiplex ELISA, likely because of the lower amount of enzyme-linked ArMV antibodies (-10%) used in the mixture in comparison with the simple ELISA (Fig. 1b).

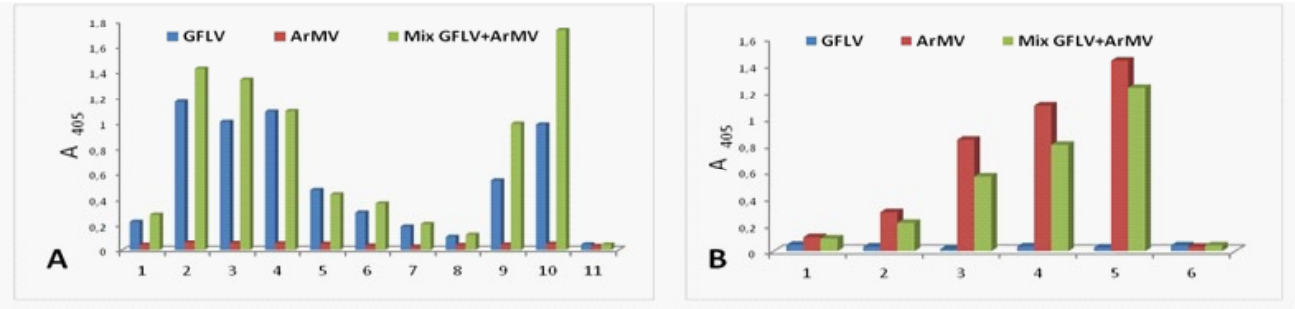

Fig. 1. ELISA readings (A_{405}) of GFLV (A) and ArMV (B) infected samples by using single antibody kits of GFLV (blue) and ArMV (red), and mix antibody kits of GFLV+ArMV (green). Samples 11 (in A) and 6 (in B) are negative controls.

Simultaneous detection of GLRaV-1 and GLRaV-3 (Fig. 2). The simultaneous detection in multiplex ELISA of GLRaV-1 and GLRaV-3 gave results comparable with those obtained with single ELISA kits. Because of the different speed of reaction of the two ELISA kits (GLRaV-3 detection was faster than that of GLRaV-1), in the preparation of the antibody-mixture the amount of GLRaV-1 conjugate antibodies was slightly increased (+ 20%) and that of GLRaV-3 reduced (-20%), thus maintaining the same total amount of enzyme-linked antibodies (table 1). This modification can explain the slight differences observed in comparative ELISA readings (Fig. 2).

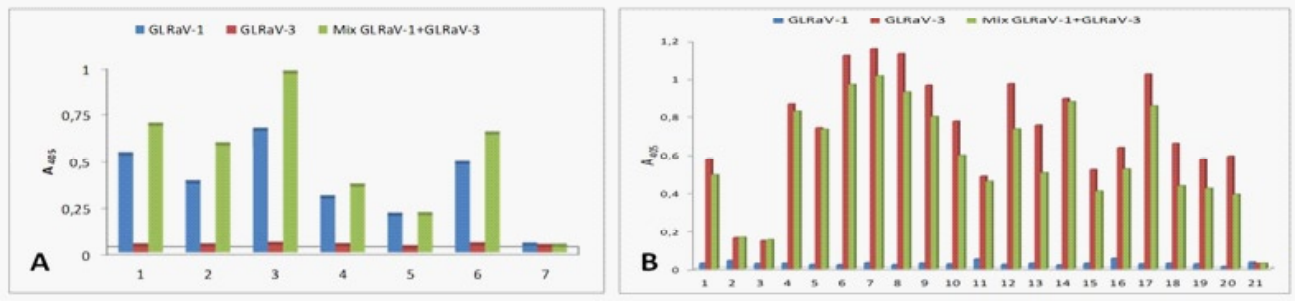

Fig. 2. ELISA readings (A_{405}) of GLRaV-1 (A) and GLRaV-3 (B) infected samples by using single antibody kits of GLRaV-1 (blue) and GLRaV-3(red), and mix antibody kits of GLRaV-1+GLRaV-3 (green). Samples 7 (in A) and 21 (in B) are negative controls.

In GLRaV-1 and GLRaV-3 mixed infections, absorbance values were higher with multiplex than single ELISA (Fig. 3).

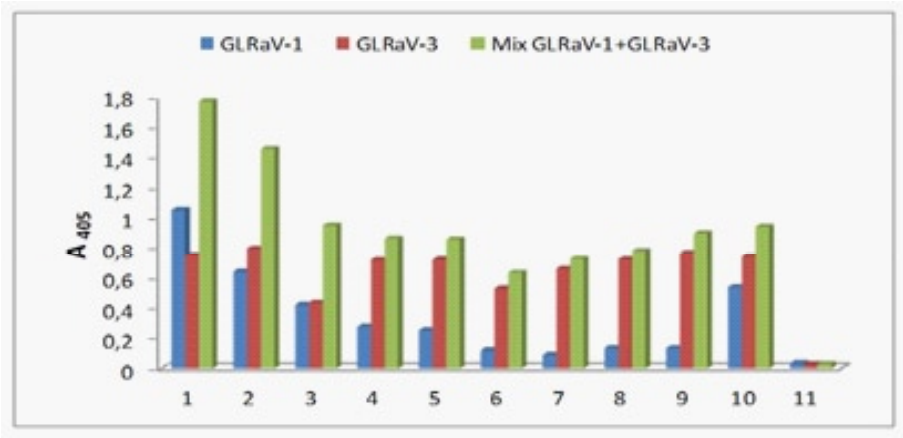

Fig. 3. ELISA readings (A_{405}) of samples infected by both GLRaV-1 and GLRaV-3 by using single antibody kits of GLRaV-1 (blue) and GLRaV-3(red), and mix antibody kits of GLRaV-1+GLRaV-3 (green). Sample11 is negative control.

Simultaneous detection of GFLV, GLRaV-1 and GLRaV-3. The possibility to test more than two viruses belonging to different genera, was evaluated by mixing the antibodies to GFLV, GLRaV-1 and GLRaV-3 Also in this case the simultaneous detection in Multiplex ELISA gave results comparable with those obtained with single antibody kits (data not shown).

The results obtained in this study clearly indicates that two or more viruses, also belonging to different genera (e.g. GFLV, GLRaV-1 and GLRaV-3), can be detected in a single multiplex ELISA test without loss of sensitivity and specificity in comparison with traditional ELISA. This result is promising in view of the possibility of extending this application to an higher number of viruses in the same test, where, apparently, the only limiting factor could be represented by the low immunogenic power of the viruses to be detected (i.e. antibody kits that need to be used at low dilution in ELISA).

In conclusion, when specific identification of single viruses is not required, as in the routine sanitary controls in clonal selection and certification programs, multiplex ELISA allows to simplify the test and reduce time and costs (lower amount of antibodies, reagents, materials and handwork).

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Simultaneous Detection of the Main Viruses and Viroids Affecting Grapevine by Molecular Hybridization Using a Unique Riboprobe or 'Polyprobe'

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INTRODUCTION

Grapevine is economically the most important fruit crop in the world, which is affected by various diseases of viral and/or viroidal etiology, whose symptoms range from asymptomatic, through chlorosis, interveinal reddening or yellowing, delayed ripening of the grapes, etc. which may affect the production of grapes with losses of up to 15% (7). Traditionally, viral infection assays in grapevine have been based on the bioassay or ELISA serological technique. However, both techniques have distinct disadvantages associated with 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 13 (low density array, TaqMan RT-PCR, 8) or 44 (Microarrays, 3) vine 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 simultaneous detection of different viruses/viroids by using a single probe or 'polyprobe' containing, fused in tandem, the different viral/viroidal sequences. This methodology permits the simultaneous detection of different viruses/viroids in one test with limit detection similar to the greater 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 virus and/or viroids affecting stone fruit (4, 9), tomato (1) and citrus (2). In the present work, we have developed a polyprobe with the capacity to detect 15 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 (5, 6). 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 amplicons corresponded to the following viruses and viroids: *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 1*, *2*, *3*, *4*, *5*, *6*, *9* (GLRaV-1, -2, -3, -4, -5, -6, -9), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus D* (GVD), *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). 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 (9).

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 (eight viruses and two viroids) using a non-radioactive molecular hybridization procedure (9). In the present work we have generated three different

polyprobes for the detection of the main viruses (15, Poly15) viroids (5, poly5) or both (poly20) affecting grapevine crops. Actually, we are analyzing the detection limit and the specificity of the new polyprobes. To our knowledge, this is the first polyprobe described with the capacity to detect twenty different pathogens.

ACKNOWLEDGEMENTS

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Detection of Dual Infection with *Grapevine Rupestris Stem Pitting-associated Virus* and *Uncinula necator* in Grapevines by Single-tube RT-PCR

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) is a foveavirus linked with the complex Rugose Wood disease which affects both graft take and longevity of vines in a productive vineyard. This virus has only been detected in *Vitis*, and is present in over 90% of grapevine samples tested in Australia (unpublished). Although most of the GRSPaV positive vines do not show symptoms, this virus has been found to be associated with grapevine vein necrosis in Italy and shown to be present in Syrah decline affected vines in Australia (Borgo et al., 2009; Habili et al., 2006). During indexing for this virus by single-tube RT-PCR using our routine primer pair we sometimes observed an amplicon smaller than that which is diagnostic for GRSPaV. Sequence analysis revealed that this smaller product belonged to the rRNA of the powdery mildew fungus (*Uncinula necator*) present on the surface of infected leaves.

MATERIALS AND METHODS

Leaf samples of *Vitis vinifera* infected with *Uncinula necator* (Un) were collected from the field or aseptically maintained on agar plates. Total nucleic acids (TNA) were extracted from leaf samples by the guanidine hydrochloride method of McKenzie et al (1997) using a SiO_2 matrix for the absorption, washing and elution of nucleic acids. The SiO_2 stock was prepared after a size fractionation by 3 x differential centrifugations at 1000 g for 1 min. Mycelia from the leaf surface were collected using a brush soaked in 20% Sarkosyl and its TNA was extracted using the guanidine buffer as above. Amplicons from single tube RT-PCR were cloned using the Invitrogen TOPO cloning system and sequenced by AGRF (Adelaide). The GRSPaV specific primer pair, RSP48 (5'AGCTGGGATTATAAGGGAGGT) and RSP49 (5'CCAGCCGTTCCACCACTAAT), was used to target a 329 bp segment of the RNA sequence on the coat protein gene. As an internal control for the assay we used the RubiscoL primers RBCL-H535 [CTTTCCAAGGCCCGCCTCA] and RBCL-C705 [CATCATCTTTGGTAAAATCAAGTCCA] which give an amplicon size of 171 bp (Nassuth et al, 2000).

RESULTS AND DISCUSSION

TNA extracts from GRSPaV infected grapevines give a virus specific amplicon of 329 bp in RT-PCR (Fig. 1, lane 1). However, if the leaf is dually infected with *Uncinula necator* (Un) two bands are produced (Fig. 1, lane 3). The lower band with a size of 258 bp was related to Un as shown in Fig. 1, lane 2. No band was present when TNA extracts from Chardonnay grapevines growing *in vitro* and subjected to thermotherapy to remove GRSPaV were targeted (Fig. 1, lane 6). Lanes 4 and 5 show that the 258 amplicon was also detected in TNA extracts from Botrytis (grown on *Capsicum* sp.) and Baker's yeast which had not been in direct contact with the grapevine. To establish the nature of the 258 bp band the DNA was subjected to cloning and sequencing (Fig 2, Un-Amp). The BLASTn analysis showed that the 258 bp band was part of the 26S ribosomal RNA present in fungi and in bacteria. To further analyse the 258 bp amplicon, a primer pair was designed from Un-Amp (Fig 2) [RPFuF Forward: CCGCTTTCTGGCATGGATTCT and RPFuR: CCACTAATAGGGAACGTGAG] giving an amplicon size of 220 bp. These primers reacted with the DNA extracts from fungi and bacteria but not from higher plants.

Multiple alignments of both the RSP primers (RSP48 and RSP49) with their 258 bp product (Un-Amp in Fig. 2) and two corresponding published sequences in GenBank showed highest sequence identity to *Artemisia* (EZ196181) and *Botrytis* (AL113843) indicating that the virus primer sequences were conserved in the two aligned DNAs, while in the primers only 50% of the sequences were conserved (Fig. 2). The *Artemisia* mRNA showed highest sequence homology and appeared on the top of the BLASTn list. However, when we used NA extracts from our locally grown *Artemisia*, no 258 bp amplicon was obtained.

Based on the above evidence we concluded that the Artemisia sequence reported by Graham et al (2010), who claimed to be associated with a low-yielding mRNA, is in fact an artifact.

Reviewing our RT-PCR gels for the detection of GRSPaV since 2000, the year we commenced using this pair of primers, we noticed the presence of double banding (see Fig 1, lane 3) in a number of our gels indicating that samples were possibly double-infected with the virus and either powdery mildew or other fungi or bacteria.

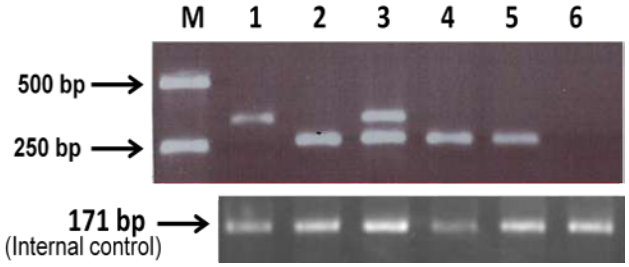

Fig. 1. Gel electrophoresis profiles of a typical RT-PCR using GRSPaV primers targeting total nucleic acid extracts from: 1, GRSPaV-infected *Vitis vinifera* grown *in vitro*. 2, *Uncinula necator* (UN) on leaf surface. 3, Chardonnay from field affected by both UN and GRSPaV. 4, *Botrytis cinerea*, 5, Baker's yeast and 6, Virus-eliminated Chardonnay grown *in vitro*.

Fig. 2. Mutiple sequence alignments of the Un-Amp sequence reported here with the two most homologous sequences: Artemisia (Acc No. EZ196181) and Botrytis (Acc No. AL113843). Our GRSPaV sequences are underlined.

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                    10        20        30        40        50        60
                    |         |         |         |         |         |
Artemisia   A-CTACGATGATGTGGGAGACAATCGCCGCTTTCTGGCATGGATTCTGACTTAGAGGCGT
Botrytis    A-CTACGATG-TGTGGGTATAAATCACCGCTTTCTGGCATGGATTCTGACTTAGAGGCGT
Un-Amp      AGCTGGGATTATAAGGGAGGTACTCGCCGCTTTCTGGCATGGATTCTGACTTAGAGGCGT
RSP48/49    AGCTGGGATTATAAGGGAGGT-----------------------------------------
                    70        80        90        100       110       120
                    |         |         |         |         |         |
Artemisia   TCAGCCATTATCCAGCAGATGGTAGCTTCGCGGCACTGCCTG-TCAGACAGCCGCCAAAA
Botrytis    TCAGCCATTATCCAGCAGATGGTAGCTTCGCGGCACTGCCTTGTCAGACAGCCGCAAAAA
Un-Amp      TCAGCCATTATCCAGCAGATGGTAGCTTCGCGGCACTGCCTGGTCAGACAGCCGCCAAAA
RSP48/49    ------------------------------------------------------------
                    130       140       150       160       170       180
                    |         |         |         |         |         |
Artemisia   CCAATTATC-GAATCAGCGGTTCCTCTCGTACTAAGCTGAATTACCATTGCGGTGACAAT
Botrytis    CCAATTATCTGAATCAGCGGTTCCTCTCGTACTAAGCTGAATTACCATTGCGGCGACGGT
Un-Amp      CCAATTATCTGAATCAGCGGTTCCTCTCGTACTAAGCTGAATTACCATTGCGGTGACAAT
RSP48/49    ------------------------------------------------------------
                    190       200       210       220       230       240
                    |         |         |         |         |         |
Artemisia   CATCAGTAGGGTAAAACTAACCTGTCTCACGACGGTCTAAACCCAGCTCACGTTCCCTAT
Botrytis    CATCAGTAGGGTAAAACTAACCTGTCTCACGACGGTCTAAACCCAGCTCACGTTCCCTAT
Un-Amp      CATCAGTAGGGTAAAACTAACCTGTCTCACGACGGTCTAAACCCAGCTCACGTTCCCTAT
RSP48/49    ------------------------------------------------------------AT
                    250
                    |
Artemisia   TAGTGGGTGAACAA-TCC
Botrytis    TAGTGGGTGAACAA-TCC
Un-Amp      TAGTGGTGGAACGGCTGG
RSP48/49    TAGTGGTGGAACGGCTGG
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Various species of fungi and bacteria were obtained from Ph D students at the Waite Campus. An aseptic powdery mildew sample maintained on leaves on agar medium was a gift from Ms. Pooja Vashist. Financial assistance of Barossa Grape Growers Vine Selection Society and Adelaide Hills Wine Improvement Committee towards N. H.'s travel is appreciated.

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Comparative Procedures for Sample Processing and Quantitative PCR Detection of Grapevine Viruses

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ABSTRACT

In this study, different instruments and methods used for tissue homogenization, RNA extraction, and quantitative PCR (qPCR) based detection of grapevine RNA viruses were evaluated. Semi-automated and automated homogenization techniques were compared to process samples from grapevine petioles and cambial tissue. Four different high throughput automated nucleic acid extraction platforms were compared with the RNeasy plant extraction kit for their capacity and efficiency of extracting viral RNA from grapevine infected tissues. The RNA prepared from each extraction platform was then used as template for a comparative analysis of qPCR by One Step RT-qPCR, Two Step RT-qPCR and low density array (LDA) detection. This study showed that a thorough homogenization of grapevine tissues using the Tissue Lyser as well as DNase digestion of the purified RNA prior to cDNA synthesis improved the virus detection and yielded the lowest quantitation cycle (Cq) values in RT-qPCR. Comparison of different RNA extraction methods showed that methods implementing the magnetic bead-based technology were superior to other methods used. Comparing different qPCR detection methods, One Step RT-qPCR showed the lowest Cq values for the same sample tested compared to Two Step RT-qPCR and LDA.

MATERIALS AND METHODS

► Grapevine leaf petioles and cambial scrapings of lignified grape cuttings were collected from grapevines originating from wide geographical regions which had tested positive by RT-PCR for one or more of the viruses listed above. To account for the possible uneven distribution of the virus within the plants, samples from at least six different branches were randomly collected, combined and divided. Two replicas of 0.1 g each to test the two different homogenization methods and were immediately frozen at -80°C (Table 1). Ten replicates of 0.3 g each were used to test 5 different methods for RNA extraction using two different lysis buffer (Table 2).

►72 samples were subjected to five different total RNA extraction methods and used for the comparative analysis of One Step RT-qPCR, Two Step RT-qPCR and LDA, using different kits for genomic DNA digestion, cDNA synthesis, 0ne step RT-qPCR and 2 Step RT-qPCR detection.

► Three different detection methods were used to target four different grapevine viruses. Viruses targeted in this project included Grapevine leafroll associated virus 2 (GLRaV-2), Grapevine rupestris stem pitting associated virus (GRSPaV), Grapevine vitivirus A (GVA) and Grapevine Fleck virus (GFkV).

RESULTS

Table 1. Homogenization of grapevine tissues (Petioles and Cambium) using semi-automated homogenization and the Tissue Lyser.

	Mean Cq value	
Tissue Type	Semi-automated homogenization	The Tissue Lyser
Petioles	19.5 ± 0.02	14.7± 0.02
Petioles	24.8 ± 0.04	15.1± 0.02
Petioles	28.6 ± 0.03	20.9± 0.03
Cambium	23.4 ± 0.01	17.8± 0.02
Cambium	28.2 ± 0.03	23.8± 0.02
Cambium	23 ± 0.03	19.2± 0.03

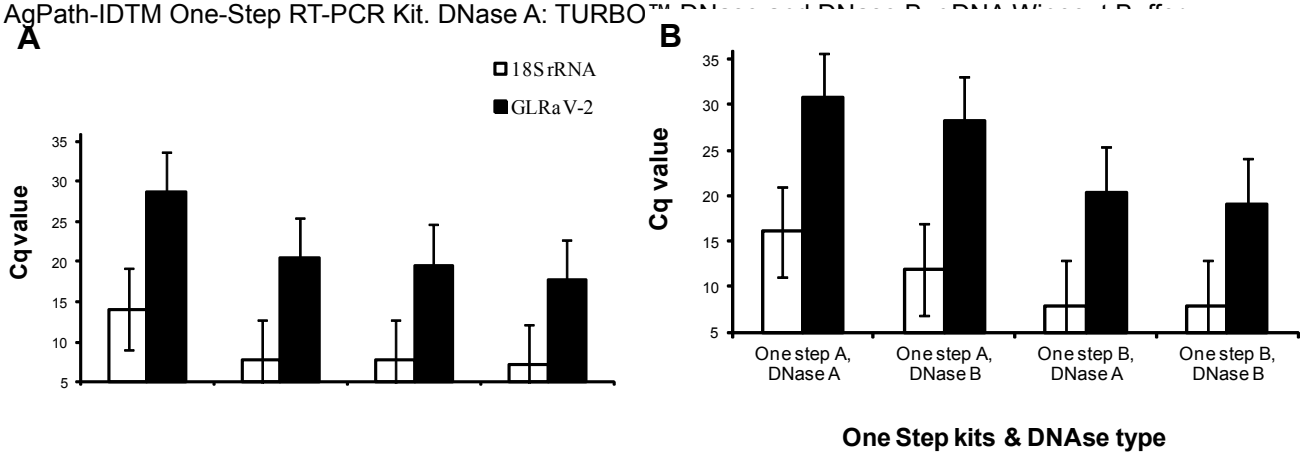
RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and qPCR tested using GLRaV-2 assay. The mean Cq values ± Standard Deviation of three replicas of the samples are shown. P value for petioles = 0.0446, P value for cambium= 0.0677

Table 2. The viral RNA yield of five different extraction methods using different lysis buffer.

Tissue type	Method A[1]		Method B[2]		Method C[3]	Method D[4]		Method E[5]	
	Guanidine	RLT lysis buffer	Guanidine	MagMax lysis buffer	Guanidine	Guanidine	QIAxtractor lysis buffer	Guanidine	AB 2X lysis buffer
Petioles	20.2* ± 1.54	23.3± 1.06	20.15±1.33	23.55±1.36	21.55±1.49	25.35±1.95	28.2±2.44	26.55±1.47	21.75±1.56
Cambium	17.9 ± 1.01	21.5 ± 1.1	17.3± 1.43	21.9 ±1.81	18.5 ± 1.30	22.3 ± 1.27	19.1±1.1	27.6±1.64	22.3±2.02

Two types of Grapevine tissues; Petioles and Cambium were used.The following five different RNA extraction platforms used were; 1: BioSprint 96 (QIAGEN), 2: MagMaxTM Express-96 (LIFE TECHNOLOGIES), 3: RNeasy Plant Mini Kit (QIAGEN), 4: QIAxtractor® (QIAGEN), and 5: ABI PRISM® 6100 Nucleic Acid PrepStation (LIFE TECHNOLOGIES). RNA extracted was qPCR tested using GLRaV-2 assay. Mean Cq value ± Standard Deviation of 12 Petioles and 12 cambium tissues are shown.

Fig. 1. Comparison of One-step RT-qPCR kits and DNase treatment in cambium (A) and Petioles (B).Two different kits were used: One step A: TaqMan® One-Step RT-PCR Master Mix Reagents Kit and One step B: the AgPath-IDTM One-Step RT-PCR Kit. DNase A: TURBO™ DNase and DNase B: rDNA Wipeout Buffer.



CONCLUSION

Thorough homogenization of grapevine tissues as well as DNase digestion of the purified RNA prior to cDNA synthesis is crucial to yield the lowest Cq values. Comparison of different RNA extraction methods showed that methods implementing the Magnetic bead-based technology in conjunction with Guanidine lysis solution yielded lowest Cq values in the shortest amount of time.

One-Step RT-qPCR yielded the lowest Cq values for the same sample tested compared to both Two-Step RT-qPCR and LDA.

High-Throughput Sequence Analysis of Small RNAs in Grapevine (*Vitis vinifera* L.) Affected By Grapevine Leafroll Disease

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INTRODUCTION

RNA silencing is a defensive strategy adopted by plants to ward off virus infections. This defensive pathway is triggered in response to virus infection and generates small-interfering RNAs (siRNAs) called virus-derived siRNAs (vsRNAs) to specifically target and cleave the viral genome into smaller non-functional fragments in a homology-dependent manner (Ding and Voinnet, 2007). Like viruses, viroids are also capable of triggering RNA silencing, but the mechanism of biogenesis of viroid-derived siRNAs (vd-sRNAs) appears to show some similarities to, as well as differences from, vsRNAs (Navarro *et al*., 2009). Virus infection and viral proteins can also modulate microRNAs (miRNAs) in infected plants leading to phenotypic changes such as symptom expression and other developmental abnormalities (Cillo *et al*., 2009). Most studies related to research on small RNAs (sRNAs) in virus- and viroid-infected plants have been conducted in model systems under controlled environmental conditions and very little information is available on sRNA profiles in virus-infected perennial crops grown under field conditions. In this study, we have used high-throughput sequencing to compare profiles of sRNA populations recovered from own-rooted Merlot grapevines with and without infection by the grapevine leafroll disease (GLRD).

MATERIALS AND METHODS

Two pairs of grapevines (*Vitis vinifera* cv. Merlot), each with one vine showing GLRD symptoms (GLRD+ve) and an adjacent non-symptomatic vine (GLRD-ve), grown under standard viticultural practices in a commercial vineyard were selected for this study. Leaves at the basal portion of canes showing typical symptoms of GLRD from GLRD+ve grapevines and comparable leaves from adjacent GLRD-ve grapevines were collected simultaneously in mid September. Samples were tested for a panel of grapevine-infecting viruses and viroids included in standard virus indexing programs (Naidu *et al*., 2006). Small RNAs were isolated from leaf samples and sRNAs of 18-28 nt size range were gel-purified from a denaturing 15% polyacrylamide gel. The isolated sRNAs were sequentially ligated to 5' and 3'RNA oligonucleotide adapters, reverse transcribed, and amplified by PCR. High-throughput sequencing of the small cDNA libraries was done using the Sequencing-By-Synthesis technology (Illumina Inc.) and computational analyses of sRNA reads were performed as described (Jagadeeswaran *et al*., 2010).

RESULTS AND DISCUSSION

Samples from GLRD+ve vines were tested positive in RT-PCR for *Grapevine leafroll-associated virus 3* (GLRaV-3), *Hop stunt viroid* (HpSVd) and *Grapevine yellow speckle viroid 1* (GYSVd-1) and those from GLRD-ve vines tested positive only for HpSVd and GYSVd-1. None of the samples tested positive for *Grapevine yellow speckle viroid 2* (GYSVd-2) and other viruses and viroids. Small RNA reads specific to GLRaV-3 (vsRNAs) were found only in GLRD+ve libraries, whereas sRNAs specific to HpSVd, GYSVd-1 and GYSVd-2 (vd-sRNAs) were found in both libraries (Table 1). Analysis of the size classes of vsRNAs and vd-sRNAs showed that the 21 nt size class of sRNAs was the most abundant in both GLRD+ve and GLRD-ve leaves, regardless of the virus or viroid species. On a comparative basis, HpSVd vd-sRNAs were more abundant in both libraries than vd-sRNAs specific to GYSVd-1 and GYSVd-2 (Table 1). Conversely, very low amounts of GYSVd-2 vd-sRNAs were recovered from both libraries (Table 1). Further analysis indicated that HpSVd vd-RNAs were present in more or less equal amounts in both libraries and twice the amount of GYSVd-1 and GYSVd-2 vd-sRNAs were recovered from the GLRD-ve library than from the GLRD+ve library (Table 1). A total of 2,299 reads represented by 1,373 unique reads showed perfect homology to the GLRaV-3 genome sequence.

Table 1. Profile of small RNAs recovered from GLRD+ve and GLRD-ve grapevine leaves.

Category of small RNA	cDNA library reads	
	GLRD-ve	GLRD+ve
Known miRNA homologs	1010327	1078224
New and candidate miRNAs	5966	2021
Grapevine leafroll-associated virus 3-derived vsRNAs	0	2299
Hop stunt viroid-derived vd-sRNAs	6118	5413
Grapevine yellow speckle viroid 1-derived vd-sRNAs	3121	1338
Grapevine yellow speckle viroid 2-derived vd-sRNAs	692	332

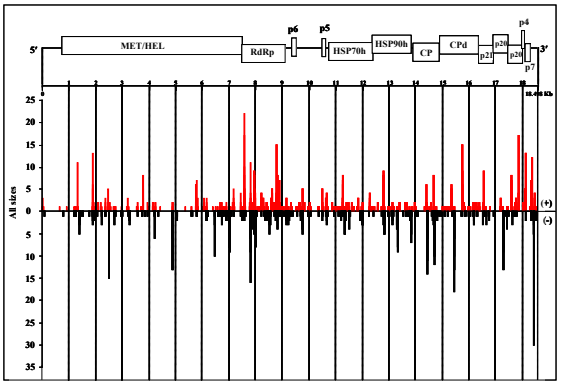

Figure 1. Genome organizationof GLRaV-3 and mapping of vsRNAs from GLRD+ve leaves. Number of unique hits at each genomic position are represented by red (+sense reads) or black (-sense reads) bars. Each bar shows normalized raw reads (TPM) of vsRNAs at each genomic position from the total pool of unique vsRNAs. GLRaV-3 genome (EU259806) and location of different ORFs were drawn to scale. MET, Methyl transferase; HEL, Helicase; RdRp, RNA-dependent RNA polymerase; HSP70h, Heat shock protein 70 homolog; HSP90h, Heat shock protein 90 homolog; CP, Coat protein; CPd, Diverged coat protein; p21, 21 kDa protein; p20, 19.6 kDa protein; p20, 19.7 kDa protein; p4, 4kDa protein; p7, 7kDa protein.

The vsRNAs of all size classes were mapped throughout GLRaV-3 genome in both sense and antisense orientations (Fig. 1). On a genome wide scale, the density of vsRNAs of both polarities and sizes showed biased distribution with relatively few reads mapping to the 5'-terminal region corresponding to nucleotide positions 1 to approximately 5,500 than to other portions of the viral genome (Fig. 1). As a result of this uneven distribution pattern along the GLRaV-3 genome, multiple vsRNA-generating hot spots (based on normalized raw reads [TPM: transcripts per million] of vsRNAs of both polarities) were located in the replicase (ORF 1a&b), HSP70h, HSP90h, CP, CPd and the 3'NCR (Fig. 1). In addition to 135 previously identified conserved miRNAs in grapevine (Vvi-miRs), we have identified ten novel and several candidate Vvi-miRs in GLRD+ and GLRD- grapevine leaves based on cloning of miRNA star sequences. Quantitative real-time RT-PCR of select conserved Vvi-miRs indicated that individual members of a miRNA family are differentially expressed in GLRD+ and GLRD- leaves. This study offers resources for further elucidation of compatible host-pathogen interactions to provide ecologically relevant information for better understanding of host x pathogen x environment interactions in a perennial fruit crop.

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GLRaV-2: Sanitation and Performance of Emblematic French Clones of Cabernet-Sauvignon

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ABSTRACT

French clones have been widely propagated in wine countries around the world since the 70's. Among this palette, clones of Cabernet-Sauvignon took a large place in the reputation of the French clonal material. Three emblematic clones of Cabernet-Sauvignon N — 191, 337 and 341 — contributed to the success of wineries in France and abroad. These three clones tested positive for GRLaV-2. In France, we still consider that effects and symptoms of GLRaV-2 on vines are of poor impact on growth and quality on the wines. 191 and 337 have been recently cleaned-up by IFV, formerly ENTAV, using the micro shoot tip culture (MSTC) method.

As agronomic and genetic profiles are required for the French official registration, in addition to sanitary testing by indexing, the "new candidate clones" were planted in an experimental vineyard, located in an INRA unit which was in charge of the vineyard management. Viticultural checking, wines and tastings by a panel have been carried out by Chambre d'Agriculture de la Gironde.

This presentation provides the final results with detailed performance of five clones involved. Two clones have been registered in 2010 by the Vine Section of the Technical Permanent Committee for Selection (CTPS), 1124 (191 MSTC) and 1125, no micro shoot tip cultured, an "offspring" of the historical and original vine of 337.

Five years of experimentation provided the following results:

- 1124 : higher vigour, slightly higher producer in comparison with 191. Its fertility is higher, bunches are bigger, anthocyanins are also more present and its wines are structured and balanced.
- 1125 : more fertile than 337, bunches are smaller, wines are aromatic and full.
- 337 MSTC did not perform as well as the original 337 (LR2 +), and, above all, wines have been less appreciated than 337. A decision for its registration has not been made.

SANITATION PROCESS

In France and EU, sanitary selection is based on the detection of main virus diseases by indexing, which is still the official and reference method.

GFLV, ArMV, GLRaV-1 and GLRaV-3 are required for the scion varieties. In addition, GFkV is required for the rootstock varieties. Other virus diseases such as KSG or RSPaV are not mandatory.

At the end of the 90's, through reliable ELISA tests, Cabernet-Sauvignon 191, 337 and 341 tested positive for GLRaV-2. This information was confirmed afterwards by additional indexing using different indicators, with weak and no evident symptoms on leaves.

Even though GLRaV-2 in France never caused severe damage (excepted incompatibility with the rootstock Kober 5BB), IFV (ex ENTAV) micro shoot tip cultured clones 191 and 337.

The whole process of sanitation is two seasons long. Starting from cuttings stored in pots in a hot chamber at 32 to 34 degrees Celcius (90–93° F) for 1 to 2 months. Then, the apex from a main shoot is collected and sterilized. The meristem is excised and grafted onto a hypocotyl from a seed of the rootstock variety Vialla. We used to obtain 8 to 10 microplants per variety or clone grown in tubes on a media culture. After several weeks in a culture room at 26 degrees Celcius (79° F), plants are moved to a greenhouse acclimation. When plants are big enough and lignified, it is time to check their virus status by ELISA or PCR. In the past decades, numerous clones of rootstocks have undergone this treatment.

TECHNOLOGICAL PERFORMANCES

The performance of these "new clones" has been tested in Bordeaux 1ères Cotes, by INRA and Chambre d'Agriculture de Gironde. Financial support of this experimentation was provided by the Regional Council of Aquitaine, FranceAgriMer and the Interprofessional Council of Bordeaux wines.

Viticultural data

Vigour and production of 191 is significantly lower than other clones. 1124 (191 MSTC) is slightly more productive than 191 due to bigger clusters and higher fertility. Berries are similar. 337 MSTC fertility is higher than 337 and 1125. Clusters of 1125 are a little bit smaller than others, and its clusters are also looser, which is an interesting characteristic for *Botrytis* tolerance.

Clones	Weight per vine (kg)	Clusters per vine	Weight per cluster (g)	Weight of canes (g)	Weight of 100 berries (g)
191	1,26	10,5	123	368	132
1124 (191 MSTC)	1,73	12,5	126	592	124
337	1,69	12,6	133	493	130
337 MSTC	2,04	13,9	135	452	127
1125	1,72	13,0	126	608	128

Berries maturity

The analysis of berries reveals that potential of anthocyanins of clones 1124 (191 MSTC) and 1125 is higher than other clones involved. Seeds of 1125 are more mature and evolved. Total polyphenol index is lower for 337.

Clones	Anthocyanins at pH 1 (mg/L)	TPI at pH3,2	EA %	Seed maturity
191	1167	46	41	42
1124 (191 MSTC)	1247	48	42	41
337	1010	43	39	44
337 MSTC	1232	47	42	40
1125	1352	46	44	36

TPI : total polyphenols index, EA : anthocyanin extractability.

Oenological data and tasting results

When we compare clones pair by pair, there are no evident differences between 191 and 1124 (191 MSTC), and between 337 and 337 MSTC. The only weak difference comes from anthocyanins, more concentrated for the clones 1124 (191 MSTC) and 1125.

Clones	Alcohol (% vol.)	TA (g H_2SO_4/L)	pH	TPI	Anthocyanins (mg/L)
191	12,2	3,4	3,78	49	521
1124 (191 MSTC)	12,5	3,5	3,75	51	550
337	12,2	3,4	3,85	47	469
337 MSTC	12,2	3,4	3,85	50	512
1125	12,4	3,5	3,77	51	584

Through tastings by panel of winemakers, scientists and growers, the main characteristics of the clones are:

- 1124 (191 MSTC): intense color, strong aromas. Much appreciated by the panel – elected by 77 % - which underlined the balance and the structure of the wines.
- 1125: also much appreciated, balanced, with aromas (flavor and mouth), colored and structured. Elected by 62 % of the panel.
- Both are well ranked for their aging aptitudes.
- 191, 337 and 337 MSTC were all less appreciated. 191 and 337 have been respectively rejected by 58% and 63% of the panel.

CONCLUSION

The vineyard experimentation confirmed that sanitation generates an increasing of vigour, fertility, size of clusters and production. However, these changes did not have a significant impact on the quality of the wines. For this reason, 1124 has been successfully submitted to the Vine section of the CTPS. With very good growing aptitudes, the GLRaV-2 virus free clones 1124 and 1125 have been recently introduced to FPS, the United States National Grapevine Importation and Clean Stock Facility. They will be available in a while.

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Field Performances and Wine Quality Modification in a Clone of 'Nebbiolo' (*Vitis vinifera* L.) after *Grapevine Fleck Virus* Elimination

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INTRODUCTION

Fleck, one of the most common virus disease in grapevine, is due to the infection of the *Maculavirus Grapevine fleck virus* (GFkV). Fleck is symptomless in *V. vinifera* and in many American hybrid rootstocks, whereas typical clearing of minor leaf veins are expressed in *Vitis rupestris*. According to the limited literature available, GFkV is reported to reduced growth of some rootstock mother vines (Credi *et al*., 1996) while, so far, only negligible influence due to GFkV was registered in *Vitis vinifera* cultivars (Credi *et al*., 1997). In other experiences GFkV was present in mixed infection with other more harmful virus such as GLRaV-3 and GVB (Golino *et al.,* 2009) or GLRaV-1 (Komar *et al*., 2007), so its specific effect could not be isolated. The aim of this study was to evaluate the effect of GFkV elimination on grapevine agronomic and enological 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' formerly infected by GFkV and tested free from GFLV, GLRaV-1, GLRaV-3 and GVA was heat-treated obtaining the eradication of GFkV. Cuttings collected from GFkV infected (MP) and from heat-treated (HT) mother plants were rooted and kept in collection. In 2005 scions of PM and HT lines were propagated by grafting on healthy Kober 5BB rootstocks and planted in two parallel rows of 35 vines each: healthy and infected vines were alternated in groups of five. Along the rows four replicates of five plants (total 20 vines) were selected for each sanitary status. The vineyard is located in Lessona (BI), a typical area for 'Nebbiolo' cultivation. Vines were vertically trained and single-cane pruned. The plantation density was 5000 vines per hectare. The virological status of every single vine under study in the vineyard (20 + 20) were controlled by DAS-ELISA on dormant cane samples collected during 2010-2011 winter and using commercial kits according to the manufacturer's instructions (Agritest Srl, Valenzano, Bari, Italy).The tests confirmed that all the diseased progeny was GFkV infected and free from ArMV, GFLV, GLRaV-1, GLRaV-2, GLRaV-3, GVA and GVB, while the healthy line was free from all the virus previously mentioned included GFkV. On the same samples a molecular diagnostic testing was performed by multiplex RT-PCR, which confirmed the ELISA results revealing the presence of GRSPaV in the MP progeny. When the vineyard reached full production in 2011, the main agronomic parameters were assessed on each replicate. Field data were statistically elaborated by ANOVA. In addition, a sample of around 300 berries was collected from both MP and HT vines in order to carry out analyses on juice composition and berry phenolic content. The total crop of the 20 vines (around 40 kilos of grapes) for each sanitary status was submitted to small scale winemaking. Chemical and sensory evaluations were then performed on the wines after few months of rest in the bottle. Sensory evaluations were carried out by a 'duo-trio' test (i.e. the panel must pick out the two identical wines among a group of three) followed by a paired-preferences test. A characterization test was also used to investigate the intensity of the different components of colour, bouquet and taste.

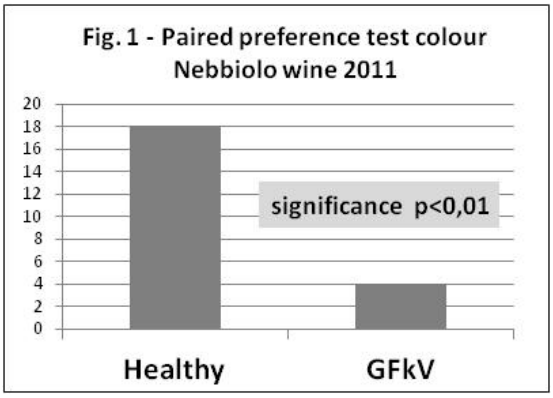
RESULTS AND DISCUSSION

The presence or the absence of GFkV did not influence the vine vigour, practically the same in both progenies (Tab. 1). Quite surprisingly the crop was lower in the HT plants due to a lower number of clusters/vine and to a lower average cluster weight. Also the berries were smaller in the GFkV-free vines. Despite the difference between the crops, juice sugars concentration was nearly the same, while the juice acidity resulted slightly higher in the HT vines. Among grape qualitative parameters, total anthocyanins (responsible of wine colour) and total phenols (responsible of wine body) resulted significantly increased by GFkV elimination (Tab. 1). The analyses of the wines showed, according to the original grape composition, a similar degree of alcohol but an higher titratable acidity in the wine of HT plants (Tab. 2). This character however was mainly due to a reduced salification of the organic acids (i.e. lower contents of potassium). In terms of enological potentiality the most interesting modification found in wine as a consequence of GFkV elimination was the content of anthocyanins,

higher in the product obtained from healthy vines. The higher amount of these compounds (and probably their better extractability related to the smaller berries) produced an increase of the wine colour intensity. This result is of particular interest for Nebbiolo wines, whose colour is usually a critical point due to its specific anthocyanin profile. Sensory analysis carried out by a trained panel of 22 tasters overcame successfully the 'duo-trio' test confirming the two wines in comparison were distinguishable (21 correct responses out of 22: statistical significance p≤0,001). In addition the panel, according with the analytical data, pointed out, as the major difference between the two wines, the depth and the shade of the colour, favouring for these important descriptors the wine made with grapes of HT vines (Fig. 1). The colour of this wine was described more intense and with a slightly more violet hue than the wine from MP plants (Fig. 2). The HT wine resulted also a little more full-bodied, while no bouquet differences were noticed.

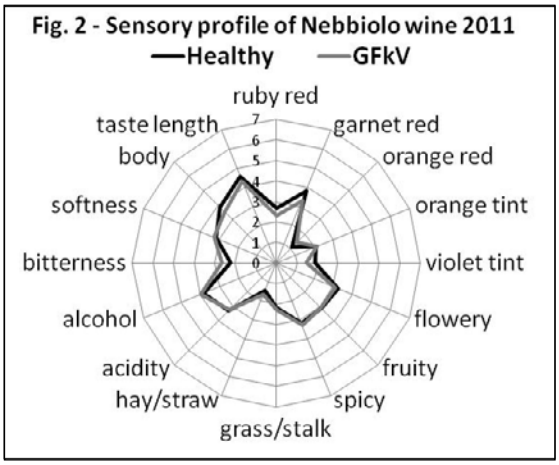
Tab. 1 - Field performances and juice composition of Nebbiolo (2011)
All data are expressed as average values.
Significance: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, ns = not significant.

	Healthy	GFkV	F
Yield (kg/vine)	1,54	2,48	*
Bunch wt (g)	143	178	ns
Berry wt (g)	1,45	1,74	*
Bunches/vine (n°)	11	14	*
Pruning wood wt (g/vine)	864	894	ns
Soluble solids (g/L)	234	236	ns
Titratable acidity (g/L)	7,3	6,9	ns
pH	3,27	3,31	ns
Tartaric acid (g/L)	7,20	5,15	***
Malic acid (g/L)	2,42	2,93	***
Total phenols (mg/kg)	3999	3249	**
Total anthocyanins (mg/kg)	667	407	***



Tab. 2 - Nebbiolo wine composition (2011)

	Healthy	GFkV
Alcohol (%vol)	13,46	13,36
Dry extract (g/L)	28,1	27,2
Titratable acidity (g/L)	5,28	4,89
pH	3,73	3,84
Tartaric acid (g/L)	0,86	0,83
Lattic acid (g/L)	1,45	1,67
Potassium (mg/L)	1505	1724
Ash (g/L)	3,65	3,91
Total phenols (mg/L)	1531	1523
Total anthocyanins (mg/L)	97	75
Colour intensity (A420+520+620)	3,88	2,91
Colour hue (A420/520)	1,05	1,10



In conclusion, the elimination of GFkV (and GRSPaV) reduced crop (around 40%) in 2011 vintage although inducing beneficial effects on the amount of grape anthocyanins (berry red pigments) and consequently on the intensity of final wine colour. The improvement of wine colour was confirmed by both chemical analyses and sensorial tests. Despite the yield reduction, the overall effect of GFkV (and GRSPaV) elimination can still be considered profitable considering that bunch thinning (i.e. removing at least 30% of clusters on plant at veraison) is a common field practice for 'Nebbiolo' cultivation in order to reduce crop and improve grape maturity.

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Small RNAs Profiling in Virus-infected Grapevines

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INTRODUCTION

Recent advances in understanding plant defense mechanisms against virus infections disclosed modifications of the small RNA (sRNAs) population during disease expression (Hu *et al*., 2011) and the existence of a novel regulatory cascade involving disease resistance proteins (Li *et al*., 2012; Shivaprasad *et a*l., 2012). These studies, although conducted on herbaceous model plants, suggested that similar regulatory mechanisms against pathogen infections may work also in grapevine. In this respect the "degeneration" of grapevines induced by *Grapevine fanleaf virus* (GFLV) constitutes an interesting model system due to the severe malformations consequent to virus infection. The present work profiles sRNAs population in the course of virus infection allowing comparisons with recent findings in plant-virus interactions in model plants.

MATERIALS AND METHODS

GFLV-infected grapevines showing symptoms of leaf malformations and stunting (s3) and healthy plants (s5) of cv Italia from the same vineyard, were selected from a collection of the University of Bari. Cuttings were rooted in pots and leaves collected in spring, at the onset of symptoms, and in summer, when these fade away. Field-grown vines of cv Montepulciano showing (P1A, P1B) or not (MH) infectious degeneration symptoms were also selected during spring. Small RNA purification, library preparation and sequencing by Illumina technology were performed according to Giampetruzzi *et al*. (2012). Secondary analysis of the libraries were performed with the UEA siRNA toolkit (Moxon *et al*., 2008) and a standalone BLAST software (Altschul,1990).

RESULTS AND DISCUSSION

Bioinformatic analysis of the sRNA libraries and routine RT-PCR showed that the s3 vine was infected by *Grapevine fanleaf virus*, *Grapevine rupestris stem pitting-associated virus* and *Grapevine yellow speckle viroid 1* and *Hop stunt viroid.* Later in the season a further library was prepared and analyzed from the s3 plant, which showed recovery from symptoms. Size distribution of sRNA molecules showed that s3 plant had a predominant 21nt sRNA population, while the opposite occurred in the healthy s5 vine in which the 24 nt size class prevailed. Surprisingly, recovered tissues of s3 plant had the same 21nt/24nt ratio as the healthy vine with a prevalence of 24 nt size class, a condition that was confirmed by gel electrophoresis (Figure 1).

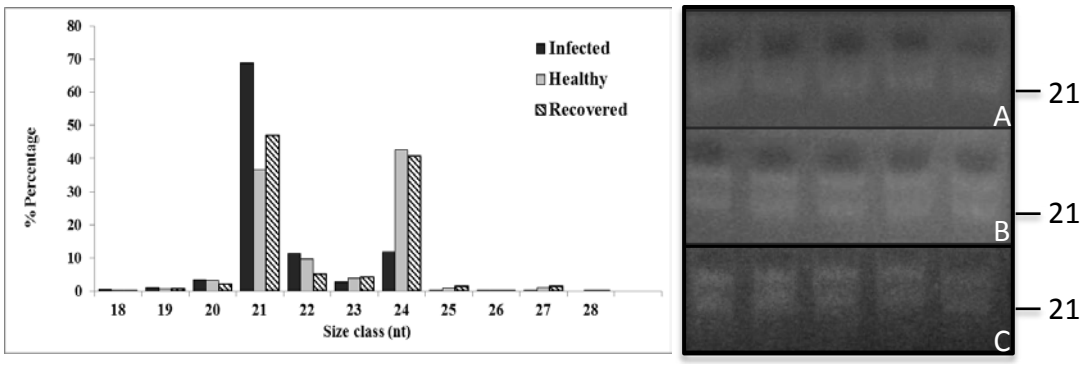

Figure 1. Size distribution and abundance of endogenous sRNAs matching the grapevine genome. Histogram indicates the relative abundance of sRNAs *per* size class. Gel electrophoresis separation of the low molecular weight RNA fraction purified from the s3 infected (A), s5 healthy (B) and recovered s3 vines(C).

To test the hypothesis that the observed sRNA size distribution occurred in plants showing fanleaf symptoms we analyzed three additional sRNA libraries from the tissues of two cv. Montepulciano vines showing infectious degeneration symptoms (P1A and P1B), and a healthy looking vine of the same cultivar (MH). Bioinformatic search of the sequenced sRNAs revealed that besides GYSVd-1, HSVd and GRSPaV, GFLV was also present

in the three plants and *Grapevine leafroll-associated virus 3* in P1A. sRNA size distribution of these three vines disclosed that the 21/24 nt ratio was 2:1 in MH and 7:1 and 4:1 in P1A and P1B. respectively. As in the previous study, this size distribution was confirmed by gel electrophoresis analysis (*data not shown*).

To investigate the origin of the observed size distribution we looked for 21 nt phased sRNAs in the sequenced libraries since recent findings on *Arabidopsis thaliana* infected with a tobamovirus (Hu *et al*., 2011) showed an increase of this sRNA size class. We found that, comparing the same number of genome loci, a higher number of phased sRNAs was generated from the s3 plant (infected) with respect to the s5 healthy vine. Furthermore, in the s3 recovered plant phased sRNAs were comparable to those generated from the s5 healthy vine, seemingly restoring a "healthy" condition. The same analysis performed on the three additional libraries, showed that the MH symptomless plant had a lower number of 21nt phased sRNAs as compared to those obtained from P1A and P1B.

In depth examination of the phased sRNAs, disclosed that the highest number was produced by two loci in the chromosome 14 identified as homologous to the *A. thaliana* TAS4 locus (Rajagopalan *et al.*, 2006). These two *Vitis vinifera* TAS4 loci were differently expressed in the two cultivars investigated (Italia and Montpulciano). Database search showed that the majority of the remaining phased sRNAs identified, were predominantly generated from putative disease resistance proteins belonging to the NB-LRR gene family. This is reminiscent of a recently discovered defense mechanism based on the pathogen-inducible expression of NBS-LRR proteins, which is under the control of a microRNA superfamily having the size of 22nt (Zhai *et al*., 2011; Shivaprasad *et al*., 2012) whose presence has already been detected in grapevines These microRNAs control NBS-LRR disease resistance proteins through cleavage at conserved domains and was proposed as a novel defense pathway against pathogen attack. Comparison of the phased sRNAs produced by the same predicted NBS-LRR loci showed that their number was three-fold higher in the s3 (diseased) with respect to the s5 (healthy) plant. The proposed mechanism would rely on the activity of viral suppressor of RNA silencing (VSR), which relieve NBS-LRR expression, thus resulting in a burst against pathogen invasion (Shivaprasad *et al*., 2012). Although the existence of a VSR has not yet been demonstrated for the viruses (GFLV and GRSPaV) infecting the s3 plant, the activation of such a mechanism cannot be excluded. In this framework the increased number of NBS-LRR-related phased sRNAs in the s3 vine (infected) could be explained by the increased expression of NBS-LRR mRNAs.

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RNA Expression Analysis in Virus Infected *Vitis vinifera* cv Chardonnay

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INTRODUCTION

Grapevine (*Vitis vinifera*) is one of the economically most important fruit crops worldwide. Due to the availability of the grapevine genome sequence, complete gene expression profiling is possible. Gene expression profiling allows for analyzing the response of grapevine to various biotic and abiotic stresses potentially influencing the plant's performance. One of these biotic stresses is infection of grapevine with grapevine leafroll-associated virus 3 (GLRaV-3). Grapevine leafroll-associated virus 3 is one of the most predominant viruses associated with leafroll disease (Jooste *et al*., 2010). By using RNASeq we investigate the grapevine response to GLRaV-3 infection. For that, virus infected and non-infected grapevine plants were grown under controlled greenhouse conditions. We present our findings of differentially expressed genes in virus infected and healthy *V. vinifera* cv Chardonnay.

MATERIALS AND METHODS

Certified healthy and GLRaV-3 infected *V. vinifera* cv. Chardonnay were grown in the glasshouse with constant temperature and natural light. These plants were tested for viruses commonly infecting grapevine. We used three healthy and three GLRaV-3 infected plants for this experiment. Total RNA was extracted using Plant RNA purification reagent (Invitrogen) following the manufacturer's protocols. The quality of extracted total RNA was assessed using an Agilent Bionalyzer 2100. RNA sequencing was performed using the Illumina HiSeq 2000 (Fasteris, Switzerland). The quality of the RNA reads was first assessed using the FastQC tool. The reads were then mapped to the *V. vinifera* 12X genome using TopHat v 1.3.2. Mapped reads were further analyzed using the Cufflinks suite v 1.1.0 and differential gene expression was obtained using Cuffdiff. We used Blast2go to annotate the differentially expressed genes.

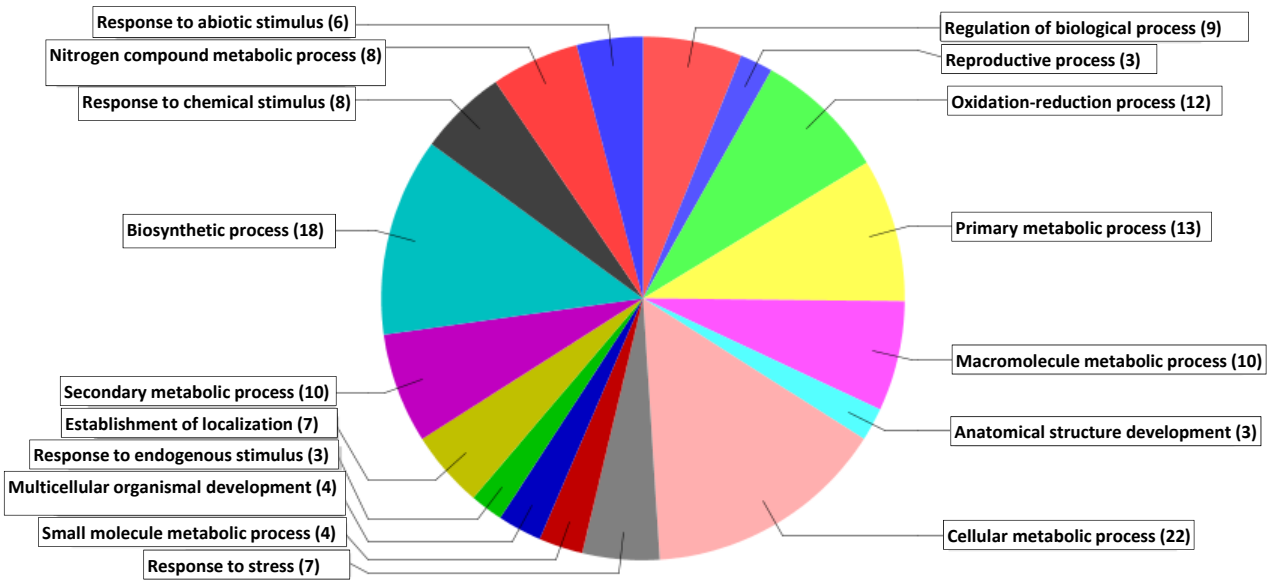

Figure 1: Annotation of differentially expressed genes using Blast2Go (biological processes level 3).

RESULTS AND DISCUSSION

About 25 to 30 million reads were generated per sample. The RNA reads were subjected to quality control using FastQC. The reads had a mean quality of 30, attesting for the high quality of sequencing. An average of 75% of total reads could be mapped to the grapevine genome. The mapped reads were submitted to Cufflinks and Cuffdiff. Fifty five genes were found to be differentially expressed of which 29 were up-regulated and 26 were down-regulated.

The differentially expressed genes were found to be involved in various biological processes. Genes involved in metabolic processes (cellular and primary metabolisms) appear to be over-represented suggesting that the plants are undergoing a metabolic switch when infected with GLRaV-3. Genes that are involved in response to biotic stress or external stimuli were also represented as well as genes involved in oxidation-reduction processes. This suggests that the plants are deploying some defense mechanism against the virus. Further downstream analysis of this data is still needed to make more solid conclusions. The differentially expressed genes will be further validated using RT-qPCR.

ACKNOWLEDGEMENTS

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ELECTRONIC RESOURCES

FastQC: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Blast2GO: http://www.blast2go.com/b2ghome

TopHat: http://tophat.cbcb.umd.edu/index.html

Cufflinks: http://cufflinks.cbcb.umd.edu/index.html

Effects of Irrigation on Leafroll Symptom Development in Cabernet Sauvignon Vines

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INTRODUCTION

Leafroll disease (LR) is an increasing problem in Israeli vineyards as well as in many grape growing areas around the world. The severity of symptoms in infected vines in a vineyard is variable: some showing early reddening that develop to clear rolling of the leaves while in others the symptoms are mild and develop late in the season, sometimes only after harvest. Neighboring vineyards blocks, planted from the same source of propagation material but grown by different people may have different incidence of symptomatic vines. Furthermore, in some instances non-symptomatic vines in infected blocks were tested by PCR and found to be positive to GVLRaV3. Such "symptomless" infection was reported before by Christov *et al*., (2007) in nurse cultures *in vitro* but to our knowledge not in field grown vines. The horticultural performance of the symptomless infected vines resembled that of healthy, not infected vines more than that of infected symptomatic vines. This raises the question whether leaf symptoms are the cause of reduced quality in LR infected vines or they are one among other effects expressed by infected plants. Several authors discuss the effect LR virus has on the photosynthetic activity of the vine. Bertamini (2005) showed a reduction in photosynthetic pigments, leaf proteins and PSII activity in LR3 infected Lagrein and LR1 infected Merzemino vines; Cabaleiro *et al*. (1999) showed that the photosynthesis rate of green leaves was nearly double compared to that of red symptomatic leaves in the same vine. This suggests that any treatment that will suppress the symptoms may result in higher assimilation rate and therefore better vine performance. The objective of the present study was to test the effect of vine water status at different phenologic stages on symptom expression, virus titer and some physiological parameters.

MATERIALS AND METHODS

An irrigation experiment was set up in a 12-year old heavily infected Cabernet sauvignon block in the Golan Heights, Israel. Two factors were examined: phenological stage (from bud-burst to fruit-set, fruit-set to veraizon and from veraizon to harvest) and irrigation treatments of (1) the vines were well irrigated to ensure minimum stress (midday stem water potential ranged from -0.6 MPa to -0.8 MPa); and (2) vines were stressed so that their midday stem water potential ranged from -1.2 MPa to -1.4 MPa. Midday stem water potential was measured weekly and irrigation rates were adjusted to maintain the threshold range. The experimental design was complete factorial design (three phenological stages X two irrigation treatments = 8 treatments). Each treatment was replicated five times with 12 vines per replicate surounded by buffer rows and vines.

Photosynthesis rate, stomatal conductance and pigment levels were measured in representative leaves, The time of symptom appearance in each vine was monitored. Maturation parameters (sugar, pH, TA and color) were measured along the season; the crop yield pruning weight were measured. Virus titer will be measured during the third season of the experiment with real-time PCR.

RESULTS AND DISCUSSION

Most of the vines showed their first symptoms from fruit set to veraizon. The irrigation treatments up to fruit set did not affect the vine symptoms, while vines from the high irrigation during the second stage had significantly more symptomatic vines compared with the low irrigation treatments. (Fig. 1). Leaves from the high irrigation treatment had higher levels of anthocyanin (501 µg/mg) compared with the low irrigation treatment (330 µg/mg). Photosynthesis rates of the low irrigation treatment were lower than the high irrigation treatment (not shown), but higher photosynthesis rates were measured in the low irrigation treatment for each value of stomatal conductance (Fig. 2).

It is not yet clear if this efficiency is due to the different water potential or to the lower symptoms expression and anthocyanin levels in the less irrigated vines.

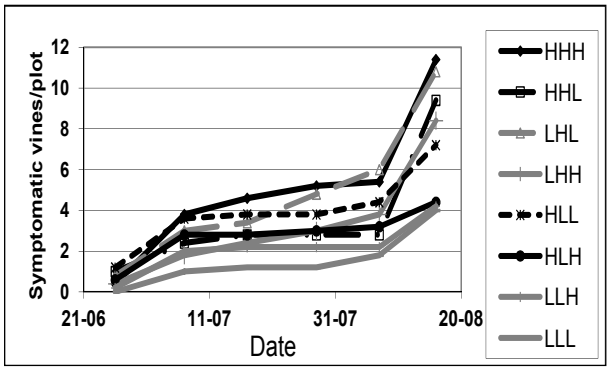

Fig. 1: Symptom development in the different irrigation treatments. H-well irrigated, L- sparsely irrigated, The letters describe the irrigation level at the 1st, 2nd and 3rd phenologic stages.

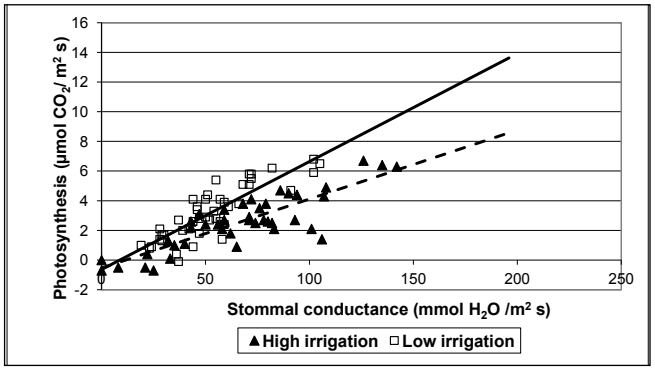

Fig 2: Effect of stomatal conductance on photosynthesis of well and sparsely irrigated vines. H-well irrigated, L- sparsely irrigated, The letters describe the irrigation level at the 1st, 2nd and 3rd phenologic stages.

Brix levels at harvest were higher in vines that received low irrigation rates before harvest (Fig. 3). Vines that received high irrigation treatment from fruit set to veraizon had higher crop yield (Fig. 4). While the effect of irrigation on fruit development is clear more research is needed to quantify the effect of the irrigation on virus development, symptom expression and their combined effect on the vine performance.

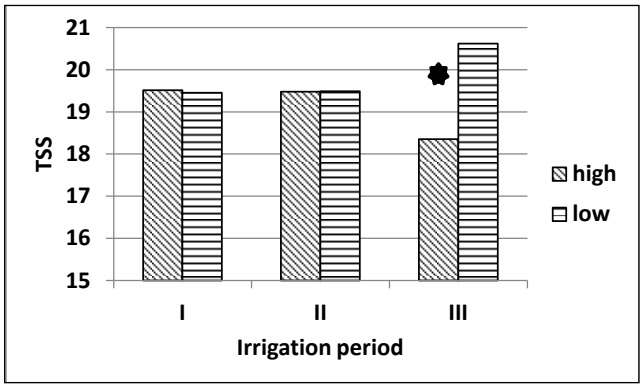

Fig. 3: Average brix level at harvest, grouped by the three irrigation periods.

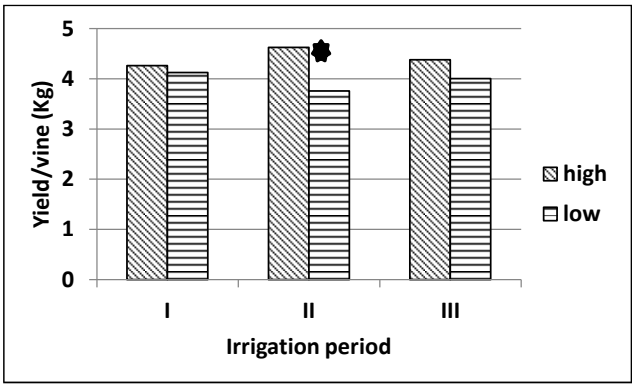

Fig. 4: Average yield (Kg/vine) grouped by the three irrigation periods.

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Major Yield Loss in Shiraz Vines Infected with Australian Shiraz Disease Associated with *Grapevine virus A*

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INTRODUCTION

Vitis vinifera cv. Shiraz (syn. Syrah) is one of the most widely planted premium red wine varieties in Australia. Shiraz is sensitive to viruses, especially to *Grapevine virus A* (GVA). GVA infected vines show restricted growth in early spring while in autumn the leaves turn red and remain on the canopy through to the winter and the wood is poorly lignified. In Australia, the disease was first reported in 2001 by Habili and Schliefert and named Australian Shiraz Disease (ASD) by Habili and Randles in 2004. It is similar to Shiraz Disease in South Africa which was reported by Corbett and Wiid in 1985.

Growers in Australia tend to graft unwanted existing varieties with desirable wine varieties by “top-working”. If Shiraz, Merlot or Sumoll are grafted to any GVA infected rootstock the graft will develop the symptoms of ASD and will eventually decline. In a survey conducted six years ago GVA was present in 6% of 3764 samples tested (Habili and Randles, 2004). White varieties like Semillon, Chardonnay, Viognier and even the red variety Cabernet Sauvignon are tolerant to GVA and do not show the ASD symptoms. We studied the effect of GVA on the yield of Shiraz plants grafted onto virus infected Chardonnay. Single-tube RT-PCR was used for the detection of GVA both in the spring and in early autumn.

MATERIALS AND METHODS

A five year old vineyard of cv. Shiraz grafted onto cv. Chardonnay rootstock at Willunga, South Australia was surveyed in the spring of 2011. About 5% of the vines showed restricted spring growth (RSG), a symptom associated with ASD (Fig 1.). Twenty randomly selected asymptomatic vines and 20 of the RSG vines were labelled for berry weight measurements later in the season. Additionally, four of the RSG vines and three of the unaffected vines were sampled for RT-PCR analysis. The samples were tested for GVA and 11 other viruses as described in the following URL: http://www.agwine.adelaide.edu.au/facilities/wdiag.html

The primer pair designed by Minafra and Hadidi (1994) and used by us were GVA-H7038: AGGTCCACGTTTGCTAAG and GVA-C7273 : CATCGTCTGAGGTTTCTACTATGVA targeting the gene for RNA binding protein of the virus and giving an amplicon size of 236 bp. Samples were tested once in the spring and once during the harvest.

RESULTS AND DISCUSSION

Fig 1 shows the Shiraz/Chardonnay vineyard in which the symptomatic vines were clustered in the middle two rows. Samples from four vines that showed RSG, an early symptom of ASD, tested positive for GVA, while the healthy vines tested negative for GVA (Fig 2). Both healthy and infected samples tested positive for *Grapevine rupestris stem pitting-associated virus* (GRSPaV), a virus which is present in nearly all Australian grapevine varieties and is not associated with symptoms. The tests were repeated at harvest in March 2012 and a similar result was obtained confirming that GVA was associated with ASD.

Grapevine berry weight analysis: The berry weight for 20 randomly selected healthy vines was 110.20 kg in 2012 while that for the 20 ASD infected vines was 2.40 kg, a reduction in yield of 98%. Since grapes are harvested by machines in Australia, the small bunches of ASD infected vines are not harvestable and easily missed by the machine. In practice the yield from infected vines is nil and the growers have to destroy the ASD infected vines. The vines at Willunga, South Australia, have been visually monitored for virus spread and none has been observed. It is believed that GVA was introduced to the vineyard via infected cuttings. GVA-associated ASD has now been detected as far away as the Hunter Valley wine region of New South Wales.

Fig.1, Five year old Shiraz vines grafted on Chardonnay at Willunga, South Australia. Vines showing restricted growth (a symptom for ASD) tested positive for GVA, while healthy vines tested negative.

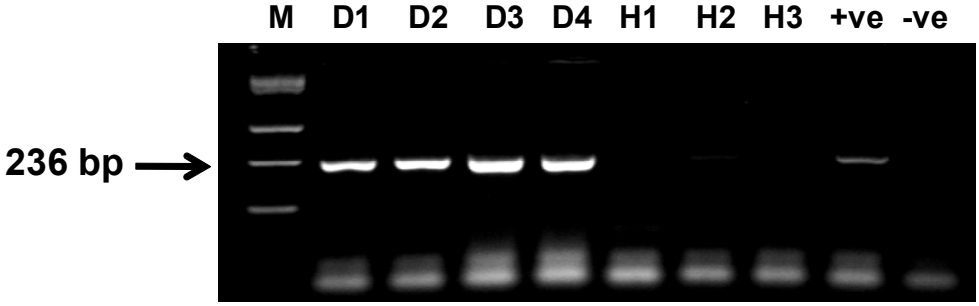

Fig. 2, Single tube RT-PCR amplicon of GVA (236 bp) from the grapevine cv. Tested Shiraz samples showing Australian Shiraz Disease (D1-D4) or not showing the disease (H1-H3). Water was used as negative, M; DNA markers.

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Elimination of GLRaV-1 and GVA Mixed Infection: Effects on Field Performances and Wine Quality in a Clone of 'Nebbiolo' (*Vitis vinifera* L.)

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INTRODUCTION

Leafroll, one of the most important virus disease 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 limited literature available, GLRaV-1, often in mixed infection with GVA, is reported to reduced growth and yield but generally without affecting fruit maturity (Credi *et al*., 1997; Mannini *et al*., 2003; Tomažič *et al*., 2005; Komar *et al*., 2007). The presence of a mixed infection of GLRaV-1 and GVA resulted also detrimental on leaf net photosynthesis and chlorophyll content (Santini *et al*., 2011). The aim of this study was to ascertain the effect of GLRaV-1 and GVA elimination on grapevine agronomic and enological 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' formerly infected by a mixed infection of GLRaV-1 and GVA and tested free from GFLV, GLRaV-3 and GFkV was heat-treated obtaining the eradication of both viruses. Cuttings collected from the originally infected (MP) and from the heat-treated (HT) mother plants were propagated and kept in collection. In 2002 scions of both MP and HT lines were propagated by grafting on healthy Kober 5BB rootstocks and planted in two parallel and facing rows of 30 vines each. Along the rows 15 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 (15 +15) was controlled by DAS-ELISA on dormant cane samples collected during 2009-2010 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 all the diseased progeny was GLRaV-1 and GVA infected and free from ArMV, GFLV, GLRaV-2, GLRaV-3 GFkV and GVB, and that the HT line was free from all the viruses previously mentioned. In 2010, the main agronomic and juice qualitative parameters were assessed on each single selected vines. Field data were statistically elaborated by ANOVA. In addition a sample of around 300 berries was collected from the same vines in order to carry out analyses of berry phenolic content. For each sanitary status, the total crop of the 15 selected vines and of other 15 non-tested contiguous 'sister' vines (around 40 kilos of grape) was submitted to small scale winemaking. Chemical and sensory evaluations were then performed on the wines after a few months of rest in the bottle. Sensory evaluations were carried out by a 'duo-trio' tasting test (i.e. the panel must pick out the two identical wines among a group of three) followed by a paired-preferences test. A characterization test was also used to investigate the intensity of the different components of colour, bouquet and taste.

RESULTS AND DISCUSSION

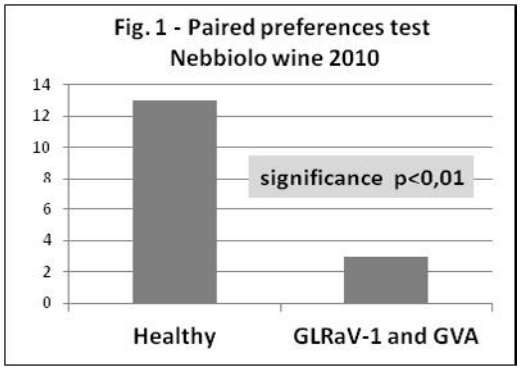
The elimination of GLRaV-1 and GVA induced an increase of vine vigor but, quite surprisingly, reduced the amount of crop due to a lower number of clusters/vine with smaller berries (Tab. 1). Juice sugars concentration was in favour of healthy vines, as expected as a consequence of lower yield, but with an higher juice acidity. Among grape qualitative parameters, total anthocyanins (responsible of wine colour) and total flavonoids (responsible of wine body) resulted increased by virus elimination (tab. 1). The chemical analyses showed the wines had a similar degree of alcohol but in the product of healthy plants titratable acidity was higher although partly compensated by a superior salification (Tab. 2). In terms of enological quality the most interesting difference as a consequence of GLRaV-1 and GVA elimination was related to the color intensity, higher in the wine obtained from healthy vines. The amount of total anthocyanins, however, was similar in the two wines so the more intensely coloured aspect of HT wines could probably be due to modifications in the berry anthocyanin profile. Santini *et al.* (2010) recently showed that GLRaV-1 eradication affected the peonidin-3-glucoside/malvidin-3-glucoside ratio in favour of malvidin-3-g, more determinant for the intensity and the stability of future wine. This result is of particular interest for Nebbiolo wines, whose colour is usually a critical point. Sensory

analysis carried out by a trained panel of 15 tasters overcame successfully the 'duo-trio' test confirming the two wines in comparison were distinguishable with the support of statistical significance. In addition, according to the previous analytical data, the panel pointed out that the major difference between the two wines was the depth and the shade of the colour, preferring for these important descriptors the wine made with grapes of HT vines (Fig. 1). In addition to the brighter ruby color with an intense violet hue, this wine showed a richer flowery and spicy bouquet and a more bodied and long lasting taste than the wine from infected plants (Fig. 2).

In conclusion the eradication of the mixed infection of GLRaV-1 and GVA induced in 2010 vintage significant modifications in the agronomic and enological performances of vines belonging to a clone of 'Nebbiolo'. In the present trial, sanitation unexpectedly reduced crop (around 40 %) unlike what reported in literature, however it produced beneficial effects on grape anthocyanins (berry red pigments) and consequently on the intensity of wine colour. The improvement of colour was confirmed by both chemical analyses and sensorial tests which evidenced also beneficial effects on wine bouquet and body. Despite the yield reduction, the overall effect of GLRaV-1 and GVA elimination can still be considered profitable since bunch thinning (i.e. removing at least 30 % of clusters on plant at veraison) is a common field practice for 'Nebbiolo' cultivation in order to reduce crop and improve grape maturity.

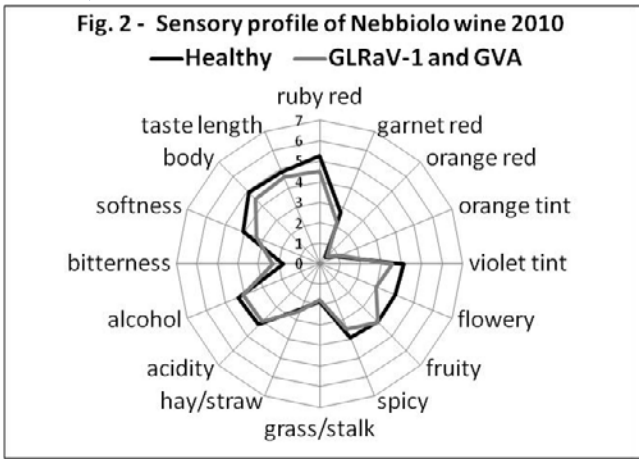
Tab. 1 - Field performances and juice composition of Nebbiolo (2010)
All data are expressed as average values.
Significance: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, ns = not significant.

	Healthy	GLRaV-1 and GVA	F
Yield (kg/vine)	1,22	1,99	*
Bunch wt (g)	197	198	ns
Berry wt (g)	1,79	1,91	*
Bunches/vine (n°)	6	10	***
Pruning wood wt (g/vine)	477	341	*
Soluble solids (°Brix)	25,3	24,4	***
Titratable acidity (g/L)	7,0	6,5	*
pH	3,08	3,11	ns
Total phenols (mg/kg)	747	689	–
Total anthocyanins (mg/kg)	2757	2492	–



Tab. 2 - Nebbiolo wine composition (2010)

	Healthy	GLRaV-1 and GVA
Alcohol (%vol)	14,34	14,21
Dry extract (g/L)	22,5	20,1
Titratable acidity (g/L)	5,93	5,27
pH	3,48	3,56
Tartaric acid (g/L)	1,06	0,99
Lattic acid (g/L)	1,64	1,60
Potassium (mg/L)	1101	976
Ash (g/L)	2,60	2,30
Total phenols (mg/L)	1561	1591
Total anthocyanins (mg/L)	126	130
Colour intensity (A420+520+620)	6,24	5,26
Colour hue (A420/520)	0,73	0,77



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Effects of Grapevine Leafroll Disease on Photosynthesis in a Red-fruited Wine Grape Cultivar

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INTRODUCTION

As obligate parasites, viruses cause pronounced changes to host plant at different levels. Apart from virus-induced physiological and biochemical perturbations, infected plants exhibit a wide range of symptoms such as mosaic, chlorosis and vein clearing that are largely dependent on specific virus-host interactions. In the case of grapevine leafroll disease (GLRD), a complex virus disease showing distinct symptoms in red- and white-berried wine grape (*Vitis vinifera* L.) cultivars (Rayapati et al., 2008), affected vines begin to show symptoms on mature leaves near the basal portion of shoots during or soon after *véraison*. These symptoms extend upwards to other leaves as the season advances. Thus, unlike other plant virus diseases, GLRD consists of asymptomatic and symptomatic phases corresponding, respectively, to two broad phenological stages, namely pre-*véraison* or berry development and post-*véraison* or berry ripening. In this study, we measured impacts of *Grapevine leafroll-associated virus 3* (GLRaV-3) on photosynthesis and associated events in own-rooted Merlot grapevines during pre- and post-*véraison* stages and compared with corresponding samples from virus-free vines under field conditions.

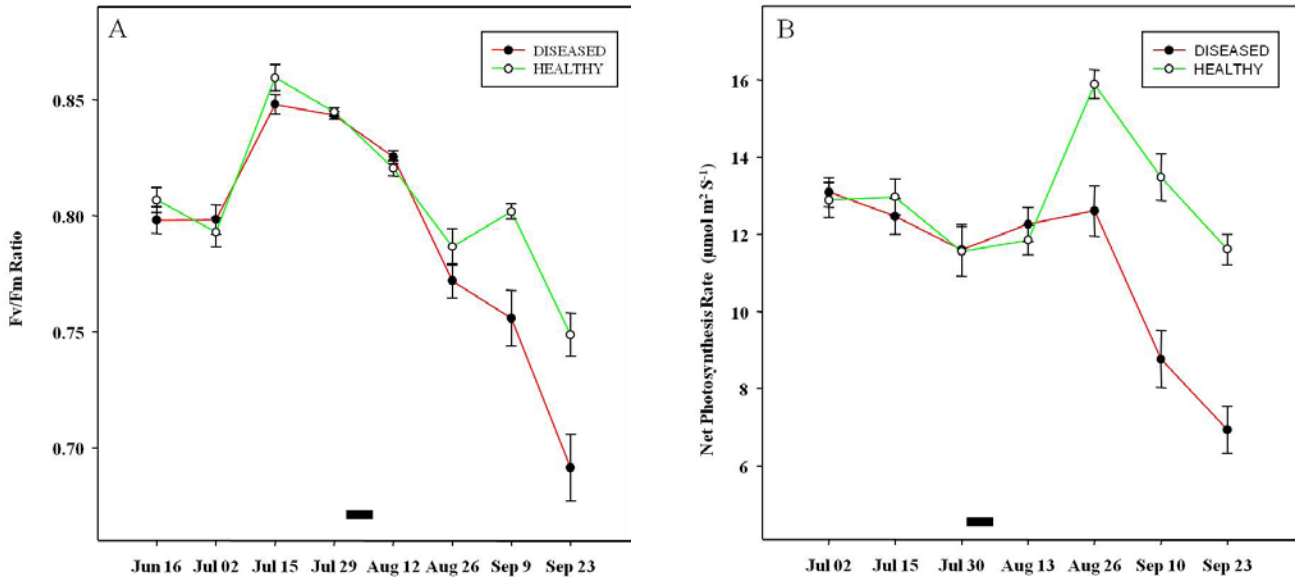
MATERIALS AND METHODS

Own-rooted grapevines (cv. Merlot) in a 10 year-old commercial vineyard block were selected in such a way that vines tested positive for GLRaV-3 and showing GLRD symptoms (GLRD+ve) and nonsymptomatic vines tested negative for the virus (GLRD-ve) are located adjacent to each other in a given row. This allowed to minimize possible errors in experimental results due to variations in soil and other growing conditions. *In situ* Chl a fluorescence was measured as the ratio of variable to maximal fluorescence (Fv/Fm) in intact leaves from GLRD +ve and –ve grapevines using a portable pulse-amplitude modulated Fluorescence Monitoring System 2 (Hansatech Instruments Ltd, UK). The rate of photosynthesis (Pn), stomatal conductance (Gs), internal CO_2 concentration (C_i) and transpiration rate (E) were measured during pre-*véraison* and post-*véraison* using a portable photosynthesis system (CIRAS-2, PP Systems, Amesbury, MA). All measurements were taken between 9.00AM to 1.00 PM during the day time using `fully expanded leaves` at the basal portion of canes at two phenological stages corresponding to asymptomatic stage (pre-*véraison*) and symptomatic stage (post-*véraison*). At each time point, measurements were taken from three separate leaves per vine and the same leaves were used for all measurements. The transcript levels of five genes involved in photosynthesis were analyzed by reverse transcription quantitative PCR (RT-qPCR) relative to three stable reference genes (Gutha et al., 2010). Sugars and starch were estimated using commercial kits (GAHK-20 for glucose, SCA-20 for sucrose, FA-20 for fructose, STA-20 for starch, Sigma-Aldrich, St. Louis, MO). Values were subjected to statistical analysis using Student's *t* test (Sigma Plot 11.0) and differences between healthy and virus-infected leaves were considered significant ($P < 0.05$).

RESULTS AND DISCUSSION

In own-rooted Merlot grapevines planted under cool-climate conditions of Washington State, GLRD symptoms begin to appear during early August, as the crop moves toward *véraison*, and typical disease symptoms become apparent during September and October. Therefore, we selected two time-points corresponding to asymptomatic (pre-*véraison*) and symptomatic (post-*véraison*) stages of GLRD for our studies and the data was compared between these two phenological stages across three seasons in (2009, 2010 and 2011).The results showed that Chl a fluorescence and net photosynthetic rate, including other photosynthetic gas exchange parameters, were indistinguishable between GLRD+ve and –ve leaves during pre-*véraison*, suggesting that GLRaV-3-infected leaves exhibit normal photosynthetic capacity during asymptomatic stage of GLRD. In contrast, an overall down regulation of Chl a fluorescence and photosynthesis occurred in virus-infected leaves concomitant with symptom

Fig. 1. (A) *Chl a* fluorescence (Fv/Fm) and (B) net photosynthetic rate in GLRD+ve (infected, red line graph with closed circles) and GLRD-ve (non-infected, green line graph with open circles) leaves of Merlot grapevines at different periods during the 2009 crop season. Each value represents the mean of values from three separate leaves and vertical bar indicates ±S.E.
'*' represents statistically significant differences at p ≤0.05. ▬ Represents approximate time of *véraison*.



development during post-*véraison* (Fig. 1). Total chlorophyll, on a fresh mass basis, was reduced by 24.90%, 10.59% and 21.67% in 2009, 2010, and 2011, respectively, in symptomatic leaves during post-*véraison*. No significant difference in total chlorophyll was observed in GLRaV-3-infected leaves at the asymptomatic stage when compared to corresponding values from GLRD-ve samples. The relative transcript abundance of photosynthesis-related genes showed reduced levels of expression of small sub unit of ribulose-1, 5-bis phosphate carboxylase (*rbcS*) and sedoheptulose-7-biphosphate (*SBP*) in GLRD+ve leaves at symptomatic stage than in asymptomatic stage when compared to corresponding values from GLRD-ve leaves. Both *rbcS* and *SBP* play a key role in carbon fixation during photosynthesis. Similarly, expression of photosystem II protein D1 (*psbA*) and photosystem I P700 apoprotein A1 (*psaA*), which encode core subunits of the reaction centers of PSII and PSI, respectively, and light-harvesting chlorophyll-binding protein of photosystem I (*Lhca3*), a nuclear encoded protein involved in light-harvesting and transfer to the reaction centre of thylakoid membrane, were repressed in virus-infected symptomatic leaves than in virus-infected asymptomatic leaves, when compared to corresponding values from GLRD-ve leaves. These results imply transcriptional downregulation of key photosynthetic genes leading to reduction of photosynthesis in symptomatic leaves. Our results also showed that sugars and starch levels were similar in virus-infected and healthy (GLRD-ve) leaves during asymptomatic stage but their levels increased several-fold in virus-infected, symptomatic leaves during post-*véraison*, implying possible link between carbohydrate status and the development of GLRD symptoms. Implications of these results for a better understanding of the symptomatology of GLRD in red-berried wine grape cultivars will be discussed.

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Impacts of Grapevine Leafroll Disease on Own-rooted Wine Grape Cultivar in Cool Climate Viticulture

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INTRODUCTION

Grapevine leafroll disease (GLRD) is one of the most serious and complex diseases of grapevines (Rayapati et al., 2008). Among the several closteroviruses associated with GLRD, *Grapevine leafroll-associated virus 3* (GLRaV-3, genus *Ampelovirus*, family *Closteroviridae*) is the most widespread worldwide. Studies have shown that viruses, in particular GLRaVs, can cause reduced plant vigor and longevity, and significant losses in both yield and quality of the fruit (Golino et al., 2009; Lee et al., 2009; Komar et al., 2010). However, these studies have been conducted with grafted vines planted in different geographical regions. In this study, we have evaluated impacts of GLRD on an own-rooted wine grape cultivar grown under cool-climate conditions of eastern Washington.

MATERIALS AND METHODS

The study was conducted during three consecutive seasons (2009 and 2011) on a 10 year-old commercial vineyard block located in Yakima Valley of Washington State. Fifteen pairs of own-rooted grapevines (*Vitis vinifera*, cv. Merlot), with each pair consisting of a healthy vine (tested virus-free) and a GLRD-affected vine (tested positive for GLRaV-3) located adjacent to each other in a given row, were selected for this study to minimize error in experimental results due to variations in soil and other growing conditions. Berries were collected from healthy and infected vines during various stages of berry development before and after *véraison* (onset of berry ripening). Juice extracted from 100 berry samples collected randomly at each sampling point from each vine was used separately to measure ºBrix, pH, titratable acidity (TA) and total anthocyanins as per standard protocols (Iland et al., 2000). At the time of commercial harvest, data on total fruit yield per vine was collected from an additional 15 pairs of vines in the same commercial block, with each pair consisting of healthy and GLRD-affected vines located next to each other in a given row.. Cane pruning weight was collected during the winter season. Small-lot wine made from grapes harvested from GLRD-affected and healthy vines in the same commercial block were evaluated for their alcohol content, polymeric pigments, total anthocyanins, total iron reactive phenolics and tannins following standard protocols (Iland et al., 2000). Wines were also subjected to sensory analyses by a panel of trained sensory analysts. All the data was analyzed statistically for significant differences between healthy and GLRD-affected vines.

RESULTS AND DISCUSSION

As shown in the Table 1, 15-30% fruit yield reduction was observed in GLRD-affected vines between 2009 and 2011 seasons. This reduction was largely contributed by less number of bunches (15-17% reduction) produced by affected vines. At commercial harvest, 4-9% reduction in soluble solids was observed in grapes harvested from GLRD-affected vines. Although the data showed variation between seasons, likely due to differences in climate conditions, the results clearly showed negative impacts of GLRD on vine performance and quality of grapes produced by own-rooted Merlot grapevines. Fruit maturity indices (soluble solids and fruit acidity) and total anthocyanins were measured during different stages of berry development in three seasons (Fig. 1).The results indicated consistently lower levels of total soluble solids (ºBrix) in berry extracts from GLRD-affected vines compared to healthy vines. The difference in these values increased with berry ripening and it was significantly higher during post-*véraison*. Extracts from berries of GLRD-affected vines showed higher levels of acidity and lower levels of TA throughout berry development and ripening. However, differences in the TA values were not as apparent as with ºBrix. The amount of total extractable anthocyanins was significantly less in GLRD-affected vines during berry ripening. At commercial harvest, the difference in total anthocyanins was less apparent between berries from GLRD-affected and healthy vines. Small-lot wines made from grapes harvested in 2010 season from GLRD-affected grapevines had significantly less amounts of pigments (anthocyanins, small- and

Table 1. Impact of GLRD on fruit yield and soluble solids (°Brix) in cv. Merlot.

Parameter	2009		2010		2011	
	Healthy	**Infected**	**Healthy**	**Infected**	**Healthy**	**Infected**
# Bunches/vine	90.73±5.57	75.13±6.62 **(-17.19%)**	83.5±4.00	69.3±2.50 **(-17.00%)**	116 ± 12.56	99 ± 16.13 **(-14.66%)**
Fruit yield/vine (kg)	4.51±0.60	3.18±0.63 **(-29.49%)**	3.84±0.73	3.25±1.47 **(-15.36%)**	5.68 ± 0.91	4.51 ± 0.89 **(-20.57%)**
°Brix	26.06±0.13	23.68±0.12 **(-9.13%)**	25.2±0.15	23.7±0.25 **(-5.60%)**	23.50 ± 0.05	22.53 ± 0.15 **(-4.11%)**

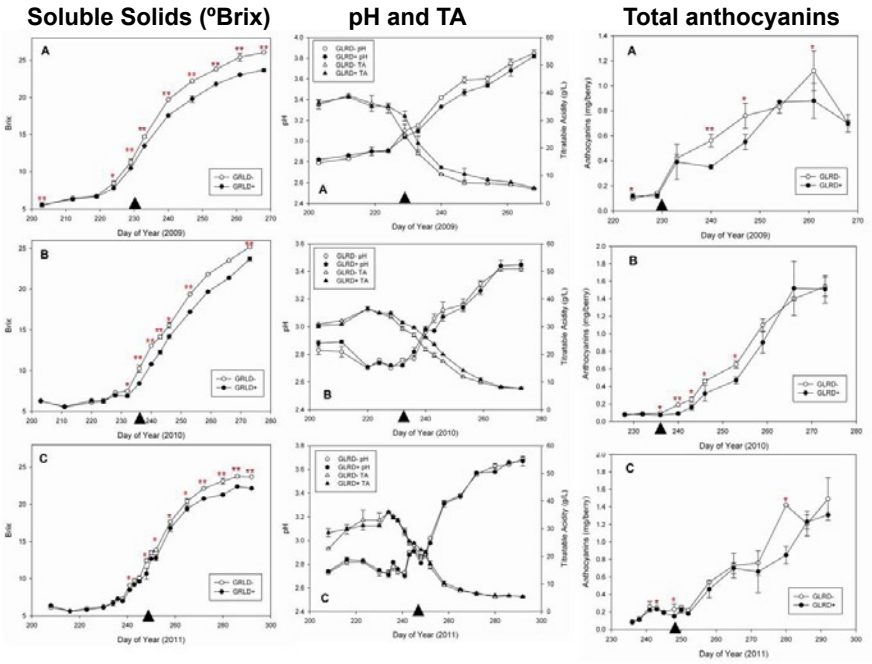

Figure 1. Time-course analyses of impacts of GLRD on fruit maturity indices (total soluble solids, pH, titratable acidity and total anthocyanins) in own-rooted wine grape cv. Merlot in (A) 2009, (B) 2010 and (C) 2011 crop seasons. Each data point represents means of 5 replicates per treatment. Approximate date of *véraison* in each season is denoted by ▲. Statistical significance determined by one-way ANOVA: (* = $p < 0.05$; ** = $p < 0.001$).

large-polymeric pigments), phenolics, tannins and alcohol than wines made from fruits harvested from healthy grapevines. A descriptive analysis indicated that small-lot wines from healthy vines were more purple, less brown and more saturated in color with a higher predominance of red fruit aroma and a lower predominance of earthy character than wines from berries of GLRD-affected vines. Negative impacts of GLRD on sensory analysis of wines were observed in 2010 vintage while no such effect was observed in 2011 vintage. In summary, our results demonstrated that GLRD decreases fruit yield and negatively impacts fruit and wine quality in own-rooted Merlot grapevines under cool-climate conditions of eastern Washington State.

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This study was funded, in part, by the Agriculture Research Center, Washington State University College of Agriculture, Human and Natural Resource Sciences, the Wine Advisory Committee of the Washington Wine Commission, USDA-NIFA -Specialty Crop Research Initiative (Award No. 2009-51181- 06027) and USDA Northwest Center for Small Fruits Research.

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The Influence of *Grapevine fanleaf virus* (GFLV) on the Yield of Vines of Cultivar Pokalca Trained by Two Training Systems

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INTRODUCTION

Grapevine fanleaf virus (GFLV), the causal agent of grapevine fanleaf degeneration disease, is the member of genus *Nepovirus*. Its genome is composed of two single-stranded positive sense RNA molecules (RNA1 and RNA2). Each of the two genomic RNA encodes a polyprotein, which is processed into functional proteins by the RNA1-encoded protease (Andret-Link et al., 2004). It has been shown that GFLV exists as a mixture of different genotipic variants (Naraghi-Arani et al., 2001). The concern on the biological and economical impact of the different GFLV variants has activated studies on the biological diversity of the virus. The genetic diversity of GFLV has been assessed of the partial or complete $2C^{CP}$ gene (Naraghi-Arani et al, 2001; Vigne et al., 2004; Fattouch et al., 2005). In our laboratory, the genetic variability of GFLV was assessed within RNA2, including $2A^{HP}$, $2B^{MP}$, and $2C^{CP}$ genes by immunocapture (IC) - reverse transcription (RT) - polymerase chain reaction (PCR) - restriction length fragment polymorphism (RFLP), followed by cloning and sequencing. Sequence analysis of cloned RNA2 ORF amplicons obtained by IC-RT-PCR showed presence of mixed infections and slightly higher nucleotide variability in the $2A^{HP}$ and $2C^{CP}$ genes relative to the $2B^{MP}$ gene. Also, gene $2A^{HP}$, unlike genes $2B^{MP}$ and $2C^{CP}$, had a variable size (765-774 nucleotides) and high amino acid diversity (up to 15%). In addition, a recombination event was identified at nucleotide position 220-225 of gene $2A^{HP}$. No clear association was apparent between symptomatology and restrictotype composition, phylogenetic clustering, or occurrence of recombination (Pompe-Novak et al., 2007).

GFLV is naturally spread by the nematode vector *Xiphinema index* and through the use of infected planting material. Although several means for the control of virus spread are possible, testing of vines in the frame of certification programmes by efficient GFLV detection methods and usage of healthy planting material is crucial. In our laboratory, a TaqMan® one-step reverse transcription real–time PCR (RT-qPCR) assay was developed for the specific detection and quantification of GFLV with the sensitivity approximately 1000-fold higher than the sensitivity of the conventional ELISA assay. The developed method is applicable for high-throughput diagnosis of GFLV in different types of grapevine material including dormant phloem scrapings. The quantitative nature of the assay was evaluated by monitoring the seasonal variation of the GFLV amount present in the plant phloem (Čepin et al., 2010).

Although the grapevine fanleaf degeneration disease was reported already in 1883 by Rathay (Raski et al., 1983) and it is recognised as one of the most important viral diseases of grapevine resulting in a progressive decline of infected vines, yield loss and poor fruit quality in all wine producing areas in the world (Pearson and Goheen, 1998), there are very little experimental data on the influence of GFLV on the yield reduction. Yield reduction by over 20% caused by GFLV infection was reported in cultivar Callet (Cretazzo et al., 2009). In our laboratory, in the recent study the influence of GFLV on the yield of vines trained by two different training systems was investigated in cultivar Pokalca.

MATERIALS AND METHODS

26 vines (16 healthy and 10 GFLV infected) of cultivar Pokalca trained in single Guyot system and 20 vines (9 healthy and 11 GFLV infected) of cultivar Pokalca trained in double Guyot system were selected for the study in a vineyard in Prepotto, Italy. At harvest, yield and the 100-berry weight were determined for each vine. Average and standard error were calculated. The degree of statistically significant difference between healthy and GFLV infected plants was calculated by Student's t-test.

RESULTS AND DISCUSSION

The results showed no difference in yield (figure 1A) and in 100-berry weight (figure 1B) between single and double Guyot training systems for healthy vines, while different results were observed when plants were virus infected. In case of single Guyot, the yield and the 100-berry weight of GFLV infected grapevines were statistically lower as compared with the healthy vines, while in the double Guyot training system no impact of GFLV on the yield and the weight of 100 berries was detected.

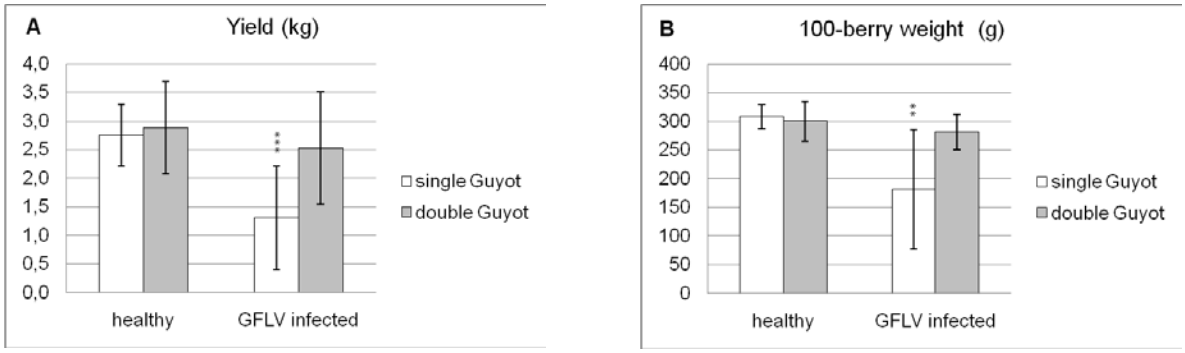

Figure 1: Yield (A) and 100-berry weight (B) of healthy and GFLV infected vines in single and double Guyot training systems. Averages for healthy and GFLV infected plants were separated with *t*-test (**, p<0,01; ***, p<0,001). Average ± standard deviation is shown.

We can conclude that GFLV infection had a different influence on the yield of vines of cultivar Pokalca when trained on two different training systems.

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Virological Problems of Native Cycladian Grapevine Varieties

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INTRODUCTION

Cycladian vineyards are characterized by the presence of native self-rooted propagative material usually spread along small areas. The study of the phytosanitary status of this material is very interesting since these cultivars are grown in the cycladian islands over centuries. During a project sponsored by the local South Aegean government 60 self-rooted grapevine plants originating from 17 different native varieties were labeled and screened for the presence of viruses.

MATERIALS AND METHODS

Screening was originally done on grapevine shoots (cambial scrapings) taken from the labeled vineyards and later on young leaves and old leaf stems originating from the plants kept in pots in the farm of the Institute of Grapevine and Vegetables located in Crete (NAGREF, Heraclion). Different ELISA versions and RT-PCR assays were applied for the detection of the most widespread grapevine viruses. More specifically previously reported RT-PCR assays were used for detecting GLRaV-1, -2, -3 (Dovas et al., 2006), GLRaV-4-9 (Maliogka *et al*., 2009), GVA (Nakaune and Nakano, 2006), GVB (Minafra and Hadidi, 1994) and GRSPaV (Terlizzi *et al*., 2011). Total RNA extraction was done according to Dovas *et al*. (2006).

RESULTS AND DISCUSSION

The results showed higher incidence of the viruses associated with the grapevine leafroll disease (GLRD) and more specifically of GLRaV-3 and the GLRaV-4-9 group (Table 1). GLRaV-7 was also found in 13 of the tested plants. GLRaV-1 was rarely encountered (3/60) while GLRaV-2 was not found in any of the local varieties tested. Apart from the GLRD associated viruses, GFLV and GVA were also prevalent in the area of Cyclades. Nevertheless, GFKV was only detected in one grapevine plant while GVB and GRSPaV were not found in any of the tested varieties.

Table 1. Virus incidence in the grapevine material collected from cycladian vineyards

Virus tested	Number of positives/number of plants tested	
	ELISA	**RT-PCR**
GFLV	14/60	nt
GLRaV-1	2/60	3/60
GLRaV-2	0/60	0/60
GLRaV-3	11/60	18/60
GLRaV-4-9	19/60	21/60
GLRaV-7	13/60	nt
GVA	12/60	12/60
GVB	0/60	0/60
GFKV	1/60	nt
GRSPaV	nt	0/60

nt: not tested

These results show that the cycladian vineyards host specific degenerating viruses. Their detection in asymptomatic grapevine plants can only be explained from the drastic elimination over time of grapevine plants showing severe disease symptoms, which had as a consequence the prevalence of mild virus strains/isolates. The absence of viruses or severe viral strains, which are inducing serious problems in other grapevine areas of the country, is mainly due to the maintenance of vineyards with self-rooted plants. It is especially interesting to mention the high prevalence of the GLRaV-4-9 *Ampelovirus* group, which includes the simplest and possibly the most ancient viruses of the genus, in these really old Greek grapevine varieties. We assume that the attempted recovery of Cyclades' vineyards with American rootstocks and mainly foreign varieties (non-certified material) will result in significant changes in the character of traditional island viticulture and will downgrade the potential promotion of local grapevine genetic material and wine production.

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Virus Effects on Vine Growth and Fruit Composition of Selected Zinfandel Field Selections

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INTRODUCTION

Many Zinfandel growers plant certified rootstocks that are bench grafted or field budded with scion wood collected from vineyards with reputations for producing high quality grapes and wines. Virus diseases are common in vines established in this manner. A trial was established to evaluate the performance of popular Zinfandel field selections believed to have variable incidence of grapevine leafroll disease.

MATERIALS AND METHODS

The trial was established in 2004 in the Dry Creek Valley American Viticultural Area (AVA) in Sonoma County, California. Dormant, certified 110R rootstock (*Vitis berlandieri x V. rupestris*) was planted in 2003 and budded May 2004 with eight Zinfandel field selections and one Primativo field selection. Scion buds were collected from well known Zinfandel vineyards in Sonoma, Napa and Santa Clara counties. Selections are commonly identified by name of vineyard owner; however they are presented as selection numbers herein. Selections 8 and 9 were sourced from one vineyard planted with certified Zinfandel FPS 03 and Primativo FPS 03 respectively grafted on *V. rupestris* ('St George') rootstock. The nine selections were planted 1.8 m by 2.4 m, vine by row, in a randomized complete block design with 6 replications and 8 vines per replicate. Vines were head trained, spur pruned and drip irrigated. At maturity, vines were pruned to 6 to 8 two-bud spurs per vine. Cultural practices were typical for head trained Zinfandel, and were performed by the grower cooperator. Crop load was reduced by shoot thinning in spring to maintain 2 shoots per spur and clusters and/or cluster wings selectively removed at veraison to balance crop load with vine size and prevent crowding which could result in Botrytis bunch rot disease.

Yield component and fruit composition data were collected in 2008-2011. In 2010, yield data were not collected due to high temperatures in August that caused fruit to dehydrate throughout the AVA. All vines, regardless of selection, were harvested on the same day as determined by the winery. All data were taken on a per-vine basis, except for berry weight and juice maturity indices. Clusters were harvested by vine, counted and weighed. Pruning weight per vine was measured each winter. Grapevine leafroll disease symptoms were observed prior to harvest in 2009. Subsequently, samples were collected on two dates for virus testing. Dormant canes were collected on 25-February 2010 from 15 vines ("Sample A") selected the previous fall and virus testing was done by conventional PCR. On 18 June 2012 one vine was randomly selected per replicate in 3 replications for testing for Grapevine leafroll associated virus-2 (GRaV-2) and Grapevine leafroll associated virus-3 (GLRaV-3) on a total of 27 vines ("Sample B") with real-time RT-PCR (Osman *et al.,* 2008). Petioles were collected from each vine utilizing disposable gloves which were discarded and replaced after each vine was sampled to prevent contamination of plant sap across samples (V. Klaassen, personal communication). For analyses of yield and fruit components, across-year treatment differences were revealed using the GLM procedure in SAS version 9.2 statistical software. Year, block, year by block interaction, and block by treatment interaction were treated as random effects. Treatment differences were also analyzed by each individual year using the GLM procedure with no random effects in the model. When treatment effects were significant ($\alpha<0.05$), treatment means were separated using Duncan's new multiple range test. For analyses to determine the effect of the presence of GRaV-2 on yield, yield components and pruning weight, t-tests were run for individual years. For the multi-year analysis of virus effects on these variables, repeated measures were run using the GLM procedure with year treated as a random variable.

RESULTS AND DISCUSSION

There were significant differences in the growth and yield parameters evaluated in field selections within years (data not shown); however, selection effects in these variables – yield, cluster weight, cluster number and pruning weight - were independent of year (Table 1).

Table 1.
Yield, yield components and pruning weights of Zinfandel and Primativo field selections, Healdsburg, CA. 2008-2011.[a]

Field Selection	Yield (kg vine^{-1})	Cluster Weight (g)	Clusters per Vine	Pruning Weight (kg vine^{-1})
Zinfandel 1	4.2 ab[b]	203 b	20 bc	0.62 cd
Zinfandel 2	4.6 a	210 ab	21 b	0.74 b
Zinfandel 3	4.3 a	213 ab	20 c	0.57 e
Zinfandel 4	4.4 a	213 ab	20 bc	0.62 c
Zinfandel 5	4.3 a	211 ab	20 cd[c]	0.59 cde
Zinfandel 6	4.1 ab	198 b	20 bc	0.62 cd
Zinfandel 7	3.8 bc	200 b	19 d	0.58 de
Zinfandel 8	4.6 a	222 a	20 bc	0.59 cde
Primativo 9	3.7 c	156 c	23 a	0.79 a
Significance				
Year	<0.0001	<0.0001	<0.0001	<0.0001
Selection	0.0003	<0.0001	<0.0001	<0.0001
Year * Selection	0.0820	0.0793	0.5612	0.7039

[a]Includes only pruning weight data in 2010. [b]Means within the same column followed by the same letter are not significantly different by Duncan's multiple range test, at p<0.05.

The 15 vines in Sample A were located in 6 replicates and 1 to 4 vines were sampled per replicate. In 5 replicates one or more vines tested positive for GLRaV-2. GLRaV-3 was negative in all samples. Two vines tested positive for GVB and one vine for GVD. All but one vine tested positive for RSPaV. Ten vines in Sample B tested positive for GLRaV-2; two of these vines were also positive for GLRaV-3.

The presence of GLRaV-2 in Sample A and Sample B vines resulted in significantly fewer clusters per vine and reduced pruning weights as compared to vines testing negative for the virus (Table 2). This is consistent with the standard practice of adjusting cluster number per vine based on vine size; diseased vines are more likely to have reduced shoot length thus more clusters are removed. Vine yield and cluster weight were not affected by GLRaV-2 in this trial suggesting that berry weight may have increased to compensate for reduced cluster number in diseased vines.

Table 2. Influence of GLRaV-2 status on vine yield, yield components and pruning weight of Zinfandel and Primativo field selections.

	GLRaV-2	2008	2009	2010	2011	Multi-Yr Ave
Yield (kg vine^{-1})	-	3.88	5.04		5.10	4.65
	+	3.54	4.21		4.77	4.16
	Pr>F	0.3143	0.1288		0.5356	0.0700
Cluster Wt (g)	-	203	232		197	211
	+	206	210		214	210
	Pr>F	0.8271	0.2003		0.3258	0.8684
Clusters per vine	-	19.0	21.4		26.1	22.0
	+	17.2	19.8		22.3	19.7
	Pr>F	0.0592	0.2119		0.0400	0.0035
Pruning Wt (kg)	-	0.55	0.58	0.57	0.63	0.58
	+	0.44	0.48	0.50	0.49	0.48
	Pr>F	0.0285	0.1801	0.3710	0.0384	0.0016

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Patterns of Virus Transmission from Hosts with Mixed Infections

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INTRODUCTION

Management practices of crop diseases typically focus on one specific pathogen, but crop plants are often infected with multiple pathogens. Two or more pathogens can have multiple interactions with each other, including competition, cross-protection, or facilitation. These interactions can occur either in the host or in the vector during transmission. With competition, one pathogen is expected to be more fit than another, and will prevail, via higher transmission rates and/or higher replication rates within the host. With cross-protection, the presence of one pathogen in a host or vector hampers the transmission and/or establishment of another pathogen. With facilitation, the presence of one pathogen actually aids the transmission and/or establishment of another pathogen. These interactions among pathogens can affect disease spread and severity in very different ways, leading to different management needs.

Both *Grapevine virus A* (GVA ,*Vitivirus*, *Betaflexiviridae*) and grapevine leafroll-associated viruses (GLRaVs*,*Ampelovirus, *Closteroviridae*) are economically important in *Vitis vinifera* (grape) growing regions worldwide, causing reduced vine vigor, yield, and fruit quality. GLRaV-3 is the most common GLRaV species associated with disease in *V. vinifera*. Furthermore, GLRaV-3 is subdivided into several genetically distinct variants (Turturo *et al.,* 2005; Sharma *et al.,* 2011; Wang *et al.,* 2011). Both mixed species infections of GVA and GLRaV-3 (Notte *et al.,* 1997; Zorloni *et al.,* 2006), and mixed infections of two or more GLRaV-3 variants (Sharma *et al.,* 2011) are common, but little is known about the interactions between virus species or between virus variants within a host or during insect transmission. Knowledge of GVA and GLRaV transmission dynamics is needed to inform management strategies.

Past research has indicated that in *V. vinifera,* GVA may require the presence of GLRaV-1, -3, or -4 in the source plant in order to be transmitted by a vector and establish infection in a susceptible plant (Zorloni *et al.,* 2006; Hommay *et al.,* 2007; Tsai *et al.,* 2010; Le Maguet *et al.,2012*). There is one report of GVA transmission alone in *Nicotiana clevelandii* and transmission of purified GVA by *Pseudococcus longispinus* (Notte *et al.,* 1997), but transmission of GVA alone has not been found in *V. vinifera*. Thus, we expect that GVA does not require other viruses for transmission, yet conclusive evidence is lacking.

We tested the interaction between two virus species, GLRaV-3 and GVA, during transmission by *Planococcus ficus* (Hemiptera, Pseudococcidae), a common vineyard pest. We used symptomatic *Vitis vinifera* cuttings with mixed infections of GLRaV-3 and GVA. We also tested the interaction between two GLRaV-3 variants during transmission by *P. ficus,* from vineyard collected *V. vinifera* with mixed variant infections of GLRaV-3a and -3e.

MATERIALS AND METHODS

To explore transmission dynamics of the two virus species, we tested transmission by *P. ficus* from symptomatic vineyard *V. vinifera* cv. Chardonnay cuttings coinfected with GLRAV-3 and GVA to test plants of *V. vinifera* cv. Pinot Noir in the greenhouse. Source plant cuttings were collected from a site in Napa Valley, and acquisition was started within 48 hours of collection. Acquisition and inoculation access periods were each 24 hours. Inoculations were performed using groups of five first and second instar insects taken from one leaf of each source plant and confining them on the underside of one leaf of each test plant. Ten replicate source plants were used with twenty replicate test plants per source plant. One replicate of twenty negative control test plants was kept in the greenhouse with the inoculated test plants for the duration of the study. Test plants were kept in the greenhouse for four months post-inoculation before testing. Three petioles were then collected from each test plant to maximize the chance of virus detection in the event of localized infections. All test plants were assayed for virus infection with GVA using primers from Minafra *et al.* (1994) and for GLRaV-3 using primers from Sharma *et al.* (2011). One step RT-PCR and fragment analysis (Sharma *et al.,* 2011) were used for virus detection.

To explore transmission dynamics of the two GLRaV-3 variants GLRAV-3a and -3e (Sharma *et al.,* 2011), we tested transmission by *P. ficus* from source cuttings of *V. vinifera* cv. Cabernet Sauvignon coinfected with GLRaV-3a and -3e, using the same experimental design and methods described above. Primers for detection of GLRaV-3a were based on Sharma *et al.* (2011). Primers for detection of GLRaV-3e were developed for this study based on Seah *et al.* (Submitted).

RESULTS AND DISCUSSION

We found all possible combinations of transmission to test plants from source plants with mixed GLRaV-3/GVA infections: no infection (43%), GVA only (2%), GLRaV-3 only (24%), and mixed GLRaV-3/GVA infections (31%). Our results demonstrate that mealybugs can transmit GVA from infected to susceptible *V. vinifera* plants without simultaneous transmission of GLRaV-3. Our findings are consistent with previous studies of transmission studies of GVA in *V. vinifera* (Zorloni *et al.,* 2006; Hommay *et al.,* 2007; Tsai *et al.,* 2010; Le Maguet *et al.,* 2012), and with the findings of Notte *et al.* (1997) based on transmission of purified GVA or GVA in infected *N. clevelandii*. Therefore, spread of GVA in vineyards with or without GLRaV may be a concern for vineyard management. It still has not been shown whether GVA can be transmitted alone in *V. vinifera* without a coinfection by GLRaVs in the source plant. Transmission experiments from singly infected plants will indicate whether the observed patterns are due to competition or facilitation between virus species.

From source plants with mixed GLRaV-3a/3e infections, 56% of test plants became infected. Transmission of GLRaV-3e alone was most common (29%), then transmission of mixed infections (25%), with transmission of GLRaV-3a alone being the least common (2%). Our results indicate that GLRaV-3e is more readily transmitted than GLRaV-3a from host plants with mixed infections; however, GLRaV-3a appears to be more common than GLRAV-3e in Napa Valley (Sharma *et al.,* 2011). Further transmission studies are needed to understand this discrepancy between GLRAV-3a being more common versus GLRaV-3e being more readily transmitted by *P. ficus*. Transmission experiments from singly infected plants will indicate whether the observed patterns are due to competition between GLRaV-3 variants during transmission. Furthermore, testing transmission by other mealybug species will determine if the transmission efficiency of each GLRaV-3 variant is dependent on vector species. Understanding transmission and disease severity has important implications for managing disease spread.

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Monitoring Distribution of Grapevine Leaf Roll Associated Viruses in Turkey

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INTRODUCTION

Northeastern part of Anatolia peninsula located between Black sea and Caspian sea region is the gene source and culture area of the most important varieties of grapevine, *Vitis vinifera* L. Turkey is one of the nations native to grapevine in the Middle East

Therefore, our nation is familiar with grapevine culture for more than 6000 years and has a very rich potential of both wild (*Vitis vinifera ssp. sylvestris*) and cultivated grapevine (*Vitis vinifera ssp.sativa*) varieties.

According to the data of FAO,Turkey has 540.000 ha of grapevine cultivation area and comes from after Spain, France and Italy and is at the 4th level. Grape production is 3.923 milyon tons and is at the 6th level after Italy, France, China,USA and Spain. Nine viruses have been reported to be associated with disease, all of which are phloem limited and belong to the family *Closteroviridae*. The family *Closteroviridae* comprises three genera; only *Closterovirus* genus contains *Grapevine leaf roll associated virus-2* (GLRaV-2), that are transmissible by mechanical inoculation. The other species belong to *Ampelovirus* genus and are not mechanically transmissible(Martelli, 2006). Recently, GLRaV-5 was detected by Buzkan et al. (2009) from southeastern part of Tukey. Severe reddening and inward curling were present in main viticulture production areas of Turkey; therefore surveys were carried out to look at virus presence and identity.

MATERIALS AND METHODS

The main viticulture production areas Aegean, Central Anatolia, Eastern and Western Anatolian parts of Turkey were surveyed in the late summer of 2009 and 2010 and 281 leaf samples were collected. Severe redness and inward curling with greening of the major veins were common on most of the samples collected.

DAS-ELISA (Clark and Adams, 1977) was applied to the extracts obtained from leaf veins and petiols, using the kits of GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4-9, GLRaV-6, GLRaV-7 obtained from Bioreba and used according to the instructions of the manufacturer.

RESULTS AND DISCUSSION:

During the surveys conducted in the main viticulture areas of Turkey in 2009 and 2010, totally 217 symptomatic samples were collected and investigated. Grapevine leaf roll associated viruses were present as mixture of all or some of them. The most prevalent infection was GLRaV-4-9 which was present in 125 of the samples and it was not detected in previous researches in Turkey. It was followed by GLRaV-3 (78 samples), GLRaV-7 (76 samples), GLRaV-1 (72 samples), GLRaV-2 (68 samples). Minimum infection rate was detected in GLRaV-6 infection, the virus was present only 6 of the samples. The virus infections were prevalent in the vineyards located in Aegean region , Central Anatolia and Thrace.This is the first report on the presence of GLRaV-4-9 in Turkey.

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The Combined Effect of Preliminary Infested Vines, Spatial Spread Pattern and the VMB Population Level on the Grapevine Leafroll Disease Infestation Rate

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INTRODUCTION

Grapevine leafroll disease (GLD) is a widespread viral disease of grapevines, reported from almost all grape-producing countries worldwide (Charles *et al*., 2009). GLD affect growth, development and longevity of the vine and couse a decrease in berry sugar content which reduces wine quality. The pathogens suspected as causing GLD are grape leafroll associated viruses (GLRaV). The prevalent species in Israeli vineyard is GLRaV-3, and with rare evidence to the presence of GLRaV-1. The virus is introduced to vineyards by infected propagation material and spread via semi persistent transmission by Pseudococcidae and coccidae. Three main infestation patterns of GLRaV-3 had been defined (Petersen, 2005); each pattern can be derived from a different infestation factor: (1) Random pattern- related to primary infestation from infected plant material. (2) Adjacent vine pattern- associated to the vector focal mobility. (3) Vineyard edges pattern- This spatial pattern suggests that GLRaV-3 is introduced to the vineyard from an adjoining, external source. The vine mealybug *Planococcus ficus* (VMB), that is prevalent in Israel's vineyards, is considered one of the most important vectors of GLRaV-3 and GLRaV-1 (Tsai *et al.,* 2010).

It is assumed that, as mealybug population increases individuals disperse and infect adjacent vines. Thus, the rate of infestation spread in the vineyards may depend on both the number of previous infected vines and the level of mealybug population in the vineyard. In the present study we measured the effects of these two factors on the virus infestation rate in the vineyard.

METHODS

The study took part in wine vineyards in northern Israel. One vineyard was used in a multi-year tracking of GLD infested vines and we analyzed the infestation pattern. 16 plots in 4 vineyards were used to measure the effect of preliminary infestation level on infestation rate. In three additional vineyards (each vineyard as a block with three 0.3 ha. sub-plots), we tested the effect of control methods on VMB population and on GLD vine infestation rate: 1) Control plot, In the 1st year we used a non-treated plot as control, but as neonicotinoid is now prophylactically applied by irrigation to most wine vineyards in Israel, in the 2nd year we used as control neonicotinoid treated plots. 2) Soil application of neonicotinoid and organic phosphorus spray on the trunk after bark removal (Drastic). 3) Pheromone application of Male Mating Disruption (MD). In both years we measured the VMB populations during the season and compared data among the three treatments. The GLD infested vines were mapped in each plot at the end of each season.

RESULTS AND DISCUSSION

In 7 years of survey, infestation level increased from 4.5% to 31%. During the first 4 years (2005-2008) the rate was low as compared with steeper slops obtained in the following 3 years (2009-2011). The rate of change, thus, can be divided into 2 preliminary infestation phases: 4-8% and 10-25%. Using this data and data of 16 other plots in 4 different vineyards reveals effect of the infection level in year one (T0) on new infestation in the subsequent year (T1). This effect is not continues but terraced.

The dominant spatial infestation pattern is the "adjacent vine pattern" mainly in the radius of 3 meter from previously infested vine: the vines in highest risk of infestation are those adjacent to an infested vine in the same row (1st vine), in somewhat reduced risk are adjacent vines in a nearby row (1st row), then the vines in a distance of one vine from the infested vine (2nd vine) and vines in the same row. In a reduced risk are the rest of the vines, those that are more than 2 vines away from previously infested vine (random) and vines in the nearby row (2nd row). The main pattern fits the mealybug mobility and indicates that the virus infestation is spreading mainly via mealybug movements between vines.

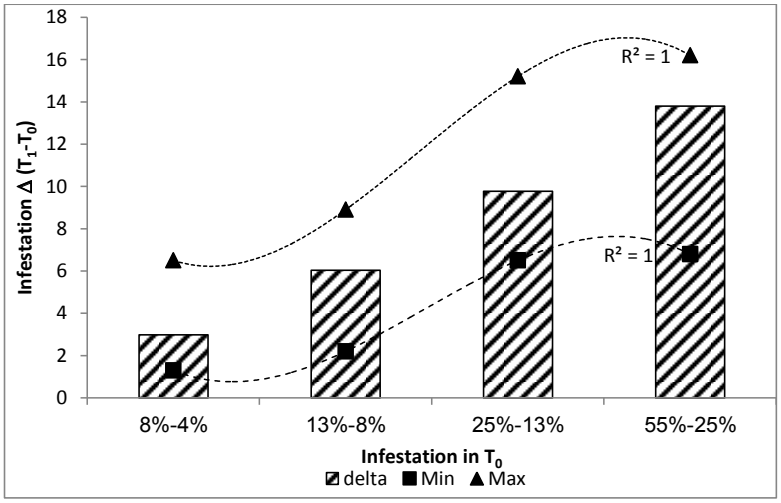

Figure. 1: The effect of the preliminary infestation level on the infestation rate (delta- [T=1]-[T=0]).

Minimum Delta ■ *Maximum Delta* ▲

All control methods reduced mealybug populations as compared with non-treated plots (Fig. 2A). In the 2nd year (Fig. 2B, C), the least effective treatment on melaybug population size was the neonicotinoid, which also had the least effect on GLD infestation rate (10%). MD had the highest effect in reducing the VMB population and keeping the lower GLD infestation rate of the vines (5%). Thus, the more efficient the treatment against the mealybugs was, the lower was the virus infestation rate.

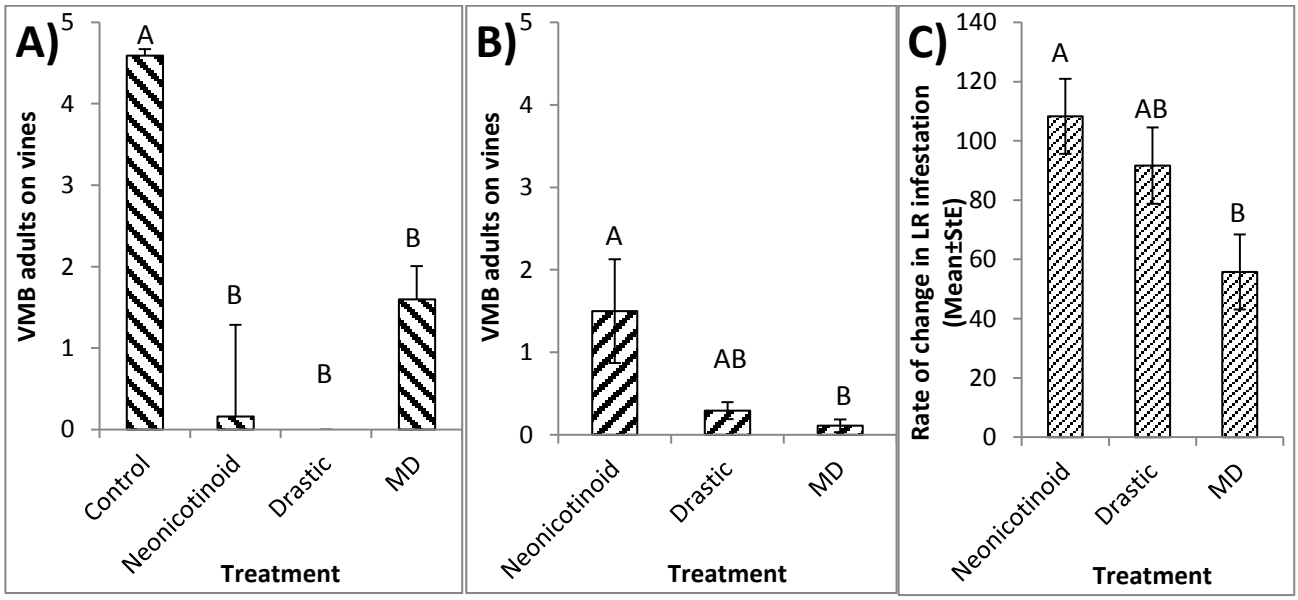

Figure 2: A) VMB number/vine under different control methods in the 1st year of the study; B) VMB number/vine under different control methods in the 2nd year of the study; C) GLD rate of change ([T1-T0]/T0) in the 2nd year of the study.Treatment: Control- non-treated; Neonicotinoid- neonicotinoid application; Drastic- application of neonicotinoid and organic phosphorus spray on the trunk after bark removal; MD- Male Mating Disruption. Different letters indicate significant differences.

CONCLUSIONS

The pattern of virus infestation spread was from infected vines to neighboring vines, which is associated with the vector focal mobility; infestation rate depends on both preliminary infestation of vines and the local mealybug level. The spread of the disease depends on the position of the infested vines in the vineyard, the preliminary level of infestation and the efficacy of control means against the vector population.

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Documentation of Grapevine Leafroll-associated Virus-2, -3, and Grapevine Fleck Virus in Wine Grape Varieties and Native Grape Species in Virginia

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Grapevine leafroll-associated virus-2 (GLRaV-2), GLRaV-3, and grapevine fleck virus (GfkV) are three of the most problematic grapevine viruses found worldwide. These viruses can cause significant crop loss and affect wine quality by reducing sugar accumulation and compromising skin color (Kovacs *et al*., 2001). Moreover, a mixed infection of GLRaV-3 and GfkV was shown to aggravate disease symptoms (Naidu *et al*., 2006). Lack of information on presence and prevalence of viral diseases of grapevines in the state of Virginia led us to conduct a statewide survey in 2009-11 seasons.

In order to identify potential regional differences, growers were selected randomly from each of five major grape growing regions of Virginia: 1) Northern Piedmont, 2) Central, 3) Eastern, 4) Southwest, and 5) Southern Piedmont. At each grower's vineyard, one to three blocks (i.e., different varieties) were selected, three consecutive vines were randomly selected from each block, and seven petioles per vine were randomly sampled (= 21 petioles/sample). Due to the uneven distribution of virus in the plant (Charles *et al*., 2006), petiole samples were collected from different locations on a vine (e.g. petioles from random shoots on the vine all over the canopy, including the top, middle, bottom and edges) and pooled for testing. Crude extract was then used in a one-tube, one-step RT-PCR protocol for the detection of different GLRaVs (Rowhani *et al*., 2000; Naidu *et al*., 2006; Rayapati *et al*., 2008). In selected vineyards, a grid of vines, each grid consisting of the same variety, was sampled. The grid was either 10 consecutive vines by 10 consecutive rows (10 x 10) or 5 consecutive vines x 20 consecutive rows (5 x 20). At least one of the vines in a grid had visual symptoms and/or a positive identification of virus-infection, previously detected by RT-PCR. On each vine, 21 petioles were randomly selected for the RT-PCR procedures described above. Similarly, wild grapevines from across the state were sampled and tested using the same method.

Over 1300 vine samples (39 different wine grape varieties) from over 130 locations in and around Virginia were tested for GLRaV-2, GLRaV-3, and GfkV. Sixty-five wild grapevines samples were also examined. Testing results showed 7.3%, 24.6%, and 0.5% of sampled vines were positive for GLRaV-2, GLRaV-3, and GfkV respectively. Figure 1 displays the sums within each variety tested of vines that were positive or negative for GLRaV-2, GLRaV-3, or GfkV. Of all vineyards surveyed, 61% were found to be positive with at least one of the tested viruses. All wild grapevines tested have been found to be free of these three viruses.

A majority of the infected vines from commercial vineyards were planted prior to the 1990's; however, some new plantings were also found to be positive, indicating movement of the viruses among vineyards and also potential contamination prior to planting. The high level of virus-infected vines emphasized the importance of clean plant materials, as well as management of vector insects (mealybugs and scale insects). The wild grapevines surveyed yielded promising results as none were outside sources of these viruses, unlike California where *Vitis californica* and *Vitis californica x Vitis vinifera* hybrids were found to be hosts to GLRaV-2 and GLRaV-3 (Klaassen, 2011). This study is the first to examine multiple grape viruses in VA, and thus, will serve as a baseline for the level of viral grape disease infections in the state.

We acknowledge the Virginia Wine Board for their support of this project.

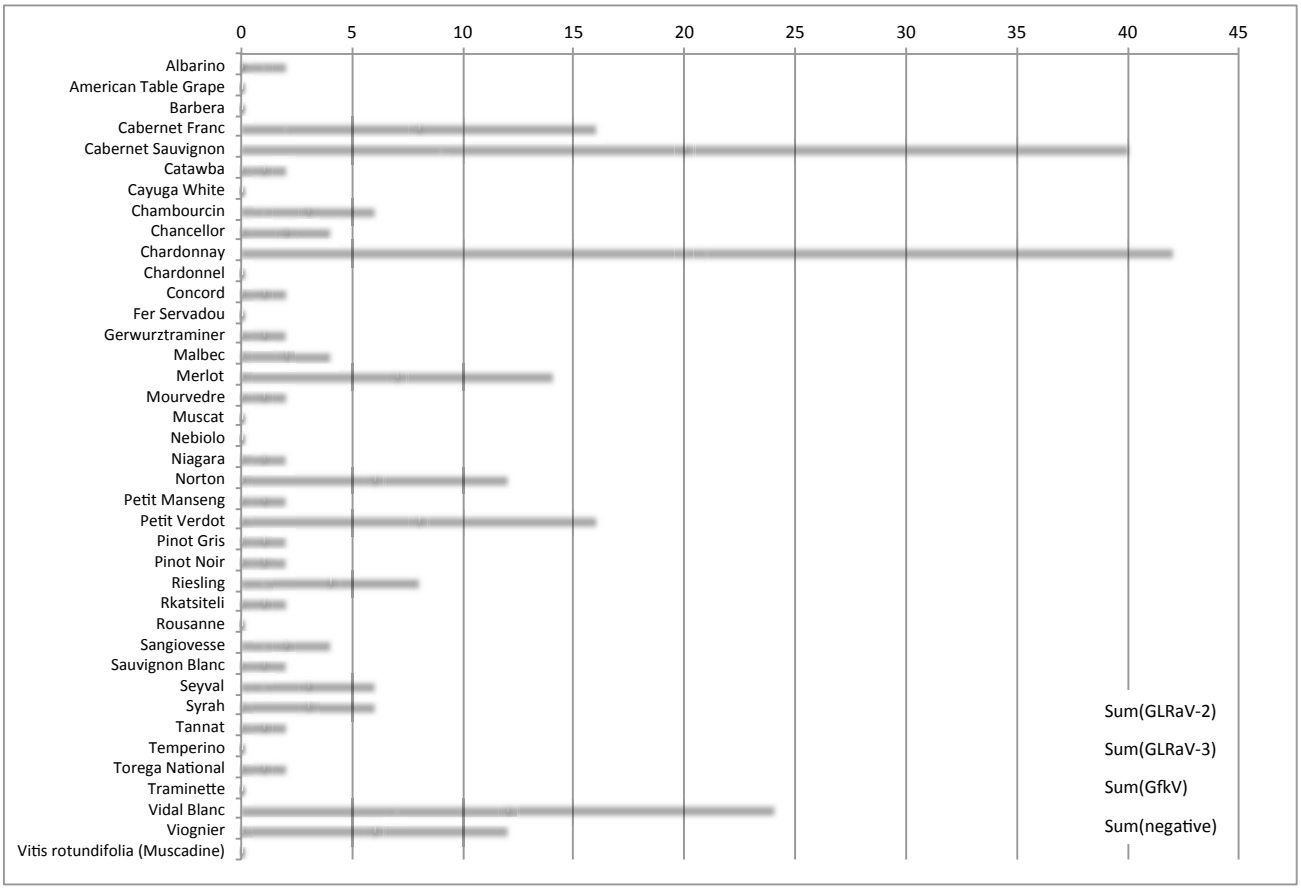

Figure 1. Sum of Positive and Negative Vines tested for GLRaV-2, GLRaV-3, and GfkV by Variety in Commercial VA Vineyards. Total length of bar beside each variety represents total number of samples taken from that variety of grapevine. Length of the blue, red, and green section of the bar represents the total number of vines positive for GLRaV-2, GLRaV-3, and GfkV respectively, in that sampled variety. Length of the purple section of the bar represents the total number of vines negative for any viruses in that sampled variety.

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"Grapevine Viruses" in Muscadines

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INTRODUCTION

Muscadines (*Vitis rotundifolia*) are grapevines (gen. *Vitis*, sub-genus *Muscadinia*) native to the southeastern U.S. from Delaware to the Gulf of Mexico. They thrive in a warm and humid climate of this region that is hostile for most European grapes. Muscadines have been extensively cultivated for several centuries and used as fresh fruits, or processed as jelly, juice or wine. Their fruits are rich in resveratrol, quercitin, ellagic acid and other polyphenolic substances that making them nature's ultimate "superfruit" concerning health benefits. However, significant commercial production of muscadine grapes is limited only to the southeastern US.

Muscadines have high degree of resistance to diseases/insects including Pierce's disease, phylloxera and nematodes. While *Vitis vinifera* and related rootstocks are susceptible to more than 60 different viruses (1), knowledge on muscadine viruses is limited to a recent paper on an isolate of *Grapevine Syrah virus 1* from this host (4).

This study, initiated in spring 2012, was aimed at identifying and studying viruses infecting this crop in Mississippi and southeastern US, and to understand their incidence and economic importance.

MATERIALS AND METHODS

Virus sources

Virus sources used in this work belonged to several commercial cultivars (i.e. Albermerle, Black beauty, Fry, Summit, Tara, etc), and muscadines of uncertain varietal identity. Samples were collected from production plots, backyards and varietal collections.

Cloning, sequencing and phylogenetic analyses

Double stranded RNAs (dsRNAs) extracted from phloem tissues were selectively treated with DNAse and RNAse and used as a template for random-primer generated cDNAs according to previously published protocol (3). PCR-enriched complementary DNAs were digested with a proper restriction endonuclease and cloned into proper vectors for sequencing. Sequence analyses were performed with on-line resources (BLAST; CD, Pfam, etc), and with various software according to the scope of analyses (i.e. Lasergene-DNAStar package, MEGA 5.05, etc).

RESULTS AND DISCUSSION

In this study we have identified several viruses naturally infecting muscadines in Mississippi: Grapevine leafroll-associated virus 2 (GLRaV-2; gen. *Closterovirus*, fam. *Closteroviridae*), *Grapevine virus B* (GVB; gen. *Vitivirus*, fam. *Betaflexiviridae*), *Grapevine Syrah virus 1* and *Blackberry virus S* (GSyV-1 and BlVS; both gen. *Marafivirus*, fam. *Tymoviridae*) and another marafi-like virus yet under characterization.

Identities of GLRaV-2 isolate from muscadine with known strains of this virus varied from 74% to 94% concerning nucleotides. GLRaV-2-musc resulted most similar to the strain GLRaV-2 93/955 from South Africa (2). Partial sequences of muscadine isolate of GVB was most similar (94%) with the "type isolate" of GVB (GVB-IT, Figure 1).

At least three distinct marafiviruses were identified in muscadines in a pool of samples tested so far. Whereas GSyV-1 was previously identified in muscadines and other native *Vitis* spp (4), identification of *Blackberry virus S* represents first report on this virus in species other-than-blackberry (3). Furthermore, an additional marafivirus was identified in cv. Tara. Based upon partial sequences of viral RdRp and CP, this virus appears to be a distinct and yet undescribed species in the taxon as it shares limited sequences identities with known marafi/maculaviruses (69-74%). Its further characterization is underway.

In conclusion, results of this study indicate that muscadines are prone to natural infections by viruses reported from European grapevines, some of them considered of economic importance in *Vitis vinifera*. Interestingly, GLRaV-2, GVB, GSyV-1 and BlVS were isolated from samples showing rough bark and decline symptoms. We have developed sensitive molecular assays for detection of all these viruses and we currently test additional samples affected by this disorder in order to understand if this problem could be of viral origin.

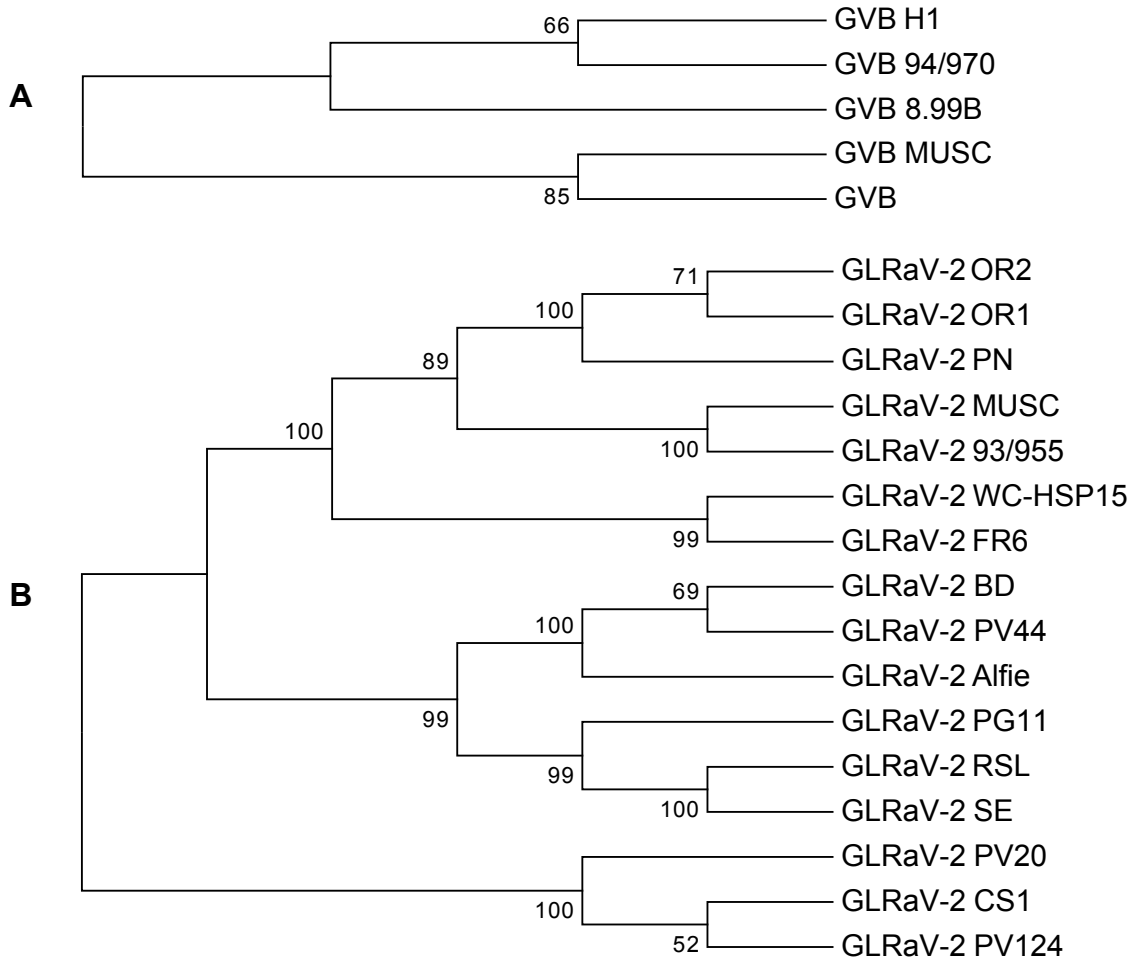

Figure 1. Phylogenetic trees showing relationships of *Grapevine virus B* (panel A) and *Grapevine leafroll-associated virus 2* (panel B) isolates from muscadines (labeled as "MUSC") with some of known isolates of these viruses reported from *V. vinifera*.

ACKNOWLEDGEMENTS

Deep thanks to various homeowners, farmers and Dr. S.J. Stringer for kindly allowing access to muscadine samples used in this study.

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Seasonal Pattern and Dynamics of Virus Acquisition by the Grape Mealybug in a Leafroll-Diseased Vineyard

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INTRODUCTION

Leafroll disease affects the profitability and sustainability of the grapevine industry worldwide. To date, five viruses have been isolated and characterized from leafroll-affected vines (3). They are readily transmitted by vegetative propagation and grafting, as well as by mealybugs (Hemiptera: *Pseudococcidae*) and soft scales (Hemiptera: *Coccidae*), with the exception of *Grapevine leafroll-associated virus 2* and -7 for which no vector is known (3). In the Finger Lakes region of New York, GLRaV-1 and GLRaV-3 are prevalent in leafroll-diseased vineyards (1) and low populations of the grape mealybug (*Pseudococcus maritimus*) are present. No specificity is associated with the transmission process of GLRaVs by mealybugs, as a given mealybug species can vector several virus species (5). Crawlers are more efficient vectors of GLRaV-3 than L2, L3 and adults (4), and a single nymph is sufficient to transmit the virus to a healthy vine and initiate infection (2). Amid advances on transmission parameters, information is scarce on seasonal patterns of virus acquisition by mealybug vectors in leafroll-affected vineyards. Similarly, the transovarial transmission of GLRaVs from female adults to progeny is poorly characterized. To address these issues, we collected grape mealybug immatures, adults and eggs from April to November over two consecutive years in a vineyard of *Vitis vinifera* cv. Chardonnay that is naturally infected with GLRaV-1 and GLRaV-3 and tested for the presence of viral genetic elements by RT-PCR using specific primers. Here we report on virus acquisition by overwintered crawlers (as early as April near bud break), as well as by summer generation crawlers, and lack of evidence of transovary virus transmission.

MATERIALS AND METHODS

Twenty-three vines of *V. vinifera* cv. Chardonnay were selected for this study in a vineyard naturally infected with GLRaV-1 and GLRaV-3 in the Finger Lakes region of New York. Grape mealybugs (immatures, adults and eggs) and plant tissue (leaves, petioles, bark) were collected from the selected vines at monthly or bi-monthly intervals in 2010 and 2011, and tested for the presence of GLRaV-1 and GLRaV-3 by RT-PCR with primers designed in the second diverged copy of the coat protein gene of GLRaV-1, heat shock protein 70 homologue gene of GLRaV-3, *Vitis* 18S ribosomal gene, and grape mealybug nuclear small subunit ribosomal RNA gene (1). Mealybugs were collected under cracked bark of one and sometimes two year-old wood. Immatures were collected in April-November and adults were sampled in June and August. Collected specimens were placed in 1.5 ml microfuge tubes and kept in RNAlater™ storage solution (Ambion, Inc, Austin, TX) at -20 °C until further processing. Data on the presence of viral genetic elements in mealybug samples were compiled for each time point over two consecutive years as indication of virus uptake. Some eggs collected in June were allowed to hatch on Petri dishes in the lab and crawlers were tested for transovary virus transmission.

RESULTS AND DISCUSSION

Vineyard observations indicated that overwintered crawlers were becoming active just prior to bud break (April) at which stage 77% (20 of 26) of the specimens tested were viruliferous, preferentially for GLRaV-1 (Table 1). In May, crawlers became more dispersed and almost all of them were viruliferous, preferentially for GLRaV-3 (96%, 25 of 26). In late May and early to mid-June, adult females moved to the trunk and beneath the bark to oviposit. At this stage, 83% (25 of 30) of the mealybugs were viruliferous, mainly for both GLRaV-1 and GLRaV-3 (Table 1). In July, the new generation crawlers moved into the new growth following hatching and 82% of them (28 of 34) were viruliferous, mainly for both viruses. In August, most crawlers and adults (91%, 29 of 32) were viruliferous for either GLRaV-3 or both viruses. By September, the second-generation was mainly found under the bark and none were viruliferous (0%, 0 of 12) (Table 1). In November, a single overwintering crawler was found viruliferous (7%, 1 of 15).

Table 1. Occurrence of GLRaV-1 and GLRaV-3 in grape mealybugs from April to November in a Chardonnay vineyard in the Finger Lakes region of New York.

			Mealybugs positive for[a]					
Month	**Development stage**	**N[b]**	**No virus**	**LR1**	**LR3**	**LR1+LR-3**	**Viruliferous/Tested**	**(%)**
April	Crawlers	26	6	19	0	1	20/26	77
May	Crawlers	26	1	0	14	11	25/26	96
June	Crawlers & Adults	30	5	2	4	19	25/30	83
July	Crawlers	34	6	1	6	21	28/34	82
August	Crawlers & Adults	32	3	3	16	10	29/32	91
Sept.	Crawlers	12	12	0	0	0	0/12	0
Nov.	Crawlers	15	14	1	0	0	1/15	7

[a]Data represent the cumulative number of mealybugs with no viral amplicon (no virus) or amplicons for GLRaV-1 (LR1), GLRaV-3 (LR3), and GLRaV-1 and GLRaV-3 over two consecutive years; [b]N: number of specimens tested.

Our results were consistent with (i) the majority of mealybugs tested from April to August carrying genetic elements from GLRaV-1 and/or GLRaV-3, (ii) a preferred virus uptake from bud break to bloom (April to June) and at a pre-veraison stage (July-August), and (iii) very few viruliferous overwintering crawlers. In addition, no viral amplicon was obtained by RT-PCR from any of the 213 eggs collected in June 2011; neither was a viral amplicon obtained from any of the 51 crawlers collected on egg masses or 33 crawlers that hatched from eggs. These results indicated no transovary transmission of GLRaV-1 and GLRaV-3, confirming a semi-persistent transmission mode (4). Together, this study suggests that leafroll management strategies based on mealybug control should target vector populations earlier (from bud break to expanding leaves) than recommended by most integrated pest management programs.

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Analysis of subjectivities about leafroll disease management among Napa grape growers and winemakers

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INTRODUCTION

Grapevine leafroll associated virus (GLRaV) has become increasingly important to grape growers in California (Golino *et. al.,* 2008). A grapevine certification program has led to the availability of certified virus tested nursery stock for growers (Olmo, 1951, Alley *et. al.*, 2000), although the program is not utilized by all grape growers in California (Golino *et. al*., 2008). Historically, the elimination of certain leafroll disease related virus pathogens from grapevines has been associated with an increase in vigor and quality (Mannini *et. al*., 1996, 2006). When Golino *et al.* (2008) discovered GLRaV-3 to be spreading in a Napa Valley vineyard; concerns arose about neighbor to neighbor spread of disease. What once was believed to be an easily resolved issue with the use of certified planting stock became a quandary which individual growers were unable to deal with: What was the value of replanting with certified stock if GLRaV-3 could spread from infected neighboring blocks? If successful management of the disease depends on collective action it is important for all involved (growers, nurseries and extension/outreach personnel) to understand the subjective views held by the decision makers who will be collaborating. To address this issue we began a study incorporating a Q-method approach to understand the subjectivities of those involved (Brodt *et. al.,* 2006) in leafroll management in today's California vineyards. The resulting analysis and understanding of the diversity of views is helping in efforts to establish neighborhood disease control groups.

METHODS

A Q-method approach was used to assess the opinions concerning leafroll management (Brodt et al., 2006). Three workshops were held in the Napa Valley at which invited participants were asked to write down their views in response to a set of open-ended questions about leafroll, its impacts and the prospects for cooperative management of the disease. Responses were collated and sorted into thematic groups (e.g. statements about financial issues, clean planting material, interpersonal trust, etc.) then a small subset of response statements was extracted. These statements encapsulated the groups of opinions which were expressed. This resulted in a set of 47 statements. Invitations were issued via email and by personal contacts to a further group of participants drawn from the Napa Valley grower/management and winemaker communities. This resulted in a participant group of 37 individuals who were interviewed and individually carried out Q-sorts of the statements. The Q-sort process involves participants ranking the statements based on their own personal ranking of agreement/disagreement with the proposition in each statement. The design of the sorting process forces a relative ranking of the statements. The interviews were conducted during the fall and winter months of 2011. The end result of the process is a two-way table of data in which each row gives the numerical rank assigned to each statement by one participant. The two-way table was then subjected to a Principal Components analysis using SimSTAT statistical software to extract information regarding the distribution of opinions over the group of participants and to identify meaningful classifications of the responses.

RESULTS AND DISCUSSION

The principal components analysis revealed a unique response profile of each of the 37 individuals over the set of statements. The results from the analysis are shown in Figure 1.

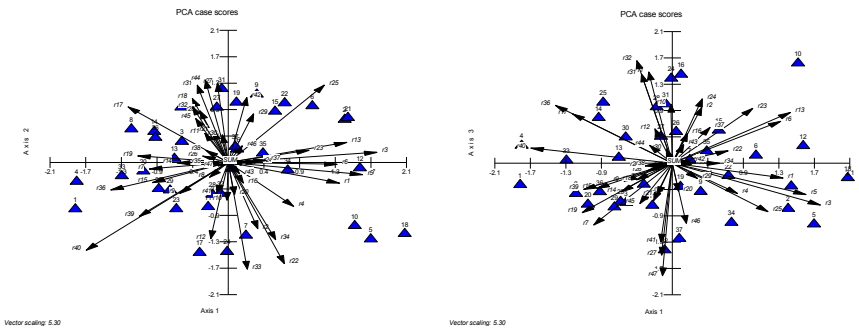

Figure 1. Triangles represent participants, arrows represent statements. The Principal Components analysis shows a broad distribution of participants on multiple axes.

By relating statement distribution to participant distribution, we have identified the qualities of individuals who were located in different volumes of the principal axis space from the analysis. This relationship can be seen in Figure 2.

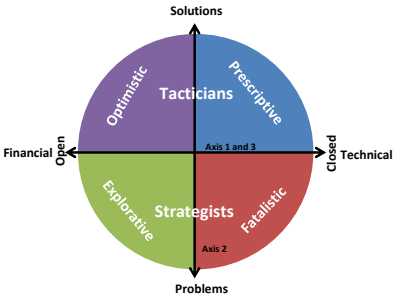

Figure 2. Schematic representation of the attitudes represented in different volumes of the principal axis space after analysis of the ranked opinion data set. Axis 1 and Axis 2 separated the individuals into 4 groups broadly determined by whether they predominantly focused on financial or technical issues and whether their thinking appeared to be strategic or tactical. Axis 2 and Axis 3 separated four groups on the basis of whether focus was on problems or solutions and the extent to which their opinions expressed a strongly normative element or a *laissez-faire* approach. Axis 1 and 3 are represented on the horizontal axis.

In addition to the Principal Components Analysis, Figure 3 shows the variance in the score for each statement (across the 37 participants) against the total score. The variance/score plot reveals several interesting features of the data. The variance in scores tends to be highest for statements in the low/medium range of total scores.

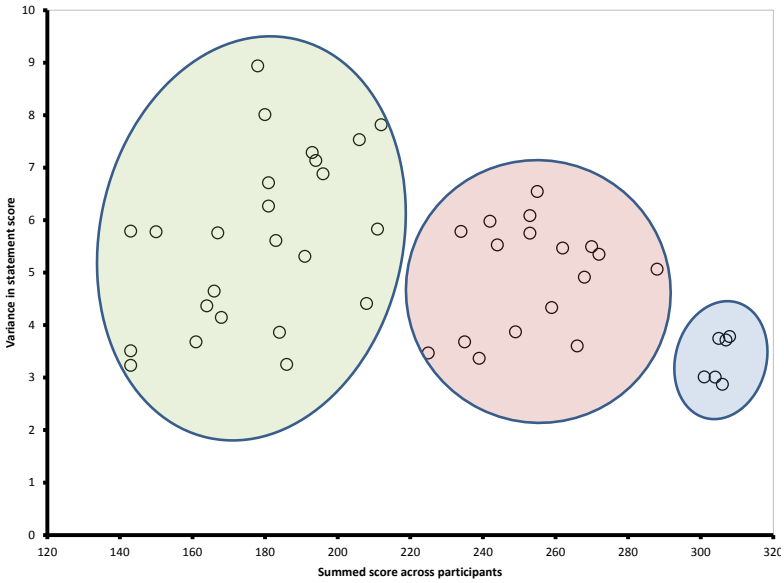

Figure 3. The variance in scores for 47 opinion statements ranked by 37 different individuals for the extent to which the statements agree with their own views, plotted against the total score for each statement. Three groups of statements are apparent (highlighted by colored areas) as is the general tendency for the variance to be lower at either end of the range of total scores.

At the high end of the scale, a set of six statements had total scores of over 300 suggesting a high level of agreement about the importance of the concepts they represent. The high ranking statements taken as a group reveal a strong focus on the quality; cost and provenance of planting material and the potential negative impact that leafroll can have on the financial value of clean planting. Additionally these statements express the ease with which it can render the effort in establishing new clean blocks pointless.

ACKNOWLEDGEMENTS

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Statistical Parameters of Spatial Patterns of Spread for Leafroll Disease

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INTRODUCTION

The grape and nursery industry has become increasingly interested in the subject of *Grapevine leafroll associated virus-3* (GLRaV-3) due to a recent discovery that GLRaV-3 is spreading rapidly in Napa Valley (Golino *et. al.* 2008). GLRaV-3 is vectored by mealybugs (*Pseudococcus, Planococcus*) which are present in many grape growing regions including California (Tsai *et. al*., 2010, Golino *et. al*., 2008). A certification program was put in place in response to the need for virus tested clean stock to be made available to growers (Olmo, 1951; Alley *et. al*., 2000), however noncertified nursery stock is still widely used in California vineyards (Golino *et. al*. 2008). The eradication of GLRaV-3 shows overall improvement of vine performance, with specific positive effects on grape qualitative parameters (Mannini, *et. al*., 1996, 2006). While the epidemiology of leafroll disease has been studied in other grape growing areas of the world, a comparative analysis is needed. The purpose of this study is to analyze historical data maps of leafroll symptoms to elucidate quantitative parameters summarizing disease progress in time and space at an individual vineyard scale.

METHODS

Disease incidence data sets were collected from the literature (Jordan *et al*., 1993 Fig. 1 and Fig. 2; Cabaleiro *et al*., 2008, Fig. 1), from previous observations (Golino *et al*., 2008), and observed in a vineyard in Napa county, CA. Maps of symptomatic/asymptomatic vines were analyzed using quadrat-based approaches (Hughes *et al*., 1997) to characterize the spatial pattern of leafroll over time. Vineyards were divided into appropriate quadrats, based on field size. Rates of disease progress and spatial variances of disease incidence were calculated and used to characterize epidemics.

RESULTS AND DISCUSSION

Irrespective of the numerous differences among the studies, the analyses revealed a consistent spatio-temporal pattern across epidemics (Figure 1). In all cases, the spread of leafroll was consistent with a highly aggregated (or patchy) spatial pattern, indicating that the dominant mechanism of dispersal operates over a short distance. The degree of patchiness in the data is revealed by the fact that the observed relationship between disease incidence at the plant scale and the quadrat scale lies along the line equivalent to a binomial distribution of diseased plants in samples with n = 3, while actual quadrat sizes ranged from n = 4 to n = 30.

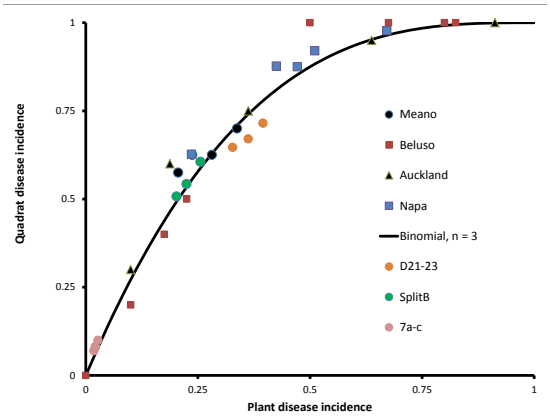

Figure 1. An incidence-incidence plot for leafroll disease incidence measured at quadrat and individual plant scales in four different studies of disease progress. Vineyard sizes, cultivars and quadrat sizes differ between studies. A common spatio-temporal pattern is observed across all studies indicating a consistent dispersal mechanism which is relatively insensitive to local variations in growing conditions. The analysis is consistent with predominantly plant to plant spread of the virus.

To analyze the temporal dynamics of leafroll incidence increase, plant disease incidence values, p, were transformed by taking the logit values [=ln(p/(1-p))] and plotting them against time (Figure 2). A fifth data set (Habili *et. al.*, 1997) which reported temporal leafroll data from a vineyard in Southern Australia was included. Because the studies involved natural leafroll epidemics and observations at multiple stages of infection we translated the different epidemics onto a common timeframe using the Beluso data set as a reference since it started at p = 0 and was also the longest series of observations. To align the data sets we took the initial value of disease incidence in each set of observations and plotted it in the same year as the closest incidence value at Beluso. All data sets consisted of consecutive observations so this procedure only fixed the initial year of observation to be equivalent to a year in the Beluso study but did not affect the estimate of the rate of increase in disease in each study.

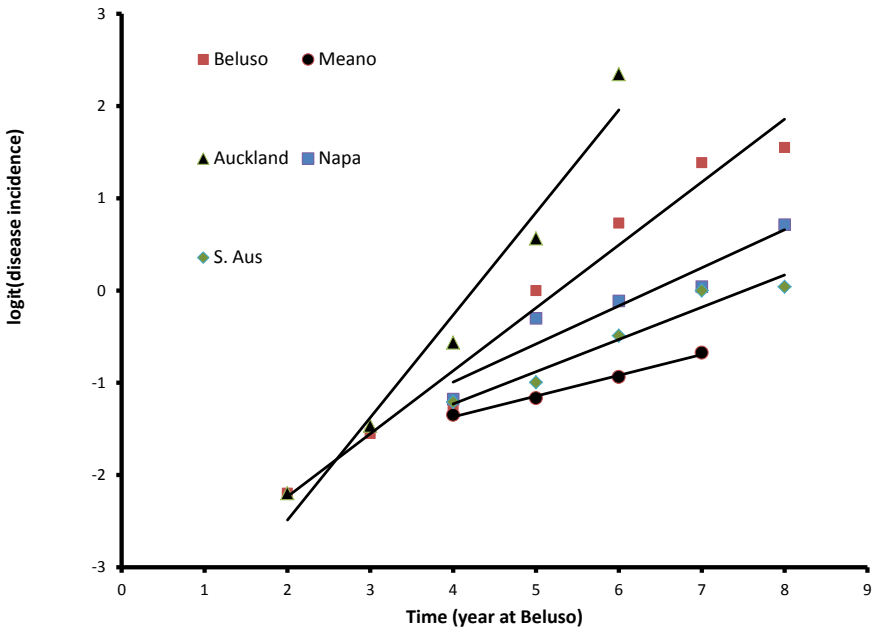

Figure 2. Logit transformed disease incidence values for multiple leafroll epidemics plotted on a common timeframe which uses the longest series (Beluso) as a reference. The fitted lines give estimates of the rate parameter, *r*, of a logistic growth curve for disease incidence in each case. The estimates range from 1.11 (Auckland) to 0.22 (Meano).

The estimates of logistic rate, *r*, of disease increase ranged from 1.11 in Auckland to 0.22 in Meano (Spain). The average value *r* was 0.55 (s.e.m. = 0.160); at this rate of increase it would take leafroll in the order of 10 to 15 years to completely infect a block of vines when initial incidence is in the order of 0.1%. Averaged over all studies the annual rate of increase in disease incidence was found to be 11% of vines per year.

Spatial patterns and temporal dynamics of leafroll disease across multiple studies revealed similar behavior consistent with spread by a vector with a mainly localized dispersal process. Additional work is needed to characterize leafroll disease incidence at a landscape scale. To assess the spatial pattern of leafroll over a large area we require the capacity for rapid collection of spatially-referenced data on disease incidence. The aggregated pattern of disease will make sampling for disease detection relatively inefficient at low disease incidence but also suggests that removal of infected vines with a suitable buffer of asymptomatic vines might be effective at stopping early infections. The stability of statistical properties for different leafroll epidemics indicates that decision tools based on epidemic characteristics should have generic applicability.

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A Preliminary Survey of Grapevine Viruses in Algeria

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INTRODUCTION

With a surface of about 91,000 Ha, the grapevine ranks fifth among the fruit crops of Algeria, after pome and stone fruits, olive and date palm. Vineyards are concentrated on the coastal area in the western and central part of Algeria (Ain Temouchent, Boumerdes, Mostaganem, Sidi Bel Abbes, Mascara, Tlemcen, Media, Tipaza). Table grape varieties are by far the most widely grown, and the new varieties imported from abroad are gradually substituting the old local varieties.

The sanitary status of Algerian viticulture is little known as few published reports are available, recording the occurrence of leafroll, rugose wood and fanleaf diseases (1). Given the paucity of information on the presence and the incidence of virus infections in Algeria, an investigation was initiated, the preliminary results of which are reported herein.

MATERIALS AND METHODS

Field inspections and collection of samples were conducted in March and September 2010. Mature canes were collected from 736 individual vines, representative of 74 varieties and 5 different rootstocks, in five grapevine collection plots generally used as sources of buds for nurseries at Skikda, Tassala El Merdja, Medea, Tighennif, Ain Temouchent, and in one nursery at Blida. About 58.1% of samples were from the most important local varieties (Ahmar de Mascara, Muscat de Fandouk, Valenci noir, Sidi Ahmed Draa Mizene, Ahmar Bou Ameur, etc.), 34.0% from varieties introduced from abroad (Italia, Alphonse Lavallée, Dattier de Beyrouth, Halawani, Cardinal, etc.) and the remaining (7.9%) from rootstocks (1103P, 140Ru, 110R, 41B, SO4). All samples were analysed for the presence of *Gr. fanleaf virus* (GFLV), *Gr. fleck virus* (GFkV), *Gr. virus A* (GVA), *Gr. virus B* (GVB), *Gr. leafroll associated virus 1* (GLRaV-1), *Gr. leafroll associated virus 2* (GLRaV-2) and *Gr. leafroll associated virus 3* (GLRaV-3). Tests were made on cortical scraping extracts by DAS-ELISA (GFLV, GLRaV-1, GLRaV-2 and GLRaV-3), DASI-ELISA (GFkV and GVB), and protein A-DAS ELISA (GVA) (2). Polyclonal antisera and monoclonal antibodies used as reagents were from Agritest (Bari, Italy). *Grapevine rupestris stem pitting-associated virus* (GRSPaV) was investigated by RT-PCR assay using specific set of primers (RSP48 5'-AGCTGGGATTATAAGGGAGGT-3'; RSP49 5'-CCAGCCGTTCCACCACTAAT-3') (A. Rowhani, personal communication [University of California, Davis, USA]).

The leaf extracts of forced cuttings were mechanically inoculated to a standard series of herbaceous hosts from about 100 samples of native varieties chosen at random.

RESULTS AND DISCUSSION

In the field the only symptoms observed and identified with reasonable confidence were those typical of leafroll (i.e. rolling and reddening of the leaves, in particular on red berried varieties) and fanleaf (i.e. yellowing, leaf deformation, short internodes, fasciations and bifurcations of the canes).

No virus other than GFLV was recovered by sap transmission assays, despite the high number (*ca.* 100) of samples tested.

Serological assays were more informative. Of 678 *V. vinifera* vines tested by ELISA, 82.6% were infected by one (30.4%) or more (52.2%) viruses. GLRaV-3 was the most widespread virus (55.3%), followed by GFkV (41%), GFLV (33.3%), GVA (19.9%), GLRaV-1 (8.4%) and GLRaV-2 (8.1%). GVB was scarcely represented (2.9%) whereas ArMV was completely absent (Tab. 1). GLRaV-3 and GVA, notoriously transmitted by pseudococcid mealybugs, and GFLV, transmitted by the dagger nematode *Xiphinema index,* showed particular high infection levels in the local varieties rather than in the imported ones (Tab. 1), thus indicating the large presence of the vector species in the Algerian vineyards.

The infection level in native grapevine varieties was *ca.* 85%, whereas it was *ca.*78% in the imported ones. The two main local varieties Ahmar de Mascara and Muscat de Fandouk had infection levels of 96% and 73.7%, respectively, whereas the infection levels of the other varieties ranged from 0 to 100%. In several important native varieties, e.g. cvs. Ahmar Mechtras, Bouabar des Aures, Aneb el kadi, Ghanez, Ain El couma, Kabyl Aldebert, Lakhzine, Muscat El adda, either not a single vine was found free from the viruses tested. Totally infected were also many other minor native cultivars of which, however, only a low number of samples was analyzed.

Of a total of 67 vines tested by RT-PCR, 28 (41.8%) were infected by GRSPaV.

Markedly better was the sanitary condition of rootstocks, of which only one of 58 samples tested by ELISA was infected by GLRaV-1, and none of 22 samples tested by RT-PCR was infected by GRSPV. This result, apparently controversial if compared with that of *V. vinifera* varieties, is due to the multiplication and use of "healthy" mother plants of rootstocks which were imported from Italy and France some decades ago.

Given the very high infection level of grapevine varieties, and in particular of local ones, the implementation of a selection and sanitation program seems highly desirable to improve the sanitary status of Algerian viticulture. Through this study some putative candidate clones of at least 8 grapevine varieties resulted negative to all viruses tested and could represent the starting points for their multiplication and distribution in the framework of the current certification program of plant propagating material in Algeria.

Table 1. Incidence of eight different viruses in Algerian grapevine varieties and rootstocks.

Virus	***V. vinifera* cvs.** **Native** (428 samples)		***V. vinifera* cvs.** **Imported** (250 samples)		**Total** (678 samples)		**Rootstocks** (58 samples)	
	No. inf. samples	%	No. inf. samples	%	No. inf. samples	%	No. inf. samples	%
GVA	120	28	15	6	135	19.9	0	0
GVB	10	2.3	10	4	20	2.9	0	0
GLRaV-1	23	5.4	34	13.6	57	8.4	0	0
GLRaV-2	43	10	12	4.8	55	8.1	1	1.7
GLRaV-3	279	65.2	96	38.4	375	55.3	0	0
GFLV	171	40	55	22	226	33.3	0	0
GFkV	162	37.9	116	46.4	278	41	0	0
ArMV	0	0	0	0	0	0	0	0
Total	**366**	**84.9**	**194**	**78.4**	**560**	**82.6**	**1**	**1.7**

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Survey and Partial Molecular Characterization of Grapevine Virus and Viroids from Valencia, Spain

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INTRODUCTION

Virus and virus-like diseases are very important among the factors that limit grape production in the world. To check the phytosanitary status of Valencian grapevines (Spain), we began a prospective study in 2011. Samples were collected in autumn from grapevines collection of the 'Escuela de Viticultura y Enología' of Requena, where is possible found different varieties including those local. We sampled grapevine rootstock plants, wine and table grape varieties and analyzed them using RT-PCR to detect and characterize the most economically important grapevine viruses and viroids. This work represents one of the first comprehensive and complete surveys of viruses and viroids that affect grapevines in Valencia Region.

MATERIALS AND METHODS

In November 2011, 127 plants, corresponding to 84 varieties, were collected and analyzed through RT-PCR to detect 16 virus and 2 viroids: *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 1*, *2*, *3*, *4*, *5*, *6*, and *7* (GLRaV-1, -2, -3, -4, -5, -6, -7), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus D* (GVD), *Grapevine fleck virus* (GFkV), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine rupestris vein feathering virus* (GRVFV), *Arabis mosaic virus* (ArMV), *Grapevine vein-clearing virus* (GVCV), *Citrus exocortis viroid* (CEVd), *Grapevine yellow speckle viroid 1* (GYSVd-1). Phloem scrapings from mature dormant canes were used for virus and viroids testing (8). Total nucleic acids (TNA) extraction was by the silica capture method (10, 11). TNA aliquots were primed with DNA random hexanucleotides and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV RT). DNA amplification was performed using target-specific primers (1, 2, 3, 4, 5, 6, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19). To characterize viruses and viroids found, following RT-PCR analyses, selected amplicons were purified using the Concert Rapid PCR Purification System. DNA fragments were cloned and putative recombinant clones were analyzed by colony-PCR using specific primers flanking the polylinker region. Three colonies per amplicons were sequenced in both directions. The viral origin of the amplicons was confirmed using BLAST tools.

RESULTS AND DISCUSSION

From all plants analyzed, 69.3% were positive for at least one virus or viroid. This study reveals the presence of GFLV, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5, GVA, GFKV, GRSPaV, GRVFV, and GYSVd-1. GRSPaV and GLRaV-2 showed the greatest infection levels (48.8% and 15.0% respectively), followed by GFKV (10.2%), GLRaV-3 (8.7%), GFLV (7.9%), GRVFV (7.1%), GYSVd-1 (6.3%), GLRaV-5 (3.2%), GVA (3.2%) and GLRaV-4 (0.8%). The other viruses and CEVd were not detected. Single and mixed infections (two to five viruses) were present. Rate of 37.8% for single infections; 20.5%, 7.9%, 0.8%, and 0.8% for mixed infections with two, three, four, and five pathogens respectively. The analysis of partial nucleotide sequences confirmed the results obtained by RT-PCR about the presence of the nine viruses and GYSVd-1. To our knowledge, GRSPaV and GRVFV are new records for Spain. Meanwhile GLRaV-4 and GLRaV-5 were detected for the first time in Valencia Region.

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Variability of Grapevine Leafroll-associated Virus 5 (GlRaV-5) in Portuguese Field Grown Grapevines

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INTRODUCTION

Grapevine leafroll disease (GLD) is one of the most harmful widespread viral diseases affecting grapevine [7]. Eight serologically distinct viruses were found associated with GLD and named Grapevine Leafroll-associated Virus (GLRaV) 1, 2, 3, 4, 5, 6, 7 and 9. Of late, three newly assigned ampeloviruses isolates serologically distinct from all other GLRaVs and associated with GLD were reported: Grapevine leafroll-associated virus Pr or GLRaV-10, Grapevine leafroll-associated virus De or GLRaV-11 [6], and Grapevine leafroll-associated virus CV or GLRaV-CV [1]. All GLRaVs are members of the family *Closteroviridae.* With the exception of GLRAV-2 and -7, GLRaVs are included in genus *Ampelovirus*. The existence of two evolutionary lineages within this genus has been discussed and two phylogenetic subgroups have been proposed [6]. In the original paper Subgroup I comprised the leafroll-associated viruses 4, 5, 6, 9, 10 (Pr), 11 (De) and CV, characterized mainly by dispersal through grafting and vegetative propagation. Overall transmission by insect vectors, namely pseudococcid mealybugs, has only been established in experimental conditions and so far only in the cases of GLRaV-4, -5 and -9 [5, 12 and references therein]. Included in Subgroup II are *GLRaV-1* and *-3*, for which insect-vectored is considered an important means of dispersal [4 and references therein]. These two viruses are included in the EU grapevine certification scheme, and virus-specific antibodies are commercially available for routine detection. Their dissemination can thus be subjected to close control either in propagation material or field situations. The viruses in Subgroup I though, have been more of a scientific curiosity and case-studied mainly at the level of genome sequencing. Research has produced information suitable for phylogenetic inference, understanding evolutionary lineage divergence, and tentative establishment of species boundaries. The overall view from the literature conveys the impression of low incidence of the above-described Subgroup I viruses, but the fact remains that information regarding propagation, prevalence and population structure in field situations is either sparse or lacking. In the last three years, reports on GLRaV-5 detection in Argentina [11], in China [10], Chile [2] and Spain [9] are closing in on the fact that, due to lack of virus-specific routine detection tools and praxis, we might be overlooking a situation where GLRaV-5 is in reality more widespread or even expanding. An accurate establishment of those circumstances and a clearer understanding of the involvement of GLRaV-5 in leafroll disease are thus required. For that purpose, research on the natural variability of the virus and the genetic structure of its populations is fundamental. This type of study will permit to identify genomic variants, clarify transmission dynamics and assess effective population size, while providing background for biological indexing and improvement of diagnostic tools.

MATERIALS AND METHODS

Plant Material: the fifteen GLRaV-5 isolates analyzed in this work were each obtained from a different field grown *Vitis vinifera* grapevine, after molecular detection initially targeting the capsid protein (CP) gene of the virus. Four of the isolates were obtained from the INIAV national collection of grapevine varieties (CAN-PRT051) situated at Dois Portos (Torres Vedras), Portugal. The CAN is located on average at 350 Km from the other sampling sites. The other eleven isolates were collected from ungrafted field grown grapevines, at various points in the Algarve (Portugal), in private small-scale vineyards.

RNA extraction and gene amplification: For each isolate total plant RNA was extracted and cDNA obtained. PCR reactions were performed using primer pairs designed in this work, based on the GenBank accession AF233934, targeting three regions of the GLRaV-5 genome: a 657 bp fragment of the HSP70h gene (nt 879- nt 1535), a 1779 bp fragment including the -3' end of the HSP70h gene and the heat shock protein 90 homolog (HSP90h) complete gene sequence (nt 1517-nt 3295), and a 1045 bp fragment including the complete sequence of the capsid protein (CP) gene (nt 3106-nt 4150).

Cloning, SSCP analysis and Sequencing: for the three genes amplicons were cloned before sequencing. An SSCP (Single Strand Conformation Polymorphism) analyzes was conducted on at least 16 clones of the CP gene for each isolate, prior to sequencing, and used to determine the heterozygosity level of the CP gene within each isolate, using Nei's *h* coefficient [8].

Sequence Data Analysis: gene-specific datasets were constructed, including homologous nucleotide sequences from GLRaV-5 and related ampeloviruses available at GenBank. Phylogenetic trees were obtained in MEGA5 using the Maximum Likelihood method.

Estimates of evolutionary divergence and selection pressures: estimates of average evolutionary divergence analysis were conducted using the Datamonkey webserver. Evolutionary analysis included Tajima's D test of neutrality and the test of natural selection, G-test statistics, of the McDonald and Kreitman test, both performed with DnaSP software vs. 5.10.01.

Recombination analysis: detection of evidence of putative recombination events in the HSP90h and CP sequences was performed with the genetic algorithms for recombination detection (GARD) available at the Datamonkey webserver and also using the RDP v.3 alpha44 software and associated programs package.

RESULTS AND DISCUSSION

During an ongoing survey of grapevine leafroll-associated viruses in field grown plants we have molecularly detected the presence of GLRaV-5 in a varietal collection at INIAV (CAN PRT051) and in private vineyards in the Algarve. This allowed us to identify a set of GLRaV-5 field isolates on which we targeted the HSP70h, CP and HSP90h genes and conducted a detailed analysis of within-isolate viral populations [3].Our results contribute with novel information on the virus diversity, and suggest a strong role of host vegetative propagation in viral variant divergence dynamics.

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Evolutionary Analysis of the Coat Protein Gene of Grapevine Leafroll Associated Ampeloviruses

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INTRODUCTION

RNA viruses are considered to be among the fastest evolving organisms. Two main evolutive forces drive the generation of sequence variants: mutation and recombination. In case of RNA viruses, mutation rate tend to be highly significant due to the lack of proofreading activity of RNA dependent RNA polymerases. However most of mutations are deleterious, and in consequence, lost in the evolutive story. The mutations produced into an ORF can be at silentious or non silentious sites, producing or not, changes in the final protein. The rate of substitutions at silentious sites (dS) and at non silentious sites (dN) are used to quantify the selection pressure. The dN/dS ratio is one of the most used tools for estimation of selection pressure. The recombination in virus evolution may act at different levels (intra or interespecific recombination, or even through the acquisition of host genes). This is a well documented process which can lead to emergence of new species or increase the variability inside a species.

The etiology of Grapevine Leafroll Disease is very complex, since several viral species belonging to the *Closteroviridae* family have been associated with this disease (mainly belonging to *Ampelovirus* genus). The *Ampelovirus* genus comprises viral species with clear genetic differences between them, leading to the conformation of two putative subgroups, referred to as subgroups I and II (Maliogka et al. 2008). The complete or nearly complete genome sequences of most of the putative species have been reported. Two of these reports that were recently published (Abou Ghanem-Sabanadzovic et al. 2011; Thompson et al. 2011) {Abou Ghanem-Sabanadzovic, 2011 #1279}agree with the proposal by Martelli (2009) of a taxonomic revision of the Ampelovirus genus to consider the grapevine-infecting ampeloviruses of subgroup I as a single divergent specie. In any case, the phylogenetic cluster of highly related GLRaV-4, -5, -6, -9, -Carn represent a group (sometimes called GLRaV-4-like) of species or strains with a particular coat protein (CP) variability behavior. The CP is one of the key genes for taxonomy of plant viruses. In this context, the GLRaV-4-like group presents a variability level and a serological behavior which difficult its taxonomy. The level of similarity of the so far sequenced CP of different putative species ranges between 79% and 87%, but all the strains share a highly conserved C-terminal region (87-99% identity) and a most variable N-terminal end (Rowhani 2009).

MATERIAL & METHODS

A total of 41 GLRaV-4-like CP sequences were analyzed. Thirty of them were obtained from twenty leafroll infected grapevine plants identified into a previous survey. dsRNA was extracted according to Zhang *et al* (1998) and used as template for RT-PCR using the primers Amp-CP-F and Amp-CP-R (5′-GCTGGATAGGTTYAGRTCNAAAGAYACYCC-3′, and 5′-TAACCTCCATATTTTCAAACG-3′). The PCR products were cloned and sequenced in both senses. As significant sequence differences were obtained in different clones from same plants, they were considered as divergent strains or multiple infections occurring into a single plant. All these sequences together with 11 available CP sequences in GenBank, were used for the generation of a multiple alignment of codons, and such alignment used for subsequent evolutive analyses using the HyPhy software package (Delport et al. 2010). In first instance, the entire dataset was subjected to GARD analysis. A putative recombination event was inferred at position 228 of the alignment of codons. This recombination event was confirmed by means of RDP software. Two datasets were then generated, one corresponding to the first 222 nucleotides (76 codons) and the other to the remaining aligned codons. The two subsets were analyzed by three methods: SLAC, REL, and FEL (Kosakovsky Pond and Frost 2005). To evaluate the presence of putative linear or continuous epitopes over the sequenced CP, the BepiPred software (Larsen et al. 2006) was used.

RESULTS

Although overall dN/dS ratios were less than 1 (0.117 and 0.119 by SLAC and REL respectively), the C-terminal region was subjected to heavier purifying selection (dN/dS by SLAC 0.046 and by REL 0.047) than the N-terminal region (0.281 and 0.351). The site-by-site analysis revealed a different pressure constrains along the CP sequence. In the first 76 codons, twelve sites were inferred as being under positive selection by REL, and 48 were identified as being under negative selection pressure by at least one method. Regarding the second dataset (codons 77–end), no method detected positive selection pressure, whereas 164 sites (over 200 codons) were inferred as being under negative selection. In general, the high prevalence of negatively selected sites begins at position 24 of the multiple alignment of codons. The Bepipred analysis using all the sequences revealed the highest probability of occurrence of a B-cell linear epitope in the first 24 amino acids, in concordance with the previously reported (Maliogka, Dovas et al. 2008). It should be noted that in the same positions, most of the sites under positive selection were inferred, and the region was also the most variable of CP.

The results obtained in this study can explain the previous records of a differential identity level across the CP sequence of GLRaV-4-like viruses (Rowhani 2009). The results of the epitope prediction analysis revealed a considerably high probability of the occurrence of a linear B-cell epitope in the N-terminal region of CP, being consistent with previous observations (Saldarelli et al. 2006; Maliogka, Dovas et al. 2008). Regarding that point, some immunological issues need to be discussed. Considering that most serological reagents available for characterizing the ampeloviruses of subgroup I are monoclonal antibodies (Gugerli 2009) and some of them possess good reactivity against the denatured CP in Western blot, it is highly probable that they are directed against a linear epitope. Moreover, considering that viruses were applied in the native form during immunization, these epitopes may be located on the virion surface. Taking account that the most immunogenic region may be a linear epitope (the most variable region), the monoclonal antibodies targeting these epitopes will not be useful for taxonomic assignation at the species level. In conclusion, there are evidence of a differential selective constrains acting in the evolutive history of GLRaV-4-like viruses. This lead to a highly variable N-terminal region, as result of the occurrence of positively selected sites. And, as this region is prone to be highly immunogenic, this may explain the variable immunological behavior observed in this cluster of viruses

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First Results on Wind Dispersal of *Parthenolecanium corni* Larvae in a Newly Planted Vineyard

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INTRODUCTION

Many species of mealybugs (*Pseudococcidae*) and soft scales (*Coccidae*) living on grapevine have been shown to vector grapevine leafroll ampeloviruses (e.g. GLRaV-1 and -3) and 'rugose wood'-associated vitiviruses (e.g. GVA) in throughout grape-growing regions in the world (Herrbach et al., *in press*) and they represent a non negligible way of virus dispersal over short distances. It has been demonstrated that some mealybug species are able to spread rapidly leafroll from infected plots to new plantations (Cabaleiro et al., 2008; Le Maguet, 2012 ; Le Maguet et al., *submitted*), but the dispersal and virus spreading by soft scales in the vineyard is not documented.

Several ways of natural dispersal can be observed in the vineyard. Larvae can easily crawl from one leaf to the other, and then to adjacent plants. They can also be transported by winegrowers and their engines during the different winegrowing works. Scale-attending ants, that carry crawlers from vine to vine, may also contribute to the spread of viruses (Daane et al., 2007; Mgocheki and Addison, 2010). However, the main factor of passive dispersal is probably the wind, as previously observed for several scale species (Greathead, 1997, Grasswitz and James, 2008). In addition, wind dispersal of fallen leaves bearing larvae is also possible (Lo et al., 2005-2006).

Parthenolecanium corni (Bouché) is a soft scale that thrives in northern European vineyards and is able to vector GLRaV-1 as well as GVA (Hommay et al., 2008). The establishment of a new plantation between plots being both virus-infected and *P. corni*-infested offered the opportunity to evaluate whether nymphs may be detached from their support by the wind, during their active dispersal after hatching ('crawler' phase) and during their migration as second instars (L2) down the stocks to hibernate. The aim of this study was to assess whether nymphs can be transported by the wind and carry leafroll viruses or GVA, susceptible to contaminate new stocks.

MATERIALS AND METHODS

The young vine plot was planted in Nothalten (Alsace, north-eastern France) at spring 2008 with certified rootstocks (34 EM), on a strip of 94 m x 11 m. The plot was arranged in four rows, south-north oriented along the slope. The plantation was surrounded by plots infested by *P. corni* and infected by GLRaV-1, -2, -3 and GVA in various combinations, the plot to the west being the most heavily infested.

Sticky traps consisted of transparent PVC cylinders (height 30 cm, diameter 14 cm), wrapped up with a 30 cm x 45 cm transparent polythene sheet sprayed with glue (Soveurode™) and staked at a height of *ca*. 1.2 m (i.e. approximately at the level where larval density was the highest on the neighboring plots). A grid of such traps was set up in the young plot: five (2009) or six (2010 and 2011) traps placed at 20 m intervals within each of the four rows of plantation. From July 2009, a control trap was placed on a neighboring plot at *ca*. 50 cm height close to a highly infested vine infected by GLRaV-1, -3 and GVA. Traps were checked every week during the crawler phase and during autumnal migration of L2. The egg-laying period of females was controlled in order to settle traps just before the first hatchings. Sheets were examined under binocular microscope and nymphs were marked and counted on a grid divided into eight sectors corresponding to wind directions.

Trapped crawlers were then collected from the glue, if possible in samples of min. 20 individals, and tested in a quadriplex RT-PCR for the presence of GLRaV-1, -2, -3 and GVA (Beuve et al., *submitted*). Populations of L2 larvae were counted at spring on each vine of the first two first rows of the neighboring plots. Infection of the most infested vines was checked by ELISA.

In spring 2011 and 2012, distribution of L2 was controlled on the young plantation. In 2011, larvae and winter canes of the most infested vines were tested by the same quadriplex RT-PCR procedure. Mean temperatures, rainfall, maximal wind speed and wind direction were obtained from a meteorological station situated at *ca*. 12 km from the experimental plot.

RESULTS AND DISCUSSION

The total number of *P. corni* crawlers caught in spring 2009, 2010 and 2011 was respectively 145, 251 and 611 in all traps set up in the young plot (7, 10 and 25,5 in average per trap). The distribution of catches in the young plot seemed to be related to the main prevailing winds and to population density in the immediate vicinity. However, the settling of *P. corni* on the young plot displayed no significant structure. In Autumn, very few L2 nymphs were caught, probably because their heavier weight.

About 30% of crawlers batches and 50% of L2 batches carried by the wind contained one virus or more. There is therefore a possibility that wind-borne larvae could contaminate young vines, provided they are able to attain a plant and feed on it. However, the relative part of such a way to disperse a virus is unknown. Moreover, our detection tests could not reveal the presence of either virus transmitted by *P. corni* (GLRaV-1 and GVA) in the colonized vines in the young plot. It is possible that the number of larvae is too low (less than 20 L2 on the most infested young vines) or that the virus, if transmitted, was not yet detectable. New monitorings are underway in 2012, as well as virus detection tests in young vines.

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Effects of Foliar Insecticidal Treatments for the Control of Mealybugs on Grape in Virginia

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Mealybugs, members of the Pseudococcidae family, are important vectors of grapevine leafroll-associated viruses (GLRaVs) that are causal agents of grapevine leafroll diseases (GLD) (Rayapati et al., 2008). GLD can cause significant crop losses and reduce grape quality (sugar content, skin color, etc) in severely infected vineyards (Kovacs et al., 2001). As a recent survey has shown (Nita et al., 2012), vineyards in the state of Virginia have a significant amount of leafroll-infected vines present. With the impending threat of the spread of GLD, management of the primary vector needed to be assessed. Therefore, two field experiments were conducted, one at the experimental farm (AHS AREC at Winchester, VA) and the other at a commercial vineyard (Orange, VA) to investigate the effectiveness of foliar insecticide sprays on controlling grape mealybug populations.

The AHS AREC vineyard block has 21-year old 'Cabernet Sauvignon' grapevines infected with grapevine leafroll disease (specifically GLRaV-2 and -3). Within each row, all but one of the infected vines were removed and re-planted with certified 'Cabernet Franc' cuttings at approximately 5 and 10 feet away from each infected vine. After planting, foliar insecticide treatments were applied in a randomized block design with six replications. The treatments were (i) an acetamiprid (Assail, 2.5 oz/A) at delayed dormant stage, (ii) an acetamiprid (Assail, 2.5 oz/A) at delayed dormant stage plus a pyrethroid (Baythroid XL, 3 oz/A) at bloom and (iii) a control (no spray). At the commercial vineyard, a single row of variety 'Chardonnay' was used. Treatments were assigned in a completely randomized design with four replications. Two foliar neonicotinoid treatments, dinotefuran (Movento, 6 oz/A) and spirotetramat (Scopion, 4 oz/A), were tested. Following treatments, mealybug numbers were visually assessed by a rater spending 5 minutes per vine.

The experiment at the AHS AREC resulted in the increase of mealybug populations over a course of a season on all vines regardless of treatment, where the vines treated with two-time application of insecticide resulted in significantly higher mealybug count ($P < 0.05$) at bunch closure (mid-July). There was also a significant difference in the number of mealybugs found on the older vines versus the number of mealybugs found on the newly planted vines in all treatments. On the other hand, treatments at the commercial vineyard resulted in a significant time and treatment interaction ($P < 0.05$) where two insecticide treatments showed significantly faster decline of mealybugs count over time. Figure 1 shows the general decline of mealybugs over time following insecticide application at the commercial vineyard.

The results indicated the importance of insecticide selection for grape mealybug management. As the commercial vineyard sprays outperformed the AHS AREC plot sprays. It is suspected that the use of a pyrethroid at bloom for the treatments at the AHS AREC field resulted in an increase in mealybug numbers by decreasing beneficial insects that would normally prey on mealybugs within a vineyard. The extent of infestation and the species of mealybugs present in Virginia is still unknown and needs to be examined. This year, three additional field trials were added to this experiment at other locations to further assess the effectiveness of foliar insecticide treatments on mealybug control.

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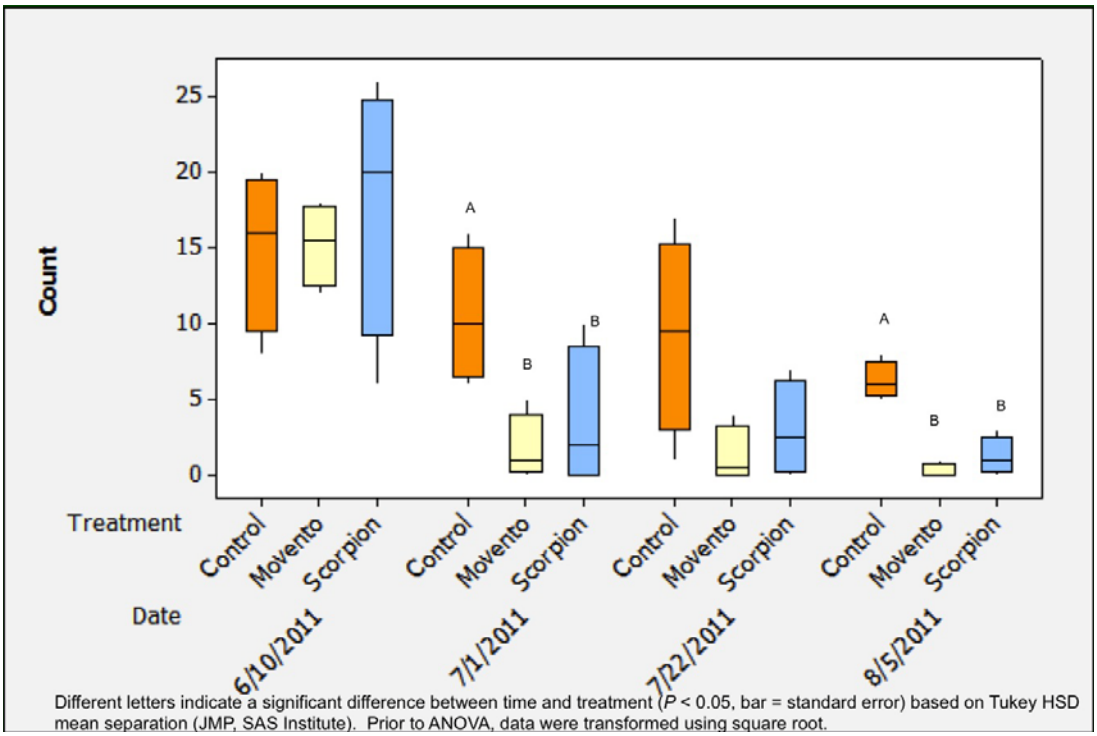
Figure 1. Mealybug Count Data over Time within Treatments



Different letters indicate a significant difference between time and treatment ($P < 0.05$, bar = standard error) based on Tukey HSD mean separation (JMP, SAS Institute). Prior to ANOVA, data were transformed using square root.

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Survey of Grapevine Viruses in Poland

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INTRODUCTION

Grapevine is one of the oldest horticultural crops and represents a highly valuable agricultural commodity. Approximately 1000 hectares of grape (*Vitis* spp.) are grown in south and south-west Poland. The main grape varieties are Aurora, Bianca, Hibernal, Johanniter, Marechal Foch, Merzling, Muskat Odesski, Regent, Rondo, Riesling, Seyval Blanc, Sibera, Solaris and Zweigelt. Like other grape-growing regions around the world, the Polish vineyards are vulnerable to many virus diseases. More than 60 viruses have been reported to infect grapevines worldwide (Martelli, 2009). The most common and economically important are *Grapevine leafroll associated virus* (GLRaV) -1, -2, -3, -4, -5, and -9, *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Rupestris stem pitting associated virus* (RSPaV), *Grapevine fanleaf virus* (GFLV) and *Grapevine fleck virus* (GFkV). Although grapevine crops have been extensively grown in the last decade in Poland, the sanitary status of the plants is unknown. Therefore, we have undertaken studies to document the occurrence of viruses in grape cultivars grown in Poland.

MATERIALS AND METHODS

Between 2010 and 2011, a number of vineyards of different area were visited to assess their sanitary status. A total of 300 wine and table grapevines were sampled. Leaf samples were collected during the growing seasons (June-October), primarily from vines that showed symptoms but also from apparently symptomless plants. Total nucleic acids were isolated using silica capture (SC) method described originally by Boom et. al., 1990. One microliter o SC nucleic acids preparations was used for RT-PCR in total volume of 10 µl. Amplification was performed using SuperScript One-Step RT-PCR kit (Invitrogen, Carlsbad CA, USA and the specific primers (Table 1). All samples were tested individually for the presence of GLRaV-1, -2, -3, -4, -5, -7 and -9, GVA, GVB, RSPaV, GFLV. GFkV, *Cherry leaf roll virus* (CLRV) and *Arabis mosaic virus* (ArMV). The RT-PCR amplified fragments from select number of samples were sequenced in order to confirm their specificity. Multiple sequence alignments were done using the online service ClustalW (Thompson *et al*., 1994). Corresponding sequences of each virus available in GenBank were included in these analyses. The RT-PCR samples positive for GLRaV-1, -2, -3, GVA, GVB, GFLV and GFkV were tested additionally by ELISA using commercial kits (Agritest, Valenzano, Bari, Italy) according to the manufacturer's instructions. To confirm detection of GLRaV-5 and RSPaV a second pair of primers for these viruses was used (Table 1). Positive controls for RT-PCR and ELISA were lyophilized leaf tissues from infected fresh plants kindly provided from Carole Balmelli (Station de recherche Agroscope Changins-Wädenswil ACW, Switzerland).

RESULTS AND DISCUSSION

During this survey, virus leaf symptoms like distortion, discoloration, downward rolling and mottling were frequently seen in most of the vineyards. Additionally, poor fruit settings, irregular ripening and reduced size of the berries were observed. The RT-PCR and ELISA results indicated the presence of GLRaV-1, -2, -3, -5, RSPaV, GVA, GVB, GFLV, GFkV and CLRV. In contrast, RT-PCR using primer pairs for GLRaV-4, GLRaV-7, GLRaV-9 and ArMV failed to amplify fragments with the expected size. GLRaV-1, -2, -3 were detected in six, nine and five samples, respectively. In majority of these samples RSPaV and GFkV were found as mixed infections. The presence of both GLRaV-5 and GFLV was confirmed in one grapevine as a single infection. The RT-PCR results showed that five grapevines were infected with GVA. In four of them GLRaV-1, GLRaV-3, RSPaV and GFkV were documented in different combinations. GVB was detected in one grapevine together with RSPaV. CLRV, GFkV and RSPaV were detected in 55, 70 and 218 tested samples with frequency of 18.3%, 23.3% and 72.6%, respectively. In majority of these samples, the three viruses were found as mixed infections with different viruses mentioned above. Overall infection in the surveyed grapevines was 82.6%. Using molecular biology

Table 1. The primers used in RT-PCR

Virus	Primers	Reference
GLRaV-1	LOV1-H47/LEV1-C447	Osman and Rowhani 2006
GLRaV-2	V2dCPr1/V2dCPf2	Bertazzon and Angelini, 2004
GLRaV-3	LC1/LC2	Osman and Rowhani 2006
GLRaV-4	HSPV-F/HSPC/R	Escobar *et al*., 2008
GLRaV-5	LR5HSPV/LR5HSPC	Osman and Rowhani 2006
GLRaV-5	G5F5 5' GGCAGCGATAGTGCAAGGGA 3' G5R5 5' CCCGAAGTAGACCCAAACGAG 3'	This study
GLRaV-7	LR7-F/LR7-R	Engel *et al*., 2008
GLRaV-9	LR9-F/LR9-R	Alkowni *et al*., 2004
GVA	H7038/C7273	MacKenzie *et al*., 1997
GVB	GVBV1/GVBC1	Minafra *et al*., 1994
RSPaV	RSP13/RSP14	Zhang *et al*., 1998
RSPaV	RSP52/RSP53	Rowhani et al., 2000
GFLV	C3310-H2999	MacKenzie *et al*., 1997
GFkV	FLCPV/FLCPC	Osman and Rowhani 2006
ArMV	ArMV-CP1202F/ ArMV-CP1313R	Osman and Rowhani 2006
CLRV	RW1/RW2	Werner *et al*., 1997

approaches, the presence of genetic variants of detected viruses was found. The results documenting the occurrence of different viruses and their variants in grapevine cultivars improved our understanding of the sanitary status of vineyards in Poland.

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Occurrence of Grapevine Leafroll-associated Viruses 1 and 3 in the Vineyards of India and their Characterization

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SUMMARY
Grapevine is a strategic horticultural crop of India. A survey was conducted during 2010-2011 in the vineyards of Nashik and Pune regions of western India. Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) study suggested the occurrence of two viruses associated with leafroll disease in grapevines. Subsequent study, using p24 protein gene specific primers in reverse transcriptase PCR (RT-PCR), revealed the association of *Grapevine leafroll-associated virus 1* (GLRaV-1) with two cultivars of grapevine. Presence of *Grapevine leafroll-associated virus 3* (GLRaV-3) was confirmed in RT-PCR using complete CP (coat protein) and partial heat shock protein 70 homologue (HSP70h) genes specific primers in the seven cultivars. Cultivar Shiraj from a vineyard showed the presence of mixed infection for both the virus species. The isolate of GLRaV-3 from cultivar Cabernet Souvignon showed incongruent grouping behavior in its phylogeny based on complete CP and partial HSP70h sequences. In a phylogeny constructed based on the partial HSP70h sequences of GLRaV-3 clustered three isolates together in group 2 while two isolates from cultivar Shiraj grouped in group 1.

INTRODUCTION
Grapevine, an emerging important fruit crop of India, assumes a strategic position among the horticultural crops in view of its production, area occupied, value addition, and job creation in both rural and urban areas. It is rated as the highest foreign exchange earning fruit crop in India (Anonymous, 2011). Because of these reasons, viticulture has become one of the most remunerative farming enterprises in India. Grapevines have been credited as a 'sink for viruses' which cause a significant reduction in the quality and quantity of the crop as well as they also reduce the productive life of vineyards (Coetzee et al., 2010, Martelli et al., 2006, Martelli, 2003). Grapevine leafroll disease (GLD), one of the most widespread and economically important viral diseases, causes 62 % losses because of virus diseases in grape production worldwide (Little et al., 2001). A survey was conducted in January-February of 2010 and 2011 in Nashik and Pune regions of India. Typical symptoms of leafroll disease i.e. downward rolling of leaf margins and reddening of interveinal areas of leaf lamina were observed in the dark fruited cultivars of grapevines in the experimental farms of National Research Centre for Grape (NRCG), Pune and in farmers' vineyards of Nashik which accounts for 94% of wine production in India. GLD, caused by a complex of about eleven virus species (*Grapevine leafroll-associated virus 1, 2* and so on), the most encountered viruses associated with the leafroll disease worldwide are GLRaV-1 and GLRaV-3 (Akbas et al., 2009). Therefore, an effort was taken to detect and characterize these two most common leafroll associated viruses i.e. GLRaV-1 and GLRaV-3 by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcriptase PCR (RT-PCR).

MATERIALS AND METHODS
Samples were collected from leafroll exhibiting symptomatic plants and a few asymptomatic plants during the survey conducted in January-February of 2010 and 2011. For negative control, samples from two tissue culture raised vines belonging to two different cultivars i.e. Pusa Navrang and Centeenial Seedless were used. Both the viruses i.e. GLRaV-1 and GLRaV-3 were detected using the commercially available polyclonal antibodies and the alkaline phosphatase conjugated monoclonal IgG following the manufacturer's instructions (Bioreba, Reinach, Switzerland). To confirm the presence of viruses further, ELISA positive samples were subjected to one step RT-PCR amplification of p24 protein (ORF9) for GLRaV-1 and coat protein (CP) and heat shock protein 70 homologue (HSP70h) for GLRaV-3.

0.25 g of samples containing petoles and vein and veinlets of of leaves were taken and subjected to crude extraction and one-step RT-PCR following the protocol suggested by Rowhani et al., (2000). Primer pair pORF9F and pORF9R (GGCTCGAGATGGCGTCACTTATACCTA and CCTCTAGACACCAAATTGCTAGCGA respectively) was used for GLRaV-1 (Little et al., 2006). For GLRaV-3, the primer pair, CPf1—ATGGCATTTGAACTGAAATT and CPr942—CTACTTCTTTTGCAATAGTTG, designed from available sequences, was used for coat protein amplification and primer pair, LC1—CGCTAGGGCTGTGGAAGTATT and LC2—GTTGTCCCGGGTACCAGATAT, was used to amplify partial HSP70h (Turturo et. al., 2005).

The amplicons were purified using commercially available gel extraction kit (QIAGEN GmbH, Hilden, Germany) and cloned in pGEM-T easy vector (Promega, Madison, WI, USA) following standard procedure (Sambrook and Russel, 2001). Three clones of p24 gene of GLRaV-1 and CP gene and partial HSP70h gene of GLRaV-3 were sequenced in both directions at either at Chromous Biotech, Bangalore, University of Delhi, New Delhi, and Scigenom Labs Pvt. Ltd., Cochin or at all of them. The specific sequences were assembled and subjected to BLAST analysis at NCBI (www.ncbi.nlm.nih.gov/) and CLUSTAL W multiple alignments using *BioEdit* 7.0.9.0 (Hall, 1999). Phylogentic tree were generated using MEGA 4.0.2 (Tamura et al., 2007). The evolutionary history was inferred using the minimum evolution (ME) method (Rzhetsky and Nei, 1992).

RESULTS AND DISCUSSION

Samples from seven cultivars namely, Cabernet Souveignon, Shiraj, Krishna Seedless, Sharad Seedless, Flame, Pinot Noir and Thompson Seedless from six vineyards reacted positively against GLRaV-3 in DAS-ELISA while samples from two cultivars, Shiraj and Pinot Noir from two vineyards reacted positively against GLRaV-1 in DAS-ELISA. Cultivar Shiraj showed the presence of mixed infection of GLRaV-1 and 3 together. Cultivars Shiraj, Pinot Noir and Cheema Sahebi from three vineyards and tissue culture-raised cultivars (Pusa Navrang and Centennial Seedless) could not react against any of the viruses tested. All the cultivars positive for respective viruses in DAS-ELISA were confirmed through RT-PCR using specific primers (ORF9F, ORF9R for GLRaV-1 and CPf1, CPr942 for complete CP of GLRaV-3 and LC1 and LC2 for partial HSP70h of GLRaV-3). After sequencing, 646 bp including 630 bp of p24 protein gene was obtained from the two ELISA positive cultivars of GLRaV-1 while 942 bp and 546 bp of CP and partial HSP70h genes were sequenced from a cultivar Cabernet Souvignon. Partial HSP70h gene was also amplified and sequenced (546 bp) from another four GLRaV-3 positive cultivars viz. Shiraj, Sharad Seedless, Krishna Seedless and Pinot Noir.

The p24 gene sequences of GLRaV-1 from India shared maximum identity of 95.8 to 96.1 % at nucleotide level and 96.1 to 96.6 % at amino acid level with Claretvine isolate of GLRaV-1 from USA (GenBank Accession No. HQ833477). Nashik isolate of GLRaV-3 from Cabernet Souvignon (Accession No. JN616386) shared 99.4 % of maximum identity with the ten isolates at nucleotide level while at aa level it shared 100 % of maximum identity with eleven isolates. This isolate of GLRaV-3 from India showed incongruence in its phylogeny based on complete CP gene and partial HSP70h gene. It clustered in group 2 on the basis of CP phylogeny while on the basis of partial HSP70h phylogeny it grouped in group 1.

All the five isolates of GLRaV-3 from India shared 97.4 to 100 % of maximum identity at nucleotide level with the GenBank submitted isolates. At amino acid level, the five isolates of India shared 99.4 to 100 % of maximum identity with the GenBank submitted isolates. All the five isolates shared 94.3 to 100 % of maximum and minimum identities among themselves at nucleotide level and at amino acid level; the corresponding values of identities were 98.3 to 100 %. When the phylogeny was constructed based on the partial HSP70h gene sequences of GLRaV-3 isolates from five cultivars of grapevine from India, the three isolates clustered in group 2 while two isolates (previously characterized Nashik isolate from cultivar Cabernet Souvignon and another isolate from cultivar Shiraj) grouped in group 1 (Fig 1).

Even though there are eleven leafroll-associated viruses, an attempt was made to detect and characterize the two most common leafroll-associated viruses of grapevine, which we detected either alone or in mixed infection. The cultivars, positive for leafroll viruses in the study, have either been introduced or they are the selection from the imported cultivars. Although India ranks first in productivity of grapevine, the effect of leafroll-associated viruses on yield and quality of both, berries and wine, in Indian context have not been studied and this needs to be investigated further.

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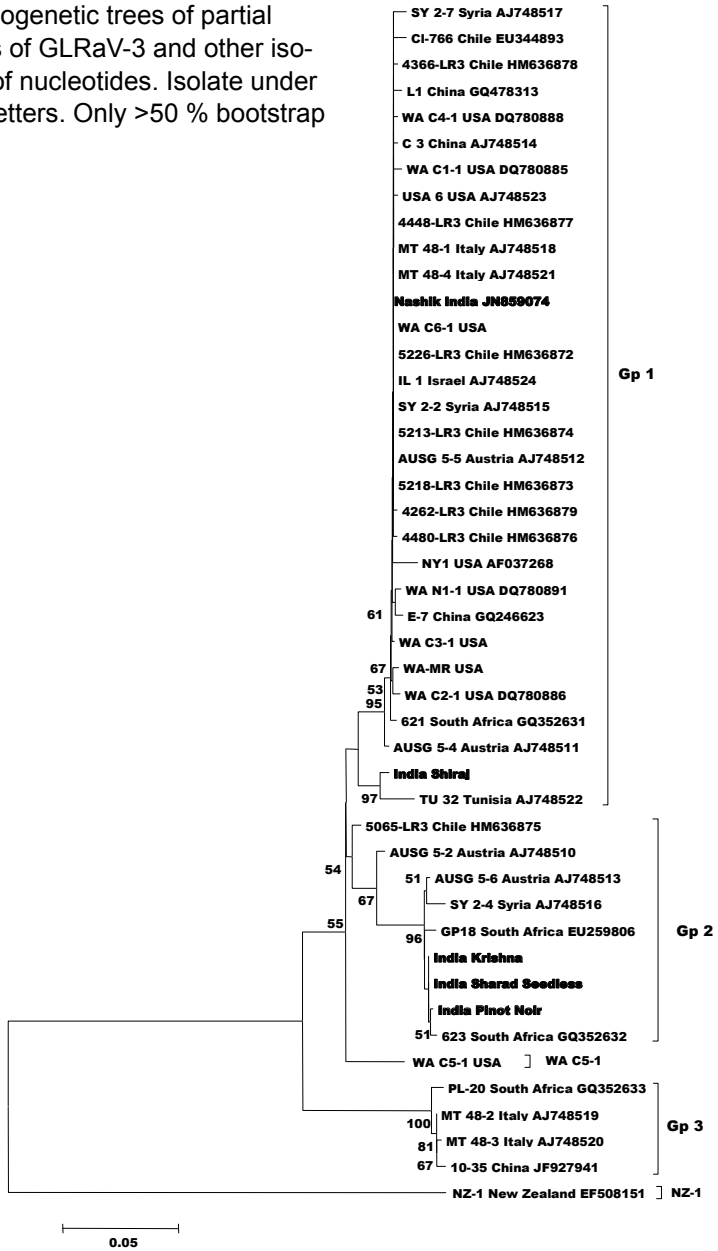

Fig. 1: Minimum evolution phylogenetic trees of partial HSP70h gene of Indian isolates of GLRaV-3 and other isolates in GenBank on the basis of nucleotides. Isolate under study has been shown in bold letters. Only >50 % bootstrap values are shown here

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Grapevine Enation Disease: First Records In Marche Regions (Central-Eastern Italy)

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SUMMARY

Many diseases infect grapevine and some of these cause serious economic damage. In particular, virus and virus-like diseases are the most dangerous because their control is very difficult and the only use of healthy propagating material is effective. Aim of this research was to clarify the detrimental effects of enation disease on cv. Sangiovese, in Marche region (central-eastern Italy). For these reasons, we carried out surveys in 2009 and 2010 in two commercial vineyards, we recorded the vines showing symptoms of enations, from which we collected cuttings, during autumn, for serological and molecular analysis. Typical proliferations on the leaves were recorded in 2009, while mild symptoms on the canopy were observed in 2010. In both vineyards the productions were dramatically reduced mainly in 2009, when the expression of symptoms were more severe. From the molecular analysis, the material selected showing enation symptoms resulted infected by other viral entities. For this reason from cloning and sequencing, we obtained mainly high homology with GRSPaV, widespread not always associated with a specific symptoms.

INTRODUCTION

Grapevine enation disease was firstly described and studied by Hewitt (1954) in California and later in Europe (Germany, Italy, France, Spain, Greece, Austria, Czechoslovakia, Moldova), South Africa, Venezuela, Australia and New Zealand (Graniti and Martelli, 1970; Prota and Garau, 1976; Martelli and Boudon-Padieu, 2006).

Even if typical symptoms had been already recorded since long time in Europe (Buchenau, 1891; Petri, 1931). Sensitive grapevines include the cvs. Italia, Panse precoce, and Primus, but symptoms were recorded on 17 cultivars in some vineyards in Sardinia and Emilia Romagna (Italy) (Prota and Garau, 1976; Credi, 1996).

When infected, these cultivars are slow to break dormancy in the spring and develop shoots with shortened internodes and convoluted and thickened leaves. The enations commonly develop on the undersides of basal leaves and appear as miniature leaf-like outgrowths in the interveinal region (Fig. 1).

Grape production is greatly affected, and loss of yield up to 70% (Prota and Garau, 1970; Credi, 1996). In some European scions and American rootstocks, the upper leaf surfaces may develop enations (Prota and Garau, 1976). Also, symptoms are erratic and may not be present every year. The etiological agent of grapevine enation disease is erratically distributed in infected grapevine, however it is graft-transmissible, and LN33, although not very sensitive, can be used as an indicator. Hence, grapevine enation is disseminated primarily through diseased planting stocks, no information are available about the agent and the way of spreading in the vineyards.

Fig. 1 – Typical symptoms of grapevine enation disease, recorded on cv. Sangiovese, in a commercial vineyard located Marche region.

Aim of this research is to verify the impact of enation disease on viticulture of Marche region (central-eastern Italy) and try to identify the causal agents of the disease.

MATERIAL AND METHODS

From May 2009 up to May 2010, in the behalf of a clonal and sanitary selection plan (Romanazzi *et al*., 2007), we carried out visual inspections in two commercial vineyards located in Ascoli Piceno province (Italy), where vines with typical symptoms of enations had been recorded. The propagating material used in the two vineyards was cv Sangiovese (clones VCR103 and VCR23), grafted on 775P rootstocks (*V. berlandieri* x *V. Rupestris*),

virus-free category. During the inspections the symptomatic vines were recorded on a bi-dimensional map, in order to verify the progression of the disease in the following year. In the winter 2009, we collected cuttings from 40 vines showing typical enation symptoms, were analyzed by ELISA with specific antibodies (Agritest, Valenzano, Bari, Italy) for the main grapevine viruses (GVA, GVB, GFLV, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-7, GFkV, and ArMV). From the same samples, total nucleic acid (TNA) was extracted from 200 mg of cortical scrapings of dormant cuttings, as described by Foissac *et al*. (2001). The RT-PCR was carried out with specific primers for virus for which were available ELISA antibodies and for GRSPaV, GLRaV-5, and GLRaV-9 (Gambino and Gribaudo, 2006; Osman and Rowhani, 2006; Engel *et al*., 2008). Leaf and cortical scrappings of tissues (15–30 g) from symptomatic and symptomless vines were used to recover dsRNAs according to a protocol of Dodds (1993). DNA and single-stranded RNA were sequentially enzyme-digested as described by Saldarelli *et al*. (1994). cDNAs, synthetized from dsRNA, were amplified in DOP-PCR, using degenerate oligo primers (Rott and Jelkmann (2001). All PCR products were directly cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions and used to transform *Escherichia coli* DH5*a* competent cells. Selected clones were subjected to automated sequencing (BMR genomics). Nucleotide and amino acid homology was done with Blast analysis.

RESULTS AND DISCUSSION

From visual inspections carried out in 2009, in the two commercial vineyards we recorded an incidence of grapevine enation disease of about 50% (1500 vines out of 3000) and 16% (1000 vines out of 6250), respectively. Sangiovese vines infected showing a bushy spring vegetation and a delayed bud opening, as previously described in other observations carried out on cvs Barbera, Cardinal, Girò, Malaga, Malvasia, Nasco, Nuragus, Regina, Vermentino, Vernaccia and Vernaccina in Sardinia (Prota and Garau, 1976) and on cv Trebbiano Romagnolo in Emilia Romagna (Credi, 1996). During the season the intensity of symptoms on canopy seemed to be mitigated.

From visual inspections carried out in 2010, we recorded mild symptoms, characterized by malformed basal leaves with cup shape, thick blade and prominent veins (Fig. 2). No typical proliferations were detected on leaves. It is known that enation symptom severity may be not constant in different years, probably for the different interaction among causal agents, environmental and plants conditions (Prota e Garau, 1976; Credi, 1996). However, considering the productions, we recorded for cv Sangiovese (clones VCR23 and VCR103) a loss of about 50% in the 2009, when the typical symptoms of enations occurred.

Fig. 2 – Enations mild symptoms recorded on cv Sangiovese in spring 2010. Upward cup-shaped leaves and shortened internodes are not linked to enations.

For highly susceptible varieties such as cvs Italia and Trebbiano romagnolo, not only the quantitative parameter resulted dramatically affected, but also qualitative parameters (titratable acidity, and sugar content) were significantly perturbed (Prota *et al*., 1980; Credi, 1996). The not constantly expression of enation symptoms, probably influenced by climatic conditions, and the co-infection with other virus, in the symptomatic vines, made very complex the identification of the causal agent of enation disease. More than 23% of vines showing enations resulted infected by three different virus and 19% of vines was infected by two viral entities. For these reasons, the amplification of cDNAs by DOP-PCR, and the following cloning and sequencing, allowed to obtained nucleotide sequences with high homology with GRSPaV, a virus widespread not always associated with a specific symptoms.

The advent of deep sequencing techniques could be solved this question as recently happened for Syrah decline (Al Rwahnih *et al*., 2009). What is not unclear is why a enation disease is not among the viral disease from which the propagating material must be free, since it determines high loss of production, although sporadical outbreaks are recorded.

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Specific Patterns of Co-occurrence of Grapevine Viruses in Washington and Virginia Vineyards

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INTRODUCTION

A survey for grapevine viruses conducted in Washington vineyards during 2005 and 2009 revealed the presence of Grapevine leafroll-associated virus 1 (GLRaV-1), -2, -3, -4, -5, and -9) Grapevine rupestris stem pitting-associated virus (GRSPaV), Grapevine Virus A (GVA), GVB and Grapevine fanleaf virus (GFLV) in many wine grape cultivars (Naidu et al., 2006; Mekuria et al. 2009). These viruses were found occurring as single and/or mixed infections in individual grapevines. In order to determine the scale of association (or lack of) of viruses in mixed infections at the individual plant level, the data sets from the survey were analyzed by the Jaccard association analysis to measure the probability of any two viruses co-occurring in individual grapevines.

MATERIALS AND METHODS

The association between two viruses with in a vine sample was determined with the Jaccard index of similarity (*J*) for 1267 paired petiole samples (Everitt 1998). The Jaccard index was calculated *as J = a/(a+b+c).* For example, when GLRaV-1 and GLRaV-2 were compared, a represents the number of cases where both GLRaV-1 and GLRaV-2 were present, b represents the number when only GLRaV-1 was present, and c represents the number when only GLRaV-2 was present. The index goes from 0 to 1 where values close to 1 suggests a high degree of association between two viruses, and 0 suggests disassociation between two.

A standard normal statistic can be calculated using: $Z = (J-J_{ran})/S_j$. Where J_{ran} is a mean Jaccard index based on randomization, and the standard error (S_j) was estimated using a jackknife procedure developed by Turecheck and Madden (2000). Values of $Z > 1.96$ indicate significant positive association, and $Z < -1.96$ indicate significant negative association (i.e., disassociation) at $P = 0.05$. The Cramer's V, which is another measurement of the association, was also examined (PROC FREQ SAS ver. 9.2, Cary NC). Means of virus infect vines per vineyard were also examined using principal component analysis of Minitab 15 (University Park, PA).

RESULTS AND DISCUSSION

Results based on the Jaccard similarity index indicated that some grapevine viruses such as GLRaV-2 and -4 were significantly positively associated ($Z \geq 1.96$) and others like GLRaV-1 and -2 were negatively associated ($Z \leq -1.96$) (Table 1). Results based on Cramer's V analysis (ranging from -1 to 1, 0 = no association) showed a very similar trend to that of Jaccard similarity index (Table 1).

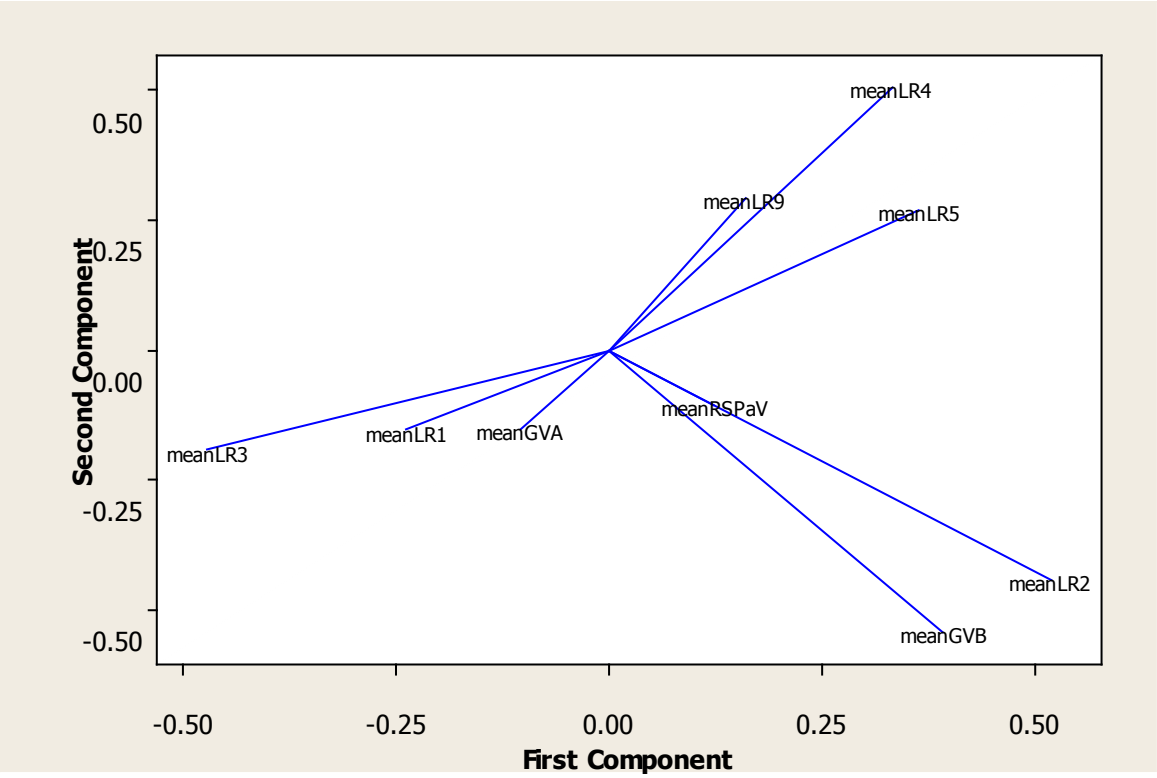
A principal component analysis of the data sets by vineyard showed that ~ 80% of variation was explained with the first five components. Examination of the first two components (factors) revealed that GLRaV-3 and GVA, which are known to be transmitted by similar vector(s), were grouped together (Fig. 1).

In addition to the data shown here, data from a similar survey done in Virginia vineyards during 2009 and 2011 will be discussed.

Table 1. Association of two viruses on the same vine, described with Jaccard index for association (upper right side), and Cramer's V statistics (lower left side).

	GLRAV-1	GLRAV-2	GLRAV-3	GLRAV-4	GLRAV-5	GLRAV-9	RSPaV	GVA	GVB
GLRAV-1		-5.91	1.19	-2.59	-1.70	-1.68	3.17	3.47	-2.74
GLRAV-2	-0.095		-2.17	4.24	2.64	-0.96	-2.76	-0.98	5.22
GLRAV-3	0.071	-0.104		-3.19	-2.85	-3.01	-6.83	-0.11	1.17
GLRAV-4	-0.055	0.170	-0.150		7.04	0.59	-3.68	-3.81	1.15
GLRAV-5	-0.033	0.112	-0.134	0.445		0.00	-3.22	0.79	-2.93
GLRAV-9	-0.024	-0.019	-0.127	-0.018	-0.035		0.00	-7.51	0.51
RSPaV	0.130	-0.066	-0.298	-0.078	-0.060	-0.065		0.51	0.03
GVA	0.141	-0.030	-0.007	-0.089	0.028	-0.074	0.015		-4.20
GVB	-0.044	0.268	0.069	0.046	-0.041	0.025	0.000	-0.083	

Figure 1. Loading plot of PCR analysis using first two components



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Multiplex Real-time RT-PCR for Estimation of the Prevalence of the Main Five Grapevine Viruses Infecting Wine and Table Grapes in Alicante, Spain

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INTRODUCTION

The designation of origin (D.O.) "Alicante" (Spain) is an important area of cultivation of grape (*Vitis* spp.) (14,200 ha) dedicated to wine production and the D.O. "Vinalopó bagged table grape" (Alicante, Spain) is another area of production (7,500 ha) totally focused on table grape. A survey in these regions for the five grapevine viruses included in the EU Directive 2002/11/EC has been carried out to evaluate the sanitary status of the grapes grown in these areas. The Directive rules the requirement that the initial plant material for vegetative propagation must be free of *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine fleck virus* (GFkV), *Grapevine leafroll associated virus 1* (GLRaV-1) and *Grapevine leafroll associated virus 3* (GLRaV-3) because long distance spread of grapevine viruses occurs primarily by propagation of infected plant material. Because of sanitary certification programs depend on a reliable and sensitive detection of these viruses a new developed multiplex real-time RT-PCR based on TaqMan chemistry was developed and used to assess prevalence of these viruses.

MATERIAL AND METHODS

Virus isolates, plant material and samples preparation.

Virus isolates of GFLV, ArMV, GFkV, GLRaV-1 and GLRaV-3 from different origins kept in collection were used as positive controls. In addition, 154 plant samples collected in a representative survey in the D.O. "Alicante" and 95 plant samples from D.O. "Vinalopó bagged table grape" were analysed. Grapevine plants of this study were labelled and georeferenced and samples collected during latency. Plant samples were collected in winter, when plants are commercialized, to assess the ability of the technique for diagnostic purposes. At least five dormant buds or bark tissues, shoots or complete spurs (winter) around the grapevine plant, were collected as samples. Extracts were prepared from cambial scrapping cuttings by grinding aprox. 1/20 (w/v) in PBS buffer, pH 7.2, supplemented with 0,2% (w/v) DIECA, and 2% (w/v) polyvinil-pyrrolidone (PVP-10) in individual plastic bags with a heavy net (Plant Print Diagnostics) to avoid contaminations among samples. The same crude extracts were used for ELISA tests and for total RNA purification. Total RNA was extracted from 200 μl of crude extracts using Ultraclean Plant RNA isolation kit (Mobio) following the manufacturer's instructions.

Real-time multiplex RT-PCR.

Real-time multiplex RT-PCR consisted on a 25 µl cocktail (final volume), containing 1 x AgPath-ID One step RT-PCR buffer (Ambion) and 1.5 x AgPath-ID One step RT-PCR enzyme mix (Ambion), 5 µl of sample, 400 nM of GFLV, ArMV, GFkV, GLRaV-1 primers and 800nM GKRaV-3 primers, and 200 nM of each probe. The amplification protocol consisted on a RT step of 45 °C for 25 minutes and a denaturation step of 95 °C for 10 minutes followed by 45 cycles of amplification (95 °C, 15 seconds; 50ºC, 15 seconds and 60 °C, 60 seconds). Table 1 shows primers and probes used in this study. Nucleotide sequences of primers for GFLV, GFkV, GLRaV-1 and GLRaV-3 were those previously published (Bertolini et al., 2010). New primers were successfully designed for detection of ArMV. In the case of the GFkV, GLRaV-1 and GLRaV-3 probes, those previously described (Bertolini et al., 2010) were used and ArMV and GFLV probes were successfully designed a used. The fluorescent dye in the ArMV probe was FAM, in GFLV probe was LC610 and for GFkV, GLRaV-1 and GLRaV-3 probes were YAK, LC640 and LC670, respectively. All probes were labelled in 3' with BHQ quencher.

RESULTS AND DISCUSSION

The newly designed primers and probes for ArMV and the probe for GFLV diagnosis were tested against several ArMV and GFLV isolates from different origins. All the isolates tested positive. To improve and discriminate correctly the different species, by real-time multiplex RT-PCR, a compensation colour assay was necessary and performed, aimed to minimize the emission interference among the five fluorescent dyes. The color compensation assay consisted on preparing five different cocktails of reaction, each cocktail included only the specific probe of one virus using as template a composite sample containing all targets. Subsequently, and after the samples reached the stationary phase of amplification, a compensation of colour was carried out, consisting of a cycle of 95 ° C for 1 second to 4.4 ° C/sec; 40 ° C, 30 seconds, to 2.2 ° C/sec; and 65 ° C (temperature of detection plus 5 ° C), with 2 acquisitions / ºC. The generated file with the data, allowed compensating interferences. Technical sensitivity obtained by real-time multiplex RT-PCR using serial dilutions of positive controls was the same to the singleplex real-time RT-PCR reactions. The analyses of samples indicated that the incidence of viruses is high in both growing areas (Table 1). Prevalences of GLRaV-3, GLRaV-1 and GFLV were lower in grape dedicated to wine production than those from the D.O. “Vinalopó bagged table grape” located in the same Spanish region (60% vs 95%, 1% vs 65% and 40% vs 95%). However, GFkV, for which the vector is unknown, reached 85% of prevalence in D.O. “Alicante”, compared to 65% in the D.O. “Vinalopó bagged table grape” survey. The presence of mixed infections were frequently found. The high rates of infections of this virus indicate that either initially infected plant material from nurseries, and/or uncontrolled traffic of propagating plant material, are key factors in the pathway requiring a review of the current control programs. The new designed real-time multiplex RT-PCR due to its high sensitivity and specificity opens new possibilities in the detection of grapevine viruses and its inclusion in sanitary grapevine programs will lead to improve their control and improve quality of certification programs.

Table 1. Percentage of incidence of viral infection

	GFkV	ArMV	GFLV	GLRaV-1	GLRaV-3
D.O. “Alicante”	85	0	40	1	60
D.O. “Vinalopó bagged table grape”	65	0	95	65	95

ACKNOWLEDGEMENTS

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The Effect of Seasonal Changes on the Titer of Grapevine Viruses in Infected *Vitis vinifera* and their Downstream Detection using RT-PCR and qRT-PCR

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ABSTRACT

A time course study was performed for two years with three months interval to understand the seasonal viral profile of various grapevine viruses in infected *Vitis vinifera*. Viruses included in the study were *Grapevine leafroll associated viruses* (GLRaV-1, -2, -3, -4, -5, -7, -9), *Grapevine leafroll associated virus*-2 Redglobe strain (GLRaV-2RG), viruses constituting the Rugose wood complex [Grapevine rupestris stem pitting virus (GRSPaV), Grapevine vitivirus A (GVA) and B (GVB)], Grapevine fanleaf virus (GFLV), Grapevine fleck virus (GFkV), and Tomato ringspot virus (ToRSV). Sixty-five grapevines varieties previously tested to be infected with a wide range of these viruses were selected as the starting material. The samples were collected early in the growing season (May) in three months intervals for two years. From May till November leaf petioles were collected, whereas in February dormant grapevine cuttings were collected. The samples were tested using conventional reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR). Results show that detection methods based on highly sensitive RT-qPCR techniques allowed for the detection of these viruses present in low concentration at various periods of the grapevine vegetation. This is a thorough study carried out to establish the best plant material and sampling times to optimize grapevine virus detection by RT-PCR, RT-qPCR and LDA.

MATERIALS AND METHODS

In order to test the effect of seasonal changes on the titer of grapevine viruses, a time course study was performed in which samples were collected in three month intervals for two years starting early in the growing season (May). From May until November leaf petioles were collected, whereas in February dormant grapevine cuttings were collected. The samples were tested using three diagnostic techniques; RT-PCR, Real-time qPCR and LDA.

For RNA extraction, samples (petioles or cambial tissues) from eight different branches within the grapevine; four from each side of the cordon were randomly collected, combined and divided into 0.3 g amounts then subjected to total RNA extraction using RNeasy Plant Mini Kit (Qiagen) as described in Osman et al., 2008. RNA was subjected to genomic DNA elimination reaction to remove the genomic DNA as describes in Osman et al., 2012. All RNA samples extracted were tested for the 18S rRNA TaqMan® RT-PCR assay to check for RNA quality and to ensure there was no PCR inhibition (Osman et al., 2007).

Two µl of the purified total RNA were used in a 12 µl final volumes for each RT-PCR and One-Step qRT-PCR. The qPCR primers and probes for all viruses have been described before (Klaassen et al., 2010, Osman et al, 2007 and 2008. One-Step qRT-PCR reactions were run using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems) and the Low Density Array (LDA) as describes in Osman et al., 2012 and Osman et al. 2008 respectively.

Twenty six samples previously tested to be infected with the viruses under investigation were subjected to a virus distribution study in which samples from all around the grapevine were collected. Each sample was collected from a single cordon, dividing the cordon into 5 locations (A, B, C, D and E) starting from one end of the grapevine cordon to another as illustrated in Fig 1. Samples have been collected from 5 different locations within the grapevine, 4 petioles from each side, combining 8 petioles per location. 0.3 g of each sample has been weighed, homogenized and the RNA extracted has been tested using LDA detection for all viruses under study.

RESULTS AND CONCLUSIONS

Real-time qRT-PCR and LDAs can be used yearlong due to their high sensitivity as they were able to detect viruses at low titer and more virus strains at different seasons.

The detection of higher virus titers on samples vines and at the end of the growing season in all tissues tested November (Time course 4 and Time course 8) and February Time course 1 and 5) suggest that the movement of virus in the phloem is fast being that the viruses are phloem limited.

Phloem of lignified canes, when available, was found to be the best source for all viruses tested, allowing 100% detection by qRT-PCR.

Grapevine leafroll viruses were found to be more heterogeneously distributed than other viruses under study.

Heterogeneous distribution, low concentration and seasonal variations of grapevine leafroll viruses in grapevines (*Vitis vinifera*) remain a main problem which prevents the introduction and standardization of molecular biology-based quick laboratory detection protocols for their routine use in certification and quarantine.

Fig. 1 Time course presentation of the virus titre for GLRaV-1,-2,-3,-5 and 2RG over two years with three months interval as detected by qRT-PCR/LDA detection.

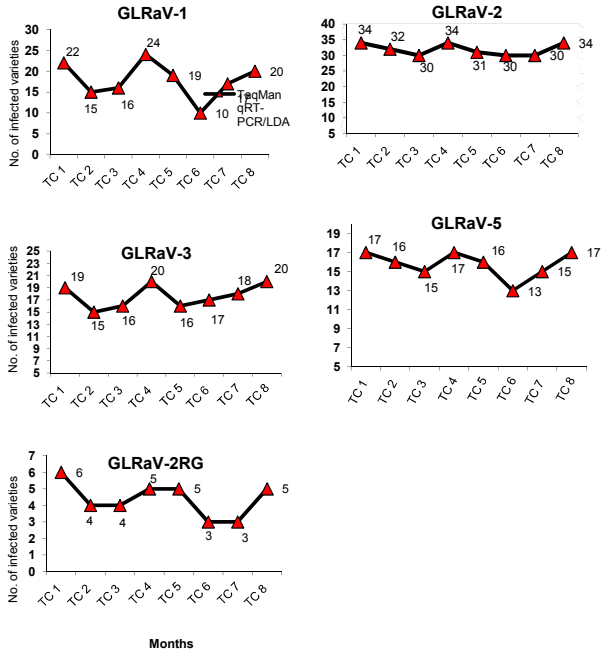
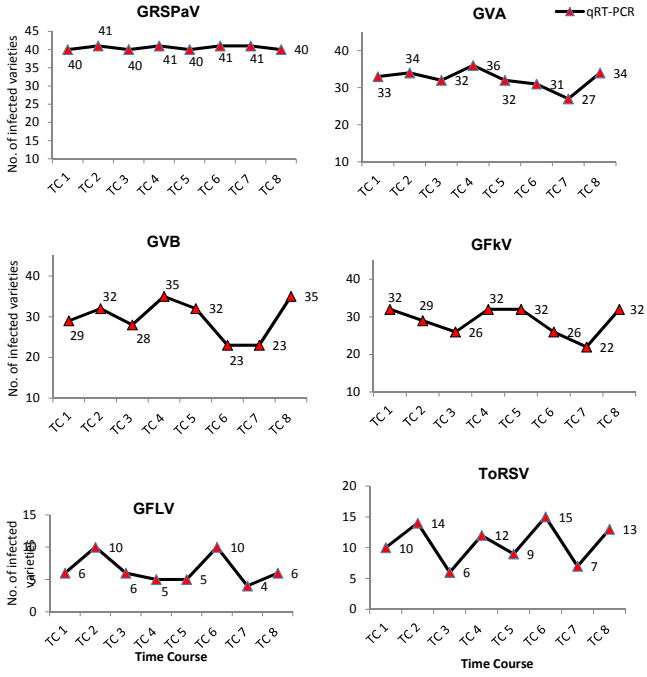


Fig. 2 Time course presentation of the virus titre for GRSPaV, GVA, GVB, GFkV, GFLV and ToRSV over two years with three months interval as detected by qRT-PCR/LDA



Elucidating Disease Epidemiology for Management of a Complex Virus Pathosystem in Cool-climate Viticulture

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INTRODUCTION

Washington State is the largest wine grape-producing state in the Pacific Northwest of the United States. As of 2011, total wine grape (*Vitis vinifera* L.) acreage in Washington State was reported to be about 44,000 acres (about 18,000 hectares), including new plantings. Of the total wine grape acreage, 46 percent were red-berried cultivars and 54 percent were white-berried cultivars. A recent study indicated that the *Washington state wine industry had an* economic impact of $8.6 billion annually in the state and $14.9 billion nationwide (Stonebridge Research, 2012). The Washington State Grape Industry Research Task Force report "Building the Future of the Washington State Grape and Wine Industry Through Research" has identified grapevine leafroll disease (GLRD) as one of the greatest biotic constraints affecting vine health, fruit quality, and economic prospects for the grape and wine industry in Washington State (WAWGG, 2010). GLRD is a complex viral disease producing distinct symptoms in red- and white-berried cultivars (Rayapati *et al*., 2008). Grape mealybug *(Pseudococcus maritimus* Ehrhorn, Pseudococcidae) is the only vector species of GLRD documented in Washington vineyards (Walsh et al., 2001). However, the status of scale insects (Coccidae) and their ability to act as vectors for GLRD is not clear. We have been conducting field studies to better understand the epidemiology of GLRD in own-rooted wine grape cultivars grown in cool-climate conditions of Washington State relative to other grape-growing regions, where grapevines are planted as grafted vines and different species of mealybugs and scale insects are involved in vectoring grapevine leafroll-associated viruses (GLRaVs).

MATERIALS AND METHODS

Leaf samples showing GLRD and GLRD-like symptoms were collected from red-berried wine grape cultivars. Since white-berried cultivars do not exhibit typical symptoms of GLRD, leaf samples were collected randomly from individual grapevines. Nearly 2500 samples were collected during 2005 and 2011 seasons between July and October from about 40 different wine grape cultivars planted in 40 commercial vineyards in different American Viticultural Areas (AVA) in Columbia Valley and a few samples were from Puget Sound AVA of Washington State. Petiole extracts were tested individually for the presence of GLRaVs by one tube-single step reverse transcription-polymerase chain reaction (RT-PCR) assay using species-specific primers. Samples were also tested by RT-PCR for other grapevine viruses and viroids. Cloning and sequence analysis of amplicons was performed as described previously (Alabi et al., 2011). The spatial distribution of GLRD was monitored in three geographically separate vineyard blocks of Cabernet Sauvignon, Merlot, and Cabernet Franc. The position of individual vines showing symptoms of GLRD was recorded and plotted in a XY matrix using the row number and vine position in each row as co-ordinates. The spatial and temporal spread of GLRD in young vineyards planted with virus-tested cuttings was studied by monitoring vines annually for GLRD symptoms. Samples from both symptomatic and asymptomatic vines were tested by RT-PCR for the presence of GLRaVs.

RESULTS AND DISCUSSION

A wide range of GLRD symptoms was observed in different red-berried wine grape cultivars indicating substantial variability in disease symptoms. Since GLRD symptoms 'mimic' those caused by nutritional deficiency- and injury-related factors and the fact that many viruses remain asymptomatic in own-rooted grapevines, we used RT-PCR, instead of visual observations alone, for reliable documentation of viruses. The results from a seven year study indicated the presence of six GLRaVs (GLRaV-1, -2, -3, -4, -5, and -9) as mixed infections in different combinations in wine grape cultivars showing GLRD symptoms or suspected for disease symptoms. Among them, GLRaV-3 was found to be the most prevalent and widely distributed. In addition, seven grapevine viruses (*Grapevine rupestris stem pitting-associated virus*, *Grapevine virus A*, *Grapevine virus B*, *Grapevine virus E*, *Grapevine fanleaf virus*, *Grapevine fleck virus* and *Grapevine Syrah Virus 1*) and four viroids (*Australian grapevine viroid*, *Hop stunt viroid*, and *Grapevine yellow speckle viroid-1* and *-2*) were detected in some wine

grape cultivars exhibiting GLRD symptoms. These viruses and viroids were found occurring as mixed infections with GLRaVs. The spatial pattern of GLRD monitored in cvs. Cabernet Sauvignon, Merlot and Cabernet Franc showed clustering of symptomatic vines along individual rows indicating secondary spread between neighboring vines within rows (Fig. 1). The spatial and temporal distribution of GLRD in new vineyard blocks planted with virus-tested cuttings in close proximity to heavily infested old blocks indicated that the disease can spread to young plantings from neighboring vineyards infested with the disease (Fig. 2). Testing of representative samples by RT-PCR from symptomatic and neighboring asymptomatic vines indicated the presence of GLRaV-3 only in symptomatic vines. These results suggested that GLRaV-3 can spread from infected vines to neighboring healthy vines or from infested old blocks to neighboring 'clean' plantings. Further studies are underway to better comprehend the complexity of GLRD epidemiology in Washington vineyards and provide science-based knowledge for growers, vineyard managers and wine makers for mitigating negative impacts of the disease on vine health and fruit and wine quality.

Fig.1

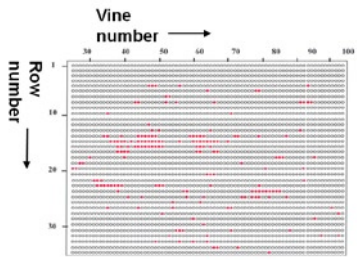

Fig. 2a

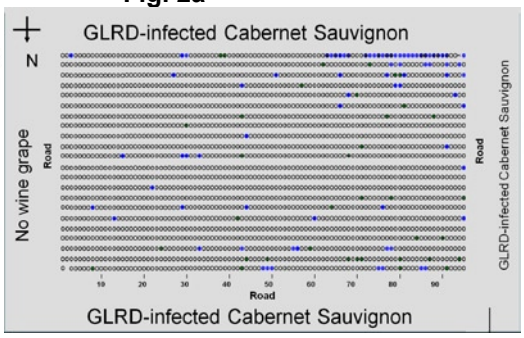

Fig. 2b

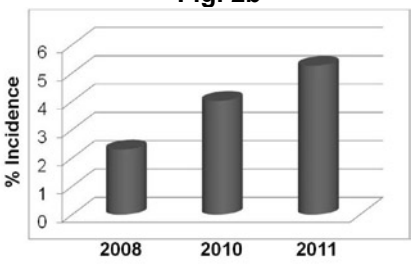

Fig. 1. Spatial distribution of GLRD-affected vines in a Cabernet Franc block. The map shows clustering of symptomatic vines in individual rows, indicating vine-to-vine spread of GLRD. Open circles indicate vines with no GLRD and solid circles represent vines with GLRD and tested positive for GLRaV-3.

Fig. 2. Spatial distribution of GLRD-affected vines in a Syrah block planted in 2004 with clean planting stock. (A) The block is surrounded on three sides by Cabernet Sauvignon blocks infested with GLRD. (B) A total of 110 vines (5.26%) in the block showed GLRD symptoms in 2011 season, an annual increase from 48 (2.3%) in 2008 and 84 (4.02%) in 2010 seasons. More number of symptomatic vines in rows at the south-west corner of the block than on other sides of the block suggests increased risk of the spread of GLRD to new blocks when planted in close proximity to heavily infested older blocks. Open circles indicate vines with no GLRD and solid circles represent symptomatic vines tested positive for GLRaV-3.

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Differential Ampelovirus Multiplication in Plants May Explain its Relative Incidence in the Vineyards

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INTRODUCTION

Grapevine leafroll complex is composed by several species of the genus *Ampelovirus* and one *Closterovirus* (GLRaV-2). Within genus *Ampelovirus*, two of them, GLRaV-1 and GLRaV-3 are apparently more common in vineyards and are also included in the EU legislation as unauthorized in nursery stock. Other ampeloviruses (GLRaV-4, -5, -6, -9,-Pr,-CV, -Car) appear to be divergent variants of a single species, GLRaV-4 (Ghanem-Sabanadzovic *et al*., 2012; Martelli *et al*., 2012). Several GLRaV-4 related ampeloviruses have been identified recently in Spain and their incidence is apparently lower than that of GLRaV-1 and -3. The availability of RT-qPCR analysis for these viruses has allowed us to initiate studies to quantify the genome copies vines to try to correlate the differential concentration of the virus as a possible explanation for the differences in the relative incidence in field. In particular, we have used as model the ampeloviruses GLRaV-3, -4 and –5, present in different grapevine materials.

MATERIAL AND METHODS

Plant material and sampling. Plants of varieties Rome, Tintilla de Rota and Gorgollasa were field collected and canes rooted and kept in pots in an insect proof greenhouse. Sampling for RNA extraction was done in late April 2012.

Isolation of total RNA and cDNA synthesis. Total RNA (from 100 mg of medium leaf petioles) was extracted using Spectrum Plant Plant RNA kit (Sigma). RNA concentration were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to normalize the nucleic acid concentration for subsequent reverse transcriptions. First-strand cDNA synthesis in RT reactions was carried out using 200 ng of RNA, random nonamers (Takara) and Mu-MLV reverse transcriptase and following the manufacturer's instructions (Eurogentec).

Real-time PCR. Primers for the amplification of GLRAV-4 and GLRaV-5 RNA dependent RNA polymerase (*RdRp*) genes were designed using Primer3 on-line software tool. For GLRaV-4: LR4-RP_F2: GGCAGTGGAATTGGAAGTGT / LR4-RP_R2: CTGCACCTGTCCTCCTTTGT; and for GLRaV-5: LR5-RP_F1: ATCGAAATCTTGGCATCCAG / LR5-RP_R1: TCTCAGCTTT AGCTGCGTCA. For GLRaV-3 *RdRp* amplification we used primers LR3qrtF and LR3qrtR (Tsai *et al.,* 2011). For the internal control we used the specific primers for *Actin* gene amplification (Gutha *et al*., 2010). qPCR experiments were performed in white 96-well PCR plates using a Bio-Rad iQ5 Thermal cycler. One μL of cDNA template from each RT reaction were added to 10 μL of KAPA SYBR Green qPCR mix (KAPA Biosystems, Cape Town, South Africa), 500 nM of each pair of primers and sterile water to complete a final reaction volume of 20 μL. Samples were subjected to the following conditions: 95°C for 3 min; 40 cycles of 95°C for 15 s, 60°C for 45 s. All qPCR assays were performed in duplicate. Specificity of the amplification products obtained were determined with software package Bio-Rad Optical System Software v.2.1 by melting-curve analysis of 60 s at 95°C, 60 s at 55°C, followed by fluorescence reading at 0.5 °C increments from 55 to 95°C.

Standard curves generation. Partial GLRaV-3, GLRaV-4 and GLRaV-5 *RdRp* genes were amplified for standard curves generation. A 1004 bp amplicon of GLRaV-4 encompassing partial *RdRp* gene was obtained with primers LR4-RP_T7F3: (T7promoter)-CTTTAGGGAGTGCTGGGTCA and LR4-RP_R1: GTATTGGCTGCACCTGTCCT, while a 936 amplicon was obtained for GLRaV-5 *RdRp* gene with primers LR5-RP_T7F2: (T7promoter)-CTGGTTTGATTGACGGTGTG and LR5-RP_R3: GCTGCCCAAGTGTCCAGTAT. Similarly was

obtained the GLRaV-3 *RdRp* amplicon following Tsai and coll. (2011). For RNA synthesis PCR amplicons were purified and used as templates using T7 RNA polymerase (MAXIscript SP6/T7 Kit, Invitrogen) according to the manufacturer instructions. Synthetic RNA obtained was quantified and serial diluted in virus free Sugar Seedless genomic RNA for reverse transcription and qPCR standard curve generation. Taking into account the molecular weight of the synthetic RNAs it was possible to calculate the number of genomic copies for each dilution and allowed to interpolate the GLRaV-3, GLRaV-4 or GLRaV-5 genome copy number for each sample.

RESULTS AND DISCUSSION

After obtaining standard curves from *in vitro* synthesized RNA of GLRaV-3, -4 and -5 *RdRp* genes we have calculated the relative and absolute concentration of viruses in leaf petioles. The efficiency of the amplification was determined for each primer pair and target resulting in 99.7% for GLRaV-3, 115.5 % for GLRaV-4 and 107.3% for GLRaV-5. For each variety, sample and virus the *Actin* gene was used for ΔCq_{virus} (Cq_{gene}-Cq_{ref}) determination and $\Delta\Delta Cq$ (ΔCq_{virusA}-ΔCq_{virusB}) was determined. In the plants analyzed, for GLRaV-4-GLRaV-3 $\Delta\Delta Cq$ averaged 2.4 and for GLRaV-5-GLRaV-3 averaged 3.3 which are around one magnitude order in the relative copy number. In addition, we could determine the absolute number of copies for GLRaV-3 and -5 in ten plants of the variety Tintilla Rota and eleven of the variety Rome which were infected by both viruses, resulting in the average number of copies per mg of tissue GLRaV-3 was 5.7×10^6. In contrast, the genome copy number of GLRaV-5 was significantly lower, averaging 1.60×10^5.mg^{-1}. On the other hand, we have analyzed plants of the variety Gorgollasa that were infected with GLRaV-4 alone or by GLRaV-3 and GLRaV-4, simultaneously. In five plants with single GLRaV-4 infection, the genome copy number per mg of tissue averaged 8.61×10^5. In the other five plants having double infection the number of copies of GLRaV-4 were similar to those having single infection averaging 6.31×10^5, while the number of copies of GLRaV-3 was greater and within the same range as in the varieties Tintilla de Rota and Rome: 5.24×10^6 copies.mg^{-1}. Data corresponding to those samples taken in mid fall 2011 and for a different gene (*hsp70*) showed equivalent relative differences (not shown). These preliminary results cannot discard interactions among ampeloviruses, but suggest that at least two variants of GLRaV-4 multiply less in plants than GLRaV-3, the most frequent in Spain, offering a possible explanation for the differences in the incidence of different *Ampelovirus* species. The relationship between symptom severity and ampelovirus concentration remains also to be investigated.

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Microscopic Localization of Grapevine Phytoplasmas: An Exciting Challenge or a Losing Battle?

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INTRODUCTION

Phytoplasma localization in grapevine by microscopic techniques has always been a big challenge for many reasons, but particularly for their very low concentration in this plant species that makes their finding an almost impossible task, at least by transmission electron microscopy (TEM) (Faoro, 2005). A literature survey, since the discovery of phytoplasmas associated with Flavescence doreè (FD) and other grapevine yellows (GY) in the sixties, shows that only three papers have been published up to now on this subject (Granata et al., 1991; Meignoz *et al*., 1992; Credi, 1994), in spite of the huge number of reports dealing with the presence of these prokaryotes in infected grapevine plants and detected by PCR techniques. Indeed, by the advent of molecular biology in the eighties, microscopic visualization of phytoplasmas in infected plants was regarded as a useless diagnostic tool, in any case providing very little information on these microorganisms, particularly from the taxonomic point of view. For all the above reasons microscopic investigations on grapevine yellows were completely abandoned. However, in the last decade the numerous efforts that have been carrying out to study the interaction of phytoplasmas with grapevine tissues, particularly in case of the recovery phenomenon, have shown that the precise localization of these prokaryotes in the tissues would be determinant to understand the underlying mechanisms. Recovery, i.e. the spontaneous remission of symptoms in diseased plants, has often been observed in FD- and GY-affected grapevines (Caudwell *et al*.,1961; Osler *et al*., 1999). This phenomenon may or may not involve the elimination of the pathogen from the host. Physiological mechanisms and possible biological factors involved in recovery are still not clear, though increased hydrogen peroxide level in the phloem of recovered plants has been observed (Musetti *et al*., 2007), together with the activation of systemic acquire resistance related genes (SAR) (Albertazzi *et al*., 2009). Moreover, other researchers hypothesized that endophytic microorganisms (bacteria, fungi, and mycorrhiza) associated with plant tissues can take a part in the recovery phenomenon (Romanazzi *et al*., 2009, Bulgari *et al*., 2011b).

While studying the role of endophytic bacteria in inducing recovery we have faced the need of verifying the distribution of both phytoplasmas and bacteria in grapevine tissues to shed light on their interaction and, in particular, to exclude their direct competition in the phloem cells (Bulgari *et al*., 2011a). For this reason we resumed microscopic techniques, such as TEM, coupled with fluorescent *in situ* hybridization (FISH), a method we previously successfully applied to co-localize grapevine phytoplasmas and endophytic bacteria in the host plants *Catharanthus roseus* (Bulgari *et al*., 2011a).

MATERIALS AND METHODS

Leaf midribs from healthy, FD-diseased and recovered grapevine plants (cv. Cabernet Sauvignon) were collected in summer 2010 and 2011 and processed for conventional TEM analysis and FISH, as previously described (Faoro *et al*., 1991; Bulgari *et al*., 2011a). Portion of the samples were also analyzed by PCR to confirm the presence of 16SrV phytoplasmas and/or endophytic bacteria. To localize phytoplasmas with FISH, a probe targeting 16SrV group, labeled with FAM (Primm, Milan Italy) or Marina Blue (MB) (Invitrogen, Milan Italy) at 5' terminus was used. Both these dyes, emitting respectively at 518 nm and 459 nm were tested, to find out the appropriate wavelengths that interfere at least with leaf auto-fluorescence. Endophytic bacteria localization was performed with a universal probe targeting bacterial 16S rDNA (but not phytoplasmal DNA), labeled with a fluorophore (Cy-5) emitting in the far-red (670 nm) (Bulgari *et al*., 2011a). Labeled sections were observed with a videoconfocal microscope (Nikon, Vico, Italy).

RESULTS AND DISCUSSION

Tem analysis, in spite of the nowadays ameliorated procedure in specimen preparation, confirmed that is not a suitable technique for phytoplasma visualization in grapevine tissues. In fact, phytoplasma detection in thin sections was an almost extraordinary event, also in heavily infected grapevine plants (Fig. 1), in spite of the severe ultrastructural alterations present in the phloem tissue. This is possibly due to the ease with which phytoplasma disruption occurs in grapevine deranged phloem. Even endophytic bacteria localization was

not easy by TEM, because these prokaryotes were scattered throughout different tissues and not grouped in enclaves. FISH technique encountered some problems in phytoplasma end endophytic bacteria visualization, mainly due to phloem autofluorescence which was particularly heavy in infected plants (Fig. 2), because of polyphenols deposition. These compounds emitted in both the wavelengths of FAM and MB, thus they were difficult to differentiate from the probes. Only Cy5 was shown to be an excellent reporter molecule for in situ hybridization analysis in grapevine tissues, as it emission was in a band far away from that of polyphenols. With the Cy5 probe it was possible to localize endophytic bacteria in all the examined samples (healthy, diseased and recovered), mostly in the xylem but also in the phloem tissues as scattered spots (Fig. 3), suggesting their random distribution, without large accumulation in specific cells. Instead, phytoplasma probes gave less clear cut results and only in a few cases it was possible to observe specific fluorescence of the probe in the phloem of diseased plants (Fig. 2), but not in healthy or recovered ones. From these results, although too preliminary to drawn any suggestion on phytoplasma-endophytic bacteria interaction in grapevine tissues, it can be concluded that FISH remains the only microscopic technique that have some chance in localizing the very few phytoplasmas present in diseased plants, thus allowing to study their interaction with endophytic bacteria. However, further investigations are needed to improve the technique, particularly for the reduction of tissue autofluorescence that would permit to label probes with a larger set of fluorophores and to co-localize phytoplasma and endophytic bacteria together in the same section.

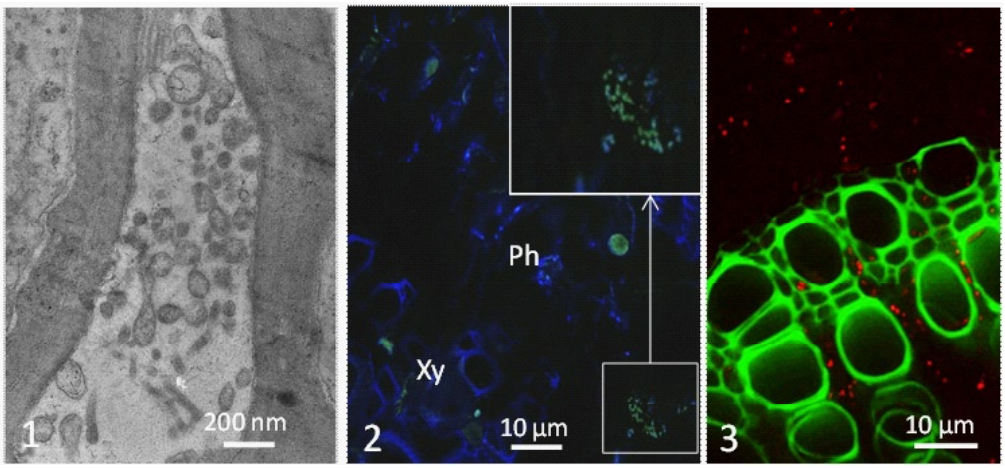

Fig. 1. Phytoplasmas visualization by TEM in FD-infected grapevine phloem: a very rare event.
Fig. 2. Phytoplasmas detected by the 16SrV probe labeled with FAM (enlarged in the inset): note the autofluorescence of both xylem (Xy) and phloem (Ph).
Fig. 3. Endophytic bacteria detected by the Cy5-labelled bacterial universal probe: scattered red spots are present in the xylem (autofluorescent) and in the adiacent phloem tissues.

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Multilocus Analyses on Grapevine 'Bois Noir' Phytoplasmas from Italy and Serbia

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INTRODUCTION

A wide world severe spreading of 'bois noir' (BN) disease is recently reported in the majority of grapevine growing areas. Recent findings indicate that molecular variability inside the BN-associated stolbur phytoplasmas is present and for some genes such as elongation factor Tu (tuf gene) it could be related with epidemic features (Langer and Maixner, 2004). Variability in five genes of BN phytoplasmas present in grapevine epidemics was therefore investigated on samples collected in infected grapevine growing areas in Italy and Serbia where BN has been molecularly identified and reported since several years.

MATERIALS AND METHODS

During summer 2009-2011, routine surveys carried out to verify identity of phytoplasmas associated with yellows symptoms in grapevine growing areas in Italy and Serbia allow to identify and partially characterize BN phytoplasmas by RFLP analyses with *Tru1*I and *Mbo*II on R16F2n/R2 amplicons (Contaldo *et al*., 2009). In Serbia 23 plants were sampled from different geographic locations i.e. Bela Crkva, Smederevo, Krčedin, Aleksandrovac and Radmilovac (Table 1), representing some of the major viticultural areas of the Country. In Italy 23 samples were collected in Emilia-Romagna from 6 out of the 9 provinces of the region (Table 1).

Total nucleic acids were extracted from midribs and phloem scrapes, and amplicon produced on 16Sr, tuf (Langer and Maixner, 2004), amp (Fabre *et al*., 2011), secY (Lee *et al*., 2010) and groEL (J. Mitrović and B. Duduk, unpublished) genes were subjected to RFLP analyses with selected restriction enzymes according with amplicon (Table 1).

Reference strains employed maintained in periwinkle were STOL (from Serbia), ASLO (from Slovenia) STOLC, STOL-PO, STOL-CH, MOL (from France).

RESULTS AND DISCUSSION

The 'bois noir' infected samples tested from Italy and from Serbia showed two different profiles (a and a+b) on the R16F2n/R2 amplicons digested with *Mbo*II; profile (A+B) was possibly indication of mixed strains/double operon presence as previously described (Contaldo *et al*., 2011) (Table 1).

All the samples amplified on 16Sr gene were also successfully amplified on tuf and stamp genes. The comparison of RFLP profiles obtained on tuf gene with *Hpa*II and on stamp gene with *Tru1*I indicated that tuf-type from Italy was always associated with identical profile on stamp gene: tuf-type a/stamp a and tuf-type b/stamp a+b. This correlation was not clearly defined in the samples from Serbia where only tuf-type b was present but it was associated with three different stamp profiles (stamp a, stamp b and stamp e) (Table 1). It is possible to hypotize a correlation between epidemiological features and tuf/stamp polymorphisms considering that common recognized vector of BN to grapevine is *Hyalesthes obsoletus*. This cixiid is very likely composed by insect populations having different characteristics not yet defined and not easy to define (J.Y. Rasplus, personal communication) therefore interaction of different insect population could have influenced the polymorphism of these genes in the Serbian samples.

Amplification of secY and groEL genes allow to amplify a number of strains but not all those tested. Polymorphism of secY gene on Italian samples with *Tru1*I and *Tsp509*I indicates that grapevine profile is distinguishable from those of all the reference strains employed. However three RFLP groups were obtained in reference strains: secY a (STOL), secY b (STOL-C, STOL-PO and STOL-CH) and secY c (ASLO and MOL). Polymorphisms on groEL gene with *Hpy188*I allow to distinguish two RFLP groups in the Serbian samples, while three different profiles were identified in the samples from Italy.

Table 1. RFLP results on BN infected samples from Serbia and Italy and from reference strains. Identical letter indicates identical profile.

Samples	16S rDNA gene		Tuf gene	Stamp gene	Samples	16S rDNA gene		Tuf gene	Stamp gene
	*Tru1*I	*Mbo*II	*Hpa*II	*Tru*I		*Tru1*I	*Mbo*II	*Hpa*II	*Tru*I
RS-Aleksandrovac 122/10	a	a	b	b	I-Ra9818	a	a+b	a	a
RS-Aleksandrovac 123/10	a	-	b	a	I-Ra9827	a	a+b	b	a+b
RS-Aleksandrovac 125/10	a	a	b	b	I-Ra 9912	a	a	a	a
RS-Aleksandrovac 127/10	a	-	b	e	I-Ra9910	a	a+b	a	a
RS-Bela Crkva 130/10	a	a	b	a	I-Ra 9830	a	a+b	a	a
RS-Bela Crkva 131/10	a	a	b	b	I-Ra9801	a	a	a	a
RS-Bela Crkva 132/10	a	a+b	b	b	I-Ra9804	a	a+b	a	a
RS-Bela Crkva 134/10	a	a	b	b	I-Ra14486	a	a+b	a	a
RS-Bela Crkva 140/10	a	a	b	b	I-Ra9709	a	a+b	a	a
RS-Bela Crkva 142/10	a	a	b	b	I-Ra9707	a	a+b	a	a
RS-Bela Crkva 143/10	a	-	b	b	I-Ra9802	a	a	a	a
RS-Bela Crkva 144/10	a	a+b	b	e	I-REV8	a	-	a	a
RS-Bela Crkva 145/10	a	a	b	b	I-REV10	a	-	a	a
RS-Krčedin 93/10	a	a+b	b	e	I-REV13	a	-	a	a
RS-Krčedin 95/10	a	a	b	a	I-MOV27	a	-	a	a
RS-Radmilovac 191/09	a	a	b	b	I-FE9805	a	a+b	a	a
RS-Radmilovac 192/09	a	a	b	b	I-FE9806	a	a	b	a+b
RS-Smederevo 65/11	a	a	b	e	I-FE9810	a	a	b	b
RS-Smederevo 66/11	a	a	b	b	I-FC10044	a	a+b	a	a
RS-Smederevo 67/11	a	a+b	b	b	I-BO14394	a	a+b	a	a
RS-Smederevo 68/11	a	-	b	b	I-BO9866	a	a+b	a	a
RS-Smederevo 69/11	a	a	b	b	I-BO9870	a	a+b	b	a+b
RS-Smederevo 70/11	a	a	b	b	I-BO9867	a	a+b	b	a+b
STOL	a	a	b	a	STOL-CH	a	-	b	c
STOL-C	a	a+b	b	c	ASLO	a	-	b	d
STOL-PO	a	b	b	c	MOL	a	-	b	c

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Stolbur Type II Phytoplasma in North Israel Vineyards: What is the Plant Source of Infection?

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INTRODUCTION

Phytoplasma is the pathogen of Grapevine Yellows disease that causes heavy damage to vineyards in most growing areas around the world as well as in Israel (Weintraub *et al*., 2007). Phytoplasmas are endo-cellular obligatory parasites that exist only in the phloem tissue of the host plant or in the digestive system of the insect vectors. The planthopper *Hyalesthes obsoletus* is the known vector, which transmits *Stolbur* phytoplasma to grapevines in Europe. This planthopper is polyphagous on both herbaceous and woody host plants. Its preference for host plant species varies according to deferent geographical area (Bressan *et al*., 2006). The dry summer in northern Israel suggests that only perennial plant could be the source for phytoplasma infection. In Israel the preferred host plant of *H. obsoletus* is *Vitex agnus-castus* (Sharon *et al*., 2005). However, this plant species was found negative to phytoplasma in past PCR analyses done in our lab. Therefore, the possibility of another source of infection (either host plant or insect vector) was questioned. The aims of this study were to survey perennial plant species as potential source for phytoplasma infection as well as searching for other possible vectors.

MATERIALS AND METHODS

In this survey, 30 - 230 specimens were sampled from perennial plants species: *Convolvulus arvensis*, *Crataegus azarolus*, *Olea europaea*, *Polygonum equistiforme*, *Quercus* sp., *Rosa canina*, *Rubus sanguine*, *V. agnus-castus*, and *Ziziphus spina-christi*. Some of these species are known as hosts of Stolbur phytoplasma, of *H. obsoletus,* or both (R. Sforza personal communication). The plants analyzed were sampled from the north, center, and south parts of the Golan Heights. In order to look for other potential vectors, we placed 42 yellow sticky traps in 14 places along ca. 50 km in the Golan Heights, ranging from 400 to 1000 meter above sea level (coordinates: 32°58′54″N 35°44′58″E). The sticky traps were replaced every 2 weeks and the trapped insects were identified and analyzed. Phytoplasma presence in the plant or insect sample was performed by nested PCR analyses with general primers P1/P7 followed by the U3/U5 primers or with the R16F2n/R2 primers to the 16S RNA polymerase gene. Positive plants and *H. obsoletus* tested for Stolbur type in additional PCR amplification of the *tuf* gene followed by sequence analysis.

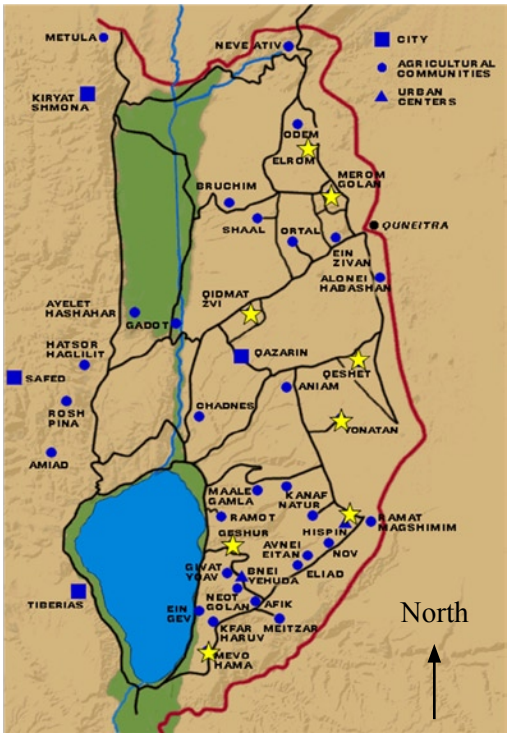
RESULTS AND DISCUSSION

The plants were sampled from one, two or three areas in the Golan Heights (Table 1). In general, the infection rate was higher in the south and center parts than in the north as revealed by PCR analyses. Out of 16 samples of *C. arvensis* from the south region, seven were infected. In contrast, no phytoplasma was found in samples from the north region. Infected samples of *V.agnus-castus* were found in the south region (3/18), and in the center region of the Golan (12/16). However, none of the 20 samples from the north part was positive. *C. azarolus*, *P. equistiforme and R. canina* that were sampled only in the north were negative. *Quercus sp*. was the only plant species in the north that was found to be infected by 9%. Trees of *O. europaea* in the Gshour olive orchard were found to harbor phytoplasma (4/17). Samples of trees of *Z. spina-christi* from the center and the south parts of the Golan were found to be infected by 10% (Table 1). PCR followed by sequence analysis of plants samples revealed that *Convolvulus* and *Vitex agnus-castus* were infected with Witches` Broom phytoplasma. In contrast, PCR followed by RFLP analysis to *tuf* gene confirmed that grapevines cv. Chardonnay and Cabernet Sauvignon are infected with Stolbur type II phytoplasma (Zahavi *et al*., 2012, submitted). The results show, for the first time, that many perennial plants in the Golan-Highest harbor phytoplasma. However, no wild or cultivated plants except *Vitis vinifera* were found to harbor phytoplasma of stolbur type II and could not explain the spread

of phytoplasma in the vineyards. In the coming summer, we plann to analyze more positive plants in order to identify the phytoplasma type. *Macrosteles quadripunctulatus*, *Circulifer haematoceps*, *Neoaliturus fenestratus* and *H. obsoletus*, were trapped in the yellow traps. These insect species are known as potential vectors for stolbur phytoplasma. Nevertheless, only *H. obsoletus* was found to harbor phytoplasma which was identified by sequence analysis of the *tuf* gene as the Stolbur Type II phytoplasma.

Table 1. PCR results for phytoplasma presence in wild and cultivated plant species in the north (N) center (C) or south (S) of the Golan Heights. Plants were analyzed in individual, groups of 2-5 (*) or in 6-7 (**) plants, and the infection rate was calculated as the number of positive groups from the total number of groups. Phytoplasma presence was tested by Nested PCR for the 16S gene with phytoplasma P1/P7 follow by U3/U5 primers. Sampling sites are presented in the map from north to south (marked with stars): Elrom, Merom-Golan, Qidmat Zvi, Qeshet, Yonatan, Bney-Israel, Geshur and Mevo Hama.

Plant species	Site name	Area	No. of samples	No. of Infected samples
C. arvensis	Geshur	S	16*	7*
	Qidmat Zvi	C	150	0
C. azarolus	Merom Golan	N	27	0
O. europaea	Geshur	S	17	4
O. europaea	Qeshet	C	4	0
P. equistiforme	Merom Golan	N	75	0
Quercus sp	Merom Golan	N	11*	1*
R. canina	Merom Golan	N	4	0
R. sanguine	Qidmat Zvi	C	5**	1**
V.agnus-castus	Yonatan	C	16	12
	Bney-Israel	S	18*	3*
	Qidmat Zvi	C	30	0
	Elrom	N	20	0
Z. spina-christi	Qidmat Zvi to Mevo Hama	C+S	30	3



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Transmission of 16SrIII-J Phytoplasma by *Paratanus exitiosus* Leafhopper

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INTRODUCTION

Phytoplasmas found in Chilean grapevines showing yellows symptoms were identified as belonging to the ribosomal subgroups 16SrI-B and 16SrI-C ('*Candidatus* Phytoplasma asteris'), 16SrIII-J (X-disease group), 16SrV-A ('*Ca.* P. ulmi'), 16SrVII-A ('*Ca.* P. fraxini'), 16SrXII-A (stolbur or "bois noir") (Gajardo *et al.*, 2009; González *et al.*, 2010). The presence of these pathogens in the plants depends on both propagation of infected plants and spreading by different insect species which feed on grapevine and also on the weeds growing near and/or in vineyards. In infected vineyards, several insects belonging to the family Cicadellidae positives to phytoplasmas were found. The most common was *Paratanus exitiosus* (Beamer) in which phytoplasmas of 16SrI-B, 16SrIII-J, 16SrVII-A, and 16SrXII-A subgroups were detected. In the present work, we verified the phytoplasma transmission ability of the leafhopper *P. exitiosus*.

MATERIALS AND METHODS

During 2011 (since September till December) and 2012 (since January till May) leafhopper survey was carried out in Chilean vineyards infected by phytoplasmas, two located in Metropolitana Region (1 and 2) and one in Valparaiso Region (3), to know biological characteristics of *P. exitiosus* and its ability to transmit phytoplasmas. The insects were captured by sweeping with an entomological net. During the sampling period adults of *P. exitiosus* captured have been released into entomological cages to let them feed on three plants of periwinkle (*Catharanthus roseus* (L.) G. Don) grown from seed and previously tested to ascertain the absence of phytoplasmas. A total of 81 plants were used. Periwinkle plants were tested starting five months after transmission trials, while dead insects were then preserved in 70% ethanol. Insects and periwinkles were tested in order to identify the phytoplasma presence. Total nucleic acids (TNAs) were extracted with chloroform/phenol methods, dissolved in Tris-EDTA pH 8.0 buffer, and maintained at 4°C; 20 ng/µl of nucleic acid were used for amplification. After direct PCR with primer pair P1/P7, nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) was performed. PCR and nested PCR reactions were carried out following published protocol (Schaff *et al.*, 1992). P1/P7 amplicons were purified using Concert Rapid PCR Purification System and DNA fragments were cloned. Putative recombinant clones were analyzed by colony PCR. Selected fragments from cloned DNAs were sequenced in both directions using the BIG DYE sequencing terminator kit. The sequences were then aligned with BLAST engine for local alignment (version Blast N 2.2.12). Identification was done using *in silico* restriction fragment length polymorphism (RFLP) analyses on sequences amplified with primer pair R16F2n/R2 with *Bst*U1 and *Hha*I restriction enzymes (Wei *et al.*, 2007).

RESULTS AND DISCUSSION

In the vineyard 1 the *P. exitiosus* capture rate remained constant during all months, with a decrease in October 2011 and April 2012. In the vineyard 2 the highest number of individuals was obtained in December 2011 and January 2012. In the vineyard 3 *P. exitiosus* was less abundant in September 2011, remained constant during the other months and increased only during February 2012. These results indicate that *P. exitiosus* tends to be more abundant during summer time. Three out of 81 periwinkles used for transmission trials were positive to phytoplasmas. Two (VC28C and VC31C) correspond to the transmission trials carried out with insects captured in the vineyard 1 in two different months (November and December 2011 respectively), the third (VC33A) was infected from insects captured in the vineyard 3 during December 2011. Cloned P1/P7 fragments were sequenced and there was no sequence difference between the cloned fragments from the three periwinkles

(1,819 bp). The similarity percentages of VC28C, VC31C, and VC33A phytoplasmas, showed a close correlation (99.6, 99.7, 99.6 % respectively) with the strain Ch10 (AF147706), corresponding to chayote witches' broom phytoplasmas (16SrIII-J) from Brazil (Montano *et al.*, 2000). After trimming the R16F2n/R2 amplicon were also subjected to *in silico* RFLP analysis that confirmed the assignment of phytoplasmas to the ribosomal subgroups 16SrIII-J (belonging to X-disease group). The phytoplasma 16SrIII-J was also detected in *P. exitiosus* specimens used for transmission assays. The three periwinkles infected with phytoplasma 16SrIII-J showed virescence, phyllody and witches' broom symptoms. This is the first report of 16SrIII-J phytoplasma transmission by *P. exitiosus*. Assays to verify the transmission ability of other phytoplasmas by the leafhopper *P. exitiosus* are in progress as well as trial to verify its ability to transmit the 16SrIII-J phytoplasma to grapevine.

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Spatial and Temporal Distribution of the Infection Potential of Stolbur Phytoplasma on a Fallow Vineyard

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INTRODUCTION

Phytoplasmas of the stolbur (16SrXII-A) group are associated with Bois noir of grapevine, a widespread and economically important grapevine yellows in Europe. The endemic pathogens are common in different elements of the wild vegetation and are transmitted by the planthopper *Hyalesthes obsoletus*. This vector spreads the pathogens not only to their natural host plants but also to various cultivated crops. Grapevine is inoculated by erratic feeding of the vector. The existence of diverse strains of stolbur that are not only restricted to different host plant species but also transmitted by host-associated populations of the vector leads to a complex epidemiological system. During the last decade, the system bound to nettle (*Urtica dioica*) emerged in various wine-growing regions of Europe and caused new or increased Bois noir problems in viticulture. The supplement of the long known 'bindweed-system' with *Convolvulus arvensis* as the principal host plant by the 'nettle-system' in Germany resulted in new disease outbreaks and the need for adapted control strategies, because both host plants are differentially distributed in the field. While bindweed is growing extensively within the vineyards, nettle is more frequent along vineyard borders and other uncultivated areas. Although the principles of the Bois noir epidemiology are quite well understood, it is still impossible to predict the actual infection pressure to grapevine emerging from nettle for a particular area. The aim of our study was therefore to describe the infection potential of stolbur phytoplasmas on a fallow field within the vineyard environment. We aimed to investigate the development of reservoirs of infection and the spatiotemporal distribution and dispersal of infective vectors that carry the inoculum for grapevine.

MATERIAL AND METHODS

The experimental plot was a fallow vineyard at Kesten (49°53"57'/6°57"07') in the Mosel wine-growing region. The plot of 1620 m^2 surrounded by vineyards was uncultivated for several years and covered by a dense vegetation of herbaceous plants and shrubs, including several patches of stinging nettle. The plot was divided into 20 sectors of 80 -100 m^2. Immediately before the emergence of the adult vectors all herbaceous vegetation except the nettle patches was mown.

Two yellow sticky traps were placed in the center of each sector at a height of 30 cm and 80 cm above the ground, respectively, and changed weekly from June to August. Planthoppers were also collected twice a week by sweep netting 30 patches of nettle of variable size (37 m^2 in total). Ten sweeps per m^2 were applied but the minimum number of sweeps per patch was 5. As far as available, 25 males and females were tested for stolbur infection by PCR with group specific primers f/rStol (Maixner et al., 1995). The tuf-type of positive samples was determined according to Langer and Maixner (2004). To analyze spatial distribution patterns, the variance-to-mean ratio (VM) was calculated as an index of dispersion. Its deviation from random distribution was tested with a chi-square statistic (Campbell and Madden, 1990).

RESULTS AND DISCUSSION

Stinging nettle is a common plant of uncultivated areas in the vineyard environment. On the experimental plot, 40 m^2 (2.5 %) were covered with *U. dioica*. The tufts were significantly aggregated (VM=4.1; Chi2=118; df=29). Four of the 20 sectors were completely free from nettle, while the coverage of the others varied between 0.1 % and 11.3 %. The size of the individual patches ranged from 0.06 to 4 m^2.

In order to estimate infection pressure, information on the presence, density, distribution and levels of infestation of *H. obsoletus* is required. The first adult vectors emerged on June 4th and the last specimens were found on August 25th. In this period, 12,459 planthoppers were caught on the nettle patches by sweep net (mean=337 specimens/m^2). The insects appeared in the same week on all patches except of three, where the flight was delayed by 11 days, although differences in plant-growth or surrounding vegetation could not be observed. The adult planthoppers were present for 13 weeks, but the critical flight period lasted for only 7 weeks

(week 4-10), when 90 % of all *H. obsoletus* were caught. The mean of the cumulative number of *H. obsoletus* per sweep over the season was 21, but the distribution was clumped (VM=11.9; Chi2=346; df=29). While two small patches were almost free from the planthopper (1/sweep over the whole season), the maximum density with 63 and 64 specimens per sweep was recorded on two bushes that grew immediately at the upper and lower border of the plot, respectively, where the lack of surrounding vegetation probably caused more favorable microclimatic conditions for *H. obsoletus.*

The average level of infestation by stolbur of the 1486 *H. obsoletus* tested was 12 %. The tuf-a type of stolbur that is associated with nettle was found in 98 % of the analyzed samples (n=166). On only one nettle-tuft no infected vectors were found at all. Although the levels of infestation ranged from 4 % to 30 %, the variation between patches was low compared to the differences in vector density (mean=12.1 %; STD=5.8 %). We found no correlation between nettle-patch size or vector density and the rate of infection. However, the average infestation of male vectors (14.9 ± 9.1; mean ± std) was significantly higher than in females (10.1 ± 6.8; Chi2=4.22; df=1; p=0.05). The number of males caught by sweep net exceeded that of females, the sex-ratio was 1.4. The males appeared earlier than the females, therefore the sex-ratio decreased from 1.7 in the first half of the flight-period to 1.0 in the second half.

The infection pressure of stolbur to grapevine depends not only on the density and infestation of the vectors, but also on their dispersal activity. To estimate the propensity of *H. obsoletus* to disperse from their host-plant stands, the sweep net catches were compared to sticky-trap data. More than 85 % of the 2086 planthoppers from the twenty trapping-stations were found on the lower traps. *H. obsoletus* was not randomly distributed on sticky traps but aggregated in some sectors, with a slight influence of the coverage by nettle on the trap catches (r^2=0.34; n=20). The vectors were found on traps in sectors without nettle, too. The numbers reached about one fourth of the average on the other traps. The distance to the next patch of nettle had an influence on the numbers of planthoppers on the traps (r^2=0.45; n=20), but some were even found on traps in a distance of up to 10 m to next nettle plant. However, the *H. obsoletus* numbers on sticky traps were correlated to sweep-netting data only for nettle patches in a maximum distance of 1 m. The male biased sex-ratio (2.9) on sticky traps compared to the life-sampling indicates a higher dispersal activity of males. The difference was less pronounced in the first half of the flight period (2.2 vs. 1.7) than in the second half (4.4 vs. 1.0), possibly because the mated females did not leave their host plants anymore. Since males of *H. obsoletus* show not only a higher dispersal activity than females but also a higher rate of infection, they may play the most important role for the spread of infection to vineyards adjacent to their host plant patches. The inspection of the neighboring vineyards supported this assumption. The incidence of BN was 12 % in the vineyard at the left (south-west) side of the plot and 14.7 % at the right side. However, a pronounced disease gradient was only recorded in the latter vineyard, where 50 % of all symptomatic vines were found in the first five of 27 rows. These rows were adjacent to the nettle aggregations in the experimental field and, furthermore, they are in the prevailing wind direction.

The experiment showed that sticky traps are useful to monitor the general presence and the flight phenology of *H. obsoletus.* However, they are only suitable to estimate the population density if they are exposed in the direct vicinity of the host plants. The results of this field study show the importance of fallow vineyards as reservoirs for stolbur inoculum. The occurrence of *H. obsoletus* is linked to the growth pattern of its specific host plants, but the distribution of the vector on the patches of those plants is aggregated, too. Further studies are in progress to monitor the microclimatic conditions at the nettle patches and the frequency of infection of the host plants and to estimate the dispersal activity in order to improve our understanding of the factors that cause the spatiotemporal patterns of infection pressure.

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Looking for Resistance to the Flavescence Dorée Disease among *Vitis vinifera* Cultivars and other *Vitis* Species

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INTRODUCTION

Despite the numerous diseases caused by phytoplasmas on cultivated and wild plants worldwide, few resistant species or varieties have been identified. Some studies performed on woody plants, like apple or coconut trees are the exception (for review Seemuller and Harries 2009). Thus, inoculations tests of different *Malus* species by the apple proliferation phytoplasma have shown that *Malus sieboldii* presents little symptoms and that phytoplasmas multiply less in *M. sieboldii* than in traditional *Malus domestica* cultivars or rootstocks (Bisognin *et al.* 2008). In the case of grapevine, intraspecific (*Vitis vinifera* cultivars) and interspecific (rootstocks) variability in plant sensitivity to the Flavescence dorée (FD) disease is well known; some inoculated rootstocks even show no symptoms of the disease (Schvester *et al.* 1967, Moutous *et al.* 1977). But this variability has not been studied in controlled conditions of inoculation and has not been characterized in term of phytoplasma titre in the plant. Our study consists in evaluating the sensitivity of major cultivars and rootstocks by recording the symptoms, the % of infected plants, and by measuring the phytoplasma titre in the plants after inoculation by the vector *Scaphoideus titanus* in high confinement greenhouse. Greenhouse experiments are completed by symptom observations and phytoplasma quantification in vineyards.

MATERIALS AND METHODS

Young plantlets (20-30 cm high) of Cabernet Sauvignon (CS), Merlot, Chardonnay and Pinot Noir cultivars, Selection Oppenheim 4 (SO4), Kober 5BB (5BB), 3309 Couderc (3309), Millardet et de Grasset 41B (41B), Nemadex Alain Bouquet, Riparia Gloire de Montpellier (RGM) rootstocks issued from in vitro multiplication were grown in high confinement greenhouse (25°C, L16:D8 photoperiod). Infectious *S. titanus* were obtained by acquisition on broad beans infected with FD phytoplasma (FDp), strain FD-PEY05 (Papura *et al.* 2009). Insects were transferred by groups of 7 onto grapevine plants for 1 week. Each experiment was performed by inoculating 14 to 16 plants per accession tested, including the sensitive CS as a positive control. After the transmission period, insect survival rates were recorded and insects were collected for further phytoplasma detection. Three, 5 and 10 weeks post-inoculation (wpi), 4 to 6 plants were collected. Stems, petioles and midribs were dissected, weighed and total DNA was extracted. Quantification of FDp cells in each plant was performed by quantitative real-time PCR on the *tuf* gene. For each time of sampling and for each grapevine accession, the symptoms, the % of infected plants and the mean phytoplasma titre were recorded.

RESULTS AND DISCUSSION

Survival of *S. titanus* on 5BB, 3309, S04, 41B and RGM was higher than or equivalent to the survival on CS which ranged between 69 and 86 %. It is not surprising as these *Vitis* species are native hosts of the vector in North America. Insects survived less on Pinot Noir, Merlot and Nemadex. In comparison with CS, the number of infected plants was higher for Chardonnay, 3309 and 41B, slightly lower for RGM Pinot N and 5BB, lower for SO4 and Merlot. No Nemadex plants were found infected. The first symptoms (coloration of leaf blades and veins, rolling of the leaves) appeared at 6 weeks post inoculation (wpi) for CS and 1 week later for Chardonnay and Pinot N. No specific symptoms could be observed at 10 wpi for the other accessions. Generally, the mean phytoplasma titre increased between 3, 5 and 10 wpi. However, for some accessions such as Chardonnay, SO4, 41B, 5BB and Merlot, it stabilized or even decreased at 10 wpi. Whatever the time, the ratio between CS and Chardonnay phytoplasma titre never exceeded 4. For 3309, RGM and Pinot N, the ratio which was high (17 to 115) at 3 wpi, decreased over the time to reach at 10 wpi ratio values similar to that of Chardonnay. For SO4, the high ratio measured at 3 wpi was slightly reduced at 10 wpi. The ratio was stable for 41B (28 to 30) but drastically increased for Merlot and 5BB (27 to 100 and 74 to 625 respectively).

In conclusion, Chardonnay like CS, can be considered as highly sensitive to FDp, Pinot N can be considered as moderately sensitive and Merlot as the less sensitive to FDp. This is in agreement with former field observations (Boudon-Padieu 1996). Furthermore, field surveys confirmed that the level of symptoms and FDp titre were significantly lower in Merlot than in CS. Although 3309 and RGM rootstocks did not present any symptoms, they exhibited high multiplication of the phytoplasma and can therefore be considered as tolerant to FDp. It was also the case for non-symptomatic "wild" rootstocks regrowth surrounding FD outbreaks. On the contrary, the low FDp multiplication in 5BB could make this rootstock a potential source of resistance to FD disease. Insect survival rate on *Muscadinia rotundifolia*-derived intergenic hybrid Nemadex was quite low and Nemadex could not be infected by FDp. Resistance to insect might explain such results as muscadine hybrids also appeared to be a good source for resistance to the nematode *Xiphinema index* (Esmenjaud *et al.* 2010).

Experiment	Accession	% of insect survival	Nb of infected plants/total inoculated	Symptom appearance at wpi	Mean phytoplasma titre in nb of cells/ug nucleic acids ± SE **(CS*/accession ratio)**		
					3 wpi	**5 wpi**	**10 wpi**
1	CS	86	13/14	6	$5x10^3$ ±$3.6x10^3$	$7.4x10^4$±$4.1x10^4$	$7.1x10^3$±$5.8x10^3$
	Merlot	62	9/14	-	$1.8x10^2$±$1.7x10^2$ **(27)**	$4.2x10^2$±$4.7x10^2$ **(175)**	$7 x10^1$ **(100)**
2	CS	69	14/15	6	$2.5x10^4$±$1.6x10^4$	$6.2x10^4$±1.3 $x10^4$	$1.6x10^5$±$7.1x10^4$
	3309	96	15/15	-	$1.5x10^3$±$1x10^3$ **(17)**	2 $x10^4$±$1.5x10^4$ **(3)**	$4.5x10^4$±$3.1x10^4$ **(3)**
	RGM	76	12/14	-	$2.2x10^2$±$1.8x10^2$ **(115)**	$5.6x10^3$±$3.9x10^3$ **(11)**	$3.8x10^4$±$2.6x10^4$ **(4)**
	SO4	84	12/15	-	$9.1x10^2$±7.4 $x10^2$ **(27)**	$1.4x10^4$±$2.4x10^4$ **(4)**	$7.9x10^3$±$6.3x10^3$ **(20)**
3	CS	83	11/15	6	2.9 $x10^4$±$3.9x10^4$	$1.1x10^5$±$1.2x10^5$	1.8 $x10^5$±$4.7x10^4$
	Chardon.	83	14/15	7	$7.4x10^3$±$7.9x10^3$ **(4)**	6.1 $x10^4$±$9.1x10^4$ **(2)**	6.2 $x10^4$±$7x10^4$ **(3)**
	Pinot N	73	10/15	7	$6.9x10^2$±$3.7x10^2$ **(42)**	$1.1x10^4$±$1.9x10^4$ **(10)**	$3.6x10^4$±$4.6x10^4$ **(5)**
	41B	91	11/14	-	$1x10^3$±$7.9x10^2$ **(28)**	$6x10^3$±$6x10^3$ **(18)**	$6.1x10^3$±$8.2x10^3$ **(30)**
	5BB	81	11/16	-	$3.9x10^2$±$2.2x10^2$ **(74)**	$3.4x10^3$±$2.7x10^3$ **(32)**	$2.9x10^2$±$2x10^2$ **(625)**
	Nemadex	41	0/15	-	-	-	-

* CS values from the same experiment were taken as a reference; wpi : weeks post-inoculation; -: no symptoms or no ratio.

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Strain Differentiation in 'Flavescence Dorée' Phytoplasmas on SecY and Tuf Genes

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INTRODUCTION

'Flavescence dorée' (FD) is a quarantine phytoplasma in EU and inspite the reduction of its impact in affected European viticultural areas, it is still of relevant importance, considering the ability of phytoplasmas associated with this disease to differentiate new strains in short periods of time. Therefore knowledge about FD strains differentiation is of major relevance towards the correct disease management. Strains were differentiated on 16S ribosomal gene and on other molecular markers (Martini *et al*., 2002; Botti and Bertaccini, 2007; Arnauld *et al*., 2007). In this work molecular characterization of a number of FD strains from diverse grapevine growing areas was performed on SecY (traslocase) and tuf (elongation factor Tu) genes.

MATERIALS AND METHODS

During 2011 grapevine samples were collected in Emilia-Romagna region (North Italy) in areas where FD epidemic was increasing. As reference strains in periwinkle elm yellows, strain EY1 ('*Candidatus* Phytoplasma ulmi', 16SrV-A) and FD strain FD-AS (16SrV-C) were used. Reference strains in grapevine were FD Veneto 8/08 and Emilia Mo2/08 (16SrV-D) and Tuscany 6, REV2, REV7, and Serbia 86/09 (16SrV-C). After total nucleic acid extraction PCR/RFLP analyses on 16S ribosomal gene plus spacer region using primers B5/P7 (Padovan *et al*., 1995; Schneider *et al*., 1995) in seminested and M1/V1731 (Martini *et al*., 1999) in nested reactions on P1/P7 amplicons were carried out. To distinguish between 16S ribosomal subgroups *Taq*I (Fast, Fermentas, Lithuania) at 65°C for 10 minutes was employed on 300 ng of amplicon. The FD-D strains were further examined by RFLP analyses on SecY and tuf genes (Angelini *et al*., 2001; Contaldo *et al*., 2011) using *Taq*I and *Tsp509*I and *Alf*I respectively.

RESULTS AND DISCUSSION

A total of 26 FD-D infected samples were selected after preliminary screening for further molecular characterization on the SecY and tuf genes. The RFLP analyses on SecY gene was carried out on 23 samples since 3 resulted not amplifiable on this gene. Two different profiles with *Tsp509*I and *Taq*I restriction enzymes were detected (Fig. 1) of which one is undistinguishable from reference strain Veneto 8/08 (profile I, Bertaccini *et al*., 2009) and from FD-88, the FD-D strain representative of the epidemic widespread in France and Northern Italy since 1990. Among the 23 samples examined 9 showed a profile that was clearly differentiable (profile II, Bertaccini *et al*., 2009). These results confirm the successful spreading of the FD-D strain identified in 2009 in the Lambrusco variety (Bertaccini *et al*., 2009) and confirm its distribution still restricted to Modena and Reggio Emilia provinces and to the same Lambrusco variety.

The RFLP analyses on tuf gene was carried out on 21 FD-D infected samples. It is interesting to underline that the samples non amplified on this gene were not corresponding to those non amplified on SecY gene except in one case. RFLP analyses with *Alf*I on tuf amplicons from grapevine and reference strains substantially confirmed the differentiation of FD-C and FD-D phytoplasmas in 16S rDNA gene (Martini *et al*., 1999), however in some cases alternative grouping was observed. In particular strain FD-AS, 16SrV-C (in collection since 1970) showed profile identical to 16SrV-D strains; strain Tuscany 6, 16SrV-C was identical to reference strains EY1, 16SrV-A and finally Serbian strain 86/09, 16SrV-C was undistinguishable from strains REV2 and REV7, 16SrV-C but also from strain Re5 and Ra3 affiliated to subgroup 16SrV-D. These latter information indicates possibility of genetic rearrangement in the tuf gene of field collected FD strains as one of the mechanism involved in 'flavescence dorée' strain differentiation.

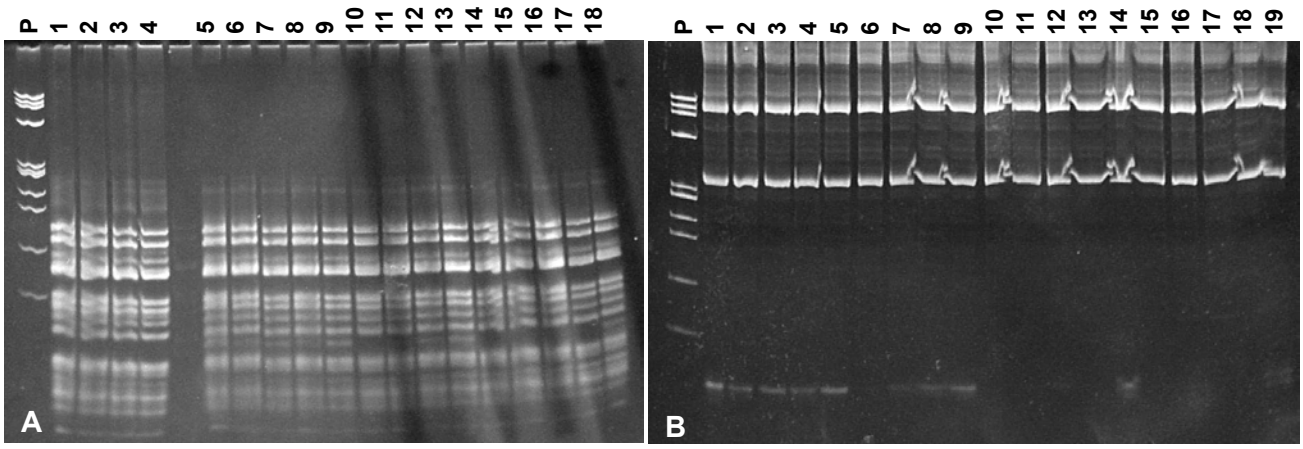

Fig. 1. RFLP profile on polyacrilamide gel 7% of FD strains amplified on SecY gene and digested with A) *Tsp509*I: P, marker phiX174 *Hae*III digested, 1, Bo1; 2, Ra1; 3, Ra2; 4, Bo2; 5, Re1; 6, Re2; 7, Re3; 8, Re4; 9, Mo1; 10, Mo2; 11, Mo3; 12, Mo4; 13, Mo5; 14, Mo6; 15, Mo7; 16Mo8; 17, Mo9; 18, Mo10; B) *Taq*I: 1, Re1; 2, Re2; 3, R33; 4, Re4; 5, Mo1; 6, Mo2; 7, Mo3; 8, Mo4; 9, Mo5; 10, Mo6; 11, Mo7; 12, Mo8; 13, Mo9; 14, Mo10; 15, Mo11; 16Mo12; 17, Mo13; 18, Mo14; 19, Mo15.

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Field Treatment with Resistance Inducers on the Canopy of Bois noir Infected Vines: Effects on the Disease Symptoms and on Grape Production

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SUMMARY
Grapevine Bois noir (BN) is widespread in all viticultural regions of Europe, determining heavy detriments of yield production and affecting grapevine quality. At the moment, an effective strategy to reduce the incidence of BN-infected vines is not known. However, the interesting phenomenon called recovery, which naturally happens, seems to be related to systemic acquired resistance. Five commercial resistance inducers (Chito Plant, Olivis, Bion, Aliette, and Kendal) were weekly applied on the canopy of BN-infected vines cv. Chardonnay from beginning of May to the end of July in two different vineyards located in Abruzzi (2007-2008) and Marche (2009-2010), respectively. At the end of a 4 year-field trial, all elicitors reduced the number of symptomatic vines, limiting the incidence of dehydrated clusters. The best and constant performances were reached applying Olivis, Kendal and Bion, allowing to obtain a production in 1-year-recovered vines not significantly dissimilar as compared with healthy ones. The increase of natural recovery rate in BN-infected vines throughout the use of elicitors seems to be effective, even if further work is needed to establish protocols that can be applied by growers.

INTRODUCTION
Stolbur phytoplasma is the agent of grapevine (*Vitis vinifera* L.) Bois noir (BN), a disease whose outbreaks are more and more frequent in several countries of the Mediterranean areas (Belli *et al*., 2010; Maixner 2011), and able to have a heavy impact on the European viticulture production, including Italy. Although, several strategies were attempted to contain phytoplasma infections, at the moment no treatment seems to be resolutive (Romanazzi *et al*., 2009a). An intriguing phenomenon, consisting in the spontaneous symptom remission, can involve plants infected by phytoplasma (Caudwell, 1961). In grapevines, this natural phenomenon, was observed in several varieties and viticultural Italian regions with a different ratio (Romanazzi *et al*., 2007). Some attempts promoted recovery by exposing grapevines to abiotic stress, such as uprooting followed by immediate transplanting (Osler *et al*., 1993) and partial uprooting or pulling (Romanazzi and Murolo, 2008), and by agronomical practices, such as pruning and pollarding (Belli *et al*., 2010). In any case, one of the few possibility to reduce consistently the number of symptomatic plants consists in the increase of hosts resistance (Romanazzi *et al*., 2009b). The aim of this research is to verify the impact of five elicitors, applied on the canopy of BN-infected grapevine under field conditions, on the incidence of symptom remission and the effects on quantitative and qualitative yield parameters.

MATERIAL AND METHODS
The experiment was carried out over four year in two commercial vineyards cv. Chardonnay: vineyard 1, located at Atri (Abruzzi, 2007-2008) (Romanazzi *et al*., 2009), vineyards 2 at Loreto (Marche, 2009-2010). The five commercial products tested in field trials in the vineyards 1 and 2 were: Chito Plant, Aliette, Kendal, Olivis and Bion. The plan of treatments was constituted by seven applications in 2007 trials (from beginning of June to the middle of July) and thirteen applications in 2008, 2009 and 2010 (from beginning of May to the beginning of August). For each year of trials, three different visual inspections, at the beginning of July, August and at the end of September, were carried out in the three experimental fields. During last inspection, leaf samples collected by symptomatic, recovered and healthy vines were subjected to molecular analyses. In vineyard 2 in 2010, grapes from replicates of each treatment, distinguishing healthy, symptomatic and recovered vines, were singly harvested at the end of September. At the same time, the number of healthy and dehydrated clusters and total production by a digital dynamometer (Handyscale, Bonso Electronics, Hong Kong, China) were recorded. The berry weight (g) was measured by *electronic balance* (*ORMA model BC* 500, Milan, Italy) and diameter (mm) by digital caliper (Metrica s.p.a., San Donato Milanese (MI), Italy). Both data were calculated by the mean of ten berries per each cluster per elicitor. Some qualitative parameters (soluble solids, titratable acidity, pH, Brix degrees) of grapes were also evaluated.

RESULTS AND DISCUSSION
The effectiveness of elicitors in the control of BN was expressed as percentage of recovered plants. All treatments increased the incidence of recovered plants, even if with different frequency, as compared with the control. A constant result in the four-year field trial was the good performance of Bion, Kendal, and Olivis which were able to significantly decrease the BN symptomatic vines in average of about 50% higher than the control. On the other side, a tendency towards a reduction of symptomatic plants was also recorded by Chito Plant and Aliette applications, that showed a lower effectiveness, as compared to the natural recovery rate. The average incidence of naturally recovered plants was about 26% in the four-year trials, with the highest value registered in 2007 (37.5%) and the minimum value (8.3%) recorded in 2009. None of these elicitors induced any phytotoxic effect.

A robust correlation (0.98) was recorded between the sanitary status (symptomatic, recovered, and healthy) assessed in September in vineyards 1 and 2, and the results of molecular detection carried out on leaf samples collected in the same period, because all healthy and recovered samples were found free of phytoplasma in leaf veins. In a very low incidence, recovered plants were found infected by stolbur phytoplasma, as also occurred in investigations carried out in Northern Italy (Bulgari *et al*., 2011).

The effects of elicitors was also evaluated recording the percentage of dehydrated clusters respect to the total number per vine. On symptomatic plants, the highest value was recorded in the control (32%). All elicitors reduced the incidence of dehydrated grapes of about 40% in symptomatic plants treated with Bion, followed by Olivis and Aliette. On the recovered vines, the incidence of dehydrated grapes resulted negligible. The production, expressed as kg/plant, was measured in September 2010, separately in recovered and symptomatic vines. Recovered plants, induced with the five elicitors had a production not significantly different respect to the healthy plants (3.8 kg/plant). Less than half production was yielded from vines treated with Olivis and Kendal but still symptomatic respected to recovered vines. Differently was the behavior of BN-infected vines elicited with Bion. Even, in symptomatic vines, Bion induced a production not dissimilar from the recovered plants of the same treatment. Other quantitative parameters such as diameter and berry weight were evaluated both for recovered and still symptomatic vines per each treatment. A significant reduction of diameter among recovered plants were recorded for all elicitors respect to healthy (12.4 mm). For the berry diameter of symptomatic vines were not recorded significant differences. Significant reduction of berry weight was recorded in recovered plants induced with Chito Plant, Aliette, and Bion respect to healthy plants (1.5 g). No differences were recorded among symptomatic plants.

The analysis of the main qualitative productive parameters (titrable acidity, pH, sugar content, and Brix degree) carried out on samples collected at the end of the trial, allowed to verify slight differences between the recovered and healthy plants. In particular, healthy vines resulted different from symptomatic plants although induced with Kendal and Bion considering titrable acidity, and from symptomatic plants induced with Chito Plant and Bion considering sugar content and Brix degree. The data obtained in the four-year experiments in the field have allowed us to verify the activity of the tested plant defence inducers in order to potentiate the induced recovery of BN-infected vines. It is important to find strategies that can help to reduce the number of BN symptomatic plants, considering the physiological perturbations and the losses of production that this disease induce to the vines (Musetti *et al*., 2007; Albertazzi *et al*., 2009; Hren *et al*., 2009; Landi and Romanazzi, 2011; Endeshaw *et al*., 2012). Therefore, a way to reduce the number of symptomatic plants was found, with three compounds, one of those, Bion, was alredy found effective in the containment of phytoplasma diseases in weed hosts (Bressand and Purcell, 2005; Chiesa *et al*., 2007; D'Amelio et al., 2010). However, further investigations are needed to move from a consistent field result to a sustainable protocol that can be introduced in the IPM strategies to control plant diseases.

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Deep Sequencing Analysis Reveals Modulated Gene Expression in Response to Aster Yellows Phytoplasma Infection in *Vitis vinifera* cv. Chardonnay

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INTRODUCTION

Aster yellows (AY) disease is associated with grapevine yellows (GY) in *Vitis vinifera*. GY-like symptoms were observed in South African vineyards in 2006, and diagnostic tests revealed the presence of AY phytoplasma (AYP) (Engelbrecht *et al*., 2010). Phytoplasmas are pathogenic, cell wall-free bacteria, confined to the phloem sieve elements of infected plant hosts and their homopterous phloem-sucking insect vectors (Christensen *et al*., 2005). AYP belongs to a widespread group known as '*Ca.* Phytoplasma asteris' (16SrI, subgroups A and B) (Lee *et al*., 2004). Studies on phytoplasma-plant interactions are limited due to failed attempts to culture them in cell-free media. Therefore knowledge of phytoplasma biology and the mechanisms of interactions with their hosts are still largely unknown. Model plants such as periwinkle have been used to study phytoplasma-plant interaction using differential display of mRNAs (Jagoueix-Eveillard *et al*., 2001). Global gene expression studies have shown that various metabolic changes occur within the host upon phytoplasma infection. With the use of microarray datasets, recent studies revealed that interactions between grapevine and Bois Noir phytoplasma affected several metabolic pathways (Albertazzi *et al*., 2009; Hren *et al*., 2009). The aim of our study was to investigate grapevine responses induced by AYP infection in terms of transcript profiles in healthy and AYP-infected *V. vinifera* cv. Chardonnay, using next generation sequencing.

MATERIALS AND METHODS

Pooled leaf and petiole material were collected during late spring from infected and non-infected Chardonnay plants in a vineyard in the Olifants River Valley (Western Cape). Plant material were processed using liquid nitrogen and stored at -80°C until use. Total RNA and DNA were extracted. The presence of phytoplasmas in plants was assayed using a conventional nested-PCR with primer pair R16mF2/mR1 in the first round followed by a second round with a modified R16F2n primer (R16vdal-F) and R16R2 (Gundersen and Lee, 1996). Plants were also screened for the following viral infections: Grapevine virus A (GVA), Grapevine virus E (GVE), Grapevine Rupestris stempitting-associated virus (GRSaV), and Grapevine leafroll-associated virus 3 (GLRaV-3). Total RNA was extracted using Plant RNA Purification Reagent (Invitrogen). RNA quality and purity was assessed using a NanoDrop ND 1000 spectrophotometer and an Agilent 2100 Bioanalyser. Whole transcriptome sequencing was performed by Fasteris SA (Geneva, Switzerland), using an Illumina HiSeq 2000 instrument. FastQC was used to confirm the quality of the raw sequence read data (www.bioinformatics.babraham.ac.uk/projects/fastqc). RNA sequencing reads were aligned to the 12X coverage assembly of the *V. vinifera* genome (Jaillon *et al*., 2007) using TopHat v2.0, followed by transcript assembly with Cufflinks v2.0. Transcript abundance was statistically estimated using Cuffdiff to analyse differential expression between healthy and AYP-infected plants (Trapnell *et al*., 2012). Finally, Blast2GO analysis was performed to assign functional annotations to the differentially expressed genes. Gene ontology (GO) slim categories were applied (Conesa and Götz, 2007).

RESULTS AND DISCUSSION

AYP and viral screening were used to obtain 3 AYP singly-infected and 3 healthy plant candidates for further transcriptome sequencing analysis. The Illumina HiSeq 2000 platform generated an average of 24 million high quality reads for each group. An average of 84% of all sequence reads mapped successfully to the *V. vinifera* 12X genome. Cuffdiff confirmed that 119 genes were induced and 56 genes were repressed by AY infection. With deep sequencing analysis we could demonstrate significant changes in the transcriptome of AYP-infected Chardonnay. The over-represented GO slim groups could be highlighted in Figure 1. The largest groups were represented by protein, carbohydrate, and lipid metabolism. A number of these differentially expressed genes may be involved in processes such as carbohydrate and starch metabolism, expression of defence-related proteins, photosynthesis, cell wall metabolism and primary -and secondary metabolism. Expression of these genes will be validated by real-time qRT-PCR. More in-depth analysis of metabolic pathways involved in such processes are necessary and may elucidate complex interactions between highly susceptible grapevine cultivars such as Chardonnay and AYP. Furthermore, our study could contribute greatly to understanding the unknown mechanisms of phytoplasma pathogenicity. Some of the abovementioned processes may refer to defence signalling pathways in susceptible plants and may assist in identifying candidate genes for resistance studies.

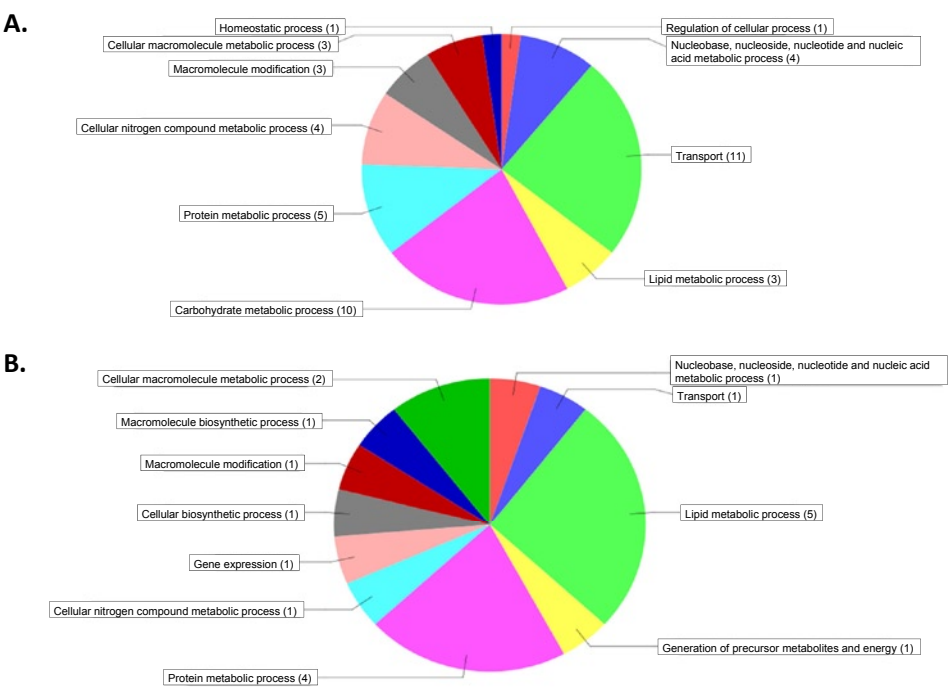

Figure 1. Charts showing the main groups of overrepresented GO slim classes in terms of biological processes modulated in response to AYP infection. The up-regulated (A) and down-regulated (B) GO processes are shown on each chart in terms of the number of genes involved.

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Incidence of Aster Yellows Disease in South African Vineyards

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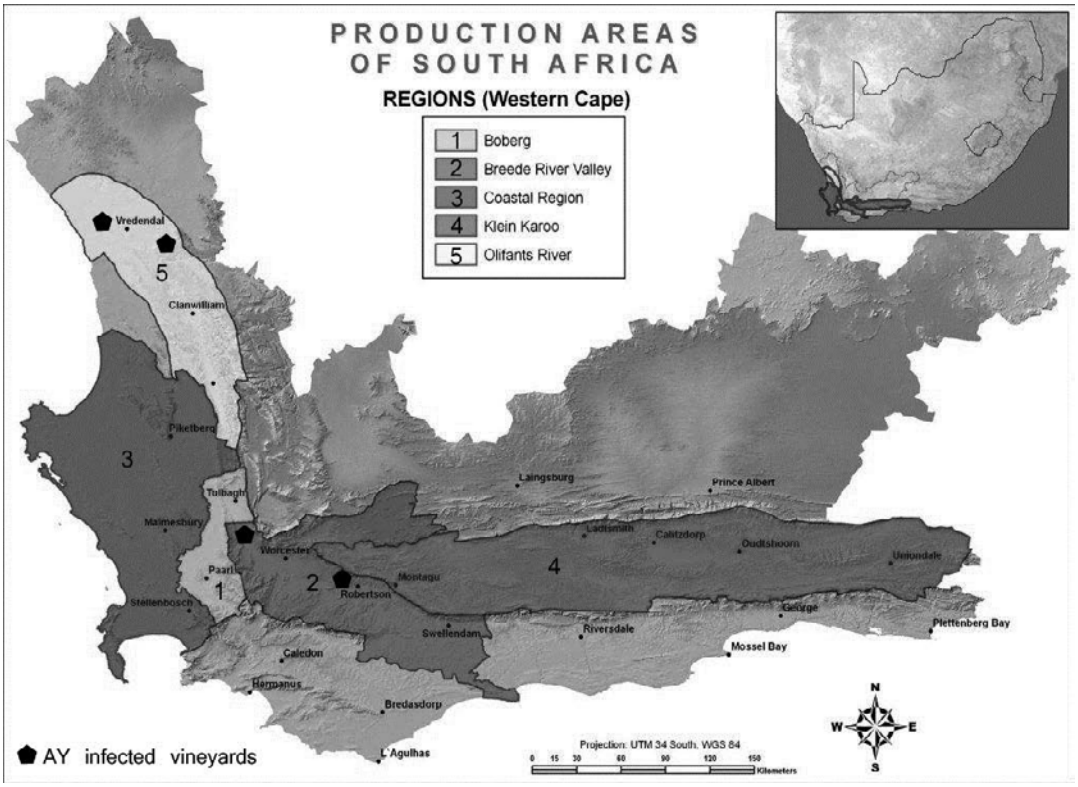
INTRODUCTION

Grapevine yellows is widespread in Europe. Phytoplasma diseases of grapevine occurs in several countries and cause serious damage ranging from lower yields to the death of vines (Magarey, 1986). In South Africa symptoms of grapevine yellows were for the first time associated with Aster yellows (AY) phytoplasma belonging to 16SrI group in 2006 (Engelbrecht *et al*., 2010). Symptoms of the disease initially occurred on grapevines in two regions, Vredendal and Waboomsrivier, but recently was also found near Robertson and Trawal in the Western Cape Province. *Mgenia fuscovaria* (Stal), was identified to be a vector (Douglas-Smith *et al*., 2010). In order to determine the impact of the disease on the South African vine and wine industry it is important to determine the incidence of the disease and the spreading tempo of the disease.



MATERIALS AND METHODS

Thirteen trial sites, which included 7 different *V. vinifera* cultivars (Chenin blanc, Shiraz, Chardonnay, Cabernet Franc, Sauvignon blanc, Pinotage and Colombar) were selected in the vicinity of Vredendal. Vineyards showing a low to medium disease incidence with ages ranging from 6 months to 18 years at the time of the initial disease evaluation (January 2010) were selected. All these vineyards fall in the area of highest disease incidence, as mapped by APIS (Agricultural Product Inspection Services) of the Department of Agriculture. Annual vine-to-vine mapping of vineyards was conducted during the past three seasons (January/February 2010, 2011 and 2012) and each vine was characterised as healthy, AY-affected or missing/dead. Vines were considered AY-affected if any one of the visual symptoms of the disease were present: (1) aborted fruit clusters, (2) downward rolling and yellowing/reddening of leaves, (3) green, immature canes and/or (4) die back of shoot tips and shoots. The

Patchy Programme (Maixner, 1993) was used to determine incidence of the disease. In order to confirm visual symptom evaluation of vines, five symptomatic and five asymptomatic vines were sampled per vineyard and subjected to PCR analysis. Total nucleic acid was extracted from leaf veins according to Angelini *et al*. (2001). The presence of AY phytoplasma was determined by using PCR-RFLP, as described by Lee *et al*. (1998), using restriction enzymes *Alu*I, *Hha*I, *Hpa*I and *Rsa*I. Nested PCR was performed using two sets of universal primers (P1+P7, followed by R16F2n+R16R2,) (Lee *et al*., 1998).

RESULTS

Previously reported results of detailed annual mapping of 13 selected vineyards in the Vredendal region (Carstens *et al.* 2011) showed that disease incidences varied between the different cultivars and vineyards. One Chardonnay vineyard showed an increased disease incidence (0.5% to 2% to 7.5% in two years).

For the verification of visual symptom evaluation of grapevines, leaf samples (symptomatic and asymptomatic) were collected every year for PCR analysis. A correlation of 83% was found between PCR and visual symptom analysis during the first two seasons.

Late summer disease symptoms differed in severity between the cultivars. All cultivars showed the typical yellows symptoms namely leaf colouring and curling, dying back of shoot tips, non-lignification of shoots and a few aborted bunches. As found in other countries, Chardonnay seems to be very sensitive to infection, with significant numbers of aborted bunches.

DISCUSSION

This study reports the status of AY disease incidence in South Africa, where the disease was detected fairly recently. Results showed that AY disease is spreading in vineyards located in a region of high disease incidence. Moreover, the disease has emerged in two new production areas in the Western Cape. Detailed annual mapping showed varied disease incidences in different cultivars and vineyards of different ages. Chardonnay seems especially sensitive, with an increase in disease incidence for one vineyard from 0.5% to 7.5% in two years. Our preliminary epidemiological data suggest that AY disease is spreading in South African vineyards. A clearer picture of the spreading tempo should emerge after data of the past season has been analysed.

ACKNOWLEDGEMENTS

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New Record of Grapevine Cultivars showing Australian Grapevine Yellows in the Riverland of South Australia

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INTRODUCTION

In March 2012, a brief post-harvest survey of foundation vineyards at Monash, South Australia (SA), revealed symptoms typical of AGY as identified by the presence of a combination of downward rolling and chlorosis of leaves (yellow or red on white or red *cvs* respectively), lack of lignification of shoots and cessation of growth and/or necrosis of shoot tips, and (where possible, post-harvest) some level of necrosis of bunches, on individual or isolated clusters of shoots on one or more arms of vines. Disease levels were, at times, high. For example, in a small varietal planting of cv. Pinot Noir, 18 of 46 vines showed AGY-like symptoms – an incidence of nearly 40%. Other cvs with similar symptoms included Muller Thurgau, Gouais and Doradillo. We had not previously observed AGY on these cvs nor in recent seasons had we seen in the Riverland, such high incidence of AGY on Pinot Noir, especially since recent incidences of AGY in the historically most affected cvs. Chardonnay and Riesling, have averaged only ~5-10% of vines. Given these observations and the wide array of cvs. present in the Monash vineyard, a more extensive survey of this and other vineyards was warranted.

METHODS

In April 2012, 47 patches of differing dimension in the above vineyard at Monash and in several others near Loxton, were visually surveyed for AGY. The number of vines showing symptoms was recorded in at least two transects made through each patch; these counts were then aggregated (Table 1).

Total nucleic acids were extracted as described by Habili et al., these proceedings. To check the validity of visual scoring AGY, cvs. Arneis and Pinot Gris symptom-bearing shoot material from Monash, and cvs Pinot Noir and six others from near Loxton, all not previously seen with AGY by the senior author, were tested via nested-PCR using phytoplasma generic ribosomal primer pair of P1 [AAGAGTTTGATCCTGGCTCAGGATT] and P7 [CGTCCTTCATCGGCTCTT] (Deng and Hiruki, 1991 and AGY specific primers (Davis et al, 1997): AUSGYF1 [ATCTTTAAAAGACCTCGCAAG] and AUSGYR2 [AGTTTTACCCAATGTTTAGTACTC: amplicon = 644 bp]. During the surveys, a single vine of cv. Chardonnay at Winkie, SA, was observed with 'little-leaf' symptoms across the entire canopy ie with otherwise healthy but uniformly small leaves (~50% smaller cf normal) (Figure 3). Leaf samples were taken for PCR analysis. Since this sample did not react with the AYG specific primers, we nested the P1/P7 product with the specific primers of Tomato big bud phytoplasma [fU5: CGGCAATGGAGGAAACT and rSPLL: AGATGATTGATTTTATTGG: amplicon = 900 bp] (Gibb et al., 1996).

RESULTS AND DISCUSSION.

In the nested PCR tests for AGY, 8 of the 9 cvs sampled proved positive (Fig. 1 shows examples for 3 cvs), affirming that visual survey in distinguishing AGY. Symptoms (Fig. 2) were seen on 29 of 47 different cultivars assessed (Table 1, only cvs showing AGY symptoms are listed). Of the 29, only cv. Sangiovese had been seen previously with AGY in the Riverland. The remaining 28 cvs are thus suspected first Riverland and South Australian records of AGY. The wider array of cvs affected and the higher incidence seen this season compared to that of the recent 6-8 (drought) years, is associated with the higher rainfall of the most recent two seasons and the subsequent observed greater growth of the candidate primary host plants for AGY eg ruby saltbush (*Enchylaena tomentosa*) and bluebush (*Maireana brevifolia*) (Magarey, *unpublished data*).In balance however, many of the cvs. newly recorded with AGY are relatively recent introductions to the region and have rarely been assessed for AGY. Given these findings, a more substantial survey during season 2012/13 would seem worthwhile, at least for the potentially commercially significant cultivars. Also, it was of interest to observe in this season with cooler than average temperatures, that the typical sectorial chlorosis usually only seen on AGY-affected leaves of red cvs (Figure 2), was more apparent on white cvs too. In 2011/12, even the white cvs. Riesling and Chardonnay displayed this symptom; these typically show little or no such chlorosis with sharply delimited by the primary, secondary and tertiary veins (Magarey, unpublished).

Table 1: Incidence of AGY in Various Cultivars, Riverland, South Australia, April 2012.

#	Cultivar	# AGY	# Vines	% AGY	#	Cultivar	# AGY	# Vines	% AGY
1	Ansonica	1	15	6.7	16	Meunier	2	15	13.3
2	Arneis	7	696	1.0	17	MullerThurgau	2	3	66.7
3	Boal	4	15	26.7	18	Parellada	2	15	13.3
4	Brachetto	3	15	20.0	19	Pinot Blanc	1	15	6.7
5	Broque	2	15	13.3	20	Pinot Gris	9	204	4.4
6	Canada-Muscat	4	39	10.3	21	Pinot Noir	18	46	39.1
7	Carignan	3	3	100.0	22	Pinotage	2	102	2.0
8	Cortese	1	15	6.7	23	Rubired	4	15	26.7
9	Doradillo	3	12	25.0	24	Sangiovese	8	60	13.3
10	Early Muscat	1	15	6.7	25	Sousao	4	12	33.3
11	Gamay	1	30	3.3	26	Tintarao	4	45	8.9
12	Gewurtztraminer	1	234	0.4	27	Tourien	16	45	35.6
13	Gouais	1	3	33.3	28	Trebbiano	6	45	13.3
14	Harslevelu	1	15	6.7	29	Xarello	1	15	6.7
15	Limberger	2	15	13.3					

Notes:
1, Scores are minimum incidence of AGY since some leaves had been lost during harvest, making detection more difficult;

2, A sample size of ≥ 50 vines/ patch allows X^2 statistical separation of differences in incidence of 5% or more.

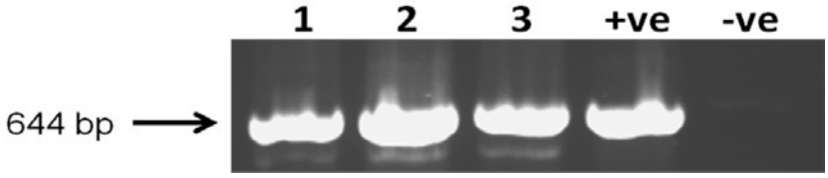

Fig 1. A gel showing nested PCR tests for AGY using DNA extracts from cvs Arneis, Pinot Gris and Canada Muscat, at Monash, South Australia. AGY positive and healthy grapevine controls used as standards.

The PCR test of the apparent 'little-leaf' symptoms on the single vine proved positive for Tomato big bud (TBB). We report possibly for the first time, the occurrence of little leaf in grapevine cv. Chardonnay in Australia which is associated with TBB. It will be of interest if this vine shows similar symptoms next season (2012/13).

Figure 2: from top left, clockwise)**:** Symptoms of AGY on vines of the white cvs. Arneis, Pinot Gris and Canada Muscat, at Monash, SA, Bottom left: Red cv. Pinot Noir infected with AGY (Loxton, SA, April 2012). Bottom right:Vine showing symptoms of 'little leaf' tested positive for TBB phytoplasma.

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First Occurrence of Aster Yellows Disease on Grapevine in the Palatinate Area, Germany

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INTRODUCTION

Grapevine Yellows (GY) are diseases caused by phytoplasmas which implicate significant crop losses in many European grape-growing countries and still are of increasing significance. Phytoplasmas are phloem-limited, wall-less prokaryotes transmitted by different species of leafhoppers, planthoppers, and psyllids from plant to plant (Weintraub and Beanland, 2006). Phloem damage is one of the reasons of the different manifold symptoms of phytoplasmas like yellowing, flower and fruit virescence, shoot deformation and dieback.

The phytoplasmas associated with GY belong to at least five different ribosomal groups. It is impossible to distinguish them by means of the symptoms exclusively. The most important GY diseases present in several European countries are Flavescence dorée (FD) and Bois noir (BN). FD is associated with Flavescence dorée phytoplasma (FDp), a quarantine pathogen in European countries that belongs to the 16SrV ribosomal group (Lee et al. 2004) whereas BN is associated with Stolbur (16SrXII-A) phytoplasma (Maixner et al. 2009). Relevant for Germany is up to now exclusively BN which caused some trouble among the winegrowers in the last years through the enforced occurrence in almost all German wine-growing areas. All other grapevine phytoplasmas are of minor importance in Germany or not yet found like FD.

Usually the first symptoms of GY appear at the earliest in the year after successful transmission by the vector due to the long period of latency. In the present study we report about a vineyard in which the first GY symptoms occurred in the planting year. In the middle of July 2009 eight grapevine plants (variety 'Portugieser', grafted on Kober 5 BB) with distinct symptoms of GY were found in a vineyard planted in May 2009. These plants were more or less evenly spread over the whole vineyard. The symptomatic vines showed a strong, nearly complete reddening of the leaf blades combined with a weak downward rolling of the leaves, limited root growth and partially dieback of growth tips. In springtime 2010 three of seven diseased young grapevines remained in the vineyard were died off whereas the others showed good shoots without any symptoms.

MATERIAL AND METHODS

Mid ribs of symptomatic grapevine leaves from the infested and adjacent vineyards were collected from 2009 to 2011. MasterPure™ Plant Leaf DNA Purification Kit (Epicentre® Biotechnologies) was used for phytoplasma DNA extraction according to the producer instructions. PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) were applied for PCR amplification. PCR/RFLP analyses were performed with the stolbur –specific primer pair f/r-Stol (Maixner et al. 1995) and on the *tuf* gene, according to Langer and Maixner (2004), using f/rTuf1 followed by nested-PCR with f/r-TufAY and *HpaII* for restriction analysis.

To detect potential vectors in the vineyard and the surroundings yellow sticky traps were used in the years 2010 and 2011 and checked weekly.

RESULTS AND DISCUSSION

With all DNA isolates used the first PCR to analyse the *tuf* gene resulted in an amplification product with a size of 940 bp. This is the expected fragment size for stolbur as well as Aster yellows phytoplasmas (16SrI) (Schneider et al. 1997). However the digest with *HpaII* gave different profiles: The DNA isolates of symptomatic samples collected in the adjacent vineyards with the varieties Cabernet Dorsa and St. Laurent resulted in the expected, typical nettle –type (tuf-a) profile of stolbur phytoplasma (Figure 1: Lane 1, 2) whereas all amplified DNA of the Portugieser vineyard resulted in one band with a size of about 500 bp (Figure 1: Lane 3 -8). The

following PCR analysis with the stolbur – specific primer pair run negative for the Portugieser isolates and positive for the others. So the evidence is that the Grapevine yellows phytoplasma in the Portugieser vineyards belongs to the Aster yellows group. The first comparison of the new "Palatinate-isolate" with a phytoplasma of the aster yellows group originally isolated from a grapevine of the Mosel valley and then transmitted to *Catharanthus roseus* (Maixner et al. 1994) showed some differences (Data not shown). Unfortunately there was not enough DNA for sequencing the PCR amplification product. In the two following years the grapevines originally symptomatic remained without any symptoms and no new infected vine could be detected in this vineyard. Checking other vineyards planted with vines coming from the same nursery respectively the same source of the scion and rootstock remained without success.

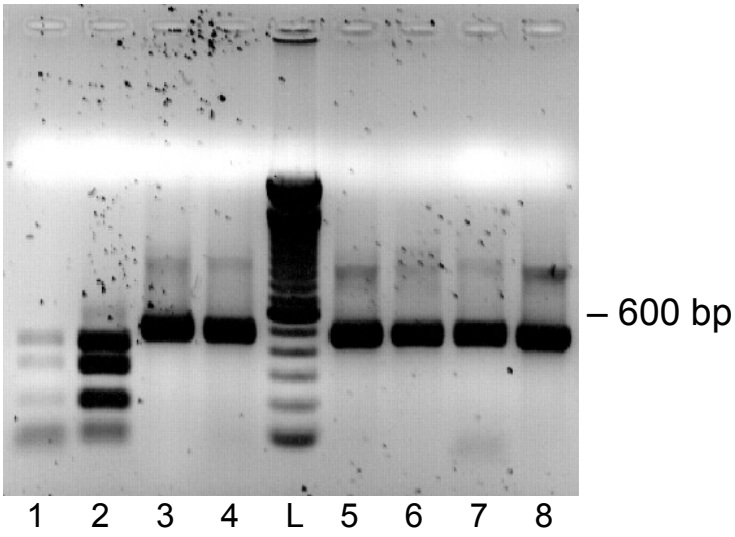

Figure 1: Agarose gel electrophoresis analysis of *HpaII* digested PCR products (*tuf* gene) obtained after 30 cycles. (L) 100 bp ladder (Invitrogen), (1) grape variety 'Cabernet Dorsa' (2) grape variety 'St. Laurent' (3 - 8) grape variety 'Portugieser'

Only a few leafhoppers could be trapped by yellow sticky traps. No *Macrosteles* species could be trapped which are known to transmit some AY-isolates (Weintraub and Beanland, 2006). All leafhoppers trapped belonged to the species *Empoasca vitis*. To our knowledge this is the first detection of a phytoplasma of the aster yellows disease group for the Palatinate. But the possible transmission pathway of the new isolate is unknown as yet.

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Emergence Of Stolbur Tuf-type-A Phytoplasma in Western Europe Relative to Dispersal of its Vector *Hyalesthes Obsoletus*: A Summary

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The dissemination of vector transmitted plant pathogens is determined by survival and dispersal of the vector, the vector's ability to transmit the pathogen and how the pathogen affects the infected plant and vector. The polyphagous planthopper *Hyalesthes obsoletus* is the major vector of stolbur phytoplasma (16SrXII-A group), which are responsible for yellows diseases in grapevine (bois noir), maize (maize redness) and various Solanaceous crops in Europe. The epidemiology of bois noir is coupled to the infection of herbaceous weeds, not to grapevine, as it is a dead-end host for stolbur and not a nymphal substrate of *H. obsoletus*. Stolbur has two major strains, defined by variants of the tuf gene (Langer and Maixner 2004). The tuf-type-a strain is associated with stinging nettle (*Urtica dioica*) while the second strain, tuf-type-b, has a broader range of natural host plants among which field bindweed (*Convolvulus arvensis*) is a dominant host. While the use of field bindweed has been recognised since stolbur was first characterised, the system bound to stinging nettle has emerged as a major source of infection, particularly in western parts of Europe. In Germany, the use of stinging nettle was observed for the first time about 20 years ago. The vector's host shift and the dissemination of two stolbur strains suggest two independent transmission cycles. Johannesen et al. (2008) showed that vector populations in Germany originated east of the European Alps but that a new immigration of vectors had reached the southern most Germany west of the Alps via France. In this Extended Abstract, we summarise data from three investigations (Imo et al. submitted; Maniyar et al. submitted; Johannesen et al. submitted) that were initiated to explore the emergence and spread of stolbur tuf-type-a in Western European viticultural regions and the vector's influence on this process.

MATERIAL AND METHODS

We studied host plant fidelity and dispersal of stolbur and *H. obsoletus* in Western Europe in a co-dispersal context using comparative gene genealogies and genotype/allele frequency distributions among regional populations. Both organisms were analysed from the two putative ancestral ranges of *H. obsoletus* (relative to Germany): 1) Italy and southern France, and 2) Slovenia and Croatia; from 3) the contact area between these two lineages in Switzerland and from 4) Germany where vectors are thought to consist of two discrete host-plant related populations. Host plant fidelity was analysed at syntopic sites in each region. Sequence diversity in stolbur tuf-type-a was characterised at four genes, *Stol-11*, *SecY*, *VMP1* and *Stamp*. The genes were amplified from DNA extracted from infected *H. obsoletus* caught on stinging nettle or field bindweed. We analysed about 70 tuf-type-a isolates with 10-16 isolates per gene per region. In *H. obsoletus*, we quantified genetic variation at seven microsatellite loci (> 1000 individuals) and for partial mtDNA sequences of COII and ND1 (175 individuals). Thus, all tuf-type-a genotypes were related to *H. obsoletus* mtDNA and microsatellite genotypes.

RESULTS AND DISCUSSION

Host plant fidelity. Tuf-type-a analysed from the four regional populations was monophyletic relative to tuf-type-b at all four genes, thus confirming previous findings of host plant specificity in this strain. By contrast, host plant specificity (microsatellites) in *H. obsoletus* was observed only in German and probably northern Swiss populations. Populations in central Switzerland, southern France, Italy and Slovenia, were not differentiated relative to host plant using the markers in this study. The specificity of *H. obsoletus* in Germany was caused by genetic divergence of stinging nettle-associated populations. These populations were genetically deprived relative their syntopic German field bindweed-associated populations and at the same time more related to these than to geographically distant syntopic host-plant populations. This and the finding that German field bindweed-associated

populations are genetically as polymorphic as ancestral populations in Italy and Slovenia strongly indicates that reduced genetic diversity in German stinging nettle-associated populations was caused by a founder effect during colonisation from field bindweed to stinging nettle and not by stepping-stone dispersal during geographic range expansion of southern nettle-associated populations. This interpretation is corroborated by the distribution of mtDNA haplotypes where both German populations have the derived haplotype “aa”.

Geographic range expansion of tuf-type-a and its vector. The diversity of three polymorphic tuf-type-a genes, *SecY* (3 genotypes) *VMP1* (13 genotypes) and *Stamp* (6 genotypes), was highest in Italy and decreased significantly towards both the eastern and western range borders. The number of tuf-type-a *SecY*, *VMP1* and *Stamp* genotypes in Italy was 3, 10 and 6, compared to 2, 2 and 3 in Slovenia and Croatia, and 1, 2 and 1 in both Switzerland and Germany, which had identical genotypes and were practically monomorphic. Nucleotide diversity in VMP1 was *c*. 20 times higher in Italy (0.00979) than in Slovenia and Croatia (0.00050) and *c*. 10 times higher in *stamp* (0.01028 vs. 0.00117). The general finding that genetic diversity is highest in ancestral populations corroborated the phylogenetic analysis of both *VMP1* and *Stamp* for which basal genotypes were observed in Italy. The regions Germany/Switzerland and Slovenia/Croatia did not share genotypes. The German/Swiss tuf-type-a genotypes were most related to French isolates in both genes. However, the phyolgenetic relationships of *VMP1* and *Stamp* genotypes differed within three Italian isolates. The difference was partly caused by diversifying selection (i.e. rates) on both genes but evidence also suggests within-strain and potentially between-strain hybridisation. Positive selection and/or hybridisation might question the phylogenetic rigor of the basal position of Italian genotypes. Despite this quandary, selection was not creating random phylogenetic signals at the geographic level because the genealogical and the geographic associations were correlated.

Mitochodrial DNA diversity in *H. obsoletus* was highest in Italy and decreased towards the west in France/Switzerland as well as the east in Slovenia/Croatia, becoming monomorphic in Germany (which was originally colonised from the east). The former region was dominated by the derived haplotype “bb” (frequency 0.85) while the latter region was dominated by the derived haplotype “aa” (0.50-0.93), as mentioned above. The frequency of the two haplotypes in Italy was 0.30 and 0, respectively, while the basal haplotype “ab” (Johannesen et al. 2008) had the frequency 0.41. Demographic analyses for *H. obsoletus* in these three regional populations (Italy, France/Switzerland, Slovenia/Croatia/Germany) indicated significant population growth for the western (Fu’s $F = -3.14$, $P < 0.05$) and the Italian (Fu’s $F = -1.25$, $P < 0.01$) populations but not for the eastern one (Fu’s $F = -0.02$). The microsatellite data supported a recent expansion of *H. obsoletus* from Italy into France and Slovenia but not into Germany (see above).

Summary. The combined data for tuf-type-a and its vector *H. obsoletus* showed a common origin south of the European Alps but also that the two organisms had incongruent co-dispersal histories. The emergence of tuf-type-a in Germany was explained by a secondary migration west of the Alps of genetically undifferentiated vectors carrying tuf-type-a, which were likely transferred to nettle-specialised vector populations of the eastern mtDNA lineage. The emergence of tuf-type-a in Germany was neither explained by resident vectors transferring tuf-type-a from field bindweed to stinging nettle in the course of a host-plant shift nor by primary co-migration from the resident vector’s historical area of origin in Slovenia. Thus, the rapid dissemination of tuf-type-a in Germany depends on the vector’s host shift but the vector’s host-plant specialisation is independent of the introduction of tuf-type-a. In Western Europe as a whole, dissemination of tuf-type-a is likely related to a general range expansion of the vector with newly acquired tuf-type-a pathogens that might be of hybrid origin.

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Identification of the Causes of Diseases of Grapes

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Enzyme-linked immunosorbent assay and polymerase chain reaction with reverse transcription have been used for harmful grapevine viruses detection in the southern regions of the Ukraine and the planting material from Republic Moldova. Our investigation conducted during 2010-2012 years allowed us to real and identify the next viruses: grapevine fanleaf virus (5,1-19,2 %), grapevine fleck virus (10,2-30,5 %), grapevine leafroll associated virus-1 (4,2-42,1 %), grapevine leafroll associated virus-3 (3,7-60,6 %), grapevine virus A (65,7 %), grapevine virus B (50,2 %). Investigation of different grapevine cultivars for latent viruses presence revealed a high level of virus diseases infection (2,4 – 100 %) due to low quality of grapevine planting material. As a result of phytosanitary inspection of vineyards of the south part of the Ukraine during 2005-2012 years the bacterial necrosis and Bois noir symptoms have been revealed for the first time. The gene expression patterns were followed in leaf midribs of grapevine cv. 'Chardonnay' naturally infected with 'Bois Noir' phytoplasma, which is associated with a grapevine yellows disease Bois noir.

MATERIAL AND METHODS

For the detection and the identification of viruses we used ELISA-test and polymerase chain reaction. For ELISA test-systems produced by Agritest (Italy) was used. For the detection and the identification of phytoplasmas we used polymerase chain reaction. Grapevine leaf samples were collected in the field from157 grapevine plants (7 cultivars: Chardonnay, Pinot nuar, Merlot, Sauvignon, Cabernet Sauvignon, Moldova, Aligotay). The clonal and regular grapevine material of different cultivars has been tested for the presence of viruses by polymerase chain reaction with reverse transcription (RT-PCR). The virus was extracted from the tips of young shoots, young leaves or wooden shoots in winter. Probes and reaction mix were prepared by method of Rowhani A. (Rowhani A. et al.,1993). For decreasing of unspecific amplification products during investigation different concentrations of Mg++ (1,3 mM, 1,7 mM та 2,0 mM) were applied. RD1 and RD2 primers were used. Reverse transcription consisted of 30 minutes at 52 ºC followed by 35 cycles of (94 ºC - 30 sec, 56 ºC - 45 sec, 72 ºC - 60 sec), with final extension at 72 ºC – 7 min (Rowhani A., personal message). Annealing temperature (Tan) was changed to improve amplification results: 52 ºC , 58 ºC, 60 ºC, 62 ºC. The optimal Tan was established (62 ºC) during investigation. Optimal Mg++ concentration in reaction mix was found out. The reaction was conducted at programmed thermostat "Tercik" (DNA-Technology, Russia). RT-PCR products were analyzed in a 1.5% agarose gel and stained with ethidium bromide which was included to tris-borate buffer (Amplisens,Russia).The results of gel electrophoresis was visualized at "Mintron" videosystem at wavelength 312 nm.

DNA samples were tested by nested PCR using two universal primer pairs P1/P7 and R16F2n/R16R2 (Lee etal., 1993). First round PCRs (20 µl) containing 1X PCR buffer (Bioline); 1.5 mM MgCl2; 200 µM of each dNTP; 0.5 µM of each primer; 1.25 units of Taq polymerase (Bioline BIOTAQ) and typically 50 ng of total DNA were cycled for one cycle of denaturation for 3 min at 94°C, followed by 35 cycles of 20 s denaturation at 94°C, 30 s annealing at 55°C and 45 s extension at 72°C, and a final extension at 72°C for 7 min. One microlitre of a 1/30 dilution of the first round PCR product was used as template in the nested reactions (20 µl). Reaction conditions were similar to the first round reactions, except the primers were annealed at 58°C.

RESULTS AND DISCUSSION

Phytoplasma was detected in all leaf samples of symptomatic vines, but not in phloem scrapings of the canes bearing those leaves.

This study revealed some fundamental aspects of grapevine interactions with 'Bois Noir' phytoplasma. In addition, the results of the study will likely have an impact on grape improvement by yielding marker genes that can be used in new diagnostic assays for phytoplasmas or by identifying candidate genes that contribute to the improved properties of grape.

After the first round of PCR with the P1/P7 universal primer pair, 7 of the samples yielded visible fragments of 1.8kb. These included mostly wine grape varieties, while two of the grape samples tested positive (cv. Chardonnay, cv. Aligotay). Interestingly, a number of samples originating from symptomatic vines did not yield any PCR fragments, possibly supporting the theory that the spatial and temporal distribution of phytoplasmas in plants are very inconsistent, thus making the diagnosis of these pathogens unreliable. None of the asymptomatic plants or any of the other negative control reactions yielded any PCR fragments.

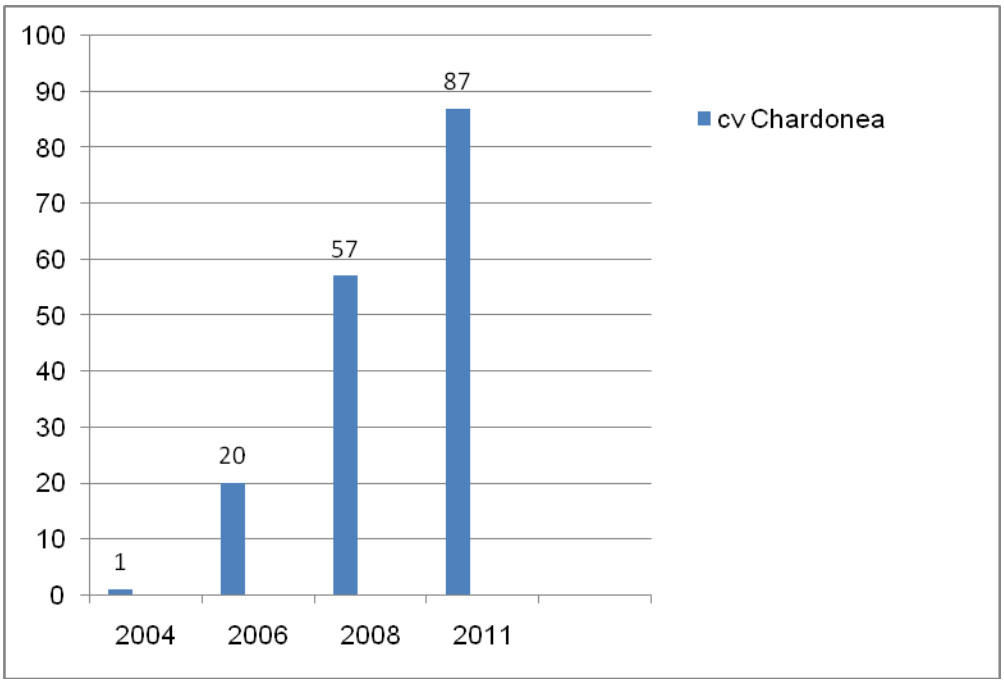

Fig.1. Dynamics of growth of morbidity of bushes of vine.

It is set that a Bois noir infection made progress and with every year the percent of defeat was increased (fig. 1).

Bois noir is identified by PCR and electronic microscopy of ultrathin section. It was established, that cv. Chardonea is most susceptible to this disease. Distribution and harmfulness of Boir nois on the Ukrainian vineyards is revealed.

The carrier of Bois noir –cicada *Hyalesthes obsoletus* is revealed and identified.

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Study of Bois noir Disease Epidemiology in Experimental Vineyards Through Phytoplasma Molecular Identification and Data Spatial Analyses

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INTRODUCTION

Bois noir (BN) is a grapevine yellows disease associated with Stolbur group phytoplasmas (Quaglino et al., 2010) transmitted plant-to-plant by the vector *Hyalesthes obsoletus* Signoret (Hemiptera Cixiide) (Maixner, 1994), a polyphagous insect living preferentially on spontaneous weeds inside and/or around vineyards (Langer and Maixner, 2004; Berger et al., 2009). Recently, several researches were focused on BN epidemiology and development of disease control strategies (Navratil et al., 2009).

MATERIALS AND METHODS

Investigation on BN epidemiology was carried out in two vineyards located in Ronco all'Adige and San Pietro di Lavagno, Verona province, North-Eastern Italy, in the years 2010 and 2011. The study was based on (i) monitoring and mapping diseased grapevines, spontaneous weeds and *H. obsoletus* specimens, (ii) BN phytoplasma (BNp) identification through real-time PCR analyses (Galetto et al., 2005) performed on leaf samples collected from grapevines and weeds and insect specimens captured by cromotropic traps and nets, (iii) statistic analyses of data spatial distribution by means of the software SADIE (Spatial Analysis by Distance Indices) (Perry et al., 1999)

RESULTS AND DISCUSSION

In the years 2010 and 2011, diseased grapevines increased (8.2% to 9.8%) in Ronco all'Adige and decreased (5.7% to 3.3%) in San Pietro di Lavagno.

Molecular analyses identified BNp in 7 and 10 weed species at Ronco all'Adige and San Pietro di Lavagno, respectively. In detail, *Convolvolus arvensis*, *Urtica dioica*, *Polygonum persicaria, Taraxacum officinale*, *Plantago lanceolata*, *Chenopodium album, Amaranthus retroflexus, Malva sylvestris, Artemisia vulgaris* and *Sonchus oleracea*, previously reported as BNp-host plants, (Langer and Maixner, 2004; Berger et al., 2009; Kessler et al., 2011), have been found frequently infected by BNp. Furthermore, BNp was identified for the first time in *Conyza canadensis, Rumex acetosa* and *Portulaca oleracea*. On the other hand, the species *Potentilla reptans*, *Solanum nigrum*, *Trifolium pratense*, *Equisetum arvense*, *Lactuca* spp., *Veronica persica*, *Sorghum* spp., *Medicago sativa* and *Calystegia sepium* did not host BNp in the examined vineyards.

Moreover, 318 *H. obsoletus* specimens (126 in Ronco all'Adige and 192 in San Pietro di Lavagno) were captured; BNp was identified in 15% of analyzed insects (3% in Ronco all'Adige and 23% in San Pietro di Lavagno).

Spatial Analysis by Distance Indices evidenced that spatial distribution of diseased grapevines and of 12 weed species were aggregated in Ronco all'Adige in 2010 and 2011. Diseased grapevine and *Urtica dioica* distributions were associated. These findings, along with the high BNp-infection rate of nettles, indicated that BNp spreading could be closely associated with *U. dioica*, the main host of *H. obsoletus* (Lessio et al., 2007). Diseased grapevines and *U. dioica* plants are aggregated mainly on vineyard borders, suggesting the possible role in BNp transmission of *H. obsoletus* feeding on weeds around the vineyard.

In San Pietro di Lavagno, spatial distribution of diseased grapevines in 2011, ten weeds and insects were aggregated. A strong statistic association was observed between BNp-infected insects, captured in 2010, and grapevines newly infected in 2011, suggesting the involvement of BNp-infected *H. obsoletus* in BN diffusion. Moreover, association between diseased grapevines and weeds *Amaranthus retroflexus, Portulaca oleracea, Plantago lanceolata*, and *Rumex acetosa* was observed in 2011, suggesting the possible role of such weeds in BN spreading. BNp was identified in such weeds. No statistical relationships were found between weeds, associated with diseased grapevines, and BNp-infected insects, suggesting that *H. obsoletus* should live on other weeds randomly distributed, such as nettle. These evidences could suggest the possible involvement of other insect vectors that could live preferentially in weeds statistically associated with diseased grapevines.

In conclusion, findings from the present work contributed to formulize the hypothesis that BN epidemics could be determined by diverse actors: (i) "*H. obsoletus* / additional vector(s) – *U. dioica* – grapevine" in Ronco all'Adige; (ii) "*H. obsoletus* – *U. dioica* / *C. arvensis* – grapevine" and "Additional vector(s) – *A. retroflexus / P. oleracea / P. lanceolata / R. acetosa* – grapevine" in San Pietro di Lavagno.

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Can Antimicrobial Peptides be Used to Engineer Resistance Against the Grapevine Pathogen Aster Yellows Phytoplasma?

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INTRODUCTION

Phytoplasmas are of increasing significance around the world, and due to the recent discovery in South African vineyards, could be highly detrimental to the local wine industry (Engelbrecht *et al.* 2010). This pathogen is known to have caused disastrous effects in vineyards in European countries, resulting in significant reductions in fruit yield and wine quality. The low base of infection at present means that the disease could currently be managed using viticultural practices. However, if the disease is not controlled, it could result in an infection incidence of 80-100% in certain regions within a short period of time. Therefore, a long term approach to control the disease through the development of resistance against the pathogen is desirable and should be investigated. Antimicrobial peptides (AMPs) can offer protection against a wide variety of bacterial and fungal pathogens in plants (Rosenfield *et.al.,* 2010). We recently showed that the AMP D4E1 shows an inhibiting effect against grapevine infecting bacteria *in planta* (Visser *et al*., 2012). Due to fact that phytoplasma lack an outer membrane they are an ideal target for AMPs. The current study intends to explore the efficacy of AMPs against Aster yellows phytoplasma (AYP) and whether AMPs could provide a mechanism to incorporate resistance in grapevine to control this devastating disease.

MATERIALS AND METHODS

Three AMPs were selected to be tested in a transient expression assay for their effect against AYP. The first, VvAMP1 was characterised and isolated from *Vitis vinifera* by the Institute of Wine Biotechnology at the University of Stellenbosch (De Beer *et. al.* 2008), the second AMP, D4E1, is a synthetic peptide which was developed by AgroMed LLC., USA, and the third AMP, Snakin1 (SN1), was isolated from potato and *V. vinifera* cv Chardonnay. Coding sequences of VvAMP1, D4E1, $SN1_{potato}$ and $SN1_{vitis}$ were amplified from available vectors or extracted plant DNA, subsequently cloned into plant expression vectors containing a CaMV 35S promotor and electroporated into *A. tumefaciens* cells. Phytoplasma infected grapevine plants (*Vitis vinifera* cv Chardonnay) were collected from a vineyard in the Vredendal district, South Africa. Single node cuttings of 4-5 cm were sterilized using washing steps with with 2% bleach, 70% ethanol and water and subsequently cultured *in vitro* in Murashige and Skoog (MS) media in tissue culture flasks, and kept in an incubator under controlled conditions. DNA was extracted from leaf and phloem scrapings at specific time intervals using the NucleoSpin® Plant II kit from Machery-Nagel. This material was then screened for phytoplasma infection by a nested-PCR procedure using primers described by Lee *et.al.* 1998. For quantification of AYP infection a semi-quantitative real-time PCR protocol was established. Semi-quantitative qPCR was performed using a SYBR® Green II detection chemistry. Primer AY-F (5'-AAACCTCACCAGGTCTTG-3') was based on a qPCR primer described by Hollingsworth *et al*. (2008) and AY-R (5'-AAGTCCCCACCATTACGT-3') based on the AY phytoplasma-specific qPCR TaqMan probe described by Angelini *et al*. (2007), to yield an amplicon size of 172 bp from the AY 16SrDNA. Additionally, transmission experiments were carried out to infect periwinkle (*Catharanthus roseus*) and *Nicotiana benthamiana* with AYP through the insect vector *Mgenia fuscovaria* (Kruger *et.al.* 2011).

RESULTS AND DISCUSSION

Cauliflower mosaic virus 35S expression vectors containing the foreign gene were constructed in order to conduct AMP activity screening against AYP *in planta.* Nevertheless, for *in planta* AMP activity screening it was necessary to establish AYP infected plantlets from infected field material. For this, infected cane material were collected from the vineyard, sterilized and transferred to *in vitro* conditions. Over 90% of these plants developed fungal contamination *in vitro,* most probably as a result of endophytic fungal infection of the grapevine material. Leaf and phloem scraping were taken from the remaining plantlets and screened using the nested PCR

procedure. Plant material was taken after two, three, four, seven and eleven weeks of *in vitro* incubation. In total, 134 *in vitro* plantlets were screened, but no AYP infection was found. Plantlets displayed a 'recovery phenotype', and until now no AY phytoplasma infected grapevine material could be established *in vitro.*

As the establishment of AYP *in vitro* material proved to be challenging, alternative AYP infected plant material needed to be established. For this purpose, the natural AYP vector in South Africa, *M. fuscovaria*, was collected in a highly AYP infected vineyard and placed on *N. benthamiana* and periwinkle plants. These plants will be tested for AYP infection. Infected plant material which can be used to test the effect of the selected AMPs in a transient expression assay still needs to established. Once phytoplasma infected plant material is available, the effect of the peptides D4E1, VvAMP1, $SN1_{potato}$ and $SN1_{vitis}$ on AY phytoplasma titres via a transient expression system will be evaluated using an established SYBR Green-based qRT PCR assay.

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The North America Plant Protection Organization Guidelines for the Movement of Stone and Pome Fruit Trees and Grapevines into a Nappo Member Country

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The North American Plant Protection Organization (NAPPO) is a Regional Plant Protection Organization (RPPO) operating under the International Plant Protection Convention (IPPC) of the Food and Agriculture Organization of the United Nations. The IPPC is an international agreement on plant health signed by 177 countries world wide (IPPC, 2012). Formally created in 1976, NAPPO has both a regional and global mandate. In support of the IPPC goal, the NAPPO mission is to coordinate the efforts of the NAPPO member countries in the area of plant protection while facilitating trade (NAPPO, 2011, 2012). The significant trade in plants for planting (IPPC, 2012b) and plant products into and between the three countries creates a substantial risk for the spread, entry, and establishment of plant pests. There are significant benefits to working collaboratively in preventing this. Regional Standards for Phytosanitary Measures (RSPM) are a major tool used by NAPPO. It is important to note that NAPPO standards are presented as guidelines and do not override country sovereignty.

RSPM No. 35, Guidelines for the Movement of Stone and Pome Fruit Trees and Grapevines into a NAPPO Member Country may be of particular interest to the ICVG. RSPM No. 35 merges and supersedes two previous import standards, RSPM No. 15 dealing with grapevines, and RSPM No. 25 dealing with fruit trees. RSPM No. 35 is a guideline for the importation into a NAPPO member country either from outside North America or from another North American country.

RSPM No. 35 outlines a systems approach for mitigating the risk of introducing regulated arthropods, bacteria, fungi, nematodes, phytoplasmas, viroids, viruses and virus-like agents in stone and pome fruit trees and grapevines without undue restriction of trade. The standard's scope does not include non-pest related items such as trueness-to-type and quality grades and standards as they are outside NAPPO's phytosanitary mandate. The objectives of this standard are to "*prevent the spread, entry and establishment of quarantine pests into NAPPO member countries, manage regulated non-quarantine pests within NAPPO member countries, facilitate equitable and orderly trade into and within the NAPPO region, and promote the use of integrated systems approaches and good plant protection practices as the basis for international exchange of stone and pome fruit and grapevine plants for planting*" (NAPPO, 2009).

The importation of vegetatively propagated crops such as grapevines carries a substantial risk of introducing plant pests. Many phytoplasmas, viruses, and viruslike agents infect these crops via pest vectors and are passed on to subsequent generations by vegetative propagation. Traditional individual phytosanitary actions such as pre or post harvest treatments or inspections are often insufficient to mitigate these risks to acceptable levels. The use of integrated measures in a systems approach for pest risk management offers a range of independent measures that may be used in combination with each other to develop an appropriate level of phytosanitary protection (IPPC, 2002; NAPPO, 2005). They may provide alternatives, where appropriate, to more trade restrictive measures such as prohibition. Certification programs are good examples of systems approaches. These programs use two or more independent measures, such as virus-testing, field inspection, isolation distances, and vector control to minimize the spread, entry, and establishment of pests. A certification program is an effective way of controlling pests within a country or area. A good foreign certification program may sufficiently mitigate pest risks to allow the importation of plants for planting with fewer restrictions.

A pest risk analysis (PRA) should be done by the importing country according to IPPC guidelines to help determine the most appropriate phytosanitary pest risk mitigation measures (IPPC, 2012b). RSPM No. 35 offers a range of suitable phytosanitary measures. These may be combined with more traditional phytosanitary actions such as inspection, fumigation, chemical sprays, hot water dips, biological control, and cold treatment.

RSPM No. 35 identifies and describes the components of a comprehensive certification program designed to control phytoplasmas, viruses, and virus-like agents (NAPPO, 2009). A certification program must be well defined and managed in order to be effective. It should clearly define requirements such as terminology, testing, eligibility, the nomenclature of certification levels, horticultural management, isolation and sanitation requirements, inspection and retesting methods and frequency, documentation, identification and labelling, quality assurance and auditing processes, and non-compliance and remedial measures. It should be administered by that country's plant protection organization or a certifying agency accredited by that organization with clearly defined roles and responsibilities for the involved individuals and entities. Staff should meet appropriate training, experience, educational and proficiency requirements. Technical proficiency should be demonstrated and documented at all steps.

The standard includes annexes with pest lists of arthropods, bacteria, fungi, nematodes, phytoplasmas, viroids, viruses and virus-like agents. Pest vectors such as nematodes or leafhoppers are also included because, even though they may not directly cause economic losses, they transmit other pests such as viruses and phytoplasmas. The lists indicate the presence or absence of a pest in each NAPPO member country. Each country is left to determine whether or not a pest is to be regulated. Some lists remain to be developed for grapevines

Two significant issues arose while developing the standard. The first issue emerged around the concepts of regulated pests and official control (IPPC, 2012b), and their implications on a country's import requirements. Under the IPPC, the application of phytosanitary import requirements should be based on the fundamental concept of non-discriminatory phytosanitary measures (IPPC, 2012b). The enforcement of phytosanitary import requirements should be equivalent to the enforcement of official domestic control programs. Only regulated pests (IPPC, 2012b), that is quarantine pests or regulated non-quarantine pests, may be regulated at importation. Official control is completely mandatory for quarantine pests, and mandatory only in certain circumstances, such as official certification programs, for regulated non-quarantine pests. One outcome from this issue was the de-regulation of some pests by Canada that did not meet these criteria. It also was an impetus behind the development of the National Clean Plant Network in the United States and the beginning of a similar program in Canada.

The second issue dealt with the pest lists. NAPPO standards usually contain only regulated pests. The NAPPO panel felt that this limitation would result in an inadequate pest list containing only a few grapevine pests meeting the regulated pest definition. The pest list was expanded to include economically significant pests in addition to regulated pests. The lists could be used by a country when developing a comprehensive certification program for either export or domestic purposes.

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French Regulation, Registration and Certification: Procedures, Controls and Perspectives

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With the contribution of the vine section of the CTPS

Adapted from a EU directive dated on 1968, 68/193/CEE revised in 2005, 2005/43/CEE once, French regulation dated September 20th 2006, defines the conditions of selection, production, propagation and distribution of vine material in France.

Considering that the prior requirement is the official registration of a variety or a clone in France or in one of the states members of the EU, then it is defined the following :

- Material produced by authorized companies, authorization delivered by FranceAgriMer.
- Definition of different categories of companies: ie., scions and cuttings producers, nurseries, breeders, selection centers,…
- Prior expertise of facilities and fields by FranceAgriMer,
- Varietal or Clonal authenticity to be verified and guaranteed,
- Official declaration to be provided.
- ….

SANITARY SELECTION AND CONTROL OF THE PROPAGATION OF VINE MATERIAL

Registration

Sanitary selection is based on the detection of main virus diseases by indexing which is still the official method. Fanleaf (GFLV and ArMV) Leafroll (GLRaV-1 and GLRaV-3) are required for the scion varieties. In addition, GFkV is required for the rootstock varieties. Any material that tested positive for one of these virus diseases cannot be registered and obviously not provided by the nurseries to the final users, the wine growers. Other virus diseases, like KSG or RSPaV are not mandatory, but the sanitary status is revealed to the CTPS vine section. Additional or complementary tests can be provided by the selection center. In 2012, only INRA and IFV are still officially recognized by the Ministry of Agriculture as selection centers.

Control of the propagation process under Ministry of Agriculture authority (FAM and PPO)

- Initial material held by selection centers has to be verified by ELISA (or PCR) every 5 years, all vines tested for GFLV, ArMV, GLRaV-1 and GLRaV-3.
- Base material is tested every 6 years, all vines tested.
- Certified material (increases blocks of scions and rootstocks) is tested every 10 years through a sampling base on the size of the single vineyard.

Since 2011, nurseries and owners of increase blocks have to take in charge this control, called "self control of the increase blocks." Sanitary tests are carried out by accredited laboratories.

GENETIC AND AGRONOMIC VALUE (VALUE FOR OF CULTIVATION USE)

Registration of varieties (2008 official technical procedure)

In addition to VCU, name and DUS (distinction, uniformity and stability) are also required for a candidate variety. DUS is carried out by ampelographers experts in the reference collection of INRA Vassal.

For a new variety or a local foreign cultivar, it is mandatory to establish 2 vineyards including a variety control. 90 vines (3 times 30) is the minimum. Data are collected during 3 years. Finally, a panel has to evaluate the interest of the candidate variety.

For a world known variety, procedures can be shortened; ie., Alvarihno registered in 2010. In such a case, bibliography and a technical report may be sufficient instead of a complete VCU.

Registration of clone (1998 CTPS protocol)
1998 protocol requires that the experimentation is carried out in the native of main wine region of the variety. There are no more than 20 candidates clones involved. 5 years of viticultural data plus 3 years of oenological data and tastings are mandatory. IFV partners are in charge of this genetic or agronomic selection.

Finally, the selection center makes the decision to submit clones to the CTPS vine section.

Perspectives of evolution of the procedures
According to the new stakes of the wine industry: opportunities of registration of additional clones, reducing of the use of pesticides, production of grape juices, global warming and experimentation of Mediterranean cultivars, probable release of an European Catalogue,… conditions might be up dated in the next future. All these procedures are being currently discussed by national experts.

Validation of Diagnostic Protocols for the Detection of Grapevine Viruses Covered by Phytosanitary Rules

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INTRODUCTION

The Italian Ministry of Agriculture funded the Finalized Project "ARNADIA", aimed at producing validated reference diagnostic protocols for the control and monitoring of plant pathogens of phytosanitary interest and, among them, grapevine viruses. In this framework, the "Working group ARNADIA – grapevine viruses (WG)", composed of 8 Universities and Research Bodies, 3 accredited Private Laboratories, one Plant Health Service and one Association of Grapevine Nurseries was established. Moreover, 5 additional Italian Plant Protection Services took part in an inter-laboratory ring test.

The aim of the WG was to produce reference and validated serological and molecular protocols allowing for the harmonization of the diagnosis of 8 grapevine viruses, namely, *Grapevine leafroll-associated virus-1*,*-2*,*-3*, (GLRaV 1, 2, 3) *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus*(GFLV) and *Grapevine fleck virus* (GFkV). Accordingly, the validation of the protocol consists in the evaluation of the processes aimed at determining their fitness for the particular use, and the validation of the assay yields test results that identify the presence of a specific target. The parameters that influence the capability of the test result to accurately predict the sample's infection status are: diagnostic sensitivity (ability of the used method to detect the presence of the pathogen in the samples truely infected by the pathogen in question - true positive) and diagnostic specificity (ability of the used method NOT to detect the presence of the pathogen in samples not infected by the pathogen in question - true negative). Other parameters that must be considered and which determine the efficiency of a protocol are: the analytical sensitivity (the smallest amount of infectious entities that can be identified by the diagnostic method), repeatability or accordance (degree of conformity of the results obtained in replications of the process, made at short time intervals, using the same reference sample and in the same working conditions i.e. equipment, operator, laboratory) and reproducibility or concordance (degree of conformity of the results obtained using the same method with the same reference samples in different laboratories). We reported the parameters obtained in the validation of a serological (ELISA) and molecular (Multiplex RT-PCR) protocols for the diagnosis of eight grapevine viruses.

MATERIALS AND METHODS

122 grapevine samples (varieties, rootstocks and pools of 5 plants, of which only one infected) have been analyzed by serological (ELISA - using 25 antisera of three commercial Companies: Agritest (8), Bioreba (9), Sediag (8) for GLRaV 1, 2, 3, GVA, GVB, GFLV, ArMV, GFkV, GLRaV 1+3, ArMV + GFLV) and molecular (multiplex RT-PCR) protocols. For ELISA, the tests were conducted carefully following instructions provided by the Companies; multiplex RT-PCR was performed using the protocol described by Gambino and Gribaudo, 2006. Moreover, three extraction methods (use of plastic bags and homogenizer, use of mortar and pestle with or without liquid nitrogen and use of milling machine) have been compared, starting from phloem tissue obtained from the bark. The tests were performed in 13 laboratories using the same samples (analyzed in blind conditions) and reagents; in each laboratory, the results have been obtained using the same threshold value calculated on

the basis of the spectrophotometer readings for ELISA and by analyzing the electrophoretic gels for the multiplex RT-PCR.

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, ELISA has proven to be a highly effective technique, comparable to the molecular method, although the latter turned out, as expected, to be more efficient for some viruses and on some specific samples (rootstocks and pool). In detail, regarding the extraction method, the use of plastic bags and homogenizer resulted less sensitive (5-8%) than the other two methods in detecting GFLV, ArMV and (2-4%) GVA. Concerning the different kind of samples, no differences have been highlighted for GLRaV-1, -2, -3 and GFkV between European varieties and rootstocks. Small and not always statistically significant differences (negative for rootstocks) were observed for ArMV, GVA and GFLV and generally good results were obtained in analyzing the pool samples, even if the accuracy was found to be lower (10-15 percentage points) for GLRaV-1, GLRaV-2 and GFkV compared to individual samples. No statistically significant differences were observed for the other viruses. Concerning the ELISA kits, all behaved absolutely equivalently in the diagnosis of GLRaV-1,-2,-3, GFLV, ArMV. Only two kits (GFkV from Sediag and GVA from Bioreba) performed worse than the respective ones from other Companies. Good results were obtained through the use of kits using mixed antisera (GLRaV-1 + -3 and GFLV + ArMV) by Bioreba, while the corresponding mixed kit GFLV + ArMV by Sediag performed worse.

In conclusion, harmonized and validated reference diagnostic protocols for grapevine viruses subjected to phytosanitary rules are, for the first time, available. The efficiency and robustness of the protocols have been proven using a large number of samples in a veriety of laboratories. On the basis of this, both serological and molecular protocols resulted valid, and their use could be as a function of different specific applications.

Virus	Diagnostic protocol	Sensitivity	Specificity	Accuracy	Analytical sensitivity	Repeatability	Reproducibility
ArMV	*Multiplex*	92 %	99 %	98 %	10^{-2}	100%	100 %
	ELISA – A/B/S	64/48/50%	85/95/96%	74/72/72%	10^{-2}	100%	95%
GFLV	*Multiplex*	68 %	100%	90 %	10^{-3}	100%	76%
	ELISA – A/B/S	75/82/77%	96/92/92%	80/84/81%	10^{-2}	100%	90%
GFkV	*Multiplex*	95%	95%	95%	10^{-2}	100%	95%
	ELISA – A/B/S	90/90/30%	100%	92/92/46%	10^{-1}	98%	88%
GVA	*Multiplex*	96 %	99 %	98 %	10^{-2}	100%	94 %
	ELISA – A/B/S	77/45/87%	100/100/96%	83/58/89%	10^{-1}	98%	82%
GVB	*Multiplex*	100%	100%	100%	10^{-2}	100%	100%
	ELISA – A/B/S	86/nt/nt%	100%	92%	10^{0} (2^{-2})	100%	85%
GLRaV 1	*Multiplex*	74 %	100 %	94 %	10^{-2}	100%	70 %
	ELISA – A/B/S	89/94/96%	100%	93/96/98%	10^{-2}	100%	92%
GLRaV 2	*Multiplex*	84%	98%	85%	10^{-2}	95%	83%
	ELISA – A/B/S	86/67/87%	100%	93/96/98%	10^{0} (2^{-2})	93%	84%
GLRaV 3	*Multiplex*	100 %	93 %	95 %	10^{-3}	100%	100 %
	ELISA – A/B/S	81/90/97%	100%	84/92/97%	10^{-3}	100%	94%

Table 1. Summary of validation parameters obtained by the ELISA test for each virus and antiserum and comparison with those obtained with the molecular protocol. A= Agritest; B= Bioreba; S= Sediag

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Virus Elimination from Grape Selections Using Tissue Culture at Foundation Plant Services, University of California, Davis

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INTRODUCTION

Micro-shoot tip tissue culture is the method of choice to eliminate virus(es) and other pathogens from many plant species. FPS first began applying this technology to grapes in 1988 and further work over the 1990s resulted in improvements in survival and success of virus elimination to the extent that this process is now routine and reliable (Golino *et al*., 2000). Molecular detection techniques for the grapevine viruses have improved, making it possible to test young plants regenerated from tissue culture, greatly speeding up the virus screening process (Osman *et al*., 2007, Osman *et al*., 2012).

MATERIALS AND METHODS

Micro-shoot tips are approximately 0.4 to 0.5 mm and include 1 to 3 pairs of leaf primordia. They are excised aseptically in a transfer hood under 50X magnification. The initial and maintenance medium is Murashige and Skoog (MS) (Murashige and Skoog, 1962) salts and vitamins with 1.0 ml/l 6-benzylaminopurine (BA), 3% sucrose, and 6.0 g/l gum agar adjusted to pH5.8. Explants are incubated in a growth chamber at 25C, 50% relative humidity,16 hour days, under cool white fluorescent and incandescent bulbs. They are transferred to fresh medium every 2 to 3 weeks. When the explants develop a shoot, they are transferred to rooting medium (half-strength MS salts and vitamins with 1.0 mg/l indole-3-acetic acid (IAA), 1.5% sucrose, and 6.0 g/l gum agar adjusted to pH5.8). When roots are well-developed plants are transplanted to sterilized potting mix.

Plants are tested by RT-PCR and/or qRT-PCR and woody index and to determine if the tissue culture treatment successfully eliminated virus. Timing of the testing varies depending on time of year the plant is acclimatized and its growth rate. Data was analyzed for 197 virus-infected grape selections that were treated by tissue culture therapy and for which virus testing was completed on the resulting tissue culture explants. Tissue culture explants were tested by woody index and RT-PCR for viruses that were previously detected in the mother plant from which they were excised. Mother plants were tested by: woody indexing on St. George, LN-33, Cabernet franc and Kober 5BB, herbaceous host indexing on *Chenopodium quinoa, C. amaranticolor, Cucumis sativus,* and *Nicotiana clevelandii*, and RT-PCR tests for *Grapevine Leafroll associated viruses* (GLRaV) -1, -2, -3, -4, -5, -6, -7, -9, -Car, *Grapevine fanleaf virus (*GFLV*)*; *Grapevine rupestris stem pitting associated virus (*GRSPaV*)*, *Grapevine fleck virus (*GFkV*)*, *Grapevine Virus A, B, and D* (GVA, GVB, GVD), Arabis mosaic virus (ArMV), *Tomato ringspot virus* (ToRSV) and *Tobacco ringspot virus* (TRSV).

Virus Status of Selection		Pre-treatment	Post- treatment
Total # selections		197	197
# Virus tested negative		0	172 (87.3%)
# Virus tested positive		197 (100%)	25 (12.7%)
Virus Detected	**GRSPaV**	153 (77.7%)	21 (10.2%)
	GFkV	59 (29.9%)	1 (0.5%)
	GLRaV-1	51 (25.9%)	0 (0%)
	GLRaV-2	41 (20.8%)	1 (0.5%)
	GLRaV-3	42 (21.3%)	3 (1.5%)
	GLRaV-5	9 (4.6%)	0 (0%)
	GVA	17 (8.6%)	1 (0.5%)
	GVB	12 (6.1%)	1 (0.5%)
	GVD	6 (3.0%)	1 (0.5%)
	GFLV	8 (4.1%)	1 (0.5%)

Table 1. Virus status of 197 grapevine selections before and after tissue culture treatment.

RESULTS AND DISCUSSION

Tissue culture therapy successfully eliminated viruses in over 87% of the grape selections that survived treatment. Of the 12.7% selections that tested virus positive post-treatment, most (10.2%) tested positive for GRSPaV. It is notable that 5% of the selections tested positive only for GRSPaV. Less than 2% tested positive for GLRaVs, GFLV, GFkV, and GVA, GVB, and GVD. No selection tested positive for GLRaV-1 or -5 post-treatment (Table 1).

Successful treatment of a selection requires that at least one explant excised from that selection tests virus negative. This negative explant is then propagated and becomes the source of 'clean' propagation material. Because more than one explant is excised from each selection, virus elimination success of a selection is not the same as success rate of individual explants. Only 79% of the total number of explants tested virus negative, which underlines the importance of producing more than one explant per selection. Table 2 illustrates the difference between virus status of a selection and an individual explant.

Selection and Mother plant (M) or Tissue Culture Explant (TCE#)	Virus Detected						
	None	GRSPaV	GFkV	GLRaV-1	GLRaV-2	GLRaV-3	GVA
Cabernet Sauvignon - M		x	x	x			
Cabernet Sauvignon - TCE1	x						
Cabernet Sauvignon - TCE2		x					
Cabernet Sauvignon - TCE3			x				
Fiano - M		x	x	x		x	x
Fiano - TCE1	x						
Syrah - M		x	x		x	x	x
Syrah - TCE1	x						
Zinfandel - M		x			x		
Zinfandel - TCE1	x						
Zinfandel - TCE2		x					

Table 2. Virus status of mother plants (M) and tissue culture explants (TCE) of four grape selections illustrating the difference between virus status of a selection as a group versus individual explants. Tissue culture treatment eliminated viruses in at least one explant in each selection, hence treatment was successful for all four selections.

Further analysis by deep sequencing of 10 explants that previously tested negative by qRT-PCR found similar results. None of the obtained reads were of virus origin; besides grapevine host reads, a few reads were found from grapevine viroids. This sequencing experiment was repeated twice.

Grape cultivar greatly affects survival and the length of time required to grow from a meristem tip to a rooted plant. An average of 75% selections survive and produce at least two rooted plants. Time from meristem excision to a potted plant averages seven months. Other variables that affect treatment outcome are technical expertise, growth medium, and season in which the selection was excised. The best season to excise meristems is in mid-spring when tips are large and rapidly growing.

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Results of Chemotherapy and Electrotherapy on Virus Elimination in Grapevine

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INTRODUCTION

Taking into consideration the large number of viruses known in grapevine, their association in viral complexes, their influence on the quality and quantity of the yield (Martelli and Boudon-Padieu, 2006), and the different behaviour of the viruses at sanitation methods, *in vitro* chemotherapy and electrotherapy are considered as alternative to thermotherapy, economic and environmental friendly methods.

The experiments described in this study were aimed to eliminate grapevine fanleaf virus (GFLV), leafroll associated virus serotype 3 (GLRaV-3), leafroll associated virus serotype 1+3 (GLRaV-1+3), and fleck (GFkV) from *V. vinifera* L. cvs. by *in vitro* chemotherapy and electrotherapy.

MATERIALS AND METHODS

Source of virus-infected material. The study concerning GFLV, GLRaV-3, GLRaV-1+3 and GFkV elimination by *in vitro* chemotherapy and electrotherapy has been done on *V. vinifera* L. cultivars naturally infected (Table 1), maintained in the grapevine virus collection of NRDIBH Stefanesti–Arges.

Table 1. Virus-infected grapevine cultivars

Grapevine cultivar	Virus	Method of virus elimination
Feteasca alba	GFLV	chemotherapy; electrotherapy
Ranai Magaraci	GLRaV-1+3	chemotherapy; electrotherapy
Caner	GFkV	chemotherapy
Cabernet Sauvignon	GLRaV-3	electrotherapy

In vitro chemotherapy. Grapevine apices (0.2 – 0.3 cm long) collected from mature virus infected plants over the growing season were cultivated on M&S (1962) basic medium (Murashige and Skoog, 1962) containing growth regulators and supplemented with one chemical drug for virus elimination. Ribavirin at three concentrations (10; 20 and 40 mg/L) or oseltamivir at three concentrations (37.5; 75.0 and 112.5 mg/L) were added to the proliferating medium for three subsequent subcultures of about 30 days each. Thus, the periods of exposure to each concentration of viricides were of 30; 60 and 90 days. Ribavirin was purchased from SIGMA, USA. Human antiviral medicine named tamiflu, produced by Hofmann - La Roche Germany, was used as oseltamivir phosphate source. After each subculture, regenerated shoots were transferred on free-drug rooting medium and than, acclimated in the greenhouse.

Electrotherapy. The infected grapevine in vegetative pots (rooted plants of 15-20 cm in length) obtained from one bud woody cuttings were subjected to continuous electric field action. The experiment consisted of the variation of the electric field intensity (10; 20 and 40 V/cm) and exposure time (5; 10 and 20 min for each intensity). Two or three apices were excised from each treated plants. The apices were grown on *in vitro* regenerative media and the regenerated grapevine were acclimated in the greenhouse.

Virus detection by ELISA. The test was performed according to the method described by Clark and Adams (1977) with commercial reagents (Sediag - France and Bioreba - Switzerland). The evaluation of virus elimination efficiency, has been done repeatedly, on leaves collected from acclimated plants. The sanitation rates / treated explant have been expressed.

RESULTS AND DISCUSSIONS

In vitro chemotherapy. Due the phytotoxic effect of ribavirin presence in the regeneration media, a reduced number of samples were ELISA tested for virus detection and no satisfactory results regarding virus elimination have been obtained. The viricide induced vitrification and necrosis phenomena during subcultures. Ribavirin was

most effective for GFLV elimination at 40 mg/L after one subculture, when 33.3% of acclimated grapevine were GFLV-free. Increasing the period of exposure did not induce high percentage of virus elimination; ribavirin at the same concentration and two subculture produced 20% GFLV-free plant. After two subcultures with ribavirin at 10 mg/L were obtained 12.5% GFLV–free vine. Ribavirin was ineffective in GLRaV-1+3 elimination; in this case no healthy plant has been obtained. The use of this chemical drug has been efficient in GFkV elimination. Thus, at the lowest concentration registered the best results; the highest percentage of GFkV-elimination was recorded after one subculture (37.5%). Although, no GFkV-free plant has been obtained after exposure to 40 mg/L ribavirin.

Oseltamivir was ineffective in GFLV elimination. GLRaV-1+3 was successfully eliminated with oseltamivir of 112.5 mg/L, after one and two subcultures (62.5% and 27.27% GLaRaV-1+3–free grapevines, respectively). The treatment with oseltamivir at 37.5 mg/L for 30 days eliminated GFkV in 25% of grapevines. 75 mg/L oseltamivir led to the GFkV elimination in increasing rate depending of the period of exposure (5.3% after 30 days; 12.5% after 60 days and, 23.5% after 90 days). However, the phytotoxic effect of oseltamivir occurred in the cases of the highest concentrations; thereby, the phytotoxic effect was not correlated to virus eradication in regenerated plants.

The treatment of apices of 1 mm on media containing 10 mg/L ribavirin for 30 days resulted in a complete elimination of GLRaV-3 and GLRaV-1+3 complex, but no GFLV-free vines have been obtained (Barba et al., 1982). Ribavirin at 20 mg/L eliminated GFLV from 94% of *in vitro* shoots (Weiland et al., 2004). The treatment with oseltamivir displayed antiviral activity on GLRaV-3 replication; all treated explants were virus-free and, in addition, no phytotoxic effects have been observed (Panattoni et al., 2006)

Electrotherapy in continuous electric field of GFLV-infected grapevines failed to induce healthy plant regeneration. If we choose the conditions that induce the best results for GLRaV-1+3 elimination, even there were some satisfactory percentage (33.3 – 50%) in several variants, they were not repeatable for explants taken from the same plant subjected to the electric field action. However, the variant 10 V/cm – 20 min was noticed with the highest percentage of GLRaV-1+3 removal (66.6%), registered for two of three initiated explants. 83.3 and 100% plants were GLRaV-3-free after exposure to electric field of 10 V/cm for 5 min and 10 or 20 min, respectively, as the most convenient variants. Also, 57.1-100% healthy vines have been identified after treatments at 20 and 40 V/cm, for different period of exposure.

The electrotherapy for GFLV-elimination in grapevine registered no positive results. However, the decreasing of ELISA values in function of the exposure period has been observed (Burger, 1989).

The possibility of inducing genetic variation by *in vitro* chemotherapy and electrotherapy in grapevine were investigated elsewhere. The RAPD profiles of micropropagated plants were found to be monomorphic and analogous to those of the control plant (Guta et al., 2010).

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Elimination of Viruses from Different Varieties of Grapevine *(Vitis vinifera* L.*)* Using Thermotherapy and Chemotherapy

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INTRODUCTION

One of the major threats to grapevine cultivation is virus infection. There are almost 60 viruses that attack grapevines. These are very harmful and cause a loss of production of millions of dollars worldwide every year. Therefore, antiviral chemotherapy and thermotherapy can be considered approaches for virus elimination in grapevine tissue culture. Ribavirin has been used to eliminate *Grapevine virus A* from *Vitis vinifera* L. (Panattoni, et al. 2007). The treatment of grapevines *in vitro* with thermotherapy using a range of temperatures between 26 and 40ºC for different durations can be appropriate for virus elimination (Skiada, *et al.* 2009). All of these approaches for elimination of grapevine viruses have had different levels of success (Martelli 2010). This study examined the simultaneous application of two control measures (thermotherapy and chemotherapy) for the elimination of grapevine viruses at the same time. A combination of chemotherapy and thermotherapy was studied for the elimination of *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine fleck virus* (GFkV) and GRSPaV isolated from declining Syrah grapevines (GRSPaV- SYD).

MATERIALS AND METHODS

Source of in vitro-material

Two varieties of grapevine *Vitis vinifera* L., Rondinella and Corvina Veronese, with unknown virus infection were obtained as *in vitro* explants from CSIRO, Australia. Virus assay using single tube RT-PCR showed that Corvina was infected with GFkV and GRSPaV while Rondinella infected with GRSPaV, GFkV and GRSPaV- SYD.

Treatments . Treatment A consisted of Rondinella explants growing in temperature controlled cabinets in media containing 25 µg/ml (w/v) Ribavirin (Sigma, Cat. # R9644-50MG) at 25ºC. In treatments B and C Rondinella and Corvina, respectively, were subjected to thermotherapy alone. In treatment D, a combination of Ribavirin and thermotherapy was used in Rondinella. The thermotherapy method used was developed in association with Clean Plant Technology (Australia) and involved an incremental increase in temperature every two weeks for 12 weeks.

Culture media were prepared according to Murashige & Skoog (1962). Media with Ribavirin followed the same process except the Ribavirin was added after a partial cooling at a concentration of 25 µg/ml. Media was changed every four weeks for each treatment.

Single-tube Reverse Transcription Polymerise Chain Reaction (RT- PCR) protocol

Total nucleic acid (TNA) was extracted from grapevines using the guanidine hydrochloride method of McKenzie et al. (1997). For the detection of GRSPaV, the primer pair RSP48 (AGCTGGGATTATAAGGGAGGT) and RSP49 (CCAGCCGTTCCACCACTAAT) targeting a 329 bp segment on the virus coat protein gene (Nolasco et al. 2000) was used in a single tube RT-PCR assay. The RubiscoL primer pair RBCL-H535 (CTTTCCAAGGCC-CGCCTCA) and RBCL-C705 (CATATCCTTTGGTAAAATCAAGTCA) with a size of 171bp has was the internal control (Nassuth et al.2000)

RESULTS AND DISCUSSION

The four independent treatments showed different effects on virus elimination from grapevines growing *in vitro*. After 8 weeks of treatment and two weeks at a temperature of 36ºC (in day) and 30ºC (at night) some explants were undetectable for GRSPaV using Ribavirin in normal growth and combined with thermotherapy (treatments A and D), whereas, samples from treatments C and B still tested positive for GRSPaV. All samples with media containing Ribavirin (A and D) were virus-free after 10 weeks while, samples from treatments B and C were still infected with GRSPaV. Minafra and Bosicain (2003) reported that GRSPaV was difficult to eliminate from grapevines in tissue culture. In this study by week 12 all samples for the different treatments were free of detectable

GRSPaV (Figure 1).This is the first report of a method to generate grapevine explants showing undetectable levels of GRSPaV by RT-PCR. The study indicates that Ribavirin is more efficient than thermotherapy and no mortality or abnormal symptoms appeared on treated explants. However, explants on media containing Ribavirin were stunted without changing in colour compared with explants treated with thermotherapy.

Further research is proposed to investigate whether lower concentrations of Ribavirin would be effective in the elimination of viruses from grapevines *in vitro* and also to determine whether the viruses have been eliminated or their replication was suppressed.

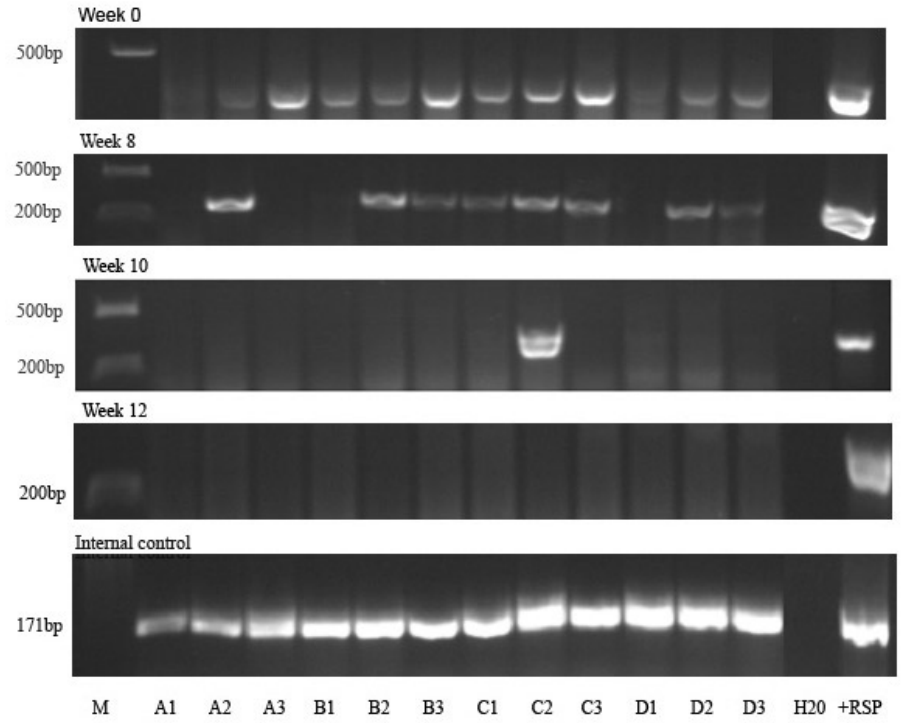

Figure 1: The progressive elimination of GRSPaV: A, Rondinella + Ribavirin; B, Rondinella +thermotherapy; C, Corvina + thermotherapy; D, Rondinella +Ribavirin+ thermotherapy; M, DNA marker

ACKNOWLEDGEMENTS

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Grapevine Leafroll Virus in Candidate Clones for Plant Certification in Spain

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INTRODUCTION

Grapevine leafroll (LR) is widespread in all the wine producing countries causing considerable damages in wine production and quality. Leafroll disease is considered a complex of several viruses belonging to the family *Closteroviridae* (Martelli and Boudon-Padieu, 2006). Among the components of the LR complex only two, GLRaV-1 and GLRaV-3, are currently considered in the EU regulations for certified material. In this work we describe the relative incidence of several LR species (1, 2, 3, 4-9 and 6) in Spain according to the results of the analysis of head clone candidates (hcc) which were received in our laboratory during the 2001-2009 period.

MATERIAL AND METHODS

Plant material come from 107 pre-selected local cultivars and 14 rootstocks from different Spanish wine producing regions, resulting in 825 head clonal candidates (hcc) which have been analyzed by ELISA , RT-qPCR and biological indexing for GLRaV species 1, 2, 3, 4-9 and 6, GFLV and GFkV.

Our analysis protocol is the following: First, we collect winter wood sent by public or private agents from clonal selection surveys and selections (hcc). Next, we perform diagnostic analysis by ELISA and RT-qPCR. Those hcc resulting negative are subjected to biological indexing, using Cabernet Sauvignon as indicator plant for Grapevine leafroll associated virus and Rupestris du Lot cv. St. George for Grapevine fanleaf virus (GFLV) and Grapevine fleck virus (GFkV) determination. Field indexing in indicator plants is monitored along three years and in parallel we run green grafting for a six months period in a growth. Visual inspection of symptoms are recorded and every positive sample is double-checked by ELISA and RT-qPCR. We evaluate symptoms 6-8 times per year.

RESULTS AND DISCUSSION

According to our analysis leafroll disease (LR) is rated in the second position when compared with the other grapevine viruses without discriminating among LR species (Table 1).Among the leafroll viruses, the most frequent virus present in the hcc samples corresponds to GLRaV-2. This is a probable consequence of the lack of limitation of this virus in certified material according to the Spanish and European regulations. In addition, it appear to be more common in temperate and mild environments in Spain rather than in warmer ones. One plausible explanation could be the possible differential distribution of the GLRaV-2 transmission vector which presently is unknown (Tsai et al., 2010). On the other hand, GLRaV-1 and GLRaV-3 appear to be the most common ampeloviruses in the Spanish vineyards. The combination GFkV/GLRaV-2 is apparently is the most frequent in Spain. Finally, we have been unable to identify by ELISA or RT-qPCR the actual virus present in some clones that induce leafroll symptoms in indicator plants (results not shown).

We must clarify that these results do not necessarily correspond with the real situation in the vineyards but come from the material that is received at our laboratory for certification. Surveys for the evaluation of the incidence of grapevine viruses in the Spanish vineyards has only be partially accomplished. However, to our knowledge there is a significant correspondence between our results and the ones in published surveys (Bertolini et al., 2010; Cretazzo et al., 2010).

Currently, the establishment of a definitive diagnosis for grapevine viruses by PCR and/or ELISA are increasingly more efficient, however, there are some situations where biological indexing is still a requirement essential to describe a given clone as GLRaV-free:

1. Low concentration of viral particles in samples can produce negative or doubtful readings.
2. New leafroll species or variants have emerged in the last years and more would appear in the future. In consequence, diagnostic tools based on the detection of pathogen molecules can be ineffective for detecting particular variants.

Thus, in the last stage we should rely on indexing for LR identification in certification programs for the grapevine.

Table 1. Number of positives in the different head candidate clones for each grapevine virus or combination according to their origin.

	Autonomous Community													
VIRUS	Andalucia	Aragón	Baleares	Canarias	Castilla y León	Cataluña	Galicia	La Rioja	Madrid	Murcia	Navarra	País Vasco	Valencia	**TOTAL**
FL		6				7								**13**
Fk	4	12		1	9	24	2	2		1			1	**56**
LR	4	2		10	10	17	4	1		6	1		4	**59**
LR1	3	1		4	7	4	3	1		1		1		**25**
LR2	3	13	2	1	24	58	11	7	3			4	1	**127**
LR3	11	2		7	4	5	1	3	2				1	**36**
LR6			2	3										**5**
FL+Fk						1								**1**
FL+LR	1	1					2							**4**
FL+LR2			3						1					**4**
FL+LR3			1			1								**2**
FL+LR1, -2					1									**1**
FL+LR 1,-3	1													**1**
FL+Fk+LR	1													**1**
FL+Fk+LR 2						1								**1**
Fk+LR					1	2		1						**4**
Fk+LR1		1			2	1								**4**
Fk+LR2		3			8	8	2		1					**22**
Fk+LR3			1			1								**2**
Fk+LR1, -3			1		1									**2**
Fk+LR2, -3			1		3									**4**
Fk+LR2, -3,-6			2											**2**
Fk+LR3, -6			2											**2**
LR1, -2		1		1	2	2	1	1						**8**
LR1, -3	4	1		3	1	1				1				**11**
LR2, -3	4	1				1								**6**
LR2, -6			2		1	2	1							**6**
LR4-9					7			1						**8**
LR2, -3, -6						1	1							**2**

FL: Grapevine Fanleaf Virus; Fk: Grapevine Fleck Virus; LR: Grapevine Leafroll associated virus; LR4-9: LR4, -5, -6 or -9

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Efficiency of Micrografting of Shoot Apices as a Sanitation Method Against Seven Grapevine Viruses (ArMV, GFLV, GLRaV-1, -2, -3, GFkV, GVA)

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INTRODUCTION

One of the main problems of viticulture industry is the presence of viruses disseminated through vegetative propagation and grafting. Production of virus-free plants is so important. In France, clones must be free of 4 to 5 viruses (GLRaV-1,-3, GFLV, ArMV, plus GFkV for rootstocks) to be certified. The detection of GLRaV-2 and GVA is often added. Different techniques to eliminate viruses in grapevine exist such as, thermotherapy, meristem culture, somatic embryogenenesis and cryotherapy (Goussard *et al*., 1991, Golino *et al*., 1998; Wang *et al*., 2003; Panattoni and Triolo, 2010). An alternative method is the micrografting of shoot apices onto hypocotyls. The latter method was used for routine sanitation for clonal selection since many years in IFV (Benin et Grenan, 1984) and numerous certified clones diffused in France were obtained by this technique. We make an assessment on the results obtained these last years, on both the percentage of regeneration and the rate of sanitation for the 7 main grapevine viruses studied.

MATERIALS AND METHODS

Woody cuttings from 139 accessions found infected by ELISA towards at least one virus were submitted to propagation. One cutting per accession was installed in a climatic chamber (32–34 °C) for at least one month. Terminal and axillary buds were collected and sterilized before being dissected aseptically under binocular to excise shoot apices. 30 apices per accession were grafted onto the side of hypocotyls from Vialla seeds. Three *in vitro* well-developed plants per accession were progressively acclimatized in a greenhouse. The spring later, two plants per accession were transplanted in bigger pots to leave them grow until an optimal development. The following winter, the woody canes were taken off from well developed vines to be submitted to ELISA test towards the 7 viruses: 241 accessions were so tested. The time schedule and organization are given in Table 1.

Table 1. Time schedule and rate of regeneration followed during three years**.** nt: not tested

Year of micrografting	Sanitized accessions (number)	Global regeneration (%)	Year of installation in the greenhouse	Year of ELISA tests (woody canes)
2009	62	12 %	2010	2011 (january)
2010	77	26 %	2011	2012 (january)
2011	78	37 %	2012	nt

RESULTS AND DISCUSSION

Regeneration rate

The regeneration rate is calculated as the number of plantlets obtained divided by the number of apices micrografted (generally 30). It has improved each year and is now about 37% (Table 1). This can be related to the new installations realized for the climatic chamber and the skill improvement of the staff. Some details of the results obtained in 2011 on the regeneration step are given in Figure 1. We must notice that the results remain very variable depending on the grape cultivar. However, they are globally very good as more than 4 plantlets were obtained in 93% of the varieties micrografted and very few cultivars were really recalcitrant to this technique.

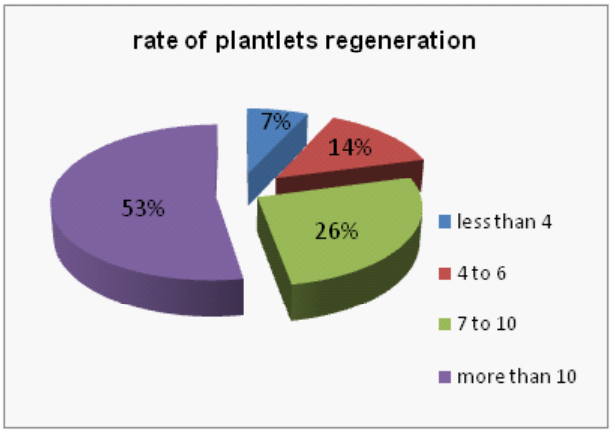

Figure 1: rate of plantlets regeneration obtained by apices micrografting (example of 2011).

Efficiency of this technique toward 7 viruses

The efficiency is evaluated virus by virus two years after micrografting on mature woody canes taken from well developed vines installed in a greenhouse (Table 2).

Table 2. Elimination of 7 viruses by apices micrografting onto hypocotyls.

Before sanitation		After sanitation		
viruses	**infected accessions (number)**	**tested accessions (number)**	**cleaned accessions (number)**	**Efficiency (%)**
GLRaV-1	30	49	49	100%
GLRaV-2	24	47	47	100%
GLRaV-3	68	115	115	100%
ArMV / GFLV	30	52	42	81%
GVA	31	55	53	96%
GFkV	23	39	39	100%

This technique confirmed its effectiveness in eliminating 7 important viruses in grapevine as all the accessions tested appeared to be free of GLRaV-1, -2, -3 and GFkV. The results appeared also very good for GVA and ArMV / GFLV with more than 80 % of the accessions obtained verified cleaned for these viruses.

ACKNOWLEDGMENTS

This study was supported by FranceAgriMer (FAM) and UMT GenoVigne ®.

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Renewing and Enlarging and the Portuguese Ampelographic Collection: Screening for Nine Viruses by ELISA

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The actual Portuguese Ampelographic Collection was established in 1988 as a result of an extensive survey all over the country to collect grapevine accessions in regional collections and in old vineyards. It also contains international references, non vinifera *Vitis* and rootstocks. Originally aiming at characterization of varieties, solving homonyms and synonyms problems, as well as preserving the overall variability of grapevine existing in Portugal, the sanitary status of the accessions was a minor concern. The lack of efficient diagnostic tools, at that time, for most of the actual known viruses and the uniqueness of a number of the accessions further contributed to the current sanitary situation [1]. Most of the original rationale of the collection was solved and exceed using classical ampelografic methods and molecular ones [2]. Renewing and enlarging the actual collection is a current project, but with a stronger sanitary element aiming at reducing the viruses present. In this paper we present the results of testing by ELISA for nine viruses the accessions already in the collection and new accessions to be introduced and epidemiological inferences.

MATERIALS AND METHODS

Plant material- cane scrapping from samples collected during the winter 2011/2012 respectively in : 680 accessions at the Portuguese Ampelographic Collection (PRT051); 30 genotypes of *Vitis vinifera* subspecies *sylvestris*; 30 genotypes obtain by hybridization aiming to get new table varieties; 18 uncharacterized accessions from recent field prospection.

ELISA tests- accessions were tested for nine viruses following the recommendations of the antisera producer Agritest (Bari, Italy) respectively from the *Secoviridae* family the genus Nepovirus- *Arabis Mosaic Virus* (ArMV) and *Grapevine Fanleaf Virus* (GFLV); from the *Closteroviridae* family the genera Ampelovirus- *Grapevine Leafroll associated Viruses 1, 3, and 7* (GLRaV 1, 3 and 7) and Closterovirus- *Grapevine Leafroll associated Virus 2* (GLRaV 2); from the *Tymoviridae* family genus Maculavirus- *Grapevine Fleck virus* (GFkV); from the *Betaflexiviridae* family the genus Vitivirus- *Grapevine virus A* (GVA) and Grapevine virus B (GVB).

RESULTS AND DISCUSSION

ELISA test results are shown in table 1.

Virus	Overall % of infections	% of infections in *sylvestris* accessions
ArMV	0.5	0
GFLV	8.6	0
GLRaV 1	8.3	0
GLRaV 2	10.5	0
GLRaV 3	59.1	25.0
GLRaV 7	0	0
GFkV	25.4	0
GVA	13.5	0
GVB	2.5	0

All plants infected with ArMV come from non Iberian accessions, a situation also verified in the certification of plant material by the National sanitary authority. Accessions infected with GFLV tend to be clustered by the original area of plant material gathering, namely Azores Islands, Madeira Island and Lisbon area accessions having the higher percentage of infection. Nevertheless at the establishing of the original collection, GFLV was already perceived as a severe sanitary problem and most of the infected accessions are rare genotypes that could not be overlooked.

Each of the viruses associated with leaf roll disease have a different distribution. GLRaV 3 stands out as the most abundant grapevine virus in Portugal and the only infecting even will vine populations [3]. GLRaV1 is present in accessions from the Northern part of the country and almost absent in the Southern ones. GLRaV 2 infection has no apparent pattern of geographic distribution. GLRaV 7 was never detected as in all other surveys already done [1].

The infection with GVA is associated with the co-infection with GLRaV 1 or 3 as reported by other authors that suggest that GVA needs the help of leaf roll viruses during transmission [4]. Plants infected with GVB are slightly dwarfed and can be visually sorted out in the ampelographic collection.

GFKV is the second most abundant virus in the ampelographic collection but this situation is not mirrored in the surveys of the National sanitary authority.

The results obtain in this survey helped us to decide on the substitution of infected accessions by healthier ones when existing to serve as truer to type references of varieties. It also supported the design of the new layout that physically separate infected accessions from healthier ones preventing field infections. In the end it also permitted the constitution of virtual core collections of accessions with certain groups of viruses that can be the base of more studies.

AKNOWLEDGEMENTS

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Harmonization of Certification Standards for National Clean Plant Network Commodities

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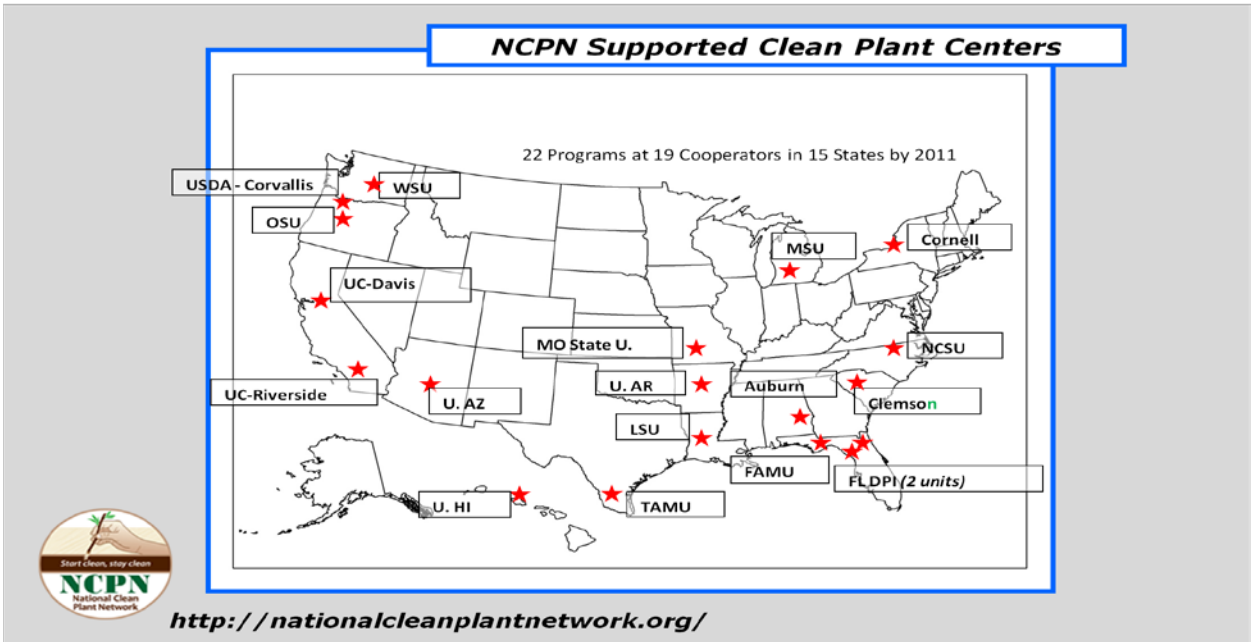
THE NATIONAL CLEAN PLANT NETWORK

The National Clean Plant Network (NCPN) was established in the 2008 U.S. Farm Bill and supports to date a total of 19 programs at 16 centers that deal with five groups of specialty crops: grapes, fruit trees, berries, citrus and hops. The three main objectives of NCPN are to: (1) develop and maintain G1 (Foundation) blocks to serve as sources of clean plant material for certification programs; (2) carry out pathogen elimination in asexually propagated crops; and (3) develop state-of-the-art diagnostic tests for systemic pathogens. A governing board consisting of researchers, representatives from industry, and state regulatory personnel was established for each of the NCPN crops to develop lists of pathogens (viruses, phytoplasma, viroids, and systemic bacteria) that need to be tested for each crop, review proposals for funding, and coordinate activities between the centers.

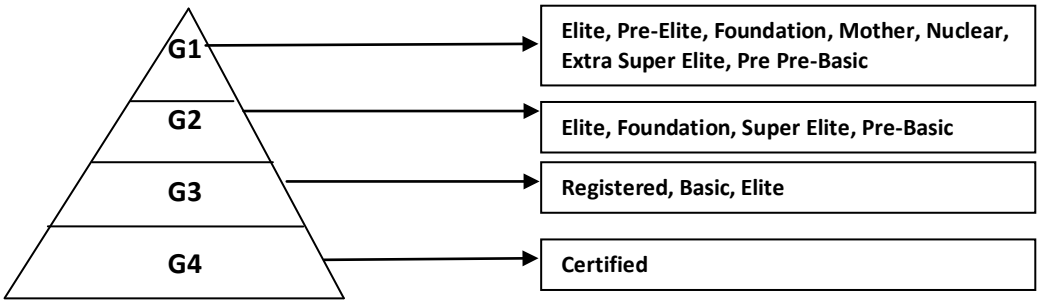


HARMONIZATION OF NURSERY CERTIFICATION STANDARDS IN THE UNITED STATES

In addition to funding for NCPN, the 2008 Farm Bill also provided funding to support nursery crop certification programs which in the U.S. are regulated at the state level rather than the federal level. A portion of this funding is being used to develop harmonized certification programs across states for NCPN crops. The pests targeted by NCPN are of regulatory significance domestically and internationally, and the commodities included in the NCPN are important to specialty crop producers and the nurseries that propagate, maintain and provide clean plants for growers. Uncontrolled, these targeted pathogens in NCPN commodities cost growers and consumers millions of dollars annually.

The development of state-level model nursery certification standards for the production, testing, and risk-based quality maintenance of clean plants is a key element in the chain of events required to provide clean plants to the growers of these crops. The model certification standards for NCPN commodities will by their very nature share many components such as: (a) the regulatory language for the certification standard; (b) the source of the clean plants for these programs (NCPN); (c) the types of pathogens included in the standard (viruses, phytoplasmas, viroids, systemic bacteria); (d) the types of tests used to detect these pathogens (biological indexing, serological tests, PCR); (e) the methods and schedules used to inspect and sample plants for the presence of pathogens; (f) the requirements for propagation and maintenance of plants; (g) product labeling and traceback requirements; (h) best management practices to avoid introduction of designated pathogens; (i) provisions for timely revision of the standard to accommodate the discovery of new pathogens and better diagnostic tests for all pathogens. Of course, the standards will differ significantly because of the diversity of plants, nursery production systems and pathogens involved in each commodity.

Part of the harmonization efforts is to have all commodities use a standard language for the various levels in certification programs. The NCPN aims to adopt the G terminology proposed by NAPPO in 2004 (RSMP 25). The use of this simple terminology that numbers generation steps from the top tier plant in a scheme should avoid confusion with the diverse terminology used by different commodities and in different countries.



G Level Terminology Equivalents

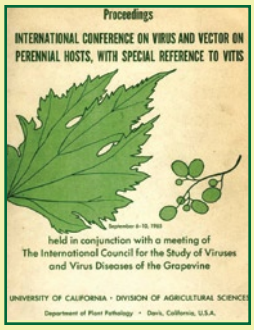
Since all of these standards will be promoted under the auspices of NCPN, efforts are currently underway to standardize, as much as possible and logical, the format and contents of these model certification standards for NCPN commodities. The goal is to have nursery certification guidelines for NCPN crops that are readily understandable and recognizable to legislators, regulators, nursery personnel, researchers and other interested stakeholders.

Author Index

–Notes–

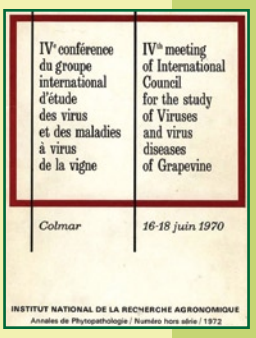
50 Years of Progress

ICVG Historic Proceedings Covers



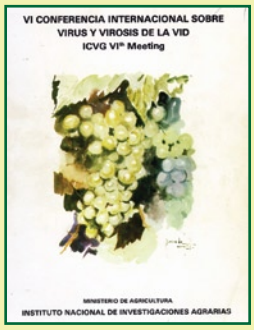
United States, 1965.

Germany, 1968.

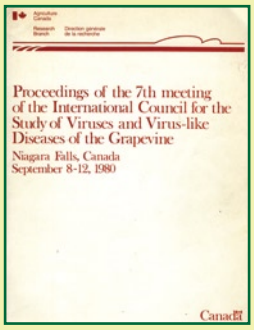


France, 1970.

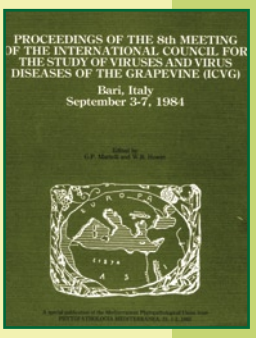
Italy, 1973.



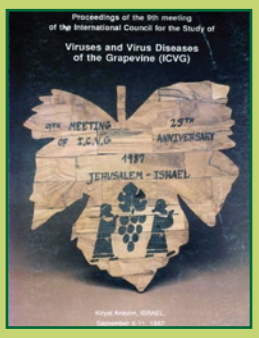
Spain, 1976.



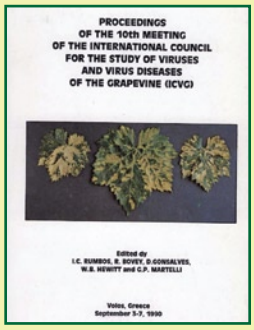
Canada, 1980.



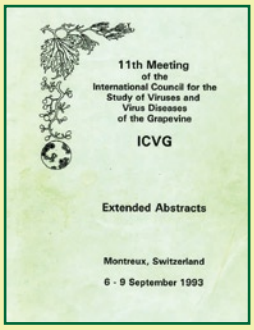
Italy, 1984.



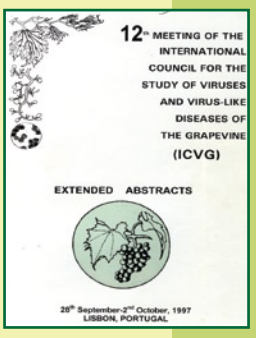
Israel, 1987.



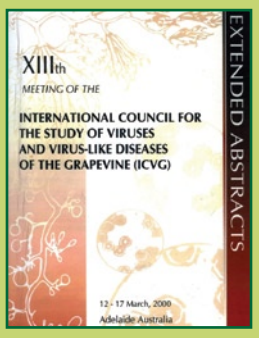
Greece, 1990.



Switzerland, 1993.

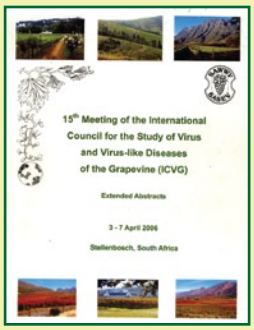


Portugal, 1997.

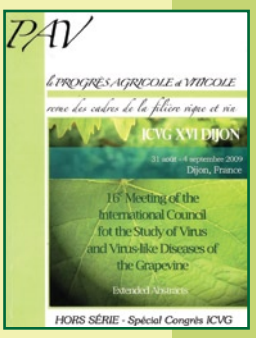


Australia, 2000.

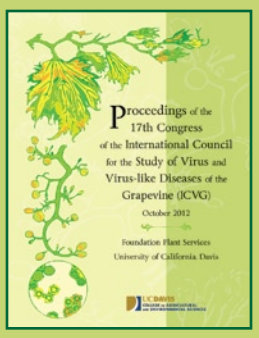
Italy, 2003.



South Africa, 2006.



France, 2009.



United States, 2012.