# ECOLOGY AND TAXONOMY OF FLAVESCENCE DOREE PHYTOPLASMAS : THE CONTRIBUTION OF GENETIC DIVERSITY STUDIES

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***Summary***

Recent insight into Flavescence Dorée phytoplasma (FDp) genome has given new tools to investigate genetic diversity of phytoplasmas members of the 16SrV phylogenetic group. Genotyping of 16SrV phytoplasmas infecting European alders and grapevines confirmed that FDp correspond to strains of alder phytoplasmas that were transmitted to grapevine and propagated by the grapevine leafhopper *Scaphoideus titanus*. Regarding to taxonomy, multilocus sequence analysis allowed a clear distinction between rubus stunt phytoplasmas and 16SrV phytoplasmas infecting alders and grapevine. Two new taxons '*Candidatus* Phytoplasma rubi' and '*Ca.* P. caudwellii' are proposed to classify the corresponding phytoplasmas.

## INTRODUCTION

South European vineyards are affected by Flavescence Dorée phytoplasmas (FDp) (Caudwell *et al.*, 1971; Boudon-Padieu, 2002). FDp belong to the phylogenetic subgroups 16SrV-C and 16SrV-D of the phytoplasma taxonomy but have not yet been officially established as a *Candidatus* Phytoplasma taxon. The difficulty in establishing a taxon to classify FDp resides in the high 16SrDNA identity between members of the 16SrV phylogenetic group (Lee *et al.,* 2004), especially between members of the subgroups 16SrV-C, -D and –E. In addition, surveys of grapevine phytoplasmas in Germany and of alder phytoplasmas in South Europe have shown the presence of phytoplasmas genetically related to FDp in alders and grapevine (Angelini *et al.*, 2001), suggesting that alders could constitute a wild reservoir of FDp. In order to clarify the taxonomy of 16SrV phytoplasmas and precisely state the genetic relationship between AldYp and FDp, phytoplasma isolates of the subgroups 16SrV-C, -D and –E were submitted to *map* genotyping (Arnaud *et al.*, 2007) and to multilocus sequence analysis (MLSA). To estimate the level of genomic differenciation between these phytoplasmas five different genetic loci were selected for MLSA from a FDp genome survey carried out by pyrosequencing of a faba bean DNA fraction enriched in FDp DNA through Bisbenzimide Cesium Gradients. Genetic diversity data get an insight into a possible classification of phytoplasmas of subgroups 16SrV-C, -D and –E and allow to compare genetic identity of FDp and AldYp.

## MATERIAL AND METHODS

Symptomatic alders and grapevines were sampled in South-Western France in three different ecosystems: FD infected vineyards, FD free vineyards and vine free areas. Genotyping was performed by sequencing the gene *map* (Arnaud *et al.*, 2007). In the case of mixed infection of different genotypes, sequences were obtained after cloning the *map* PCR products amplified using the DYNAZYME EXT proofreading polymerase. Phytoplasma isolates analyzed by MLSA are described in Table 1. 16srDNA was obtained by sequencing the P1P7 PCR fragments amplified as described by Schneider *et al.* (1995). Sequences of *map*, *degV-uvrB* and *rplV-rpsC* were obtained as previously described (Arnaud *et al.*, 2007, Lee *et al.*, 1998) and sequences of other genetic loci have been amplified by nested-PCR and sequenced using FDp specific primers. Non ribosomal sequences were concatenated for MLSA and phylogenetic reconstruction using maximum parsimony was performed using MEGA4 (Tamura *et al.,*2007).

***Table 1.*** List of 16SrV phytoplasma samples analyzed by MLSA

| Isolate | Disease - host | Origin |
|---|---|---|
| E04-D708 | Yellows - *Ulmus glabra* | Haute-Vienne, France |
| E04-D438 | Yellows – *U.minor* | Loire Atlantique, |
| EY1 | Yellows - *U. americana*– | NY, USA |
| EI04-2-2 | Yellows – *U. minor* | Friuli Venezia Giulia, |
| WJ1296-30 | Yellows – *U. carpinifolia* | Pyrénées Orientales, |
| RuS | Stunting - *Rubus fruticosus* | South Italy |
| RI04-2-6 | Stunting – *R. fruticosus* | Friuli Venezia Giulia, |
| WJ1295-21 | Yellows - *Rosa canina* | Pyrénées Orientales, |
| WJ1295-31 | Yellows - *Rosa canina* | Pyrénées Orientales, |
| WJ1295-78 | Stunting – *Rubus sp.* | Pyrénées Orientales, |
| FD70 | *S. titanus – C. roseus* | Landes, France |
| FD92 | *S. titanus– V. faba* | Landes, France |
| VI04-Lig1 | FD - *V. vinifera* | Liguria, Italy |
| VI04- | FD - *V. vinifera* | Toscana, Italy |
| VI04-C28 | FD - *V. vinifera* | Veneto, Italy |
| VI04-D004- | FD - *V. vinifera* | Veneto, Italy |
| VI04-248-04 | FD - *V. vinifera* | Piemonte, Italy |
| V04-11-44 | FD - *V. vinifera* | Drôme, France |
| V04-11-55 | FD - *V. vinifera* | Charente, France |
| V04-11-02 | FD - *V. vinifera* | Saône-et-Loire, France |
| V04-11-25 | FD - *V. vinifera* | Tarn-et-Garonne, |
| V04-11-17 | FD - *V. vinifera* | Gers, France |
| EY17-49 | PGY-A, *V. vinifera – C.* | Rheinland- |
| EY38 | PGY-C, *V. vinifera – C.* | Rheinland- |
| V04-11-1 | Yellows - *V. vinifera* | Haut-Rhin, France |
| A06-30-3 | Yellows - *Alnus glutinosa* | Gironde, France |
| A06-20-20 | Yellows - *A. glutinosa* | Gironde, France |
| A06-30-25 | Yellows - *A. glutinosa* | Gironde, France |
| A06-30-27 | Yellows - *A. glutinosa* | Gironde, France |
| WJ1444-32 | Yellows - *A. glutinosa* | Pyrénées Orientales, |
| AI04-2-4 | Yellows - *A. glutinosa* | Friuli Venezia Giulia, |

## RESULTS AND DISCUSSION

Alder yellows phytoplasmas (AldYp) were detected in more than 85% of the alder trees sampled in the three different ecosystems. Mixed infections were frequently revealed. Phylogenetic analysis including reference strains of the 16SrV group showed the existence of six genetic clusters namely FD1, 2, 3, PGY-A, B and C. AldYp isolates showed diversity with up to 13 substitutions on 674 bp. They did not form a homogenous phylogenetic group but were distributed in every cluster, except FD3. The FD1 cluster comprised four AldYp isolates of which three had the same *map* sequence as the FD1 strains from Gironde. FD2 comprised three AldYp isolates presenting 3 to 5 mutations with the sequence of the FD2 reference strains from Gironde. The FD3 Italian cluster did not comprise any French AldYp isolates. One of the two AldYp isolates present in the PGY-A cluster had a sequence identical to that of the reference strain described in Germany. One alder isolate was identical to the German PGY-B strain cluster. Finally, the PGY-C cluster comprised three identical AldYp isolates presenting 3 mutations with the reference strain PGY-C. Clustering of isolates was not related to their geographical origin because isolates from one site could be distributed in four different clusters and isolates from different places could be found in one cluster. Moreover, different isolates present as mixed infection in one plant could be distributed in two different clusters. The high genetic diversity of AldYp which include genotypes identical to those described as FD isolates in grapevine and the transmission of AldYp to grapevine by *Oncopsis alni* (Maixner *et al.*, 2000) are in agreement with existing or past exchanges between vineyard and wild ecosystems, and support the hypothesis that alders could have constituted the original source of Flavescence dorée outbreaks of genotypes FD1 and FD2 in France.

The genetic relationship between AldYp and FDp could lead to the description of a common taxon to classify these phytoplasmas. According to the rules edicted to describe new species into the genus '*Candidatus* Phytoplasma' (Firrao *et al.,* 2004), the isolates representing the new species must have less than 97.5 % 16SrDNA identity or show a clear genomic differenciation by comparison to other described species. Sequencing of 16SrDNA from alder, grapevine, *Rubus*, wild rose (*Rosa canina*) and elm confirmed the high level of sequence homology between members of the 16SrV phylogenetic group. Rubus stunt phytoplasmas detected in *Rubus* and wild rose (subgroup 16SrV-E) had all the same 16SrDNA sequence, different from the 16SrDNA of grapevine and alders phytoplasmas (subgroups 16SrV-C and –D) (figure 1). These results are in agreement with previously published PCR-RFLP data (Angelini *et al.,* 2001) and 16SrDNA sequence analysis (Lee *et al.*, 2004).

In the group 16SrV, the genomic differenciation of AldYp, FDp and Rubus stunt phytoplasmas have not been fully established. Five genetic loci were amplified and sequenced for 31 members of the taxonomic group 16SrV. The five sequences were concatenated as a single sequence and compared to conduct a phylogenetic analysis (Figure 2). Maximum parsimony analysis showed the existence of 3 monophyletic groups supported by boostrap of 100 %.

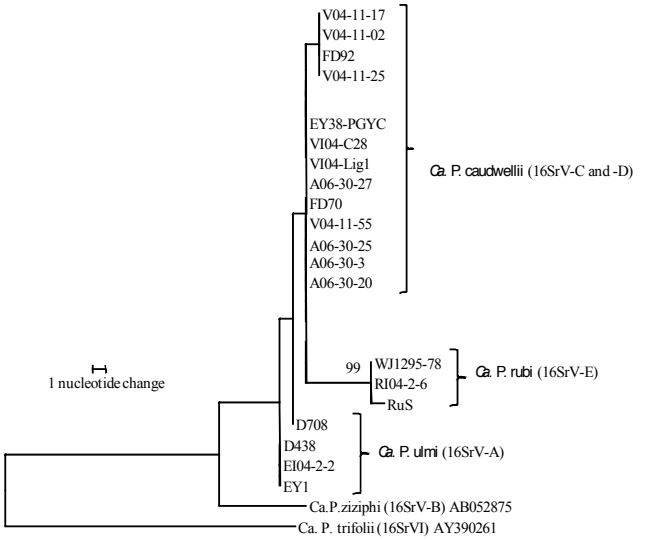


***Figure 1.*** Phylogenetic analysis of 16SrV phytoplasmas based on 16SrDNA sequences.

The first group is constituted by elm phytoplasmas distributed into two phylogenetic subgroups which correspond to '*Ca.* P. ulmi' (16SrV-A). The second group corresponds to members of the rubus stunt phytoplasmas (16SrV-E) which are clearly distinct from other members of the group. These phytoplasmas can therefore be classified as a new species for which we propose the name '*Ca.* P. rubi'. Finally, MLSA confirm the common phylogenetic origin of AldYp and FDp which are also grouped on a phyllum supported by high bootstrap of 100 %. As a consequence, we propose the classification of AldYp and FDp into a new species named '*Ca.* P. caudwelli'.

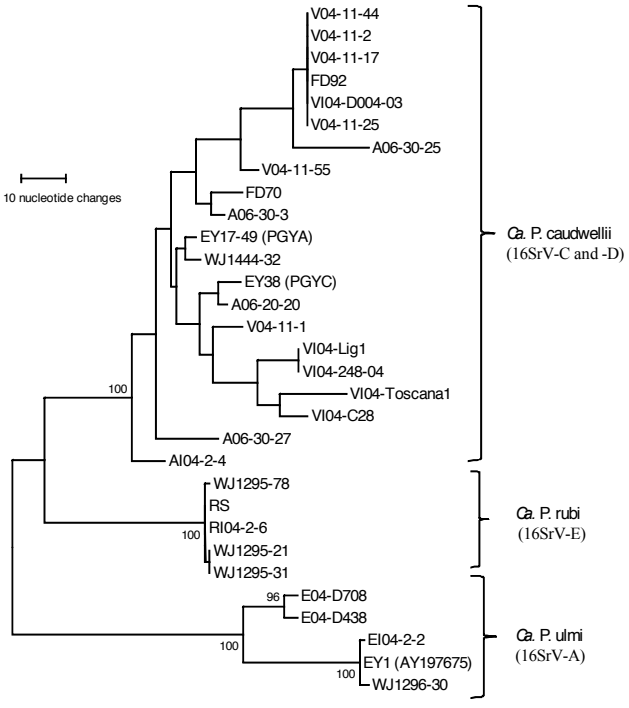


***Figure 2.*** Phylogenetic analysis of 16SrV phytoplasmas based on MLSA of genes map, degV-uvrB, tuf, rplV-rpsC and rplF.

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# MOLECULAR IDENTIFICATION AND GEOGRAPHIC DISTRIBUTION OF FLAVESCENCE DORÉE PHYTOPLASMA STRAINS

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***Summary***

Molecular analyses were carried out using two markers for Flavescence dorée (FD) phytoplasmas covering 16S ribosomal plus spacer region and translocase genes, in order to study FD strains present in Italy, Serbia, and France. The results confirm that Serbian FD strains belong to 16SrV-C subgroup. Both 16SrV-C and -D subgroups were still detected in Italy, and strains from both subgroups were studied in Veneto, while in Emilia and Tuscany 16SrV-D and 16SrV-C strains were respectively studied. Three of the four FD strains from France belong to 16SrV-D subgroup, while one was differentiable on 16S ribosomal gene from all others studied. By RFLP analyses on translocase gene it was possible to differentiate subtypes in both ribosomal subgroups confirming ability of FD phytoplasmas to differentiate genotypes.

## INTRODUCTION

Flavescence dorée (FD) is still a dangerous phytoplasma disease despite all the quarantine measures that over the last 20 years allow reducing its impact in affected European viticulture areas. The major problem viticulturists are facing is the great ability of phytoplasmas associated with this disease to differentiate new strains in short periods of time. The molecular differentiation of FD strains present in diverse grape growing areas where the disease is present, is therefore of major relevance towards a correct disease management. Several molecular markers were employed to differentiate FD strains after the first identification of two subgroups in 16S ribosomal gene (Bertaccini *et al*., 1997; Martini *et al*., 1999); FD strains were molecularly and geographically differentiated in Italy (Martini *et al*., 2002; Botti & Bertaccini, 2007) and in France (Arnauld *et al.,* 2007). In this work a molecular comparison among FD strains from major viticulture areas FD-affected in Serbia, Italy and France was carried out.

## MATERIAL AND METHODS

During 2006-2008 samples were collected in three areas of Serbia (11 samples in Irig, Niš, and Aleksandrovac), in three areas of Italy (30 samples in Veneto, Tuscany, and Emilia) and in four areas of France (4 samples in Gironde, Dordogne, Lot et Garonne, and Lasseube in Pyrénées atlantiques) (Tables 1 and 2). As reference strains TV-54, Liguria 3, FD-70, and FD-88 (Martini *et al*., 2002) were employed respectively for subgroups 16SrV-C and 16SrV-D. After total nucleic acid extraction PCR/RFLP analyses on 16S ribosomal gene plus spacer region using primers F1/B6 in nested reaction on P1/P7 amplicons (Duduk *et al*., 2004) were carried out to distinguish between 16S ribosomal subgroups. The strains were further examined by RFLP analyses on *rpS3* (ribosomal protein) and *SecY* (translocase) genes (Martini *et al*., 2002; Angelini *et al*., 2001).

## RESULTS AND DISCUSSION

*Molecular characterization of FD-C strains.* The examined strains showed RFLP polymorphisms with *Tru*I and *Taq*I restriction enzymes on *SecY* gene that resulted partially related to their geographic origin (Table 1). In particular strain differentiation was achieved from samples from Serbia and Italy and their tentative grouping showed identity between strains from Aleksandrovac (RS) and Tuscany (I) (profile IV in Table 1). RFLP profiles identity was present also among samples from Niš, Irig, and Veneto (mainly Treviso province) (profile III in Table 1). Further RFLP profiles differentiable from each other and from all the previous were also identified in samples from Niš and Irig (profiles I, II and VII in Table 1). RFLP analyses on *rpS3* gene did not allow the amplification of all examined samples and did not show polymorphisms.

***Table 1.*** RFLP results on FD-C amplicons obtained with primers FD9f3/r2 in nested PCR on FD9f2/r products. Identical letter = identical profile, in bold phytoplasma strain V30 showing profiles in common with FD-D strains.

| Location (Country) sample/year | Restriction enzyme | | Profiles |
|---|---|---|---|
| | *Tru*I | *Taq*I | |
| Niš (RS) 154/08 | A | A | I |
| Niš (RS) 155/08 | A | A | I |
| Niš (RS) 156/08 | B | B | II |
| Niš (RS) 157/08 | C | A | III |
| Niš (RS) 158/08 | C | A | III |
| Aleksandrovac (RS) ARR5/06 | E | C | IV |
| Aleksandrovac (RS) AS2/06 | E | C | IV |
| Aleksandrovac (RS) AP1/06 | E | C | IV |
| Irig (RS) 64/08 | C | A | III |
| Irig (RS) 66/08 | C | A | III |
| Irig (RS) 67/08 | A | B | VII |
| Veneto (I) 38/08 | C | A | III |
| Veneto (I) 53/08 | C | A | III |
| Veneto (I) 87/08 | C | A | III |
| Veneto (I) 107/06 | C | A | III |
| Veneto (I) 108/06 | C | A | III |
| Tuscany (I) 1/08 | E | C | IV |
| Tuscany (I) 66/08 | E | C | IV |
| **Lasseube (F)V30** | **D** | **D** | **VI** |
| TV-54 (I) | C | A | III |
| FD-70 (F) | D | A | V |
| Liguria 3 (I) | C | A | III |

*Molecular characterization of FD-D strains.* The RFLP analyses on *SecY* gene from 16SrV-D strains collected in Veneto region show identical profiles with *Tru*I, *Tsp509*I and *Taq*I restriction enzymes with reference strain FD-88 from France (profile I in Table 2). This profile was clearly differentiable from the one identified in the majority of samples from Emilia (Lambrusco varieties) (profile II in Table 2). One of the strains from Emilia was different from all others (profile III in Table 2). These results show for first time the strain differentiation in *SecY* gene of FD-D type phytoplasmas according with both geographic distribution and variety.

***Table 2.*** RFLP results on FD-D amplicons obtained with primers FD9f3/r2 in nested PCR on FD9f2/r products. Identical letter = identical profile, in bold phytoplasma strain V30 showing profiles in common with FD-C strains.

| Location (Country) sample/year | Restriction enzymes | | | Profiles |
|---|---|---|---|---|
| | *Tru*I | *Tsp509*I | *Taq*I | |
| Veneto (I) 8/08 | A | A | A | I |
| Veneto (I) 40/08 | A | A | A | I |
| Veneto (I) 51/08 | A | A | A | I |
| Veneto (I) 59/08 | A | A | A | I |
| Veneto (I) 74/08 | A | A | A | I |
| Veneto (I) 86/08 | A | A | A | I |
| Veneto (I) 101/08 | A | A | A | I |
| Veneto (I) 198/06 | A | A | A | I |
| Veneto (I) 203/06 | A | A | A | I |
| Veneto (I) 207/06 | A | A | A | I |
| Veneto (I) 210/06 | A | A | A | I |
| Veneto (I) 212/06 | A | A | A | I |
| Emilia Mo (I) 2/08 | B | B | B | II |
| Emilia Mo (I) 10/08 | B | B | B | II |
| Emilia Mo (I) 20/08 | B | B | B | II |
| Emilia Mo (I) 21/08 | C | C | B | III |
| Emilia Re (I) 5/08 | B | B | B | II |
| Emilia Re (I) 4/08 | B | B | B | II |
| Emilia Re (I) 2/08 | B | B | B | II |
| Emilia Re (I) 3/08 | B | B | B | II |
| Emilia Re (I) 7/08 | B | B | B | II |
| Emilia Re (I) 8/08 | B | B | B | II |
| Emilia Ra (I) 2/08 | B | B | B | II |
| FD-88 (F) | A | A | A | I |
| Gironde (F) V1 | E | D | B | IV |
| Dordogne (F) V11 | E | D | B | IV |
| Lot et Garonne (F) V20 | E | D | B | IV |
| **Lasseube (F) V30** | **D** | **E** | **B** | **V** |

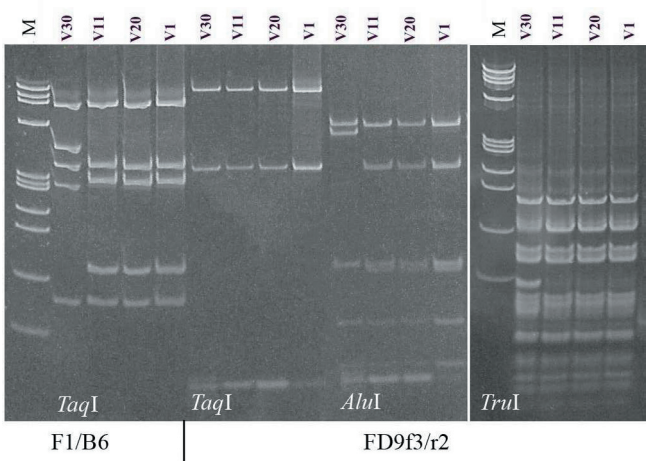


***Figure 1.*** Polyacrylamide gel showing RFLP profiles of nested F1/B6 and FD9f3/r2 products .from French FD phytoplasmas. Sample acronyms are as in Table 2. M, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

*Molecular characterization of strains from France.* Three of the samples from France were infected by 16SrV-D phytoplasmas, while the fourth (V30) showed a 16SrDNA/spacer region profile with *Taq*I different from both FD-C and FD-D profiles (Fig. 1). The three strains V1, V11, and V20 showed profiles identical to each other also in RFLP analyses on *SecY* (Fig. 1), and on *rpS3* gene. Their collective profiles on *SecY* gene were however distinguishable from that of reference strain from France FD-88 and from strains from Veneto region (profile I in Table 2) as well as from all the strains from Emilia region (profiles II and III in Table 2). These data confirm that FD-D type phytoplasmas are differentiating in strains mainly according to the geographic distribution of the disease. RFLP analyses of *SecY* gene of sample V30 showed a collective profile distinguishable from all other belonging to both ribosomal subgroups 16SrV-C and 16SrV-D (profiles V in Table 1 and VI in Table 2). Similar comparison on *rpS3* gene indicate identity of *Tru*I profiles with those of reference strain FD-88 from France and those of strains from Veneto region belonging to subgroup 16SrV-D. Further analyses are in progress to verify 16SrDNA sub-grouping of strain V30.

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# COMPARISON OF PREVALENCE, GEOGRAPHICAL DISTRIBUTION AND BIOLOGICAL PROPERTIES OF TWO FLAVESCENCE DORÉE PHYTOPLASMA STRAINS

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***Summary***

In South-West european vineyards, Flavescence dorée phytoplasma strains froms the genetic cluster FD1 (16SrV-C subgroup) are present in minority with a location in South-West France whereas FD2 strains (16sRV-D subgroup) are predominant and distributed in all vineyards. We showed that these differences in prevalence and geographic distribution seem to be due neither to differential diffusion by the insect vector *Scaphoideus titanus* nor to different biological properties of the strains in terms of multiplication, as evaluated in experimental host plant (*Vicia faba*) and vector (*Euscelidius variegatus*). Molecular typing of Flavescence dorée in affected vineyards, new outbreaks and nurseries between 1994 and 2005, strongly suggests that FD2 strains have been extensively diffused by human trading of infected plant material.

## INTRODUCTION

A Multi Locus Sequence Typing study on Flavescence dorée phytoplasma (FDp) collected in French vineyards from 2003 to 2005 has shown the existence of two genetic strain clusters (Arnaud *et al.,* 2007): the cluster FD1 (16SrV-C subgroup) displayed some genetic variability and represented 17% of the disease cases, with a preferential location in South-West whereas the non variable cluster FD2 (16SrV-D subgroup) represented 83% of the cases and was distributed in every infected vineyard of France. These differences in prevalence and geographic distribution of FD1 and FD2 do not appear to be due to differential diffusion by the insect vector. Indeed, recent studies have shown that both strains are efficiently transmitted by *Scaphoideus titanus* (Papura *et al.* submitted) and that there is no apparent co-diffusion between specific *S. titanus* genetic sub-populations and FDp strains (Papura *et al.* 2007; Papura *et al.* submitted). In this study, we analysed the possibility of differential diffusion by way of the plant. In order to evaluate if the predominance of FD2 in vineyards could be due to a better *in-planta* multiplication, we compared the multiplication kinetics of FD1 and FD2 strains in an experimental host plant (the broadbean *V. faba*) after *Euscelidius variegatus* transmission. In order to evaluate the possibility of FD2 diffusion by transport of infected grapevine planting material we realised the typing of FDp strains involved in former and new outbreaks.

## MATERIAL AND METHODS

Measurements of FDp multiplication kinetics *in planta* were realised as follows. Fourth-instar *E. variegatus* nymphs were placed for one week on broadbeans infected either by FD1 (FD-CAM isolate) or by FD2 (FD92 isolate) strains. Plants were previously selected for having equivalent numbers of FDp cells per g of midrib fresh weight (FW). After FDp acquisition, insects were placed for 4 weeks on healthy broadbeans, then transferred by groups of 6 specimens to small broadbean shoots for FDp transmission (for 7 days). After transmission, all insects were collected for phytoplasma quantification. On day 7, 14, 21, 30, 36 and 43 from the beginning of transmission, six plants were collected and total DNA was extracted from all midribs, petioles and stem of each plant. Quantification of FDp cells in plants and insects was realised by real-time PCR on the *map* gene with measurement of SYBR® Green incorporation. Mean numbers of phytoplasma cells were expressed per g of plant midrib or insect FW.

Molecular typing of FDp strains on total DNA extracts from plants and insects collected in infected vineyards was realised by nested-PCR followed by sequencing of *map* (674 bp) and *uvrB-degV* (1037 bp) loci as described by Arnaud *et al.* (2007).

## RESULTS AND DISCUSSION

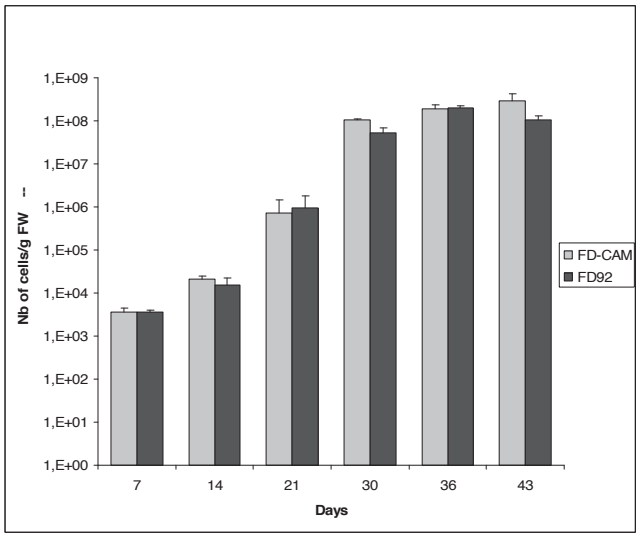


***Figure 1.*** Multiplication kinetics of FD1 (FD-CAM) and FD2 (FD92) strain types in broad bean.

*Multiplication kinetics.* Figure 1 represents the evolution of the mean number of FDp cells per g of midrib FW in FD1 and FD2-infected broadbeans from day 7 to 43 after the beginning of transmission. At the removal of insects (day 7), there was no significant difference between

the titers of FDp in FD1 and FD2-infected broadbeans: we may assume that the number of cells injected by the insects in the plants were equivalent. Furthermore at day 14, 21, 30, 36 and 43, there was no significant differences between the mean titers of FD1 and FD2 strains in inoculated plants. Both strains presented an exponential multiplication phase until day 30 followed by a plateau. No difference of symptom evolution could be observed between strains. There was also no significant difference in the mean FDp titer of FD1 and FD2-infected *E. variegatus*. The latter result is in accordance with Bressan *et al.* (2005) who showed that infection with isolates FD2000 (FD1 type) and FD92 (FD2 type) had similar effect of reduction of survival and fecondity on *E. variegatus.* As a conclusion, even though FD1 and FD2 strain types can be differentiated with serology (Boudon-Padieu *et al*., 1990) and molecular biology, no difference in symptom expression in grapevine or broadbeans, or in biological properties such as insect transmission or multiplication in the plant, have been evidenced.

*Typing of FDp in former and new outbreaks.* The geographic origin and the year of collection of 34 grapevine samples are presented in Table 1, and results of isolate typing are shown in Figure 2. Except for 5 samples (15 %) in South-West France that were infected with FD1 strain types, all vineyards from France, Italy, Switzerland and Spain were infected by FD2 strain types. This is in accordance with results obtained by Arnaud *et al.* (2007). Interestingly, samples collected in nurseries in South-East and South-West France in 1995 and 2001 respectively, were FD2-infected as well as samples taken in one-year plantations in Burgundy and Champagne in 2005. The first outbreaks in Savoie, Catalonia, Ticino and Veneto (Verona) regions were also provoked by FD2 strains. These data support the process of diffusion of FD2 by planting material in the 1990ies and 2000 in South-West Europe.

***Table 1.*** List of FDp-infected samples collected from 1994 to 2005 used in the study.

| No | Origin | Year | No | Origin | Year |
|---|---|---|---|---|---|
| 1 | Italy Veneto | 1994 | 18 | France Gers | 2001 |
| 2 | Italy Veneto | 1994 | 19 | France Hte Gar. | 2001 |
| 3 | France Aude | 1994 | 20 | France Landes | 2001 |
| 4 | France Aude | 1995 | 21 | France Tarn et Gar. | 2001 |
| 5 | France Landes | 1995 | 22 | France Tarn et Gar. | 2001 |
| 6 | France Vaucluse | 1995 | 23 | France Hte Savoie | 2003 |
| 7 | Spain Catalonia | 1996 | 24 | France Hte Savoie | 2003 |
| 8 | France Aude | 1997 | 25 | France Hte Savoie | 2003 |
| 9 | France Landes | 1997 | 26 | France Hte Savoie | 2003 |
| 10 | France Pyr. Orient. | 1997 | 27 | France Rhône | 2004 |
| 11 | France Hte Saône | 1999 | 28 | France Vaucluse | 2004 |
| 12 | France Charente | 2001 | 29 | France Vaucluse | 2004 |
| 13 | France Charente | 2001 | 30 | France Vaucluse | 2004 |
| 14 | France Charente | 2001 | 31 | Switzerland Ticino | 2004 |
| 15 | France Dordogne | 2001 | 32 | Switzerland Ticino | 2004 |
| 16 | France Dordogne | 2001 | 33 | France Marne | 2005 |
| 17 | France Gers | 2001 | 34 | France Saône et Loire | 2005 |

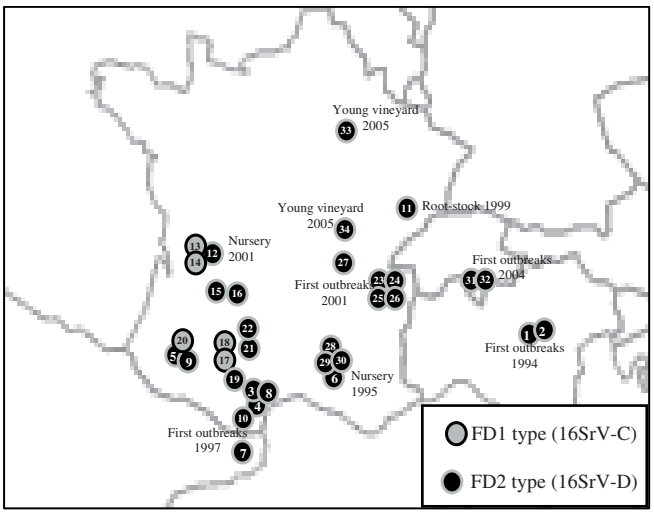


***Figure 2.*** Localisation and typing results of FDp-infected grapevine samples collected from 1994 to 2005.

As a conclusion, the differences in prevalence and geographic distribution of the two FDp strain types FD1 and FD2 do not appear to be due to differential diffusion by the insect or to different biological properties of the strains but seem to be the due to the diffusion of infected material by trading of planting grapevine material.

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### *ACKNOWLEDGEMENTS*

We thank Jessica Jean-Jacques and Claire Charenton for their help in kinetic measurements. We thank our colleagues Jean-Luc Danet, François Ferrer and Kaelig Guionnaud for maintaining healthy and phytoplasma-infected plants and insects. We gratefully acknowledge Jean Larrue and the French regional “Services de Protection des Végétaux” for providing samples. Samples from Italy and Switzerland were kindly provided by Michele Borgo and Paul Gugerli, respectively.

# GENETIC POLYMORPHISM OF STOLBUR PHYTOPLASMA IN GRAPEVINE, WILD PLANTS AND INSECTS

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***Summary***

The genetic polymorphism of stolbur phytoplasma was investigated in isolates identified in grapevines, wild plants and insects. PCR/RFLP data from the *tuf* gene showed the presence of VKI and VKII types. The sequencing of the *rplO-secY* genomic fragment showed the presence of several single nucleotide polymorphisms, which allowed 17 different genotypes to be identified. Three main clusters were identified by the phylogenetic analyses: two included most VKI and all VKII isolates, respectively; the third distinct cluster included a few VKI isolates identified in grapevines from North Western Italy. No strong association between genotypes and geographic areas was noticed.

## INTRODUCTION

The stolbur phytoplasma is the etiological agent of *Bois noir* (BN), a grapevine yellows disease which occurs in many European countries. Many other host plants of this phytoplasma have been identified. The stolbur phytoplasma is known to be transmitted by a few vectors, like *Hyalesthes obsoletus*, *Reptalus panzeri* and *Pentastiridius* sp.

Despite the high number of plant and insect hosts, the genetic variability of stolbur phytoplasma seemed to be quite low. In Germany three stolbur isolates, associated with different natural host plants, were found. VKI was associated with nettle (*Urtica dioica*), VKII with bindweed (*Convolvulus arvensis*), VKII and VKIII were found in hedge bindweed (*Calystegia sepium*), while in grapevine and in *H. obsoletus* all the three types were found (Langer and Maixner, 2004). Recent studies showed a greater variability of stolbur phytoplasma in the *vmp* gene, which however did not seem to be associated with host specificity (Cimerman *et al.,* 2009).

Previous studies on *Candidatus* Phytoplasma asteris showed that the gene encoding the translocase protein SecY was useful for the differentiation of isolates associated to different ecological niches (Lee *et al.,* 2006). Moreover, recent findings showed that the stolbur *secY* gene possesses some molecular variability (Cimerman *et al.,* 2009).

The aim of this work was to study the genetic polymorphism of the *secY* gene in stolbur isolates from grapevines, a few other wild plants and insects and to assess if its variability can be associated with host specificity.

## MATERIAL AND METHODS

More than 300 stolbur isolates were used in this study. They were identified in grapevine (298), wild plant (20) and insect (18) samples collected mainly in Italy, which were positive in PCR specific for stolbur phytoplasma.

Phytoplasma DNA extraction from plants and insects was performed according to Angelini *et al.* (2001). A TaqMan real time specific PCR assay was utilized for the diagnosis of stolbur phytoplasma (Angelini *et al*., 2007). PCR/RFLP analyses were performed on the *tuf* gene, according to Langer and Maixner (2004).

Ten new primers were designed on the most conserved portions of the sequences obtained by the alignment between the *rplO-secY* regions of three phytoplasma genomes phylogenetically close to the stolbur phytoplasma (OY-M, AYWB and *Candidatus* Phytoplasma australiense). Using these primers, all samples were amplified and double-strand sequenced, together with 7 reference stolbur isolates obtained from different research institutions (BA, A-SLO, StolC, Moliere, LibanP7, LN-IV, PO). Sequences obtained were aligned and compared and a NJ (Neighbor-Joining) phylogenetic tree was constructed.

## RESULTS AND DISCUSSION

*Analyses of tuf gene.* Samples which were stolbur-positive in real time PCR were analyses in the *tuf* gene using PCR/RFLP. The results showed that 55% of the samples were infected with the VKI type and 45% with the VKII type (Table 1). Italian vines were infected with VKI or VKII according to the place of collection: in Southern Italy VKII was predominant, while in Northern Italy both isolates were present at the same level. Most vines from abroad were infected with VKII. Nettle and bindweed were always infected with VKI and VKII, respectively, in agreement with literature data (Langer & Maixner, 2004).

***Table 1.*** Number of grapevines, wild plants and insects infected by VKI or VKII type phytoplasmas, according to the classification in the *tuf* gene.

| Sample | *tuf* type | |
|---|---|---|
| | VKI | VKII |
| *Vitis vinifera* | 164 | 134 |
| *Urtica dioica* | 1 | 0 |
| *Convolvulus arvensis* | 0 | 10 |
| *Calystegia sepium* | 2 | 4 |
| *Sambucus nigra* | 1 | 1 |
| *Parietaria officinalis* | 1 | 0 |
| *Hyalesthes obsoletus* | 14 | 2 |
| *Reptalus sp.* | 1 | 0 |
| *Dictyophara europaea* | 1 | 0 |

Samples from hedge bindweed and *Sambucus nigra* (elder) were found to be infected with both types; this is the first report of VKI infecting hedge bindweed and a further characterization of stolbur phytoplasmas infecting elder (Filippin *et al.,* 2008). One sample from *Parietaria officinalis* was infected with VKI. As regards insects, specimens of *H. obsoletus* were infected with VKI or VKII, specimens from *Reptalus* sp. and *Dictyophara europaea* were infected with VKI. The VKIII type was never found in the tested samples.

*Sequencing in the secY gene.* Seventy-six representative samples were chosen for sequencing. The *rplO-secY* gene fragment showed to be useful for the study of the polymorphism of stolbur. After alignment and comparison of the sequences, 23 single nucleotide polymorphisms (SNPs) were discovered, which allowed 17 different genotypes to be identified. Ten of them belonged to the VKI type, while 7 belonged to the VKII type. The genetic variability was similar in the two VK types, differently from data reported in Europe, where a higher diversity between the VKII type isolates compared to VKI was recorded (Johannesen *et al.,* 2008). Fifteen out of the 17 genotypes identified were found in Italian grapevines; this situation proved to be quite different from France, where only 3 *secY* genotypes were found in grapevines (Foissac *et al.,* 2008). No strong association between genotypes and geographic areas was noticed in the present work. Two genotypes belonging to the VKII type were found both in bindweed and hedge bindweed. One of them was found also in elder. The genotype found in *P. officinalis* was not found in other wild plants. As regards insects, the same genotype was found in *D. europaea*, *Reptalus* sp. and most *H. obsoletus* specimens.

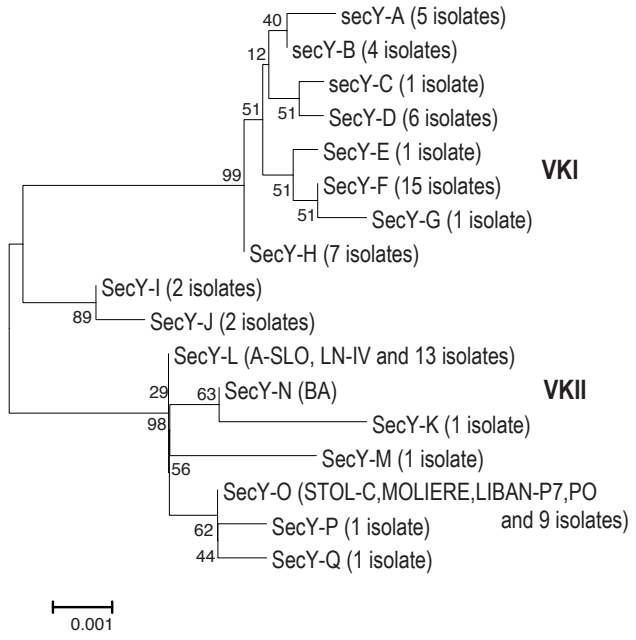


***Figure 1.*** NJ phylogenetic tree constructed with the nt sequence data obtained from the *secY* gene of stolbur isolates identified in grapevines, wild plants and insects. Bootstrap values obtained from 1000 replicates are shown.

*Phylogenetic tree in the secY gene.* The phylogenetic tree was constructed on a 873 bp fragment from the *secY* gene which contained all the SNPs. Two main clusters, which correspond to the VKI and VKII types, were identified (Figure 1). Four stolbur isolates identified in grapevines from Piedmont and Aosta Valley, which belonged to the VKI type according to the RFLP in the *tuf* gene, clustered separately in the phylogenetic tree. These three clusters were supported by high values of bootstrap (BT). Most of the minor nodes into the clusters were not well resolved (low BT values); the analysis of further genes is required to resolve the phylogeny completely.

*Conclusions.* All the different genotypes were found in the tested grapevine samples, while only some of them were found in the wild plants and insects. A possible reason for this result could be that only a lower number of stolbur-infected wild plants and insects were analysed. A more extensive survey focused on plant and vector hosts is needed in order to clarify if the genetic polymorphism of stolbur phytoplasma in the *secY* gene can be associated with the ecological niches.

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### *ACKNOWLEDGEMENTS*

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# ASPECTS OF THE INTERACTION OF STOLBUR PHYTOPLASMA, VECTORS AND HOST PLANTS IN THE TWO EPIDEMIC SYSTEMS OF BOIS NOIR

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***Summary***

Traits of the two epidemiological systems associated with Bois noir were further investigated in Germany. *Hyalesthes obsoletus* populations on nettle require higher temperature sums for completion of the life cycle, leading to an average delay of the emergence of adults of 13 days. Only tuf-type 'b' was detected in vectors at the beginning of the flight period and only tpye 'a' during the last third of this phase. Feeding on different host plants as well as phytoplasma infection had an influence on the longevity of *H. obsoletus*. Tuf types 'a' and 'b' occurred in 9 and 7 viticultural regions, respectively. Type 'a' was predominant in areas of new outbreaks of BN. PCR-RFLP analysis of the marker VMP1 revealed four different profiles in infected plants and vectors as well. An exclusive association of specific VMP1-types to tuf-types or host plants was not observed.

## INTRODUCTION

Stolbur (16SrXII-A) phytoplasmas are associated to Bois noir (BN) of grapevine throughout Europe and the Mediterranean area. They are maintained in natural epidemiological cycles consisting of different herbaceous plant hosts and the planthopper vector *Hyalesthes obsoletus.*This planthopper is the only vector known to transmit Bois noir to grapevine in the field (Maixner *et al*., 1995; Sforza *et al*., 1998). Typing of stolbur isolates from grapevine using the non-ribosomal tuf-marker allows the differentiation of at least three types of the pathogens (Langer & Maixner, 2004). The association of these types to different natural plant hosts (nettle, *Urtica dioica* for type 'a'; field bindweed, *C. arvensis* for type 'b'; hedge bindweed, *Calystegia sepium* for type c) together with phenological and biological differences between vector populations affiliated to these plants (Maixner, 2007) led to the assumption, that different and distinct natural epidemic cycles of stolbur phytoplasmas causing BN are present in the field, although no clear genetic differences between host populations where found yet (Johannesen *et al* 2008).

The "nettle-type" extended its range recently and caused severe outbreaks of BN. Knowledge about the specific interactions of phytoplasma strains, host plants and vectors are required in order to develop appropriate management strategies for BN. Therefore, additional data about the distribution and regional prevalence of stolbur strains in the German viticultural regions were collected, and the interaction of vectors with their host plants and phytoplasma strains were studied further.The variability of the VMP1 gene encoding a putative membrane protein of stolbur phytoplasmas was analyzed by PCR-RFLP (Fialová *et al*., in press; Pacifico *et al*., 2007) to achieve further information about the genetic diversity of BN phytoplasma (BNp), isolates from grapevine, herbaceous plants and vectors in different viticultural areas.

## MATERIAL AND METHODS

Average dates of the emergence of adult vectors of nettle and bindweed populations were calculated based on weather and flight data (17 datasets of different years and locations for each host plant). Day-degrees (dd) were summed up from March 15 to the average dates of emergence. In 2006, both host plants were swept weekly for *H. obsoletus* throughout the complete flight period at Bacharach, Middle-Rhine, where the bindweed and nettle system occur together. Tuf-types of the phytoplasmas were determined according to Langer & Maixner (2004) and linked to the distribution and the temporal occurrence of the insects on their respective host plants. The life span of *H. obsoletus* was detemined with field collected specimens that were caged in groups from 5 to 20. The insects were fed on non-homologous plants (vectors from bindweed on nettle, and vice versa) in 2004 and 2005, while in 2007 and 2008 experiments were carried out with the homologous hosts. Cages were checked at least every second day. Dead insects were removed and tested for phytoplasma infection. Means and medians of longevity were calculated for the host-vector combinations and for infected and non-infected vectors. Data were compared by a Mann-Whitney U-test.

Samples of symptomatic plants and *H. obsoletus* collected in nine viticultural regions during 2003 to 2008 were checked for stolbur-infection with specific primers STOLf/r and tuf-types were determined. For the analysis of the VMP1 gene, products of nested PCR assays (Fialová *et al*. in press) were digested with *Rsa*I, and the profiles were assigned to VMP1-types (Foissac, pers. communication).

## RESULTS AND DISCUSSION

The phenological differences between *H. obsoletus* populations on nettle and bindweed are correlated with temperature. The emergence of adults from bindweed varied between different years and locations from May 24 to June 17 (average: June 6), while nettle populations occurred from June 4 to July 4 (average: June 19), with a mean delay of 13 days (7-22 days). Nettle populations require 257 dd more to complete their life cycle. Whether this difference is due to intrinsic factors of the host populations or a result of nutritional differences between plant hosts is not clear.

First adult *H. obsoletus* in 2006 were found on June 5 on bindweed and June 21 on nettle at the Bacharach site.

***Table 1.*** Longevity (days) of field collected *H. obsoletus* in relation to host plant and infection by stolbur phytoplasma (*experiments of 2007/08; **experiments of 2004/05).

| Original host | C. arvensis | | U.dioica | |
|---|---|---|---|---|
| Feeding host | C. arv.* | U. dioica** | C. arv.** | U. dioica* |
| N | 180 | 351 | 350 | 453 |
| Range | 1-39 | 1-8 | 1-11 | 1-37 |
| Median | 8 | 3 | 4 | 8 |
| Mean | 13.8 | 4.1 | 5.0 | 12.4 |
| Std | 13.9 | 3.5 | 3.9 | 11.2 |
| Std.Err. | 1.03 | 0.19 | 0.21 | 0.53 |

| Host plant | C. arvensis* (tuf-b) | | U.dioica* (tuf-)a | |
|---|---|---|---|---|
| Stolbur | neg | pos | neg | pos |
| N | 51 | 32 | 169 | 94 |
| Range | 1-26 | 1-55 | 1-31 | 1-29 |
| Median | 4 | 13 | 6 | 8 |
| Mean | 8.6 | 18.3 | 9.8 | 10.9 |
| Std | 9.3 | 14.7 | 9.3 | 9.6 |
| Std.Err. | 1.3 | 2.6 | 0.7 | 1.0 |

The last vectors were caught July 12 and August 8, respectively. The relative frequency of tuf-types 'b' and 'a' during the flight period was correlated with the activity of bindweed and nettle populations, respectively. Only type 'b' was detected in the first three weeks and only type 'a' during the last four weeks. Both types were found together for only two weeks, during the transition period between the two host populations. Average proportions of 98 % of the respective tuf-types detected in the two host-populations between 2005 and 2008 affirm the specific association of the two tuf-types to their plant hosts.

*H. obsoletus* from bindweed lived significantly ($p<0.001$) longer on this plant than those caged on nettle and vice versa (Tab. 1). The survival of phytophagous insects is influenced by the suitability of host plants as food sources. Therefore, the differences in the longevity of *H. obsoletus* on the two hosts could be taken as an indication of host adaptation. Interestingly, infected vectors (tuf-type 'b') of bindweed populations lived significantly longer than healthy specimens ($P<0.001$), while no singnificant effect of infection by tuf-type 'a' ($p=0.12$) was observed in nettle populations. A 'beneficial effect' of phytoplasma infection on the vector could suggest a long co-evolution of the phytoplasma with its vector. More studies are necessary to prove this phenomenon, since tests were carried out in different years with insects of rather inhomogeneous origin.

Samples of symptomatic vines, weeds, and *H. obsoletus* from nine different viticultural regions were analyzed since 2003, when the first severe outbreaks of tuf-type 'a' occurred. In total, 474 vines were infected by tuf-type 'a', 160 by type 'b', and only 2 vines by type 'c'. Type 'a' was found in all viticultural areas and was clearly predominant in areas with new disease outbreaks (e.g. Württemberg: 225 of 246 infected vines). Tuf-type 'b' was not detected in two regions. Stolbur infection was detected in 28% of 151 specimens of *C. arvensis* (tuf-type 'b' only) and in 4% of 266 *U. dioica* (tuf-type 'a' only).

For a further differentiation of the isolates a subset of 88 plants and 150 *H. obsoletus* was characterized by PCR-RFLP analysis of the VMP1 marker. Four different restriction profiles were found and identified as V1, V2, V4 and V15 (Tab 2). V1 was the most frequent profile. It was associated with both tuf-types, but the only one observed in plants infected by tuf-type 'a'. The predominant VMP1-type in tuf-type 'b' infected specimens was V4 with 38 % of all samples. Combined profiles of V1+V2 and V1+V4 indicating double infections by different VMP1-types were found both in plants and insects and in both tuf-types. No regional patterns of VMP1-types were observed beside those due to the regional prevalence of different tuf-types. Type V1 was found in *U. dioica* (3 of 3), *C. arvensis* (1/5) and *C. sepium*(3/3). *C. arvensis* was also infected by V4 (3/5) and V15 (1/5).

Our results confirm the specific association of stolbur tuf-types with different host plants as well as differences between *H. obsoletus* host populations. They further support the hypothesis of distinct epidemiological cycles of stolbur phytoplasmas associated with BN. The VMP1 marker proved more variabel than tuf, but none of the profiles detected in Germany was diagnostic for host plants or tuf-types, while diagnostic differences in the VMP1 gene were identified by sequence analyses (Johannesen *et al*. 2009). However, RFLP-analysis of VMP1 could be a useful tool for further studies of the genetic diversity of BN isolates and their interaction, particularly with respect to the spread of the 'nettle- system' and new outbreaks of BN.

***Table 2.*** Tuf- and VMP1-types of stolbur phytoplasma from grapevine, herbaceous plant and insect hosts.

| VMP1-type | tuf-Type a | b | c | total |
|---|---|---|---|---|
| V1 | 86 | 27 | 6 | 119 |
| V2 | 4 | 20 | 1 | 25 |
| V4 | 1 | 40 | | 41 |
| V15 | 3 | 18 | | 21 |

***LITERATURE***


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# ON HOST RACES AND CO-EVOLUTION OF THE GRAPEVINE YELLOWS VECTOR *HYALESTHES OBSOLETUS* AND STOLBUR PHYTOPLASMA.

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***Summary***

Populations of the planthopper *Hyalesthes obsoletus* and stolbur phytoplasma, vector and pathogen of the grapevine disease Boir Noir, associated to the host plants *Convolvulus arvensis* and *Urtica dioica* were studied for host-race differences of *Wolbachia* infection of *H. obsoletus* and genetic diversity of stolbur. *Wolbachia* infection differed slightly between host-plant populations of the vector, thus being in agreement with the host-race hypothesis. Stolbur strains were highly diverged between host-plant populations, lending no support for the hypothesis for a recent host-plant shift from *Convolvulus arvensis* to *Urtica dioica*. Both data sets provide new evidence for separated disease cycles of Boir Noir.

## INTRODUCTION

The grapevine yellows disease "Bois Noir" (BN) is caused by infection of stolbur 16SrXII-A phytoplasma (Maixner *et al.*, 1995). Main vector of stolbur phytoplasma and cause for BN dissemination in Europe is the planthopper *Hyalesthes obsolethus* (Cixiidae) (Maixner *et al.*, 1995; Sforza *et al.*, 1998). Transmission of stolbur to grapevine is accidental as the vector does not reproduce nor can it acquire the phytoplasma pathogen from grapevines, while grapevine is a dead-end host for phytoplasma (Boudon-Padieu, 2000). In Europe, the main host- and reservoir plants of *H. obsoletes* and stolbur are field bindweed (*Convolvulus arvensis* L.) and stinging nettle (*Urtica dioica* L.). The epidemiology of BN is thus shaped by interactions between the vector, the stolbur pathogen and their herbaceous host plants.

The traditional disease cycle of BN in Germany, *C. arvensis – H. obsoletus – Vitis vinifera*, has been enlarged by the *U. dioica – H. obsoletus – V. vinifera* cycle. Stolbur strains tuf-Type-II and tuf-Type-I on each of the host plants *C. arvensis* and *U. dioica* (Langer & Maixner, 2004) indicate separate host-races of the pathogen. This suggests further separate vector host races. Phenological (Maixner, 2007) and wing size (J. Johannesen, unpublished data) differences of host-plant associated vector populations have been found, whereas genetic differences between the populations are ambiguous due to little variation (Johannesen *et al.*, 2008).

The plant-related associations point towards independent epidemiological cycles that are shaped by host-race evolution of both *H. obsoletus* and stolbur. We are mapping genetic variants of both *H. obsoletus* and stolbur relative to their geographic distributions and plant affiliations to investigate the spread of BN in Europe and the evolution of host-race relevant epidemiological cycles. Genetic diversity in stolbur is investigated with phylogenetic analyses of several stolbur genes. Because nuclear genetic polymorphism in *H. obsoletus* is low and mtDNA cannot discriminate host-plant associations, we have started to study infection by, and genetic variants of, *Wolbachia* bacteria in *H. obsoletus*. *Wolbachia* is a genus of female inherited bacteria that often influence genetic incompatibility mechanisms (Kozeka *et al.*, 2007). In the co-evolution analysis *Wolbachia* is a proxy for host-race evolution of *H. obsoletus*.

Here we report infection rates of *Wolbachia* and sequence diversity in the *wsp* gene, and genetic diversity in two stolbur genes, *SecY* and *VMP1*. Because the gene *VMP1* is highly polymorphic, consisting of several repeat motifs, we assessed regions of this gene that could be useful for co-evolutionary analysis.

## MATERIAL AND METHODS

The *Wolbachia* gene *wsp* (Baldo *et al.*, 2006) and the stolbur genes *SecY* and *VMP1* (Fialová *et al.*, in press) were amplified and sequenced by direct PCR from stolbur positive *H. obsoletus* sampled from different host plants in Europe and Israel. Genetic variability was estimated for all genes as number of genotypes and their mutational differences. *Wolbachia* infection rates were estimated for males and females separately at seven localities in Germany. For the stolbur *SecY* gene, genetic differentiation between five regional populations (Romania, Croatia/Slovenia, Italy, Spain/France and Germany) of each host plant (*C. arvensis* N = 38, *U. dioica* N = 33) was estimated with an analysis of molecular variance (AMOVA, $F_{ST}$). We did not perform AMOVA for *VMP1* due to too high levels of polymorphism. Instead, genetic variability of *VMP1* was assessed with phylogenetic analyses.

## RESULTS AND DISCUSSION

*Wolbachia infection:* A 540 bp fragment of the *wsp* gene was sequenced from 24 individuals. Only one genotype, i.e., no variation, was found in specimens from Europe and Israel. The result is remarkable because populations of *H. obsoletus* in Israel and Europe are phylogenetically highly separated and host-plant ranges differ. Whether this indicates spread of a single *Wolbachia* strain across the distribution of *H. obsoletus* or is caused by gene conservation remains to be tested with additional genes. In contrast to *wsp* polymorphism, infection rates differed between host-plant populations in Germany. Although infection rates, especially for males, differed between two data sets a general trend towards less infection of *H. obsoletus* associated to *C. arvensis* was present in both data sets (Table 1). Different infection rates are in line

with the notion of two host-race populations of *H. obsoletus*. Infection was highest in *H. obsoletus* of the expanding *U. dioica — V. vinifera* cycle. Infection of *Wolbachia* and stolbur were non-correlated.

***Table 1.*** Infection by *Wolbachia* in males and females of *H. obsoletus* caught on *C. arvensis* and *U. dioica.* N = sample size. The two data sets were analysed in the author's laboratories in Mainz (1) and Bernkastel-Kues (2).

| Data set | *C. arvensis* | | *U. dioica* | |
|---|---|---|---|---|
| | **N** | **Positive** | **N** | **Positive** |
| 1. Female | 25 | 0.52 | 61 | 0.93 |
| Male | 40 | 0 | 53 | 0.28 |
| 2. Female | 264 | 0.92 | 81 | 1.00 |
| Male | 408 | 0.36 | 132 | 0.61 |

*Stolbur genetic diversity:* Diagnostic mutations were found between stolbur strains in both the *SecY* (826bp) and the *VMP1* (1311bp) gene. Diagnostic differences in each gene correspond to tuf-type I and II strains. These differences were constant across geographical regions in Europe. For *SecY*, we observed eight genotypes in 71 isolates. Host-plant related strains were separated by four mutations. Differentiation among regional European stolbur populations associated to *C. arvensis*, $F_{ST} = 0.01$, was much lower than for *U. dioica* regional populations, $F_{ST} = 0.20$. Two *C. arvensis* stolbur genotypes (C, D) and one *U. dioica* genotype (A) were found all of across Europe. Relative high diversity in Italy caused the high differentiation estimate for *U. dioica* stolbur.

For *VMP1*, we observed 34 genotypes in 72 isolates. Sequence divergence (uncorrected p-distance) between the most diverged genotypes of *C. arvensis* stolbur ~ 0.104 was much higher than in *U. dioica* stolbur ~ 0.015. *VMP1* genotypes for stolbur associated with *U. dioica* were nested within those associated with *C. arvensis*. Hence, and in contrast to *SecY*, stolbur isolates associated to *C. arvensis* are paraphyletic. Paraphyly is caused by rearrangements in the *VMP1* repeat motifs in *C. arvensis* stolbur, not by unfinished lineage sorting. The rearrangements make *VMP1* problematic for co-evolution analyses in the *C. arvensis* lineage. By contrast, the *VMP1* has evolved much less in the *U. dioica* population, making *VMP1* suitable here for co-evolutionary analyses. As for the *SecY* gene, *VMP1* genotypes of *U. dioica* stolbur are most polymorphic in Italy and show geographic-genetic concordances in Europe, whereas *C. arvensis* stolbur genotypes show little geographic-genetic relationships. The combined results for the distribution of *SecY* and *VMP1* genotypes show highest diversity in Italy making this area a putative origin of *U. dioica* stolbur (tuf-type-I). *U. dioica* is in Italy a traditional host (Alma *et al.*, 1985; Lessio *et al.*, 2007).

*Implications for co-evolution:* The recent increase of BN in Germany has been associated to the use of *U. dioica* by *H. obsoletus*. This led to the hypothesis that *H. obsoletus* may in Germany have changed host plants, thereby transferring stolbur phytoplasma from the original host, *C. arvensis*, to the new host *U. dioica*. Due to the many and Europe-wide specific mutations in *SecY*, *VMP1* and *tuf* genes this hypothesis can be rejected. Instead, the tuf-type I and II strains are independent evolving lineages with separate epidemiological cycles. A question is to what degree, and for how long, *H. obsoletus* has vectored both tuf-strains independently? *H. obsoletus* has only recently spread through Europe from a putative source population in south eastern European or Italy (Johannesen *et al.*, 2008). Lack of mtDNA related host-plant differentiation in *H. obsoletus* (Johannesen *et al.*, 2008) suggests that the vector originally had the potential to utilize both host-plants. We hypothesize that *H. obsoletus* perhaps is a secondary vector of either *U. dioica* or *C. arvensis* strains which might have evolved due to transmission by different and monophagous original vector species, maintaining strictly separated disease cycles. In this case, *H. obsoletus* might have intruded later (after separation of strains) as a new and more polyphagous species into the two original, separated disease cylces. Ecological data suggest that *H. obsoletus* is now specializing on either *U. dioica* or *C. arvensis*.

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# MOLECULAR MARKERS AMONG STOLBUR PHYTOPLASMA (16SRXII-A) STRAINS AND THEIR ASSOCIATION WITH NATURAL ECOLOGIES OF GRAPEVINE BOIS NOIR IN ITALY

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***Summary***

Sequence analyses of 15 genes from Bois noir phytoplasma strains affecting Italian vineyards uncovered a range of new molecular markers. Emerging relationships between these markers and natural ecologies of BN disease suggest a possible role of *tuf* and *hlyC* genes in phytoplasma-plant interactions.

## INTRODUCTION

Bois noir (BN), a grapevine disease associated with infections by stolbur phytoplasma, produces typical grapevine yellows (GY) symptoms, inducing severe crop losses in almost all vine-growing European countries (Boudon-Padieu, 2003). The biological complexity of BN disease has stimulated research on molecular markers of grapevine-affecting stolbur phytoplasma genetic diversity. Three *tuf* gene sequence variants (VK-I, VK-II and VK-III) of '*Ca.* Phytoplasma solani' were found consistently associated with different herbaceous hosts and three natural ecologies of BN phytoplasmas (Langer & Maixner, 2004). Recently, *vmp1*, a gene encoding a stolbur phytoplasma transmembrane protein, was found to be more variable than other house-keeping genes and was proposed as a candidate gene involved in phytoplasma-host interactions (Cimerman *et al.*, 2009). In the present study, comprehensive molecular characterization of BN phytoplasma strains from Italian vineyards was carried out by analyses of *tuf* gene and other 14 previously uncharacterized genes. Correlation between newly identified molecular markers and biological features of BN disease suggests *tuf* and *hlyC* genes may play important roles in interactions between phytoplasma and their hosts, and therefore are potential virulence factors.

## MATERIALS AND METHODS

Analyses of *tuf* gene sequences from more than 230 BN phytoplasma strains, detected in grapevine plants of 43 Italian vineyards from 2004 to 2007 (Tab. 1), were carried out by PCR followed by *Hpa*II-RFLP assays, as previously described (Langer & Maixner, 2004). Nucleotide sequences of *tuf* gene and of other 14 previously uncharacterized genes of stolbur phytoplasmas were determined from eight selected BN phytoplasma strains (four VK-I and four VK-II) through PCR-amplification by using primer pairs designed on the basis of phytoplasmal sequences deposited in the GenBank by Cimerman *et al.* (2006). Nucleotide sequences compiled in FASTA format were searched for SNPs through sequence alignments by software ClustalX.

Presence of SNPs in recognition sites for restriction enzymes was determined by *in silico* digestion assays. Amino acid sequences were deduced by software ExPasy and aligned by ClustalX.

## RESULTS AND DISCUSSION

*Geographic distribution of 'Ca. Phytoplasma solani' tuf gene lineages.* Our work revealed differential occurrence of BN phytoplasma strain lineages VK-I and VK-II across 43 vineyards in northern, central and southern Italy (Table 1). While VK-I was found prevalent in Franciacorta (North Italy) and in Abruzzi (Central Italy), VK-II was more common in Oltrepò pavese (North Italy), Marche (Central Italy), Puglia and Sicily (Southern Italy). However, within the same vineyards in a given location, temporal shifts in the frequency of VK lineages were observed. A possible explanation of these observations is the composition and dynamics of alternative host plant species, within and near vineyards, in the epidemiology of BN disease.

***Table 1.*** Occurrence of '*Ca*. Phytoplasma solani'-related strains in Italian vineyards.

| Region | No. BN phytoplasma strains | *tuf* gene lineage | |
|---|---|---|---|
| | | VK-I | VK-II |
| Lombardy-Fc | 95 | 60 | 35 |
| Lombardy-Op | 42 | 9 | 33 |
| Marche | 28 | 3 | 25 |
| Abruzzi | 47 | 27 | 20 |
| Puglia | 7 | 0 | 7 |
| Sicily | 4 | 0 | 4 |

*Possible role of EF-Tu in host species determination.* Full protein sequences (344 amino acids) of Elongation Factor-Tu from *tuf*-I and *tuf*-II lineages of '*Ca*. Phytoplasma solani' were aligned. Analysis revealed differences at amino acid position 243 (Val/Ile, corresponding to SNP at nucleotide position 941) and amino acid position 364 (Asp/Asn, corresponding to SNP at nucleotide position 1304). Previous studies on plant and human genes suggested that single substitution between Val and Ile or between Asp and Asn could alter binding activity of affected receptor proteins (Berg *et al.*, 2008) or catalytic activity of affected enzymes (Bruner *et al.*, 2001; Kawachi *et al.*, 2006). Conceivably, critical amino acid substitutions in phytoplasma *tuf* genes could also alter the activities of the gene products and/or modify the interactions between the phytoplasma EF-Tu and its binding protein(s).

***Table 2.*** *Distinct SNP genetic lineages among BN phytoplasma populations affecting vineyards in Italy.*

| BN phytoplasma strain | SNP lineage | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *tuf* | *hlyC* | *rplS-csdB* | *cbiQ-glyA* | *potC-potD* | *trxA-truB* | *gyrA-gyrB* | *aspS-mesJ* | *pnp* |
| BN-Fc6 | I | I | I | I | I | I | I | I | I |
| BN-Op70 | I | I | I | II | I | II | I | I | I |
| BN-Ma191 | I | I | II | II | I | II | I | I | I |
| BN-Ab145 | I | I | II | II | II | I | I | I | I |
| BN-Fc3 | II | II | I | I | I | I | I | I | I |
| BN-Op40 | II | II | I | I | I | I | I | I | I |
| BN-Pu279 | II | II | II | I | II | I | I | I | I |
| BN-Si238 | II | II | I | I | I | II | I | I | I |

VK-I and VK-II lineages appear to be associated with mutually exclusive plant hosts. Thus, it is interesting to learn whether EF-Tu protein play a role in determining plant host specificity. It is worth noting that although EF-Tu is well known as a cytoplasmic protein involved in translation process, it has been reported that EF-Tu can: (i) be cell surface-localized and act as a virulence factor (Kunert *et al*., 2007); (ii) be released from bacterial cells under osmotic stress (Berrier *et al.*, 2000 ); and (iii) interact, within the bacterial cytoplasm, with virulence factors (Archambaud *et al.*, 2005). Plausibly, EF-Tu proteins of VK-I and VK-II BN phytoplasma strains, different in primary structure, may act as a fitness factor in host selection, and account in part for the mutual exclusivity of VK-I and VK-II in *U. doica* and *C. arvensis*.

*New SNP genetic lineages in BN phytoplasma strains in Italy.* Fifthteen SNPs in *hlyC* gene sequences distinguished two *hlyC* SNP genetic lineages among eight analyzed BN strains; these SNP lineages were consistent with those identified on the basis of *tuf* gene sequences (VK-I and VK-II, Table 2). Additional SNPs were detected in PCR-amplified genomic segments carrying *aspS-mesJ*, *cbiQ-glyA*, *potC-potD*, and *trxA-truB* genes. For each of those genes it was possible to define two distinct SNP lineages among the eight analyzed BN phytoplasma strains but were not consistent with *tuf-hlyC* SNP lineages (Table 2). Several SNPs of *tuf*, *hlyC*, *aspS-mesJ*, *cbiQ-glyA*, *potC-potD*, and *trxA-truB* genes were positioned within recognition sites of restriction enzymes and were confirmed with wet-laboratory RFLP assays (data not shown). Since *hlyC* gene product, hemolysin III, was previously reported as a virulence factor (Goebel *et al.*, 1988), the finding that the host specificities (or preferences) of BN phytoplasma lineages delineated based on *hlyC* gene SNPs coincide with that of VK lineages identified based on *tuf* gene sequences points to a possible involvement of hemolysin III in the interaction of phytoplasmas with specific hosts. SNPs identified in the other gene sequences can be exploited as molecular markers to study different aspects of BN disease such as strain typing, population structure determination, and epidemiology.

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# LONG TERM EFFECTS OF STOLBUR PHYTOPLASMA ON GRAPEVINES IN ISRAEL

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## INTRODUCTION

*Stolbur* is the main phytoplasma causing "yellows diseases" in grapevines in Israel. Disease incidences vary mainly according to the grape cultivar and to a lesser extent according to the rootstock used and the region of the vineyard. Remission of disease symptoms is very clear in the infected vineyards but the long term horticultural effect on the performance of previously diseased vines have not been reported so far. The aims of the present work were: 1) To characterize the growth and production of healthy, diseased and recovered vines and 2) To monitor for phytoplasma particles in different organs of those vines.

## MATERIALS AND METHODS

We have been mapping a four hectare block of young Cabernet Sauvignon vineyard (8800 vines) for the last 6 years during which disease incidence increased from 0.3 to 3 percent of the vines. During those years, reappearance of symptoms occurred in only 64 (twice) and 32 (three times) vines. For the present work vines were characterized as "healthy" (not symptomatic since the beginning of the mapping in 2003), "symptomatic in 2006" (recovered for 2 years), "symptomatic in 2007" (recovered for 1 year) or "symptomatic" (current year). 15 vines of each "type" were harvested measuring yield and number of clusters. In winter we counted on each vine the number of thick (>5 mm) and thin canes, measured cane length (4 canes per vine), counted the number of internodes for each cane and weighted the pruning of each vine. For the molecular work two canes were sampled from each vine in autumn 2008. Samples of three vines of each "type" were batched making three replicates of each vine type. DNA was extracted in CTAB buffer separately from leaf mid ribs or phloem scrapings of the canes. The universal primers P1P7 followed by U3U5 were used for nested PCR reactions. Mean differences, analyses of variance, and Duncan significant tests were performed by SAS software.

## RESULTS

Yield and number of clusters were highest in healthy vines, followed by vines that were symptomatic two years before and vines that were diseased in the previous year. The lowest yield by far was in vines showing yellow symptoms in the current year (Table 1). Pruning weight, an indicator for vine vigor, was significantly higher in healthy vines as compared to recovered or diseased vines. This difference was a result of both a decrease in cane diameter – "healthy" vines having significantly higher number of thick canes, and a decrease in cane and internode length in symptomatic and recovered vines (Table 1).

In the molecular work, all the healthy vines gave negative results (leaves and canes) while the three samples of leaves from symptomatic vines were positive. On the other hand phloem scrapping from the diseased vines gave negative results. Phytoplasma DNA was detected in canes in two of the three batches of recovered vines, both one and two years after symptom expression but in leaves of only one batch which was also positive in the canes (Table 2).

## DISCUSSION

While the symptoms of yellow diseased vines are well documented and include fruit shriveling and poor growth, less attention was given to the performance of recovered vines. The results of this work show that one year after recovery the vines bear 30% less yield as compared to healthy vines but two years after recovery there is no significant difference from the yield of healthy vines that were not symptomatic before. The yield differences obtained in our work between diseased and healthy vines are bigger then those reported by Moron *et al.,* (2007). The vigor of recovered vines was less then that of healthy vines. This was both because of shorter internodes resulting in shorter canes and thinner canes. Fruit to pruning weight ratio, a parameter used to describe vine balance, was effected, only in infected vines, suggesting there should be no effect on fruit quality in the recovered vines.

Phytoplasma was detected in all leaf samples of symptomatic vines, but not in phloem scrapings of the canes bearing those leaves. This could be due to a lower titer of phytoplasma particles in the stems compared to the leaves as suggested by Christensen *et al.* (2004) for phytoplasma in *Euphorbia pulcherrima*. Still, phytoplasmas were detected in four out of six samples of canes from recovered vines. Constable et al. (2003) detected phytoplasma in shoots of 2 out of 6 recovered vines while Terlizzi & Credi (2007) and Morone *et al.* (2007), who used only leaf samples from recovered vines, did not get any positive results. Closer look is needed to understand the dynamics and maybe the difference in concentration of phytoplasma particles in different organs of the symptomatic and recovered vines.

***Table 1:*** Fruit and vegetal characteristics of healthy, symptomatic and recovered vines

| Vine type | Yield (kg?) | | Prun weight (Kg) | | Ratio* | | Canes | | | | Node Length (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | nu of >5mm | | Length (cm) | | | |
| Healthy | 8.0 | a | 1.4 | a | 5.4 | a | 24.6 | a | 111 | a | 5.51 | a |
| Rec. 2 years | 6.9 | ab | 0.9 | b | 6.2 | a | 15.3 | b | 86 | bc | 5.41 | b |
| Rec. 1 year | 5.6 | b | 0.9 | b | 8.4 | a | 14.6 | b | 100 | ab | 5.59 | b |
| Symptomatic | 1.1 | c | 0.8 | b | 2.1 | b | 11.9 | b | 74 | c | 4.25 | b |

*Ratio between yield and cane weight

***Table 2:*** PCR results from leaves and canes of 3 batches (3 vines in each batch) of the different vine types

| Vine type | Leaves | Canes |
|---|---|---|
| Healthy | 0/3 | 0/3 |
| Rec. 2 years | 1/3 | 2/3 |
| Rec. 1 year | 0/3 | 2/3 |
| Symptomatic | 3/3 | 0/3 |

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# 'BOIS NOIR' PHYTOPLASMA INDUCES SIGNIFICANT REPROGRAMMING OF GENES INVOLVED IN CARBOHYDRATE METABOLISM AND PHOTOSYNTHESIS IN THE FIELD-GROWN GRAPEVINE

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***Summary***

Phytoplasmas (class Mollicutes) are intracellular plant pathogens causing diseases in hundreds of economically important plants including grapevine but the mechanisms of their interactions with hosts are largely unknown. We show here a global transcriptional profiling in grapevine responses to phytoplasmas. 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. We established a field ecological expression system in a productive vineyard that offered both molecular tools and plant natural histories. The two year long experiment revealed that genes involved in primary and secondary metabolic pathways were changed. A hypothesis that phytoplasmas interact with the plant carbohydrate metabolism was proven and some possibilities how the products of this pathway might be utilized by phytoplasmas were shown. In addition, several photosynthetic genes were largely down-regulated in infected plants, whereas defense genes from the metabolic pathway leading to formation of condensed tannins and PR-5 proteins were significantly induced. Genes involved in defense-signaling were differentially expressed in healthy and infected plants. A set of ten selected genes from several differentially expressed pathways was confirmed to be suitable for a reliable classification of infected plants. Collectively, our results indicate that gene expression changes in response to infection by phytoplasmas may support their nutrition by promoting alterations in host metabolism. In addition, these studies provide novel markers for the characterization of defense pathways and susceptibility features under field infection condition.

## INTRODUCTION

Phytoplasmas are bacteria without cell walls from the class Mollicutes (Christensen *et al.*, 2005; Oshima *et al.*, 2004; Bai *et al.*, 2006; Tran-Nguyen *et al.*, 2008). They are obligate intracellular plant pathogens which cause diseases in hundreds of economically important plants including the grapevine (*Vitis vinifera*). Knowledge of their biology and the mechanisms of their interactions with hosts is largely unknown because they are uncultivable and experimentally inaccessible in their hosts. We detail here the first global transcriptional profiling in grapevine responses to phytoplasmas. The gene expression patterns were followed in leaf midribs of grapevine cv. 'Chardonnay' naturally infected with phytoplasma 'Bois Noir', which is associated with the grapevine yellows disease 'Bois noir'.

## MATERIAL AND METHODS

We established an on field experimental system in a productive vineyard that allowed application of molecular tools in a plant natural environment. Global transcription profiles of infected samples were compared with the healthy ones using microarray datasets (Rotter *et al.*, 2008) and metabolic pathway analysis software (MapMan) (Thimm *et al.*, 2004). A set of ten selected genes from several differentially expressed pathways was additionally analyzed with quantitative real-time PCR (Hren *et al.*, 2007; Hren *et al.*, 2009).

## RESULTS AND DISCUSSION

The two-year-long experiment revealed that plant genes involved in primary and secondary metabolic pathways were changed in response to infection and that these changes might support phytoplasma nutrition. A hypothesis that phytoplasmas interact with the plant carbohydrate metabolism (Maust *et al.*, 2003) was proven and the model how the products of this pathway might be utilized by phytoplasmas is proposed. In addition, several photosynthetic genes (Bertamini *et al.*, 2002) were largely down-regulated in infected plants, whereas defense genes from the metabolic pathway leading to formation of flavonoids and some PR proteins were significantly induced. Few other genes involved in defense-signaling were differentially expressed in healthy and infected plants (Baebler *et al.*, 2009). A set of genes from several differentially expressed pathways was confirmed to be suitable for a reliable classification of infected plants and for the characterization of susceptibility features in the field conditions.

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.

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# DETECTION AND IDENTIFICATION OF A NEW PHYTOPLASMA DISEASE IN GRAPEVINES IN THE WESTERN CAPE

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***Summary***

Phytoplasmas are insect-transmitted pathogenic agents responsible for more than 700 diseases, many of which are lethal, in hundreds of plant species. The three major grapevine phytoplasma diseases are Flavescence dorée, Bois noir and Grapevine yellows (Lee *et al.*, 2004). Until recently South Africa was free of any known phytoplasmas but screening of phytoplasma symptomatic grapevine material led to the discovery of Aster Yellows in the vineyards of the Olifants River Valley.

## INTRODUCTION

Phytoplasmas are unculturable, wall-less, eubacteria that belong to the Class *Mollicutes* (McCoy *et al.,* 1989). They are obligate parasites of most crop plants, where they mostly cause "yellowing" or "witches broom" symptoms, which potentially translate into severe economic losses. Phytoplasmas are transmitted by phloem-feeding insects, predominantly by leafhoppers (McCoy *et al.,* 1989). Grapevines throughout all the major viticultural regions of the world suffer from one or more of the various manifestations of Grapevine Yellows disease. The three major grapevine phytoplasma diseases are Flavescence dorée (subgroups 16SrV-C and -D), Bois noir (subgroup 16SrXII-A) and Grapevine yellows (subgroups 16SrXII-A and -B) (Lee *et al.,*, 2004). Especially Flavescence dorée has been reported to cause massive yield losses in European wine producing countries. Until recently, South Africa was a rare exception with no phytoplasma diseases reported at all. In 2006, the presence of "Candidatus Phytoplasma aurantifolia" (subgroup 16SrII-B) and a Stolbur phytoplasma (ribosomal subgroup 16SrXII-A) were reported from a vineyard in South Africa (Botti & Bertaccini, 2006). This report remains unconfirmed, however.

During the growing seasons of 2006/7, 2007/8 and 2008/9 symptoms typical of phytoplasma disease have been observed in routine monitoring of vineyards in the Olifants River Valley. Symptomatic grapevine material was collected and screened for the presence of phytoplasmas. The aim of this work was to report the detection and identification of the phytoplasma responsible for South African Grapevine Yellows disease.

## MATERIAL AND METHODS

Symptomatic shoots with leaves were collected during the early growing season (November) of 2006, 2007 and 2008 from different grapevine varieties in the Olifants River Valley. Veins of mature leaves and petioles of young leaves from field collected plant samples were removed and stored at -80°C until DNA extraction. Extractions were performed using the Invisorb Spin Plant Mini Kit (Invitek), or the DNeasy Plant Minikit (Qiagen), as described by the manufacturers. Total DNA concentrations were determined in a Nanodrop ND-1000 UV-VIS spectrophotometer (Nanodrop Technologies).

DNA samples were tested by nested PCR using two universal primer pairs P1/P7 and R16F2n/R16R2 (Lee *et al.*, 1993). First round PCRs (20 µl) containing 1X PCR buffer (Bioline); 1.5 mM $MgCl_2$; 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.

Subsequent sets of DNA samples were tested by nested PCR using combinations of four primer pairs rpR1/F1 (universal) (Lim & Sears, 1991), rp(I)R1/F1 (Phytoplasma group I specific), rp(I-A)R1/F1 (Aster Yellows subgroup A specific) and rp(I-B)R2/F1 (Aster Yellows subgroup B specific) (Lee *et al.*, 2003). Fifty nanogrammes of total DNA were cycled for one cycle of denaturation for 5 min at 94°C, followed by 35 cycles of 20 s denaturation at 94°C, 30 s annealing at 50°C and 45 s extension at 72°C, and a final extension at 72°C for 7 min. Two microlitres of a 1/30 dilution of the first round PCR product was used as template in the nested reactions (10 µl). Reaction conditions were similar to the first round reactions, except for the cycle conditions which were one cycle of denaturation for 2 min at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 2 min annealing at 55°C and 3 min extension at 72°C, and a final extension at 72°C for 10 min.

For the second and third nested reactions 1 µl of a 1/30 dilution of the previous round PCR product was used as template in the nested reactions (10 µl). Reaction conditions were similar to the second round reactions, except the primers were annealed at 64°C (rp(I-A)R1/F1) or 60°C (rp(I-B)R2/F1).

PCR products were analyzed in a 1.4% (w/v) agarose gel. Ethidium bromide (0.25 µg/ml) was added to the agarose gel prior to electrophoresis for DNA visualization with a UV transilluminator. A DNA size standard (O'GeneRuler 1 kb DNA ladder, Fermentas) was run to indicate the size of PCR fragments. After the second round

of PCR, fragments of the expected size (1245 bp) were excised from gels and purified using a NucleoSpin Extract II kit (Macherey-Nagel) according to the manufacturer's instructions. Sequencing of these fragments was performed in an ABI 3130XL Automated Genetic Analyzer at the Core DNA Sequencing Facility at Stellenbosch University. Sequence data were compiled using BioEdit Ver. 7.0.4.1 sequence alignment editor software (Hall, 1999) and aligned using ClustalW Ver 1.4 (Thompson *et al.*, 1994), embedded in the BioEdit package.

## RESULTS AND DISCUSSION

After the first round of PCR with the P1/P7 universal primer pair, 17 of the samples yielded visible fragments of 1.8kb. These included mostly wine grape varieties, while two of the four table grape samples tested positive. As expected, the nested PCR yielded a number of additional positive samples – one of these is a table grape variety. 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.

Initial sequences obtained from PCR fragments were BLAST searched on the NCBI GenBank database for homology to phytoplasma sequences. All of the results showed high sequence similarities (95-99%) with the Aster Yellows phytoplasma group (16Sr I). In a comparison of the sequences of the 1.2 kb PCR fragments of 24 isolates, sequence similarities of 97-99% were observed. These results clearly illustrate that the vines that were screened were all infected with only one phytoplasma and identified it as Aster Yellows phytoplasma. The second set of PCR reactions performed with the four primer sets produced better results than the universal primer sets on the few samples tested in 2008. The third and fourth nested PCR reactions also identified the Aster yellows phytoplasma found in South African vineyards as belonging to subgroup B.

Aster Yellows occur world-wide and have been reported to infect grapevine - South Africa can now be added to this list. Towards the end of the current study, vineyards displaying typical symptoms were observed in the Slanghoek district of the Western Cape. Plant samples were collected and confirmed to be infected with Aster Yellows phytoplasma (results not shown). The Aster Yellows phytoplasma group is reported to be spread by a leafhopper. A study to determine the biological vector of Aster yellows phytoplasma, as well as the extent to which vineyards in the Western Cape are infected, is underway.

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# BIOCHEMICAL PATHWAYS IN PHYTOPLASMA–PLANT INTERACTIONS IN LEAVES OF SYMPTOMATIC AND RECOVERED BOIS NOIR AFFECTED GRAPEVINES

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***Summary***

Plant–pathogen interactions trigger numerous mechanisms of local and systemic defences. In the present study, we have analyzed the expression patterns of a number of genes potentially correlated with grapevine–phytoplasma interactions. Leaf tissues from grapevine (*Vitis vinifera* L. cvs. Sangiovese and Chardonnay) with Bois noir (BN) symptoms, asymptomatic and as recovered plants were collected in September 2006 and 2007 (visible symptoms) and in June 2007 (symptomless). Polymerase chain reaction (PCR) analysis revealed that the pathogen associated with BN was detected in the leaf tissues of symptomatic plants, but was not detected in the asymptomatic or recovered plants. The comparative real-time PCR method was used to investigate the gene expression of the following enzymes: phenylalanine ammonia-lyase, chalcone synthase, flavanone 3-hydroxylase, superoxide dismutase, catalase, class III peroxidase, NADPH dehydrogenase, class III chitinases, and β-1,3-glucanase. The results show the involvement of several genes related to hydrolytic enzymes and the phenylpropanoid pathway in both symptomatic and recovered plants. No significant expression was detected for the superoxide dismutase, catalase and class III peroxidase genes. Significant differences were recorded between the cultivars and according to the presence of leaf symptoms.

## INTRODUCTION

Bois noir (BN) is one of the most important grapevine yellows (GYs) in Europe and the Mediterranean area (Boudon Padieu, 2003; Maixner, 2006), and the most important phytoplasma disease of grapevine (*Vitis vinifera* L.) in the Marche Region (Romanazzi & Murolo, 2008). Plants infected by phytoplasma can show an array of symptoms that suggest profound changes in the balance of plant hormones and/or growth regulators. Typical symptoms of BN appear on leaves, shoots and clusters of grapevines. Plants infected by GYs can show recovery, as the disappearance of the disease symptoms (Caudwell, 1961). This phenomenon can be spontaneous or induced (Perica, 2008; Romanazzi & Murolo, 2008). The factors involved in this recovery of plants infected by these phytoplasma are not completely understood. Musetti *et al.* (2007) suggested that $H_2O_2$ and its related metabolites and enzymes are involved in a reduction in pathogen virulence and disease symptom expression. This suggests that a metabolic pathway involved in the recovery phenomenon is similar to that involved in the mechanisms of defence responses to pathogens. The plant–pathogen interaction triggers numerous mechanisms of local and systemic defences. Plant–pathogen recognition is known to induce activation of different enzymes, such as the pathogenesis-related (PR) proteins, phytoalexins, and reactive oxygen species (ROS) that are involved in defence mechanisms. Recently, the effect of phytoplasma infection on host secondary metabolites were analyzed in herbaceous host-plants (Choi *et al*., 2004) and in woody perennial crops like the grapevine (Musetti *et al*., 2007; Hren *et al*., 2009). The interaction mechanisms induced in grapevines by stolbur phytoplasma associated with BN remain mostly unknown. The aim of this study was to investigate the expression patterns in symptomatic, asymptomatic and recovered plants of a number of genes that are potentially correlated with grapevine–phytoplasma interactions.

## MATERIAL AND METHODS

*Plant materials* - This study was carried on two cultivars, Sangiovese and Chardonnay, grown in an experimental vineyard in Petritoli (AP), central eastern Italy. Leaf tissues of five symptomatic, five asymptomatic and five BN-recovered plants were collected in September 2006 and 2007 (with symptoms visible on the canopy) and in June 2007 (with no symptoms visible).

*Phytoplasma identification* was performed after extraction of DNA using the DNeasy plant mini kit (Qiagen). The 16SrXII-A phytoplasma subgroup was detected using direct polymerase chain reaction (PCR) amplification with the P1/P7 universal phytoplasma primer pair, followed by nested R16(I)F1/R1 (Lee *et al*., 1994) and restriction fragment length polymorphism (RFLP) analysis of the amplified fragments using the *Mse*I endonuclease enzyme.

*Gene expression study* - RNA was obtained from the leaves according to Iandolino *et al.* (2004), with some modifications. Total RNA was used for cDNA synthesis with reverse transcription PCR using the QuantiTect Reverse Transcription kit (Qiagen). Gene expression analysis on symptomatic, asymptomatic and recovered plants was performed by quantitative real-time PCR (q-RT-PCR) using the SYBR-green dye system. Specific primer sets were designed from *V. vinifera* mRNA sequence deposited with the NCBI, according to phenylalanine ammonia-lyase, chalcone synthase, flavanone 3-hydroxylase, superoxide dismutase, catalase, class III peroxidase, class III chitinase, NADPH dehydrogenase and β-1,3 glucanase genes. Expression of constitutive 18S rRNA, β-tubulin and actin housekeeping genes was used to normalize expression levels according to the comparative ΔΔCt method (Vandesompele *et al*., 2002). Expression levels of each sample are expressed relative to those detected from asymptomatic plants. The analysis was performed using iCycler iQ Multicolor RT-PCR Detection system (Bio-Rad), with the conditions used by Costantini *et al*. (2007).

## RESULTS AND DISCUSSION

For this study, a methodological procedure was defined to determine relative gene expression levels of symptomatic and asymptomatic BN infected plants and recovered BN plants of the grapevine cultivars Sangiovese

and Chardonnay. Preliminary studies showed the phytoplasma associated to the 16SXII-A subgroup as primarily associated to symptomatic leaves. No specific phytoplasma fragments were detected on either asymptomatic or recovered leaf tissue. Generally, similar gene expression trends were seen in the two cultivars analyzed in symptomatic leaf from September 2006-2007, with enhanced expression of some genes of hydrolytic enzymes, like β-1,3 glucanase (Fig 1a), and phenylpropanoid metabolism enzymes, like chalcone synthase (Fig. 1b).The variability of expression levels of each of these genes showed significant differences between cultivars and seasonal patterns. This study also revealed differences between cultivars according to presence and absence of symptoms. In June 2007, when symptomatic leaves were not seen, the symptomatic plants of Sangiovese showed enhanced expression of hydrolytic and phenylpropanoid genes, in comparison to asymptomatic plants, while in Chardonnay grapevine similar expression levels were seen for both symptomatic and asymptomatic plants.

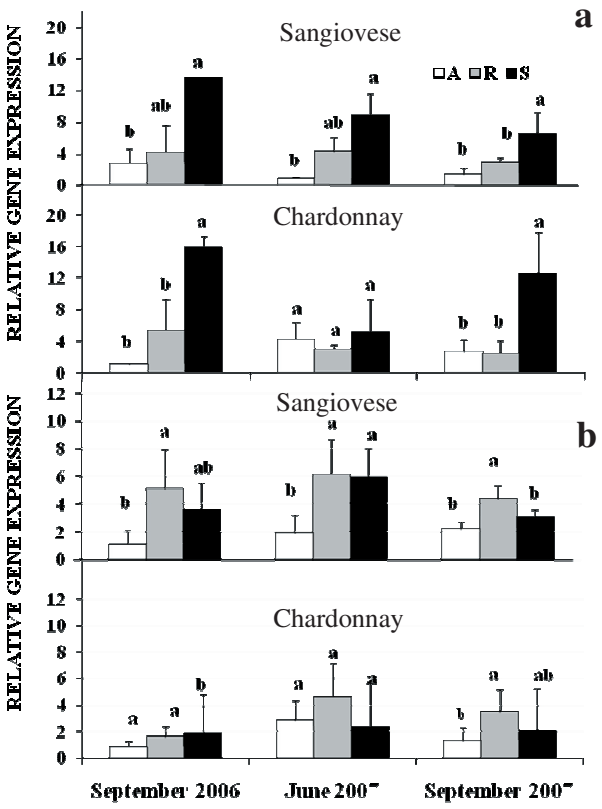


***Figure 1*** - Relative expression patterns of β-1,3 glucanase (a) and chalcone synthase (b) genes in leaf tissues from asymptomatic (A), recovered (R), and symptomatic (S) plants of Sangiovese and Chardonnay cultivars at the different seasonal sampling times.

No significant differences were seen between asymptomatic and symptomatic plants according the oxidant-scavenging-gene systems. These results could be affected by the specific characteristics of each grapevine cultivar related to the presence of constitutive or induced tannins and phenol, or to different cultivar susceptibilities to phytoplasma (Borgo and Angelini, 2002). Spontaneous complete and stable symptom remission is an interesting phenomenon that improves the physiological relationships between phytoplasma and their hosts. In this study, we saw involvement of genes of the phenylpropanoid metabolism in the recovery phenomenon. This report thus improves our knowledge of the metabolic compounds activated during the phytoplasma–plant interaction and on BN recovered plants, and provides new information towards management strategies for the control of grapevine BN.

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# PROTEOMIC ANALYSIS OF GRAPEVINE (*VITIS VINIFERA* L. cv. NEBBIOLO) MIDRIB TISSUES INFECTED BY FLAVESCENCE DORÉE PHYTOPLASMA

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***Summary***

Flavescence dorée is a serious disease of grapevine caused by a phytoplasma, an un-culturable phloem-limited plant pathogen, which belongs to the class *Mollicutes*. So far, few studies have been focused on phytoplasma-grapevine interaction and the effects of phytoplasma infection on gene expression in the host plant, but none has investigated the interaction at a proteomic level. In this work, two dimensional gel electrophoresis coupled to mass spectrometry (MS) analysis was used to examine the effect of Flavescence dorée phytoplasma infection in naturally-infected *Vitis vinifera* cv. Nebbiolo. Preliminary analysis of the 2-D maps of three biological replicates of healthy and infected samples, evidenced a series of spots displaying differential expression, i.e. over-expression, in infected grapevines and spots exclusively present in infected samples. MS analysis and protein identification showed differential expression levels of proteins with putative function as pathogen and disease resistance related proteins, and involved in aminoacid-, ROS-, carbohydrate- and secondary-metabolism.

## INTRODUCTION

Two-dimensional gel electrophoresis (2-DE) couples isoelectrofocusing (IEF) in the first dimension, with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, unabling the separation of protein extracts according to isolectric point, molecular weight, solubility and relative abundance (O'Farrel, 1975). Several applications of 2-DE to the study of grapevine physiology have been reported (Castro *et al*., 2005; Vincent *et al*., 2007; Giribaldi *et al*, 2007; Jellouly *et al*., 2008). To date, proteomic approaches comparing 2-D maps of phytoplasma-infected and healthy grapevine plants have not been reported yet. We are currently applying this technology to the study of the interaction between grapevine and Flavescence dorée phytoplasma (FDp), a phloem-limited phytopathogenic bacterium in the class *Mollicutes*, associated to an economically important disease in several European countries. Recently, the grapevine genome has been fully sequenced and also FDp genome is undergoing sequencing; this characteristic facilitates the identification of the spots of interest by multiple alignment of the peptides obtained by mass spectrometry. Our goals are: i) the identification of proteins which are differentially or exclusively expressed in infected grapevine plants, in order to study the response of the plant host to phytoplasma infection, and ii) the identification of phytoplasma proteins which are specifically expressed during infection in grapevine.

## MATERIALS AND METHODS

*Plant material:* Grapevine samples, healthy or clearly showing typical phytoplasma symptoms (Margaria *et al*., 2007), were collected in several vineyards of the Piedmont region (north-west Italy) from July to August 2008. The sanitary status of the collected material was checked: each sample was tested for phytoplasma infection (Flavescence dorée and Bois noir phytoplasma) and for other grapevine viruses (i.e., GLRaV-1, GLRaV-2, GLRaV-3, GFLV, GFkV, GVA, GVB, ArMV). FDp isolates were characterized and corresponded to subgroup FD-C. Healthy and FDp-infected samples were selected. Three biological replicates/thesis (healthy-infected) were considered; samples were plants collected in the same period and, in the case of infected samples, plants showing symptoms at the best stage. Total proteins were extracted from midribs isolated from symptomatic or healthy leaves following the protocol described by Rabilloud *et al.* (1998) with some modifications and quantified using BSA as standard.

*2-DE*: Isoelectrofocusing (IEF) was carried out with 400 µg of protein extract using immobilized pH gradient (IPG) strips 17 cm-long, pH interval 4-7 (ReadyStrip IPG Strips, BioRad). Sample loading was performed by passive rehydration for 1 h followed by active rehydratation for 12 h at 50 V in a Protein IEF cell apparatus (BioRad). IEF migration was performed at 20°C using a scalar gradient until reaching 10.000 V; the final value was 60.000 Vh. Focused strips were then equilibrated using dithiothreitol and iodoacetamide solutions. Second dimension SDS-PAGE was performed in 12% acrylamide gels using the PowerPac Universal apparatus (BioRad) in a buffer containing 25mM Tris, 0.192M glycine and 0.1% SDS. Running conditions were: 30 min at 16 mA *per* gel and 6 hours at 35mA *per* gel.

*Protein staining and analysis of gels*: After electrophoresis, gels were stained using a colloidal Coomassie brilliant blue (CBB) G-250 procedure and scanned with Versadoc Imaging System (BioRad). Image elaboration and analysis were carried out with the PDQuest Software (Biorad). Spots showing significant variation between healthy and infected samples were selected according to the results of Student's t-tests (p=0.05). Molecular weights and pI of spots were predicted according to migration of 2-D standards (BioRad).

*Mass spectrometry*: Gel fragments were excised and washed twice with MilliQ water (Millipore). MS analysis and protein spots identification was performed at the Genomics & Proteomics Laboratories Technology Facility, University of York.

## RESULTS AND DISCUSSION

Few studies are available on application of proteomics to phytoplasma-host interactions. The unique reports are accumulation of a thaumatin-like protein in phytoplasma infected Garland Chrysantemum (Zhong & Shen 2004), and description of a comparison between different extraction methods on palm coconut wood infected by Lethal yellow phytoplasma (Reyes-Martinez *et al*., 2007). The high potential of proteomics offers a tool to study the specific interaction grapevine-FDp from a wide point of view. Among the responsive proteins, plant defenses were stimulated as revealed by induction of proteins belonging to the "pathogenesis related" (PR) class and other families. Induction of several enzymatic anti-oxidant systems was observed, probably as result of an oxidative stress, beside changes in aminoacid and carbon metabolism. Some proteins were also only expressed in infected plants and may correspond to proteins which are specifically expressed during phytoplasma infection. We are currently investigating their role and expression by proteomic and RealTime RT-PCR techniques.

Our work is particularly interesting for increasing the knowledge about grapevine natural defense mechanisms against phytoplasma infection, that in a long term view could permit the identification of factors for the selection of cultivars resistant or tolerant to phytoplasmas. Moreover, the alteration of the proteomic pattern in grapevines has direct consequences in the final product, as proteins play an important role in the overall wine quality. Some of the proteins expressed by the plants in response to pathogen infection are known to be allergens in grape and wine (Mur *et al*., 2006), therefore their study is critical also for sanitary status and quality control of the final product.

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# STUDY OF *rplP* GENE FOR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF PHYTOPLASMA STRAINS WITHIN THE 16SrV GROUP

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***Summary***

Phytoplasma strains belonging to the elm yellows group (16SrV) have been previously characterized on the basis of 16S rDNA, *rplV-rpsC, map, uvr-degV* and *secY* gene sequence analyses. A complex genetic variability was specially underscored among strains associated to grapevine yellows disease (GY). In order to clarify the phylogenetic relationships among these distinct phytoplasma strains and to develop new tools for their detection, we have sequenced and analyzed *rplP* gene, that codes for the ribosomal protein L16, an essential component of bacterial ribosome.

## INTRODUCTION

The elm yellows group (16SrV) represents the third largest phytoplasma group (Lee *et al.*, 2000) and includes phytoplasma strains with various biological niches (plants and insects species) (Arnaud *et al.*, 2007). Flavescence dorée (FD) is the most dangerous disease of a complex of diseases known as grapevine yellows (Boudon-Padieu, 2003). Analysis of 16S rDNA showed the presence of phytoplasma strains associated with FD in 16SrV-C and 16SrV-D subgroups, and transmitted by the cicadellid *Scaphoideus titanus* Ball. Within the taxonomic 16SrV group, phytoplasma strains associated with diverse diseases such as Elm yellows (EY), Flavescence dorée (FD), Rubus stunt (RuS) and Alder yellows (ALY), appeared to be homogeneous on the basis of 16S rRNA gene sequence, showing a similarity from 98.6% to 99.9% (Lee *et al.*, 1998). However, they are spread by different insect vectors, inhabit diverse plant hosts and are identified in distinct geographical regions (Lee *et al.*, 2004). In the last years, different studies investigated the phylogenetic relationships among phytoplasma strains belonging to 16SrV group on the basis of analysis of different gene sequences (Angelini *et al.* 2001 ; Martini *et al.*, 2002, Lee *et al.*, 2004; Arnaud *et al*. 2007). For example, Arnaud *et al*. (2007), on the basis of analysis of three non-ribosomal genes, hypothesized a possible common origin of phytoplasmas from grapevine and alder, showing FD, PGY and ALY as being members of the same phylogenetic subclade.

In this work, *rplP* gene has been isolated and characterized from 8 different phytoplasma strains (FD92, FD-C, FD70, PGY-A, EY1, ULW, HD1 and ALY. We have obtained further information about genetic variability inside group 16SrV and relationships between FD phytoplasma strains and other close relative phytoplasmas sporadically found in grapevine.

## MATERIAL AND METHODS

Nucleic acids were extracted as previously described (Angelini *et al*., 2001) from 1g of fresh leaf tissues of periwinkle plants infected by grafting with isolates EY1, ULW, FD92, FD70, HD1, ALY and from leaf veins of grapevines naturally infected by FD-C and PGY-A. DNA was amplified as previously described (Durante *et al*., 2007). The PCR fragment obtained from all strains analyzed was sequenced by a commercial service (Primm, Milan, Italy). The ORF of *rplP* gene and corresponding amino acid sequence were found by means of ExPasy Proteomic Server (http://us.expasy.org/tools/dna.html). The nucleotide and amino acid sequences were aligned using ClustalW software and only nucleotide sequences were converted to MEGA format for cladistic analysis. The phylogenetic analysis were conducted using neighbor-joining (NJ) algorithm through software MEGA (version 3.1) and it included also the *rplP* gene sequences of phytoplasma strains previously published in GenBank (Table 1). The bacterium *Acholeplasma laidlawii* represented the outgroup of the tree.

Finally, the trimmed *rplP* sequences of FD92, ALY and EY1 were exported to pDRAW32 software for *in silico* digestion.

***Table1:*** List of strains used in this study and *rplP* sequence accession number when available

| Species-strains | Abbreviation | Accession no° |
|---|---|---|
| '*Ca*. P. ulmi'-EY1 | EY1 | Unpublished |
| '*Ca*. P. ulmi'-ULW | ULW | Unpublished |
| FD-70 | FD70 | Unpublished |
| FD-C | FDC | Unpublished |
| FD-92 | FD92 | Unpublished |
| HD-1 | HD1 | Unpublished |
| PGY-A | PGY-A | Unpublished |
| ALY | ALY | Unpublished |
| '*Ca*. P. mali' | CaPmal | NC_011047 |
| '*Ca*. P. asteris'- AYWB | Astyel | CP000061 |
| '*Ca*. P. australiense' | CaPaus | NC_010544 |
| '*Ca*. P. asteris'- OY-M | Oniyel | NC_005303 |
| *Acholeplasma laidlawii* | Achlaid | CP000896 |

## RESULTS AND DISCUSSION

Nucleotide sequence of *rplP* gene is highly conserved in FD phytoplasma strains (FD92, FD-D, FD-C and FD-70) and also in other phytoplasma strains genetically related to "flavescence dorée" (PGY-A and ALY). All FD phytoplasma strains and PGY-A shared the identical nucleotide sequence and only one SNP (A/G), located at position 72, differentiated both of them from ALY. In addition, the nucleotide sequences of *rplP* gene of FD phytoplasma strains were more similar to HD1 phytoplasma (a member of 16SrV-C subgroup) than EY1 and ULW (membera of 16SrV-A subgroup); furthermore, two strains shared a nucleotide similarity value of 100%. In detail, we have found 2 SNPs (C/A and C/G) located at positions 57 and 411, respectively, that distinguished FD phytoplasma strains and HD1, while 7 SNPs distinguished them from EY1 and ULW. Three of these 7 SNPs, differentiating FD and EY1, determined changes in the amino acid composition of L16 protein. These mutations cause variations in the polarity of the polypeptide side chain and hence, they probably induce alterations in the stucture of protein L16.

The phylogenetic tree obtained (Fig.1), showed that all phytoplasma strains genetically related to flavescence dorée form a distinct cluster closer to HD1 than EY1 and ULW. Interestingly, phytoplasma strains belonging to 16SrX group ("*Ca.* Phytoplasma mali"), clustered togheter with members of group 16SrV, while "*Ca*. P. australiense" (16SrXII-B) and "*Ca*. P. asteris" (16SrI-A and B) formed a separate clade.

Finally, *in silico* digestion showed that it is possible to distinguish FD phytoplasma strains from EY1 but not from ALY and PGY-A using the restriction enzymes *BspH*I, *BstB*I, *Cvi*AII, *Fat*I, *Hpy188*III, *HpyCH4*V, *Rsa*I, *Ssp*I and *Taq*I (Fig.2). These results confirm earlier conclusions of Angelini *et al*. (2001), Martini et al. (2002) and Arnaud *et al*. (2007), using a new region of phytoplasma genome.

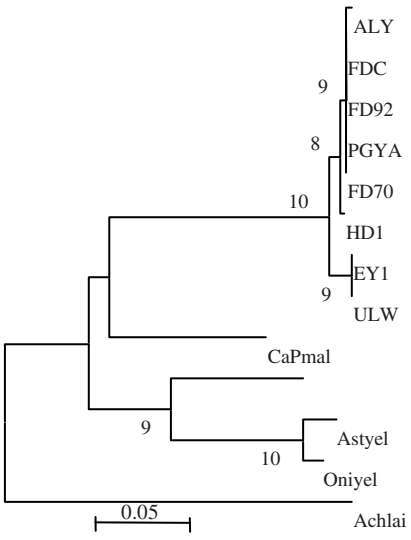


***Figure 1.*** Phylogenetic tree constructed using NJ method analysis of sequences of *rplP* gene. Bar = 5% phylogenetic distance.

Further studies will search for more detailed information on the genetic relationships between these phytoplasma strains, from other sequences belonging to S10-spc operon.

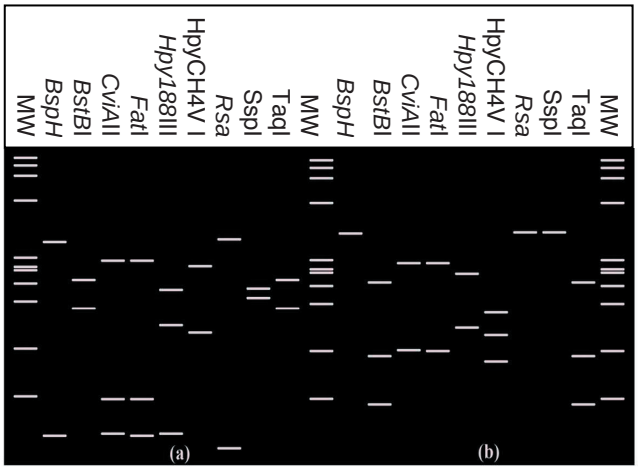


***Figure 2***. Virtual *in silico* RFLP patterns of *rplP* gene from a) EY1 b) FD92, FD70, FD-C, ALY and PGY-A.

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# REAL-TIME PCR ASSAY FOR DIAGNOSIS OF FLAVESCENCE DORÉE (16Sr-V)

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***Summary***

Flavescence dorée (FD) of grapevine is a serious disease caused by phytoplasmas. The conventional assays protocols for FD detection, based on PCR and RFLP, are complex and time consuming; so far, the new tools based on real-time PCR are not specific for FD phytoplasma detection. This report describes the attempt to develop a TaqMan allelic discrimination assay for distinction FD phytoplasma strains from other phytoplasmas sporadically found in grapevine. The primers and probe were designed using PrimerExpress software through the analysis of a nucleotide fragment located in the S10-*spc* operon, sequenced in a previously work. Results were compared with those obtained using the current diagnostic methods that utilizes nested PCR and RFLP. To date, our assay allows to detect FD and other phytoplasma strains belonging to 16SrV as EY1, ULW, ALY and PGY-A.

## INTRODUCTION

Grapevine yellows (GY) is a complex of diseases of grapevine associated with phytoplasmas. Flavescence dorée (FD) and Bois noir (BN) are the most important GY diseases in Europe, causing severe crop losses and they are characterized by similar symptomatology on *Vitis vinifera*. Nevertheless these disorders are caused by different phytoplasmas distinguished on the basis of molecular evidence (Boudon-Padieu, 2003, 2005) and transmitted by different vectors. Flavescence dorée, is the most economically important disease of grapevine in the principal wine-production areas in Europe (Boudon-Padieu, 2003). Furthermore, FD is efficiently propagated in field by *Scaphoideus titanus*, and is strongly epidemic. For these reasons FD is recognized by the European and Mediterranean Plant Protection Organization (EPPO) as a quarantine pest (EPPO/CABI, 2003). Then it is important to have riable tools for phytoplasma detection and identification. Actually, several methods for FD detection have been developed ; they include conventional PCR/RFLP methods based on analysis of ribosomal and non-ribosomal genes (Lee *et al.,* 2004 ; Botti & Bertaccini, 2007) and more recently real-time PCR assays (Bianco *et al*., 2004; Galetto *et al*., 2005 ; Angelini *et al*., 2007 ; Hren *et al*., 2007). Nevertheless, none of these protocols are able to distinguish FD phytoplasma from other phytoplasmas sporadically found in grapevine (EY, ALY, PGY-A).

In this work, we found a SNP, located on *rplN* gene, that distinguished phytoplasma strains associated with FD from other phytoplasmas (not associated with FD). Also we reported the attempt to design a suitable Taqman real-time PCR on basis of this SNP.

## MATERIAL AND METHODS

Total DNA from mid-vein leaf samples of 48 symptomatic grapevines was extracted as previosly described by Angelini and colleagues (2001). The same protocol was used for DNA extraction from fresh leafs of periwinkle plants infected by phytoplasma strains belonging to 16S group I, II, III, V, VI, VII, X and XII, used as controls to establish the specificity of the real-time assay. The DNA was diluted 10 and 50-fold in steril water and used as template for the assay. To design a real-time assay we have analyzed several ribosomal protein genes, obtained during a project of sequencing of the S10-*spc* operon, from 7 phytoplasma strains (FD70, FD92, FD-C, ALY, PGY-A, EY1 and ULW). A nucleotide sequence alignment of these phytoplasma strains belonging to group 16SrV, was performed by CLUSTALW. In detail, we have selected a SNP that distinguished FD phytoplasma strains (FD92, FDC and FD70) from phytoplasmas associated with EY1 and ALY. This SNP site, present in *rplN* gene, was selected to develop the Taqman allelic discrimination using "Primer Express" software (Version 3.0; AppliedBiosystems, Foster City, CA, USA). To optimize the conditions of reaction several assays were performed at different concentrations of primers (50nM, 150nM and 300nM). The real-time PCR reactions were performed on an ABIPRISM® 7300 (Applied Biosystem) using the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Efficiency of real-time assay was established using a PCR fragment. The concentration (ng/µl) of the PCR fragment was estimated by NanoDrop and the number of target sequences was calculated. Then, through serial dilutions (10-fold) of amplified fragment, in water and in healthy grapevine extract we have obtained a range of target molecules (from 10^6 to 1 copy of target). The estimate number of copy of target allowed also to establish the sensitivity of Taqman assay. Finally, to determinate the infection occurs in grapevine samples conventional PCR/RFLP analysis were performed as described by Martini *et al.* (1999). The RFLP profiles were resolved by electrophoresis through 6% polyacrylamide gel and staining with ethidium bromide.

## RESULTS AND DISCUSSION

Taqman real-time assay, obtained in the present work, result to be highly sensible and efficient. In fact, we have detected a fluorescent signal until 100 molecule of target also in DNA target diluited in extract of healthy grapevine. The reaction have a slope value of -3,35 and calculate efficiency was 99.8% (Figure1). $R^2$ value was of 0,99 indicating high precision during the pipeting phase.

In our assay 50-fold template dilution samples reacted in 43 cases out of 48, while only 40 samples reacted when 10-fold dilution template were used. This last result was obtained also with conventional PCR/RFLP assay. Probably, in the lowest dilution replicates, the higher concentration of PCR inhibitors negatively influences the amplification reaction. Moreover, 3 samples that were negative in conventional PCR, were positive when diluted 50-fold, in real-time assay. Further, we have to remind that PCR/RFLP methods involve two reactions of amplification (direct and nested PCR); while only one step of amplification is necessary in real-time PCR. This point underscore the high sensitivity of real-time PCR assays. The probe designed to be potential specific for FD, showed that the our assay was specific for phytoplasma strains belonging to 16SrV (Table1). However, we point out that our probe, designed in a region where is still lacking gene sequence information, does not react with the phytoplasma strains listed in table 1. In our experiment, so far, the presence of the SNP, located in the middle of the probe, is not able to distinguish the FD phytoplasma from the other phytoplasma strains belonging to 16SrV taxonomic group. Therefore, further experiments will be conducted in order to optimize a suitable protocol for specific detection of FD phytoplasma strains.

***Table 1.*** Specificity of the FD real-time assay developed in this work

| Strain | Phytoplasma subgroup | Ct Values |
|---|---|---|
| AY | 16SrI-B | - |
| SUNHP | 16SrII-A | - |
| VAC | 16SrIII-B | - |
| EY1 | 16SrV-A | 26.8 |
| ULW | 16SrV-A | 27.1 |
| ALY | 16SrV-C | 24.5 |
| PGY-A | 16SrV-C | 21.02 |
| FD70 | 16SrV-C | 19.5 |
| FD92 | 16SrV-D | 20.7 |
| BLTVA | 16SrVI-A | - |
| TBB | 16SrVI-A | - |
| AshY | 16SrVII-A | - |
| PD | 16SrX-C | - |
| AP | 16SrX-A | - |
| STOL | 16SrXII-A | - |

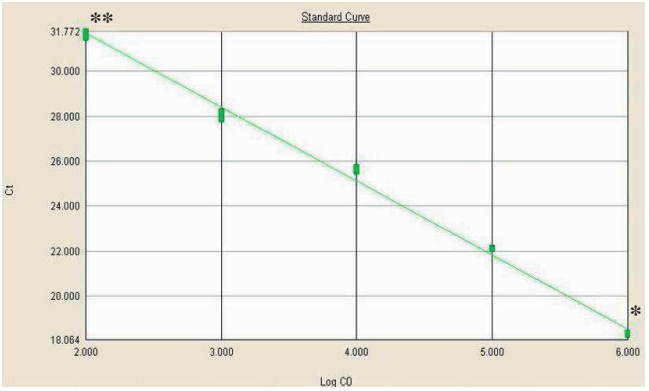


***Figure 1.*** Standard curve obtained from real-time PCR assay. * = $10^6$ molecules of target; ** = 100 molecules of target.

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# DETECTION OF GRAPEVINE FLAVESCENCE DORÉE AND BOIS NOIR PHYTOPLASMAS BY MULTIPLEX REAL-TIME PCR (TAQMAN®)

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***Summary***

Flavescence dorée (FD) and Bois noir (BN), represent a serious threat to grapevine cultivation in Europe. Reliable molecular identification of the disease-associated phytoplasmas is a fundamental control tool. However, so far, phytoplasma-DNA extraction and amplification have been based on labour-intensive and time-consuming protocols. In this work we developed a multiplex real-time PCR assay to achieve a simultaneous detection of FD and BN pathogens in the same infected vine extracts.

## INTRODUCTION

Flavescence dorée (FD) and Bois noir (BN) of grapevine (*Vitis vinifera* L.) are the most important yellows in Europe. The two diseases display similar symptoms, but they considerably differ in their aetiology and epidemiology. FD is induced by a phytoplasma classified in the Elm yellows group (16SrV) and is transmitted in nature by the leafhopper *Scaphoideus titanus* Ball. BN is also caused by a phytoplasma; however, it is classified in the Stolbur group (16SrXII) and is vectored by the planthopper *Hyalesthes obsoletus* Signoret (Martelli and Boudon-Padieu, 2006).

FD and BN are a serious threat to grapevine cultivation in most Italian viticultural areas. So, appropriate control strategies should be adopted to limit their impacts. There is thus a need for a rapid, specific, sensitive and reproducible diagnostic method. The aim of the present study was to develop a real-time PCR technique for a simultaneous routine detection of the FD and BN phytoplasmas and of an endogenous control.

## MATERIAL AND METHODS

Several grapevines from viticultural areas of Emilia-Romagna (Northern Italy) were sampled. Leaf samples were collected from plants showing typical symptoms and, as negative controls, from asymptomatic vines. Total DNA was extracted following a modified DNA extraction method (Angelini *et al.*, 2001). All extracts were resuspended in 100 µl of RNase free water and diluted 1:300 prior amplification.

Nested-PCR was performed with universal phytoplasma primers P1 and P7 (Maixner *et al*., 1995) followed, after dilution 1:20 in water, by group 16SrI and 16SrXII-specific primer pair R16(I)F1/R1 or group 16SrV specific primers R16(V)F1/R1 (Lee *et al.*, 1994).

TaqMan® probes and primers for detecting FD and BN phytoplasmas were designed, using the Primer Express™ software supplied by Applied Biosystems, within a variable region of the highly conserved 16SrRNA gene (GenBank accession numbers X76560 and AF248959, respectively). A grapevine-specific DNA control was designed on the grapevine chloroplast chaperonin 21 (ch-Cpn21) gene (Acc. Num. AY680699). All fluorogenic probes were 3' modified with a non-fluorescent quencher (NFQ) combined with a MGB (minor groove binder) moiety and 5' with VIC (for FD), 6-FAM (for BN) and NED (for ch-Cpn21) fluorophores. Multiplex real-time PCR reactions were performed on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Branchburg, NJ, USA) that detected and recorded, in real-time, the fluorescence emission during the amplification process. After an initial denaturation step at 95°C for 10 minutes, the samples were so cycled: 40 cycles of 95°C for 15s plus 60°C for 1 min. Grapevine samples were tested with both conventional nested-PCR and multiplex real-time PCR.

## RESULTS AND DISCUSSION

A one-tube multiplex real-time PCR assay was developed to detect simultaneously FD and BN phytoplasmas in DNA extracts from symptomatic leaf material. No VIC or FAM signals were observed when symptomless grapevines or plant samples infected by different phytoplasmas were tested. NED signal of the internal amplification control was always observed, indicating the correctness of the entire PCR process.

Real-time PCR offers several advantages. The output from a real-time reaction is processed using standard calculations, thereby minimising user interpretation. These assays are also performed within a single tube, minimising cross contamination between samples. Finally, the methodology is less time consuming because it does not require post-PCR manipulations.

In conclusion, our multiplex real-time PCR protocol distinguished BN or FD phytoplasmas in the same reaction. With the internal control it was possible to better discriminate real uninfected samples from a PCR inhibition condition. This should be considered a great improvement in the diagnosis of the FD and BN phytoplasmas.

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# DETECTION OF PHYTOPLASMAS ASSOCIATED WITH GRAPEVINE YELLOWS IN ROOTSTOCKS

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***Summary***

The occurrence of grapevine yellows phytoplasmas in grapevine rootstocks in Italy was investigated. Most rootstocks appeared symptomless. However, some rootstocks displayed symptoms suggesting phytoplasma infection, which were more difficult to identify than in *Vitis vinifera* varieties. Molecular tests confirmed that all the symptomatic plants were infected with *Flavescence doreé* phytoplasma. Phytoplasmas associated with BN were not found.

## INTRODUCTION

Most varieties of *Vitis vinifera* are very susceptible to grapevine yellows (GY), which in Europe are mainly *Flavescence dorée* (FD) and *Bois noir* (BN). The typical symptoms consist of yellowing or reddening and downward rolling of leaves, abortion of flowers, shrivelling of berries and incomplete lignification of 1-year-old wood. On the contrary, rootstock varieties are usually thought to be tolerant. However, the absence of typical symptoms of GY does not mean that the rootstocks do not host the phytoplasmas, which are the etiological agents of GY. Infection with FD phytoplasma was shown in France with molecular detection assays (Daire *et al*., 1993) or indexing of several varieties (Caudwell *et al*., 1994).

The presence of phytoplasmas in the rootstocks poses serious problems about their possible transmission during grafting in the nursery. Another risk concerns the presence of infected wild vine rootstocks in grapevine growing areas, often as the result of abandoned vineyards. The possible transmission of phytoplasmas from the wild rootstocks to the cultivated vineyards by the insect vector, *Scaphoideus titanus*, can constitute a risk for the viticulture. However, only a few data are available in the literature about the visual and molecular diagnosis of infected rootstocks.

This study aims to investigate the symptomatology of different cultivars of grapevine rootstocks. Molecular analyses were carried out to confirm the association between symptoms and occurrence of phytoplasmas.

## MATERIAL AND METHODS

During 2006 and 2007, visual observation of symptoms and collection of leaf samples were carried out in Susegana (Treviso, Italy), in the ampelographic collection at CRA-VIT, planted in 1995. More than 20 species of rootstocks, for a total of more than 200 varieties and clones and approximately 1300 plants were observed.

Extraction of nucleic acids from vein tissues of rootstock plants and PCR/RFLP analyses were carried out as described elsewhere (Angelini *et al*., 2001).

## RESULTS AND DISCUSSION

*Visual observations.* Most rootstocks did not show any symptoms. However, some rootstocks displayed GY symptoms, which were more difficult to identify than in *V. vinifera* varieties. Symptoms observed in autumn included partial yellowing or reddening and/or downward rolling of leaves, depending on the different varieties. In winter, infected accessions always showed some 1-year-old branches with incomplete lignification and black pustules. Visual observation in spring proved that most of the infected plants showed stunting, low number of shoots and delay in sprouting. Delay in sprouting and incomplete lignification are considered typical FD symptoms on rootstocks also by other authors (Caudwell *et al.*, 1994; Moutous, 1977).

Observations in the ampelographic collection at CRA-VIT showed that only 15 plants out of 1300, belonging to 10 rootstock biotypes, displayed symptoms of GY (Table 1). In general, only one accession per biotype was symptomatic. Other biotypes or clones of the same species or variety were not symptomatic; for instance, all the other *V. rupestris* and 420A (*V. berlandieri x V. riparia*) biotypes did not show any symptom. Some biotypes clearly showed a higher susceptibility than the others, as more than one accession was infected: for example, 125-2 Millardet and *V. rupestris* Constantia. In previous FD transmission trials carried out with *S. titanus,* symptoms were shown by all the rootstocks tested, *i.e. V. riparia* and *V. rupestris* and cvs K5BB, 420A, R99, 3309C and SO4 (Moutous, 1977). However, plants belonging to *V. riparia* and cvs K5BB and SO4, planted in the CRA-VIT collection, did not show any symptoms during the period of observation.

*Molecular analyses.* PCR analyses showed that all the symptomatic plants collected were infected with FD phytoplasma. Symptomless neighbouring and randomly-collected rootstocks were always negative. RFLP analyses showed that 11 out of 34 tested plants from 7 varieties were infected with FD-D, whereas 3 out of 15 plants from 2 varieties were infected with FD-C (Table 1). Phytoplasmas associated with BN were not found, confirming previous observations carried out in France (Boudon-Padieu, personal communication), although this phytoplasma was present in the surrounding and neighbouring *V. vinifera* vineyards. It would be interesting to investigate if the absence of BN-infected vines could be associated with the

fact that BN phytoplasma is generally less aggressive than FD in grapevine or, rather, with the ecological behaviour of the BN vector *Hyalesthes obsoletus.*

***Table 1.*** Results of symptom observations and PCR/RFLP analyses carried out in the ampelographic collection of rootstocks and American grapevines at CRA-VIT (Susegana, Italy).

| Species or variety and clone | Infected/ Total | Phytoplasma type |
|---|---|---|
| *Vitis rupestris* Constantia | 3/5 | FD-D |
| 125-2 Millardet | 3/4 | FD-D |
| 1202 Couderc | 2/5 | FD-C |
| 140 Ruggeri clone ISV1 | 1/5 | FD-D |
| 420 A clone ISV1 | 1/5 | FD-D |
| 202-4 | 1/5 | FD-D |
| Grezot 1 | 1/5 | FD-D |
| 1201 Couderc | 1/10 | FD-C |
| 16-18 Prosperi | 1/5 | FD-D |

## CONCLUSIONS

This work proved that it is possible to identify GY symptoms by visual observation also in rootstocks. The occurrence of FD phytoplasma in rootstocks, even if sporadic, poses serious problems about the possible transmission of FD phytoplasmas in the surrounding vineyards by *S. titanus*. Indeed, it was proved that the FD vector can occur in wild grapevine rootstock in Europe (Lessio *et al.,* 2007, Forte *et al.,* 2009).

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# HIGH OCCURRENCE OF *SCAPHOIDEUS TITANUS* ON WILD ROOTSTOCKS IN NORTH EASTERN ITALY

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***Summary***

The present paper aimed to investigate the occurrence of *Scaphoideus titanus*, the vector of *Flavescence dorée*, on wild rootstocks located close to treated vineyards. *S. titanus* adults were found in all the localities surveyed, even where the nymphs were not found. Generally, the number of adults captured in the wild rootstocks was higher than the number of insects captured in the cultivated vineyards beside. The trend of the flight decreased from July to September in all localities, except in a treated vineyard located close to wild and very infested rootstocks.

## INTRODUCTION

*Scaphoideus titanus* Ball (Auchenorrhyncha Cicadellidae) is a nearctic species associated with both American grapevine and *Vitis vinifera* (Maixner, 1993). This insect is the vector of *Flavescence dorée* (FD), an epidemic quarantine yellows disease of grapevine caused by phytoplasmas belonging to the 16SrV phylogenetic group (Boudon-Padieu, 2003).

The only possible preventive control strategy against FD epidemics is the insecticide treatment against the vector, which proved to be very effective. In Italy mandatory treatments in vineyards have been and are currently applied in all the FD-infected areas. Indeed, the presence of *S. titanus* in cultivated vineyards has decreased hugely. However, untreated and abandoned vineyards or wild grapevines and rootstocks can be a reservoir of the leafhopper (Pavan *et al.,* 2004; Beanland *et al*., 2006; Lessio *et al.,* 2007), which could then migrate towards cultivated vineyards and spread the FD epidemic, in the case that FD-infected plants are present.

The presence of high population of *S. titanus* in neglected vineyards and brushwood with wild rootstocks has been already observed in North Western Italy (Lessio & Alma, 2006; Lessio *et al.,* 2007) and sporadically in North Eastern Italy (Pavan *et al*., 2004). The aim of the present work was to investigate the occurrence of the leafhopper in wild rootstocks located close to treated vineyards in North Eastern Italy.

## MATERIAL AND METHODS

Observations and sampling were performed in 2008 in 4 localities in the province of Treviso, North Eastern Italy, where wild rootstocks were present close to cultivated vineyards treated with insecticides.

The presence of *S. titanus* nymphs was checked on 300 leaves at the beginning of July in plots where the nymphs had been already observed at the end of May. The flight activity of adults was surveyed using yellow sticky traps that were placed both in the wild rootstocks and in the border rows of the cultivated vineyards beside (Table 1). Traps were installed from July to September and substituted every 15 days. Captured vector specimens were counted and divided by sex using a stereomicroscope.

***Table 1.*** Number of traps placed in the four localities.

| | Number of traps in wild rootstocks | Number of traps in cultivated vineyard |
|---|---|---|
| Plot 1 | 4 | 2 |
| Plot 2 | 3 | 4 |
| Plot 3 | 4 | 4 |
| Plot 4 | 2 | 2 |

## RESULTS AND DISCUSSION

*Sex ratio.* Observation of adults captured by traps showed that the sex ratio was male biased. At the end of July - beginning of August the males were more abundant than the females in all the localities, pointing out the higher flight activity of males. Later on the number of males decreased, while that of the females increased at the same time, so that at the end of August – beginning of September the females were more abundant than the males. The results were in agreement with other reports (Lessio & Alma, 2004a; 2006).

*Differences in population levels.* Nymphs of different larval stages were present in 3 out of 4 wild rootstock plots, but were absent in the cultivated vineyards (Table 2). On the contrary, the traps captured *S. titanus* adults in all wild and cultivated grapevines. In total, the average number of insects per trap captured in the wild rootstocks was significantly higher ($p<0.05$) than the average number of insects per trap captured in the cultivated vineyards beside (8.9±14.1 and 3.1±3.1 specimens/trap, respectively). In the wild rootstocks where nymphs were not observed (plot 3), the number of adults was not different from that in the cultivated vineyard beside (average 1.1 and 1.4 specimens/trap, respectively), however it was lower than in the other plots. In all the other localities, the number of adults captured in the wild rootstocks was roughly the double of the insects captured in the cultivated vineyards. The plots 2 and 4 showed similar numbers of captured insect (average in wild rootstocks and cultivated vineyard: 6.4 and 3.8 in plot 2; 6.3 and 2.8 in plot 4, respectively). In the wild rootstocks in plot 1, the highest number of adults was captured (average 19.8 specimens/ trap). The trend of the flight of *S. titanus* decreased from July to September, in

***Table 2.*** Number of *S. titanus* nymph specimens counted per 300 leaves (July) and number of adult specimens counted per trap (August and September), captured on wild rootstocks and on the cultivated vineyards beside.

| | **Nymphs – 5 July** | | **25 July - 5 August** | | **5-18 August** | | **18 August - 1 September** | | **1-16 September** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Wild rootstocks** | **Cultivated vineyard** | **Wild rootstocks** | **Cultivated vineyard** | **Wild rootstocks** | **Cultivated vineyard** | **Wild rootstocks** | **Cultivated vineyard** | **Wild rootstocks** | **Cultivated vineyard** |
| **plot 1** | 28 | 0 | 32,5 | 3,0 | 16,0 | 1,5 | 17,3 | 6,0 | 13,2 | 11,0 |
| **plot 2** | 9 | 0 | 13,3 | 7,0 | 6,0 | 3,5 | 5,7 | 2,5 | 0,7 | 2,0 |
| **plot 3** | 0 | 0 | 1,5 | 1,5 | 1,7 | 2,0 | 1,0 | 1,7 | 0,2 | 0,5 |
| **plot 4** | 5 | 0 | 9,0 | 5,5 | 6,5 | 2,5 | 9,5 | 3,0 | 0,0 | 0,0 |

agreement with previous data that showed that the peak of the flight is at the end of July- beginning of August (Lessio & Alma, 2004a). Only in the cultivated vineyard in plot 1 the number of captured insects increased as the season advanced. This vineyard was close to the wild rootstocks where the highest number of adults was captured. Thus, a migration of the adults from the wild rootstocks to the cultivated vineyard could be suggested.

*Conclusions.* The number of adults captured in the wild rootstocks located close to the cultivated vineyards pointed out that high populations of the FD vector are still present in the areas where insecticide treatments have been carried out for more than 10 years. This could be a risk for the cultivated vineyards, as the insect could migrate between the two ecosystems. In Virginia (USA) a seasonal movement of *S. titanus* from woodland grapevines into the vineyard was observed (Beanland *et al.,* 2006). In North Western Italy the insect did not show great spreading capability (Lessio & Alma, 2004b). Further studies are therefore needed in order to investigate the possible movement of the vector into the cultivated vineyards from neighbouring wild rootstocks.

Furthermore, the occurrence of FD phytoplasma in the insects living in the wild rootstocks should be investigated in Eastern Italy. In the USA, an infection rate of 12% was detected by ELISA in *S. titanus* captured both on *V. riparia* and *V. vinifera* (Maixner, 1993). In North Western Italy the infection rate of the vector captured on grapevine rootstocks in brushwood was higher than 50% using PCR (Lessio *et al.* 2007). This finding highlighted the potential risk associated with the presence of reservoirs of the FD vector and phytoplasma.

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# SPATIAL CORRELATION OF *SCAPHOIDEUS TITANUS* BALL ADULTS ON EUROPEAN GRAPEVINE AT A PLOT SCALE: A CASE STUDY

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***Summary***

The spatial correlation of adults of *Scaphoideus titanus* Ball was studied in an experimental vineyard by applying geostatistical analyses. Yellow sticky traps placed at different distances from each other were used to capture *S. titanus* adults. Sample semivariograms of leafhopper captures were calculated according to both directional (across and along grapevine rows) and global approaches. The spatial correlation existed for across and along-row variograms, the latter being stronger and more persistent, whereas the global variogram revealed no spatial correlation. It is likely that *S. titanus* adults rely mainly on vineyard rows for movement and dispersal.

## INTRODUCTION

The nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) is monophagous on grapevine (*Vitis* spp.), and is known as the only vector of Flavescence dorée, caused by 16SrV phytoplasmas (subgroups C and D) (Boudon-Padieu, 2003). Both nymphs and adults show aggregated spatial distributions in the field (Lessio & Alma, 2006; Bosco *et al*., 1997). However, little is known about the mutual influence of sampling points of adults within a vineyard (that is, if the differences between the number of leafhoppers sampled at different positions increase along with the distance between sampling points). This research focuses on the spatial correlation of *S. titanus* adults within the vineyard, in order to better understand their dispersal capability.

## MATERIAL AND METHODS

Data were collected during 2006 in an experimental vineyard at the Faculty of Agriculture in Grugliasco (Piedmont, Italy). The study site consisted of 18 rows of different varieties (1500 $m^2$), with no other vineyards nearby. The experimental vineyard has never been treated with insecticides. 114 sticky traps were placed in a regular grid: rows were 1.5 m apart, and traps were placed at 6 m intervals along the rows. Adult *S. titanus* were counted on the traps every 15 days from mid-July to mid-September. Data were analyzed via geostatistical analyses: sample variograms were obtained plotting the semivariance γ of leafhopper captures set at different distances from each other as a function of their distance *h*:

$$\gamma(h) = \sum [n(x+h) - n(x)]^2 / 2N$$

where *n* is the number of insects captured in a certain position *x*, and *N* is the total number of pairs of sampling points that fall into the range [*x, x+ h*] (Liebhold *et al*., 1993). Variograms were calculated considering three different spatial arrangements of the traps: along (0 ± 5°) rows, across (90 ± 5°) rows, and without regard to row structure.

Sample variograms were fit to a spherical model:

$$\gamma(h) = C_0 + (C_1 - C_0) \cdot \left[ 1.5 \cdot \frac{h}{a} - 0.5 \cdot \left( \frac{h}{a} \right)^3 \right] \quad \text{if} \quad h \le a$$

$$\gamma(h) = C_1 \quad \text{if} \quad h > a$$

where $C_0$, $C_1$ and $a$ represent the nugget, the sill and the range of the variogram, respectively (Liebhold *et al*., 1993).

## RESULTS AND DISCUSSION

A total of 1601 *S. titanus* adults were captured (mean per trap: 14.04; standard deviation: 13.53). Captures showed no clear spatial correlation in the omnidirectional (no row structure) case; however, we did observe spatial correlations both along and across the vineyard rows. The along-row variogram shows a persistent correlation between captures over the whole sampling space, with a nugget of 62.04 (Fig. 1), but because the slope of semivariance on lag distance increases without asymptote over the entire region sampled, it was not possible to detect an appropriate variogram range or fit a spherical model. The across-row variogram was fit to a spherical model with the following parameters: nugget=116.32, sill=205.51, range=10.12.

Our results suggest that adult *S. titanus* move mainly along vineyard rows. On the other hand, movement among different rows is less prevalent. This is in accord with previous results showing that the density of *S. titanus* adults is related to that of grapevines, and that the gaps between plants limit their movement (Lessio & Alma, 2004). This leafhopper does not rely on wind dispersal (Lessio *et al.,* 2008), reinforcing the hypothesis that its movement is active, and occurs mainly along grapevine's rows. In America, *S. titanus* is often more abundant on wild vines in forest than in vineyards treated with insecticides, where it is caught mainly in the latter part of the season (Beanland *et al.,* 2006; Lessio *et al.,* 2007). This temporal pattern may be due either to immigration of adults from outside, or to late egg-hatching inside the vineyard. Our results suggest that the second hypothesis is more likely correct when plots are at least 10 m apart.

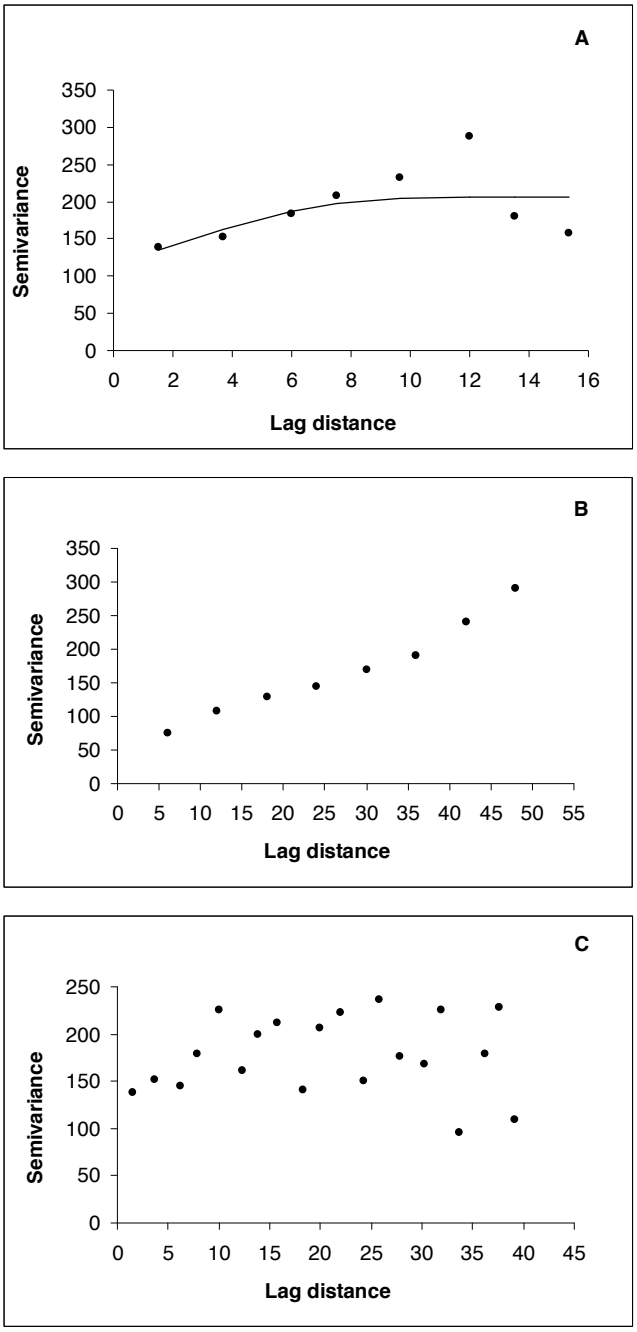


***Figure 1.*** Sample variograms obtained for captures of *S. titanus* adults at different distances from each other. A: across rows (solid line shows fitting through the spherical variogram); B: along rows: C: omnidirectional

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# DO *SCAPHOIDEUS TITANUS* LARVAE AGGREGATE FOR FEEDING?

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***Summary***

The leafhopper *Scaphoideus titanus* is the harmful vector of the phytoplasma causing the Flavescence dorée, one of the current major threats in European viticulture. The ecology of this insect is however poorly known even if better understanding of this species' life history traits and behavior will improve our ability to foresee the epidemiology of the disease. We investigated the aggregative behavior of the larvae. We conclude from different experiments that larval aggregation occurs at the plant scale, but we could not clarify the factors cueing such an aggregation. Also aggregative oviposition patterns may occur and reinforce such aggregation under young leaves. These aggregation patterns should be studied in more details in order to gain knowledge in the epidemiology of Flavescence dorée and eventually to develop control strategies based on inter-individual epideictic regulation.

## INTRODUCTION

Aggregative patterns are rather common in various insect families. For example, aggregative behavior of juvenile stages may participate in collective defensive behaviors like in several species of *Corythuca* (Aldrich, 1988). Aggregative patterns of juvenile stages may also influence the dispersive behavior and in the case of insect vectors it influences the spatial dispersion of the diseases. In *Scaphoideus titanus* (Homoptera: Cicadellidae), the vector of Flavescence dorée on grapes, aggregative patterns have been observed at vineyard scales for larvae (Lessio *et al.*, 2006) as well as for imagoes (Bosco *et al.*, 1997). These apparent aggregative patterns have however never been clearly attributed to plant quality differences nor to specific aggregative behaviors at different scales (intra stock or intra plot). In field study, it is difficult to distinguish between larval aggregation due to their behavior and aggregation linked to adults ones, especially caused by the females egg-laying. To study the aggregation of the neonate larvae, we made aggregation tests to determine if the aggregation observed in the vineyard could be due to their feeding site choice and what plant stimulus could determine their choice. The present work investigates intra stock aggregative patterns in newly hatched and developing larvae.

## MATERIAL AND METHODS

To test whether the L1 tend to aggregate, four identical grapevine cuttings were placed in each corner of an Altuglass® cage (60 x 60 x 60 cm). 140 – 350 larvae were placed in the centre of the cage at equal distance of each plant. After 9 hours, the number of larvae on each plant was numbered. Six repetitions were made.

To study the aggregation behavior of neonate larvae on plant scale, two 8/9 leaves grapevine cuttings were placed in each hatching cage and were the only food source. Four categories of leaves were distinguished from their position on the plant and their size: 1) small leaves on the top which were young leaves with leaf area < 11 cm², 2) small leaves on the bottom which were old leaves with leaf area < 23 cm², 3) large leaves in intermediate position with leaf are > 60 cm² and 4) buds shoots on woods with bud burst. The number of L1 on each leaves were checked daily and the leaf area index of each leaf were measured with a LAI meter.

To check the influence of the color on food choice, we placed on the lid internal side of hatching cages without any food resource, 4 colored traps 8 x 23.6 cm representing grapevine organs (brown: bark, green: chlorophyllian organs, red and yellow: mature berries an/or symptoms of Flavescence dorée on leaves) sprayed with insect glue. Larvae glued on the traps were daily counted. After each monitoring, traps were randomly rotated to avoid any position effect. Traps were changed as soon as they were not sufficiently sticking anymore.

Results were analyzed with Friedman Anova using R software and the "agricolae" package.

## RESULTS AND DISCUSSION

*Aggregation test:* As it was observed in vineyards, the larvae were aggregated. Indeed, on average, almost the half of the larvae was chosen the same plant to feed (Table 1).

***Table 1.*** Preferences of ***S. titanus*** neonate larvae between identical grapevine cuttings. Rows with different letters are significatively different under the Friedman Anova and LSD post hoc tests at 1 % treshold.

| | Percentage of larvae | | | |
|---|---|---|---|---|
| | *Mean* | *Standard deviation* | *Interval* | |
| 1st favorite plant | 50.51 | 7.5 | 39.02-60.38 | a |
| 2nd favorite plant | 27.15 | 4.6 | 21.49-33.02 | b |
| 3rd favorite plant | 19.37 | 6.4 | 10.22-29.27 | c |
| 4th favorite plant | 8.49 | 2.5 | 1.63-12.69 | d |

*Intra-plant distribution of larvae.* Densities of larvae were not uniform (Fig. 1). Higher densities were observed on the youngest organs: the buds shoots and the apical leaves.

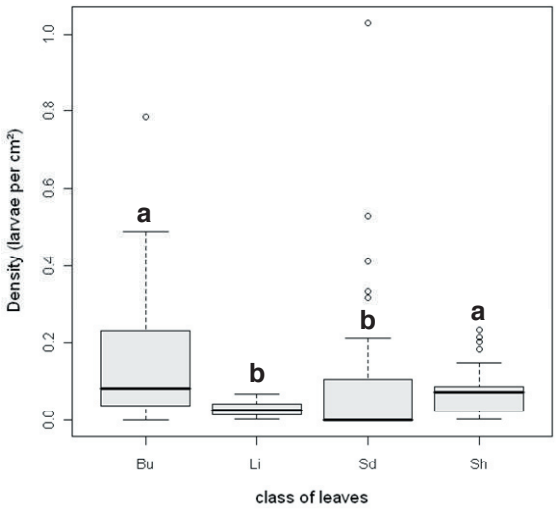


***Figure 1.*** Larval density on different leaves class**.** Bu: bud shoots; Li: large and intermediate leaves; Sd: small and down leaves; Sh:small and high leaves. Boxplots with different letters are significatively different under the Friedman Anova and LSD post hoc tests at 1 % treshold.

*Color attraction.* The major part of the larvae was caught on yellow traps (Fig. 2). These results are in contradiction with Lessio & Alma (2004) who caught more imagoes with red traps. On the other hand, these results could be related with the L1 preferences for the youngest organs. Indeed, young leaves are yellowish and have higher nitrogen content (Mooney & Gulmon, 1982) which in turn increases the fitness of other leafhoppers (Rossi & Strong, 1991) and yellow wavelengths are known to be attractive for sap-sucking insects (Saxena *et al.*, 1974; Prokopy & Owens, 1983; Todd *et al.* 1990).

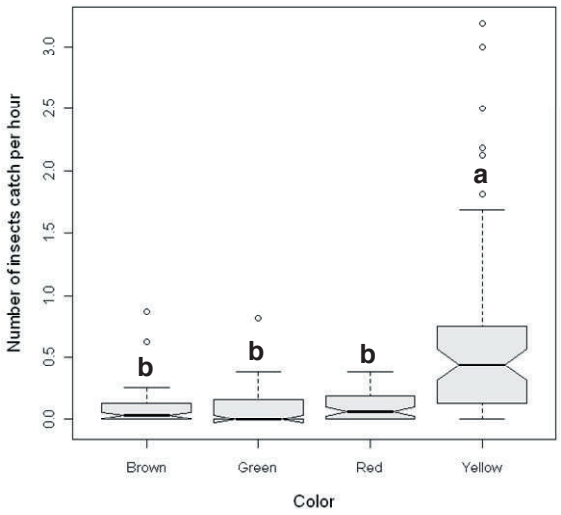


***Figure 2.*** Color choice by neonate larvae. Boxplots with different letters are significatively different under the Friedman Anova and LSD post hoc tests at 1 % treshold.

Larval aggregation could lead to a better efficiency for the phytoplasma acquisition. With aggregation, an infectious individual could transmit, via the leaf where it feeds, the phytoplasma to other individuals without the need for the infectious agent to drop in the roots, multiply and rise in the aerial organs on the following year (Boudon-Padieu, 2000).

Our results for *S. titanus* do not allow us to establish what stimuli cause gathering of the larvae. This work is a first step toward understanding the aggregation behavior of the Flavescence dorée vector and how it influences the epidemiology of the disease.

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# ACQUISITION OF FLAVESCENCE DORÉE PHYTOPLASMA BY *SCAPHOIDEUS TITANUS* BALL FROM RECOVERED AND INFECTED GRAPEVINES OF BARBERA AND NEBBIOLO CULTIVARS

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***Summary***

The capability of *Scaphoideus titanus* in acquiring Flavescence dorée phytoplasma from recovered and infected grapevines of Barbera and Nebbiolo varieties was investigated. Sanitary status of source grapes was confirmed by nested PCR. Leafhopper nymphs were caged on grapes in different viticultural areas of the Piemonte Region. FD isolates from grapes and leafhoppers were characterised by RFLP analysis of partial ribosomal and secY genes. *S. titanus* acquired FD from infected grapes only, with higher efficiency from Barbera (26%) than Nebbiolo (14%). Recovered plants did not represent a source of inoculum for the vector and therefore do not impact on FD spread.

## INTRODUCTION

Flavescence dorée (FD) phytoplasma is associated with grapevine yellows, a serious threat for wine production in Europe. FD is epidemic in north-western Italy and is transmitted by *Scaphoideus titanus* Ball, a monovoltine neartic leafhopper, monophagous on grapevine.

Infected grapevines usually show symptoms the year after inoculation, but according to the variety and possibly the age of the plant, longer latencies have been reported (Caudwell *et al*., 1987). Following the first year of symptom expression, the development of the disease may vary according to the grapevine cultivar. A spontaneous remission of symptoms, recovery, may occur and the recovered plant remains symptomless if not exposed to new infective vectors. In recovered grapevines the phytoplasma is absent or below the detection limit. Recovery is influenced by host genotype and environmental conditions (Morone *et al*., 2007).

Since 1998, compulsory insecticide treatments and roguing of infected plants were enforced by the Plant Protection Service of Piemonte Region to control vector population and reduce FD epidemics. All affected viticultural areas in the Region were declared "FD-outbreak areas". In 2007, the disease was so spread that some sites were declared "FD-settlement areas". In these areas, roguing of infected plants is no more required and recovery of grapevines becomes part of the disease management.

The main aim of this work was to determine the role of FD-recovered grapevines of the two most important Piemonte varieties, Barbera and Nebbiolo, as source of inoculum for the vector. The results are relevant to rely on recovery for disease management. To this purpose, FD-acquisition efficiencies of *S. titanus*, following feeding on recovered or infected vines with symptoms, were estimated and compared. Moreover, this work also provides clues on the difference of FD acquisition efficiencies from different grapevine cultivars (Barbera vs. Nebbiolo).

## MATERIAL AND METHODS

Source grapes were from vineyards located in four viticultural areas: Montegioco (AL), Cocconato (AT), Castagnito (CN) and Vezza (CN). In the last three years (2006 to 2008), FD was detected and characterized in these vineyards in three different periods, early June, late July and early September. Phytoplasma diagnosis was performed by DNA extraction and PCR. Every year plants were classified and labelled as "FD-infected", if they tested PCR positive in the current season, or "recovered", if symptomless and PCR-negative, but FD-positive in the previous year.

*S. titanus* eggs were obtained from grapevine branches collected in winter in vineyards with high population densities according to yellow sticky trap captures. Branches were kept in the green-house inside cages with healthy grapevines for egg hatching and nymph development.

For field acquisition experiments, 4$^{th}$ and 5$^{th}$ instar nymphs from *S. titanus* laboratory colonies were isolated inside nylon net cages on grapevine branches for seven days. About 50 insects were caged on a single branch of "recovered" or "FD-infected" grapevines, according to PCR diagnosis. At the end of the acquisition access period, caged branches were cut from the plant and brought to the lab. Insects were separately collected and maintained for three weeks on healthy grapevines in the greenhouse to complete the latent period. Insects fed on infected grapes were caged onto broadbean plants to transmit FD phytoplasmas. Single insects were then tested for the presence of FD. Field acquisition experiments were performed in 2007 and 2008, in early June and late August, to avoid mortality due to insecticides applied against *S. titanus* at mid-June and mid-July. Acquisition experiments were performed on 15 Barbera (seven recovered and eight FD-infected) and eight Nebbiolo grapevines (one recovered and seven FD-infected).

Total DNA was extracted from grapevines, broadbeans and insects according to Galetto *et al.* (2005). For FD diagnosis, two µl of DNA was used in direct PCR with

universal primers P1/P7 (Schneider *et al*., 1995). Reaction products were used as templates in nested PCRs driven by primers R16(V)F1/R1 (Lee *et al*., 1994). Reaction and cycling conditions were as detailed in the original papers. For FD characterization, two µl of DNA was used in direct PCR with universal primers FD9f2/r followed by nested assays with FD9f3/r2, according to Angelini *et al*. (2001) and Martini *et al*. (2002). Amplicons from both nested PCRs were digested with *Taq*I endonuclease for 1 h at 37°C, and the restriction profiles visualized after electrophoresis on ethidium bromide-stained acrylamide gels.

## RESULTS AND DISCUSSION

Seven and five isolates from FD-infected Barbera and Nebbiolo, respectively, were identified as 16Sr-C/secYC; one isolate from Barbera was 16Sr-C/secYD; two isolates from Nebbiolo were 16Sr-D/secYD.

Following acquisition on seven recovered Barbera, all 176 *S. titanus* were FD-negative. Similarly, all 22 leafhoppers fed on the recovered Nebbiolo vine tested negative in nested PCR. Following acquisition on eight FD-infected Barbera vines, 47 out of 142 (26%) insects tested positive in PCR assays and six out of 24 exposed broadbean plants were infected. Following acquisition on seven FD-infected Nebbiolo plants, 10 out of 70 (14%) insects tested positive in PCR tests, and two out of 21 inoculated broadbean plants were infected. The same FD isolates characterised in the source grapes were identified in leafhoppers and recipient broadbeans.

Our results suggest that recovered grapes do not represent a source of inoculum for the vector and therefore do not impact on FD spread. The results obtained for the Nebbiolo cultivar need further experiments, since they refer to only one recovered plant. Productivity of recovered plants, although lower than that of healthy ones, is satisfactory (Morone *et al*., 2007). Quality of berries from recovered grapes of Barbera and Nebbiolo cultivars is currently under evaluation. Results from these different investigations will provide evidence of the efficacy of recovery in managing FD.

*S. titanus* acquired FD with higher efficiency from Barbera than from Nebbiolo ($\chi^2 = 7.512$, P = 0.006). These results are in agreement with those of Bressan *et al.* (2005) who found significant differences in FD acquisition efficiency of *S. titanus* from Merlot and Pinot blanc cultivars. Phytoplasma multiplication in different grapevine varieties is currently under investigation.

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# GRAFT TRANSMISSION TRIALS OF FLAVESCENCE DORÉE PHYTOPLASMA FROM RECOVERED GRAPEVINES

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***Summary***

A graft transmission experiment was carried out using grapevines recovered from Flavescence dorée (FD) as mother plants producing buds for grafting. Positive and negative controls were respectively FD-infected and healthy grapevines. All the mother plants were PCR tested for two consecutive years. 4327 plants were obtained: 2721 from FD-recovered, 1046 from FD-infected, 560 from healthy grapevines. They were transplanted under screen-houses and observed for symptoms appearance for three consecutive years. Part of them was PCR tested. No symptoms were observed among all the plants and the tested ones resulted healthy to PCR analyses. During the first season, the mortality observed among the grapevines derived from FD-infected mother plants (39.7%) was significantly higher than the mortality among the plants from both recovered (15.9%) and healthy (16.1%) grapevines. The obtained results showed that the FD-recovered grapevines were similar to the healthy plants.

## INTRODUCTION

The recovery, i.e. the spontaneous remission of symptoms in plants previously symptomatically infected (Osler *et al*., 2000) is a phenomenon present and known for different phytoplasma diseases, including grapevine yellows (Caudwell, 1964). In the case of Flavescence dorée (FD) some aspects of the recovery have been studied. In particular, the objective of our experiment was to verify and confirm the absence of the pathogen in FD-recovered grapevines. For this purpose, recovered plants were both PCR analysed and used as mother plants for buds to be grafted on healthy rootstocks. The obtained grapevines were observed for the presence of grapevine yellows symptoms and PCR tested.

## MATERIAL AND METHODS

41 grapevines – 12 cv Merlot, 19 cv Prosecco, 9 cv Barbera, 1 cv Uva rara – recovered from at least two years and previously symptomatically infected by FD phytoplasma were identified in Lombardy, Veneto and Friuli Venezia Giulia regions (north Italy). As positive controls, 14 grapevines – 4 cv Merlot and 10 cv Prosecco – symptomatically FD-infected from at least two years were used. During 2004, all the plants were tested by PCR (Martini *et al*., 2002); in winter, buds were collected from each plant and successively grafted on healthy rootstocks 1103 Paulsen using the bench grafting technique. As negative controls, 560 Chardonnay buds were grafted on the same rootstocks. A total of 4327 plants were obtained: 2721 from recovered grapevines, 1046 from infected grapevines, 560 from healthy Chardonnay grapevines. The number of plants derived from each mother plant varied from 30 till 140. During May, all the plants (rooted grafted cuttings), including also the apparently failed grafts, were transplanted under two insect-proof screen-houses. The plants were observed during the vegetative season for the presence of symptoms; part of them was PCR tested. During 2005, the mother plants in the vineyards were observed for symptoms presence and retested for the presence of the phytoplasma. The grapevines under screen-houses were maintained until 2007 for two other vegetative seasons. Each year they were observed for the presence of symptoms and randomly tested by PCR.

## RESULTS AND DISCUSSION

*Recovered and symptomatically infected mother plants in the vineyards*. In year 2004, the 41 recovered grapevines resulted negative to PCR analyses for the presence of FD phytoplasma. The following year, 40 grapevines confirmed their status of recovered plants and only one plant cv Merlot resulted symptomatically infected by the pathogen. In year 2004, the 14 grapevines used as positive controls resulted infected by FD phytoplasma; in 2005, three of them – 1 cv Merlot, 2 cv Prosecco – confirmed their symptomatic infection and the remaining 11 resulted asymptomatic and negative for the presence of the pathogen, therefore becoming recovered grapevines.

*Plants under screen-houses*. During the three seasons of observations, no symptoms of phytoplasma infection were observed among the grapevines derived from both recovered and FD-infected mother plants. At least three grapevines obtained from each mother plant, for a total of 180 plants, were PCR tested choosing, if present, plants with reduced growth; all these plants resulted phytoplasma free. Interestingly, during the first year under the screen-houses, the plants derived from symptomatically infected grapevines showed high mortality (39.7%), significantly different from the mortality among both groups of plants obtained from recovered grapevines (15.9%) and from healthy Chardonnay (16.1%). During the following years, the mortality resulted the same (1-2%) among the three groups of grapevines.

The obtained results showed, first of all, that the grapevines recovered from FD from at least two years and used in this work can be considered similar to healthy plants because they were not infected in the canopy.

Moreover, they were not sources of inoculum for the transmission of the pathogen using the bench grafting. In this experiment, also the survived grapevines derived from FD-infected mother plants resulted healthy, thus indicating that the transmissibility of FD phytoplasma was like zero, while in other cases was low (Osler *et al*., 2002; Borgo *et al*., 2007). Probably all the infected grapevines, obtained from FD-infected mother plants, died during the first season under screen houses. This fact is demonstrated by the high mortality observed among them. The recovery is certainly an interesting aspect of phytoplasmoses and its study can be important and useful; if understood and clarified, this phenomenon can play a role as possibility of control of these diseases.

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# MICROBIAL DIVERSITY IN HEALTHY, YELLOWS INFECTED AND RECOVERED GRAPEVINES

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***Summary***

The composition and the structure of endophytic bacterial community were analyzed in healthy, yellows infected and recovered grapevine plants by Length Heterogeneity-PCR (LH-PCR). The statistical analysis, carried out on LH-PCR profiles, highlighted a relationship between composition of endophytic bacterial community and sanitary status of plants.

## INTRODUCTION

Flavescence Dorée (FD) and Bois Noir (BN) are grapevine yellows (GY) diseases, caused by genetically diverse phytoplasmas. In detail, FD is caused by phytoplasmas of the phylogenetic group 16SrV and BN is caused by those of 16SrXII-A group. As GY induced severe crop losses and depreciate the wine quality, their containment is a priority in the European wine producing areas. Up to now, none *Vitis vinifera* varieties have been found immune or resistant to the phytoplasma infection. Consequently, the only effective measures for FD control are chemical treatments against the insect vector, *S. titanus,* and eradication of the diseased plants.

Recently, increasing interest has been attributed to phenomena of recovery of grapevine from GY diseases Physiological mechanisms and possible biological actors involved in the recovery from phytoplasma diseases are still not completely known. Up to now there are two main hypothesis for explaining this phenomenon: the possible involvement of the systemic acquired resistance (SAR) (Musetti *et al*., 2007) and the potential role of endophytes (Musetti *et al*., 2007, Bulgari *et al.*, 2008). In fact, is known that in nature endophytic microrganisms (fungi, bacteria) colonized plants and protect them from pathogens (Lodewyckx *et al.,* 2002). Endophytic bacteria can protect plants by (i) competition for root niches or nutrients (Duffy, 2001); (ii) synthesis of allelochemicals (Bais *et al.*, 2004); (iii) induced systemic resistance (Van Lonn *et al*., 1998). To date, analyses of endophytic bacterial species have been mainly performed through cultivation-dependent approaches. The development of cultivation-independent fingerprinting molecular methods allowed obtaining a more specific, replicable and detailed description of the diversity in complex bacterial communities. Length Heterogeneity (LH)-PCR can be successfully used to describe the bacterial communities (Brusetti *et al*., 2006). In this study, the endophytic bacterial communities associated with healthy, phytoplasma-infected and recovered grapevine leaves have been characterized by LH-PCR.

## MATERIALS AND METHODS

In September 2007, leaf samples were collected from each of thirty grapevine plants of Barbera variety in a vineyard in Lombardy region (Northern Italy). Plants were chosen among asymptomatic (ten plants), symptomatic (ten plants) for grapevine yellows (GY) disease, and recovered grapevine (ten plants). Detection of phytoplasmas associated with FD and BN diseases was carried out by PCR-RFLP analyses of 16S rDNA nucleotide sequences as previously described (Deng and Hiruki, 1991; Lee *et al.*, 1998; Davis & Dally, 2001). Bacterial DNA was extracted from 20 g of sterilized grapevine leaves as previously described by Bulgari *et al.* (2009). Length Heterogeneity-PCR was used to amplify the template bacterial 16S rDNA. The LH-PCR reaction was done with the primers 27F/338R as previously repoerted (Brusetti *et al.*, 2006), with the primer 27F labelled at its 5' end with the phosphoramidite dye (6-FAM). Quantified PCR products (25 ng) were added to 0.8 µl of 500 ROX-labelled internal size standard and 15 µl of deionized formamide. Samples were denatured at 95°C for 8 min, rapidly put into ice for 5 min, and loaded on the ABI Prism 310. LH-PCR data were analysed with Genescan 3.1.2 software, and a threshold of 50 fluorescent units was used. Peak sizing and peak matrix were done with the Genescan 3.1.2 software. The same PCR amplification was run three times and three separate PCR were also run to confirm the LH-PCR peak sizing throw different PCR reactions. Univariate indices of diversity were used to characterize community structure under the different sanitary condition of plants. In particular, principal component analysis (PCA) was conducted using the NTSYS statistical software.

## RESULTS AND DISCUSSION

*Phytoplasma detection.* PCR-RFLP analysis allowed to identify '*Ca.* Phytoplasma vitis' in the GY symptomatic grapevine plants. On the other hand, phytoplasmas were not detected in asyptomatic and recovered grapevine plants.

*Bacterial community analysis.* Diversity and structure of bacterial endophytic community associated with healthy, yellows symptomatic and recovered grapevine plants were analyzed with PCA. The statistical analyses showed a distinct separation of samples on the basis of their sanitary status. In order to reduce the influence of environmental factors (crop managements, variety and climatic conditions), grapevine samples were collected in the same

vineyard at the same conditions. For that reason, we hypothesize that diversity among bacterial endophytic communities could be influenced by grapevine sanitary conditions. The LH-PCR electropherogram showed bacterial community profiles associated with healthy, GY diseased and recovered grapevine plants. In detail, the bacterial community associated with healthy plants was characterized by a greater diversity (major number of LH-PCR peaks) than that present in symptomatic and recovered plants. On the other hand, the bacteria living inside diseased and recovered plants were quite homogeneous (comparable number of LH-PCR peaks). Similar diversity patterns were reported by cultivation-dependent methods (data not shown). These findings were supported by a recent study by Kniskern *et al*. (2007) demonstrating that Salicylic Acid-mediated response defenses reduced endophytic bacterial diversity in plant. For that reason, in depth studies will be carried out in order to identify the bacterial species frequently associated with symptomatic and recovered grapevines, for clarifying the possible role of endophytes in recovery phenomenon.

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# FLAVESCENCE DORÉE IN THE EMPORDÀ (CATALONIA): RESULTS OF *SCAPHOIDEUS TITANUS* CONTAINMENT THROUGH AERIAL TREATMENTS

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***Summary***

A survey of flavescence dorée disease, including the *Scaphoideus titanus* distribution, has been conducted in Catalonia since 1996 when the first outbreak occurred. The disease incidence has drastically decreased, from 101.25 ha of productive vineyards in 1996 to 5.4 ha in 2008; this could be related to the effectiveness of the containment measures adopted against *S. titanus* and to the complete elimination of affected grapevines.

## INTRODUCTION

Flavescence dorée (FD), one of the most devastating phytoplasma diseases in Europe, has reached Spanish vineyards during the last decade.

The first FD outbreak occurred in the Alt Empordà, a northeast Catalan region, in 1996 (Laviña *et al*., 1996), therefore the 'Generalitat de Catalunya' (Catalan Government) legislated the fighting against FD (Rahola *et al.*, 1997). Basically it is mandatory to carry out treatments against *Scaphoideus titanus* Ball and to pull out and destroy any grapevine plant showing FD symptoms, if the percentage of affected plants in a plot exceeds 20%, all the plants in that plot must be pulled up. Since 1997 that measures were compulsory in the Alt Empordà, and ten years after were extended to the Baix Empordà as a consequence of the diffusion of FD towards that joining region. To control *S. titanus*, the affected area is helicopter-sprayed, before the emergence of adults, with the pyrethroid insecticide Lambda-Cihalotrin, and after this two more treatments are done by the farmers under the direction of the Servei de Sanitat Vegetal (Generalitat de Catalunya). The first aerial treatment against the insect is carried out between the 3rd and 4th week following the first recorded egg hatch of the year (considered when 2 or more larvae are captured in 2 or more vineyards). The aerial treatment was applied in the Alt Empordà since 1997 over 1,900 hectares that increased to 2,030 in 1999, 200 ha of the Baix Empordà were also included since 2007. Some vineyards, located in protected areas, are not submitted to aerial treatment and are treated exclusively by the vine grower.

The FD phytoplasmas detected in the Alt Empordà have been identified and related to the ribosomal subgroup 16SrV-D and to rp subgroup E (Torres *et al*., 2007).

The present work reports FD disease and *S. titanus* incidence in Catalonia since 1996 to 2008.

## MATERIAL AND METHODS

Vineyards of 28 localities from the Alt Empordà and the Baix Empordà (Fig. 1) covering more than 3,000 ha, were screened by visual inspection, in order to detect grapevines with symptoms of yellowing or reddening.

190 samples from symptomatic grapevines were collected within July and September from 1996 to 2008. Leaf mid-vein tissues were subjected to DNA extraction and amplification with phytoplasma-universal primer pairs P1 (Deng & Hiruki, 1991) and P7 (Schneider *et al*., 1995) followed in nested-PCR with R16(V)F1/R1 specific primer pairs for phytoplasma group 16SrV (Lee *et al*., 1994).


***Figure 1.*** Location of the Alt and Baix Empordà regions.

Captures of *S. titanus* on leaves were performed using a vacuum cleaner with a 110mm x 600mm opening for suction. The *S. titanus* captures started by the end of April, and were repeated once a week until the first aerial treatment was applied. An average of 50 plots from the affected area have been included in the study, one plot from a Baix Empordà location without FD presence and where aerial treatments have never been done has been surveyed as control. Five vines stems from each vineyard located on the edges or in places protected from wind and direct sunlight had been aspired.

## RESULTS AND DISCUSSION

*Scaphoideus titanus:* The captures of *S. titanus* since 1997 to 2008 showed that the date of the first egg hatching ranged from 4$^{th}$ to 26$^{th}$ of May in the Alt Empordà and from 11$^{th}$ to 29$^{th}$ in the Baix Empordà. This date was the reference to prepare the treatment calendar.

The percentage of vineyards with presence of *S. titanus* prior to first treatment reached values down the 5% in the areas where the treatment was aerial, and decreased until zero by 2007. The average value in the areas where the treatment was exclusively done by the farmer was 20% but increased to 50% in 2006. Only 100 ha of Alt Empordà were treated by the farmer and were not covered by aerial insecticide spraying. In the control location, *S. titanus* was found in 100% of the vineyards with annual density values ranging from 4.8 to 15.9 individuals in five leaves. Figure 2 shows the *S. titanus* incidence in the 10 years of the survey.

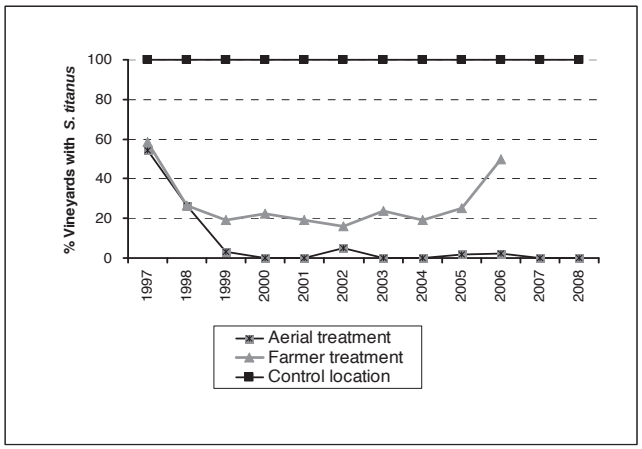


***Figure 2.*** Percentage of vineyards with *S. titanus* population in the Alt Empordà.

The spatial distribution of *S. titanus* in the Alt Empordà is not extensive where aerial treatment began in 1999: the comparison of population distribution between treated vineyards and untreated controls clearly indicates the effectiveness of the treatment. The use of insecticides at the established frequency was containing vector populations.

*Flavescence dorée:* Samples showing yellowing or reddening symptoms were analyzed to confirm FD presence. Flavescence dorée phytoplasma was detected in the 47% of grapevines analyzed; 36% of symptomatic plants gave no results to phytoplasma presence and bois noir phytoplasma was detected in the 27% of the samples tested.

In the Alt Empordà the number of foci (plots in which FD was detected in more than 20% of the grapevines) turned to zero by the year 1998, from values of 29 in 1996 (23.85 ha), and 20 in 1997 (15.38 ha). From 1998 onwards, there has only been one focus in 2001 (0.45 ha). Since all symptomatic grapevines must be eliminated, the number of plots and hectares holding symptomatic grapevines correspond to new cases of FD appearing each year. Scattered plants affected by FD were detected in several municipalities until 2005; no more symptomatic grapevines have been detected from 2006 to 2008. Figure 3 shows the hectares with foci of FD and with plots having less than 20% of affected grapevines; the number of diseased and scattered grapevines is also indicated.

In the Baix Empordà the first outbreak occurred in 2006, when two foci were detected (1.5 ha). In 2008 the affected grapevines did not reached the 20% in each plot and only 47 scattered plants in an area of 5.4 ha were observed.

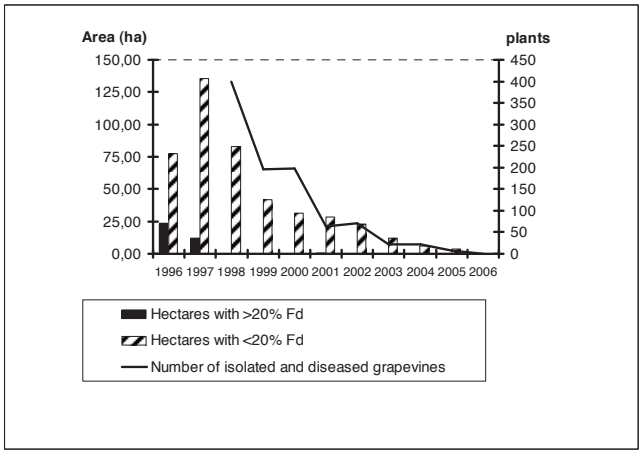


***Figure 3.*** Occurrence of flavescence dorée in the Alt Empordà.

The FD incidence has decreased from 101.25 ha of productive vineyards in 1996 to 5.40 ha in 2008. The results indicate that containment measures adopted against *S. titanus* are effective in limiting flavescence dorée spreading.

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We thank Reyes Vallejo for her technical assistance.

# EPIDEMICS OF FLAVESCENCE DORÉE DISEASE IN PORTUGAL

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***Summary***

Flavescence dorèe (FD), the most dangerous phytoplasma disease within grapevine yellows disease complex, is an recent emerging threat for viticulture in Portugal. FD epidemics have been reported in important areas for the production of high-quality D.O.C. wines such as 'Vinhos Verdes'. Moreover, large populations of the FD-phytoplasma insect vector *Scaphoideus titannus* have been found in the region of 'Vinhos do Porto'. Molecular identification of FD-phytoplasma in numerous grapevine plants lead official services to destroy 10,90 hectares of vineyards in 2008. Mandatory measures were taken for containing the spread of insect vector populations and Flavescence dorée epidemics.

## INTRODUCTION

Flavescence dorée (FD) is the disease that causes major damage in vineyards. FD is the epidemic form of grapevine (*Vitis vinifera* L.) yellows (GY) in Europe, transmitted by *Scaphoideus titanus* Ball (ST), and associated with 'group 16SrV phytoplasmas, subgroups 16SrV-D (FD-D) and 16SrV-C (FD-C) (Martini *et al*., 1999).

FD is an emerging disease in Portugal. Presence of ST was reported for the first time in 1998 in the region of Entre-Douro-e-Minho (Quartau et *al*., 2001), and in 2000 in the region of Trás-os-Montes, northern Portugal. ST individuals from Trás-os-Montes (Vila-Real) were found infected by FD-D in 2003 (De Sousa *et al*., 2003). Dispersion of ST populations is increasing in the North of the country (Fig. 1A). Evident FD epidemics, associated with large diffusion of ST populations, were reported in regions where Protected Designation of Origin (D.O.C.) wines such as 'Vinhos Verdes' and 'Vinhos do Porto' are produced. These findings suggested the potential damaging impact of FD on Portuguese viticulture. Phytosanitary measures have been implemented by the official services towards a rapid and effective control of the disease spreading.

## MATERIALS AND METHODS

From 2001 to 2006 yellow traps were placed in vineyards and grapevine nurseries for capturing ST; surveys on GY symptoms were carried out in the vineyards where ST was reported. Leaf samples were randomly collected from grapevines and tested for phytoplasma detection through nested-PCR. In 2007, during an official survey, desiccation of inflorescences, withering of clusters, irregular maturation of the wood, leaf rolling, vein banding, leaf yellowing on white variety 'Loureiro' and leaf reddening on red variety 'Vinhão' were observed in vineyards of Amares region, northern Portugal. In the last year, 1121 yellow traps were placed in 354 vineyards. Cuttings and leaves were collected from 432 plants showing typical symptoms and asymptomatic plants.

Total DNA was extracted (Angelini *et al.,* 2001) and assayed for amplification of 16S rDNA through nested PCR using primer pair P1/P7 (Deng & Hiruki, 1991) followed by primer pair R16F2n/R16R2 (F2n/R2) (Gundersen & Lee, 1996) and R16F1(V)/R16V1731r (F1/V1) (Martini *et al*., 1999). Phytoplasma strains Elm yellows (EY1) and Rubus stunt (RUS), group 16SrV, were used as reference controls. Healthy periwinkle plants and no template DNA were used as negative controls. F1/V1 amplicons were digested by restriction enzyme *TaqI* for identifying the FD phytoplasma subgroup.

## RESULTS AND DISCUSSION

ST populations were reported in 18 vineyards from 2001 to 2006, in 24 vineyards in 2007, and in 102 in 2008, when 4243 individuals were captured (Fig. 1B). In the last two years, surveys on FD symptoms were carried out in 374 vineyards; 585 samples were collected from plants for molecular analyses. Despite the presence of ST in vineyards from 2001 to 2006, GY symptoms were not observed in examined plants, and all collected samples were negative in nested PCRs performed for phytoplasma detection. Seventy -four symptomatic plants (observed from 2007 to 2008 in northern Portugal) and positive controls yielded nested PCR products (F2n/R2 and F1/V1), absent in asymptomatic samples and in negative controls. Two representative F2n/R2 amplicons, obtained from grapevines, were cloned, sequenced and deposited in GenBank (Acc. no. FJ611961 and FJ611962). Phytoplasma 16S rDNA sequences were identical and shared 99-100% identity with those of group 16SrV. All FD phytoplasma isolates from Portugal exhibited the characteristic FD-D *Taq*I RFLP pattern (Fig. 2). More than eleven hectares of FD-affected vineyards from Minho region in northern Portugal, where FD-D phytoplasma was identified, were destroyed. Furthermore,

it was observed that the dieback of FD-affected plants were faster when vineyards were also affected by other pathogens such as fungi.

The large diffusion of ST populations, here reported, constitutes a serious risk for increasing FD epidemics.

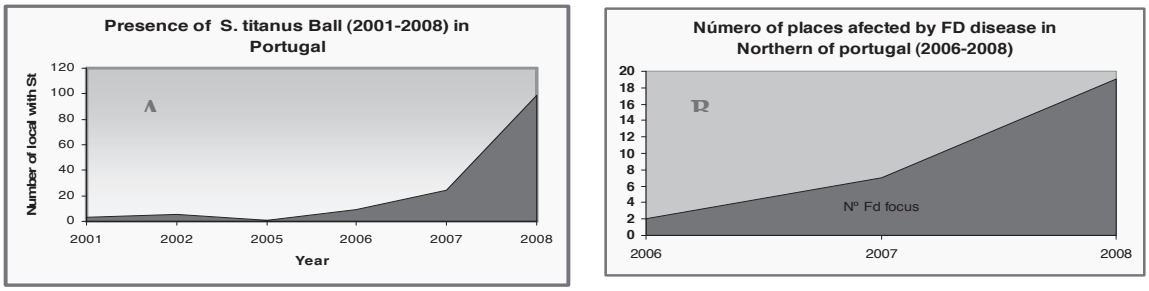


**Figure 1**. (A) Evolution of the presence of ST (2001-2008) in northern Portugal; (B) Number of locations affected by FD disease (2006-2008) in northern Portugal

With the aim to eradicate from national territory the FD phytoplasma and to avoid the spreading of ST, Portuguese Ministry of Agriculture published additional emergency measures (Portaria n º 976/2008, 1 September) for the implementation of control strategies (http://www.dgadr.pt).

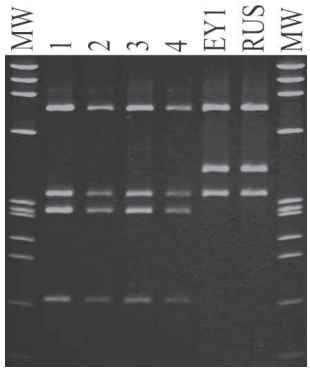


**Figure 2:** *Taq*I-RFLP patterns of PCR amplicons primed with 16SrV group-specific primers F1/V1. MW: molecular marker ΦX174 (Invitrogen, Carlsbad, CA, USA); 1-2: Loureiro var; 3-4: Vinhão var; EY1: elm yellows; RUS: rubus stunt.

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# GRAPEVINE CULTIVAR PLOVDINA – IS IT REALLY AN INDICATOR PLANT FOR FLAVESCENCE DORÉE DISEASE ?

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***Summary***

Grapevine cv. Plovdina, as already presented three years ago, is very sensitive to Flavescence dorée (FD) disease. That cultivar is very suitable as indicator plant for Flavescence dorée disease. In this paper we present our result about viruses and phytoplasma infections grafted grapevines. Our results showed that Grapevine leafroll associated viruses -1, -2 and -3 were not present in the investigated samples. But FD phytoplasma was detected in all grafted grapevines.

## INTRODUCTION

One of the most sensitive cultivar to GY disease appeared to be cv. Plovdina, a traditional local variety (Kuzmanović, 2007, Kuzmanović *et al*., 2008). This cultivar is in our condition a host to the FD phytoplasma only. Grepevine cv. Plovdina, as already presented three years ago (Kuzmanović *et al*., 2006), is very sensitive to Flavescence dorée (FD) disease. The symptoms developed very quickly four weeks after the green grafting. In that paper we suggested that cv. Plovdina was a suitable indicator plant for FD detection. On that occasion, the question was asked if the cultivar Plovdina was infected with GLRaVs before grafting. The reason for this suspicion were the symptoms which were very similar to those caused by GLRaVs (Martelli & Boudon-Padieu, 2006), especially in mixed infections (Katis, pers.com).

## MATERIAL AND METHODS

In order to remove this suspicion, during 2006 and 2007 we collected the following samples from the grafted grapevines: 7 from Sicevo region, 2 from Trstenik region and 10 from Zupa region. Leaves were collected during September for PCR identification of phytoplasmas, and a year old branches during November for PCR detection of GLRaVs

*PCR phytoplasma detection*. Total nucleic acids were extracted from 1 g of fresh plant tissue (leaf midribs) ground in liquid nitrogen according DNA extraction protocol as described by Prince *et al*. (1993). Plant nucleic acid was diluted in sterile deionized water and 20 ng DNA was used for PCR assays. Total DNAs were processed with nested PCR using two phytoplasma universal primer pairs, P1/P7 and R16F2n/R2 (1,200 bp) (Lee *et al*., 1995). Each reaction was performed in a total volume of 50 µl as described by Martini & Murari (1999), with standard procedure (annealing at 50°C) and modification for annealing at 55°C for second primers set. Amplification products were subjected to electrophoresis in a 1,2% agarose. To identify type of phytoplasmas, five microliters of the PCR products amplified using the R16F2n/R2 in modified nested PCR (with higher annealing temperature) were digested with 2 U of *Alu*I and *Tru1*I (Fermentas , Lithuania). The RFLP fragments were visualized by electrophoresis in a 2.2% agarose gel in 0.5× TBE buffer followed by staining with ethidium bromide and visualization under a UV transilluminator. Direct PCR analysis of FD infected samples were performed with specific chromosomal primers FD9f/r (Daire *et al*., 1993.).

*PCR detection GLRaV-1,2,3.* For PCR detection of GLRaV-1, 2 and 3 methods described by Minafra & Hadidi, (1994) has been used to extract total nucleic acids from leaf samples. The viral RNA has been amplified by PCR with specific primer pairs LQV1-H47/LEVI-C447 for GLRaV-1, LR2U2/LR2L2 for GLRaV-2 and LC1/LC2 for GLRaV-3 followed by random primed reverse transcription. As positive control for Grapevine leafroll associated viruses 1,2 and 3 detection and sensitivity of applied method were used GLRaV1,2 and 3 infected samples of grapevine cv. Prokupac.

## RESULTS AND DISCUSSION

*Phytoplasma detection.* Nested PCR using universal primers P1/P7 and R16F2n/R2 detected the presence of phytoplasmas in 20 grapevine samples, all cv Plovdina, and not in cv. Prokupac. On the basis of RFLP analysis the infected samples showed the presence of FD phytoplasmas (16SrV group) collected in Tuleš, Ljubava, Oreovac, Botunja, Gornja Rzenica and Sićevo vineyards. No phytoplasma presence was detected in (cv. Prokupac) (Fig. 1). The presence of FD type phytoplasma in phytoplasma infected samples was confirmed with specific chromosomal primer FD9f/r and amplicon of 1300 bp. Control samples used in all analysis was Pl 27, earlier detected as 16SrV-C in cv. Plovdina (Kuzmanovic, 2007; Kuzmanovic *et al*., 2008).

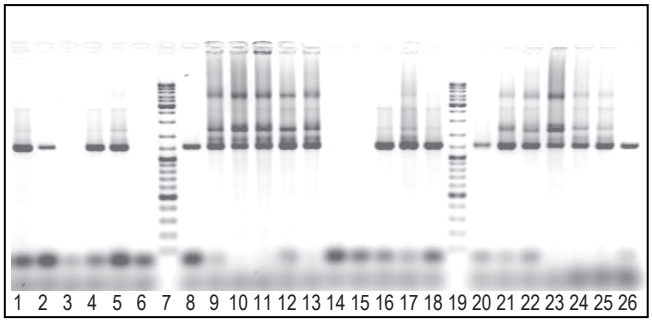


***Figure 1.*** Nested PCR products obtained by two phytoplasma universal primer pairs, P1/P7 and R16F2n/R2 (1200 bp) from grapevine infected and the Pl 27 control with 16SrV-C, Pr 10 and Pr 11- grapevine cv. Prokupac; grapevine cv. Plovdina from Tules (Tu), Ljubava (Lj), Oreovac (Or), Botunja (Bt), Gornja Rzenica (GR), Rataje (Rt) and Sicevo (Si). *1*.Or5; *2*.Or6; *3*.Pr26; *4*.Lj8; *5*.Lj9; *6*.Pr25; *7*.M; *8*.Tu12; *9*.Tu13; *10*.Tu14; *11*.Tu15; *12*.GR16; *13*.GR17; *14*.Pr10; *15*.Pr11; *16*.Rt16; *17*.Bt21; *18*.Pl27 control; *19*. M: GeneRuler DNA Ladder Mix SM0331 Fermentas, Lithuania; *20*.Si2; *21*. Si3; *22*. Si4; *23*.Or4; *24*. GR18; *25*. GR19; *26*. GR20

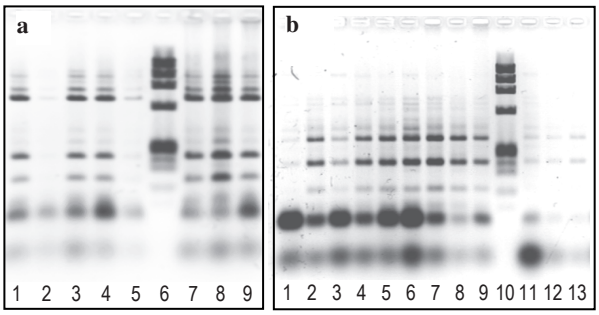


***Figure 2.*** Detection of Flavescence dorée (FD) in different samples of grapevine cv. Plovdina using RLFP analysis. Agarose gel electrophoresis of 16SrDNA fragments of 1200bp obtained with R16F2n/R2: a) *Alu* I patterns: *1*. Or5; *2*. Si2; *3*. Lj8; *4*. GR20; *5*. Tu12; *6*. M: ϕX174 *BsuR*I digested, SM0251 Fermentas, Lithuania; *7*. Pl 27 control; *8*. Bt21; *9*.; b) *Tru1*I patterns: *1*. Si1; *2*. Or5; *3*. Or6; *4*. Lj8; *5*. Lj9; *6*. Tu12; *7*. GR20; *8*. Bt21; *9*. Pl 27 control; *10*. M ; *11*. Si2; *12*. Or4; *13*. GR18.

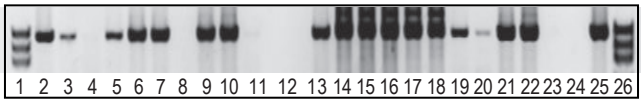


***Figure 3.*** Detection of Flavescence dorée (FD) in different samples of grapevine cv. Plovdina using specific chromosomal primer FD9f/r (fragments of 1300bp). *1*.M: GeneRuler DNA Ladder Plus SM0321 Fermentas, Lithuania; *2*.Or5; *3*.Or6; *4*.Or7; *5*.Lj9; *6*.Or4; *7*.Rt16; *8*.Pr10; *9*.Lj8; *10*.Pl27 control; *11*.Si3; *12*.blanck; *13*.Pr11; *14*.Tu12; *15*.Tu13; *16*.Tu14; *17*.Tu15; *18*.GR19; *19*.GR17; *20*.Si1; *21*.Si2; *22*.Bt21; *23*.GR18; *24./*; *25./*; *26*.GR20; *26*.M

*PCR detection GLRaV-1,-2,-3.* Our results of amplification have shown that Grapevine leafroll associated viruses 1, 2, and 3 were not present in the investigated samples. Figure 4 shows results of tested grafted grapevine cv. Plovdina (line 3-7), for GLRaV-3.

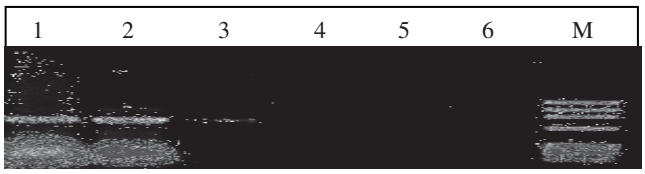


***Figure 4.*** Detection of GLRaV-3 in different samples of cv. Procupac, as positive control (lane 1-2) using specific primer pairs LC1/LC2 (fragment 550 bp), grapevine cv. Plovdina (lane 3-6), M: pBR322/AluI, SM0121 Fermentas, Lithuania.

These results confirmed that grafted grapevine cv. Plovdina were not GLRaVs infected, and that it is a really sensitive indicator plant for Flavescence dorée disease.

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# PHYTOPLASMA DIFFUSION THROUGH GRAPEVINE PROPAGATION MATERIAL AND HOT WATER TREATMENT

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***Summary***

Phytoplasmas can be transmitted by the propagation of scions and/or cuttings collected from diseased plant during a period of symptom latency. Although propagation itself usually results in a strong reduction of the infection in the progeny, some infected plants may survive bringing the pathogen in a new vineyard. Hot water treatment of propagation material could prevent from this danger.

## INTRODUCTION

Flavescence dorée (FD) and Bois noir (BN) are serious grapevine diseases caused by two distinct phytoplasmas, although showing identical symptoms in the vine. FD is transmitted by the leafhopper *Scaphoideus titanus*, while BN is transmitted by the leafhopper *Hyalesthes obsoletus*. FD and BN diffusion in the commercial vineyards is mainly due to these vectors, however the propagation of infected plants by nurseries may also contribute. Although propagation itself usually results in a strong reduction of the infection (Credi *et al*., 2007), only few survived infected plants could bring the disease in a viticultural area or in a vineyard previously free becoming a source of infection. In this respect the role played by propagation is not of minor interest. Hot water treatment (HWT) has been proposed since 1966 by Caudwell to cure dormant woody plant material from phytoplasmas. Afterward other experiences showed the effectiveness of the treatment against these pathogens (Caudwell *et al*., 1990; Tassart-Subirats *et al.,* 2003), although sometime the BN resulted more difficult to be completely eradicated than FD (Borgo *et al.,* 1999; Bianco *et al*., 2000; Mannini & Marzachì, 2007). In addition HWT must be carefully applied because it could reduce the vitality of propagation material. This is the main reasons why HWT did not meet the favour of nurseries, especially in Italy. Recent experiences carried out in Piedmont however confirmed the reliability of HWT applied to scions and cuttings when done with a proper equipment and treating the propagation material at 50°C x 45' (Mannini & Marzachì, 2007; Mannini, 2007).

## MATERIAL AND METHODS

*Effect of hot water treatment on nursery grafted rootlings*. In 2006 a nursery parcel containing around 3000 grafted rootlings belonging to several wine grapevine cultivars (Barbera, Nebbiolo, Vermentino, etc.) were stopped from commercialisation by Phytosanitary Regional Service because of the presence of several rootlings, showing phytoplasma related symptoms which tested positive to both FD or BN. The following year (2007) these grafted rootlings (except the symptomatic ones which were discarded) were used for a trial focused to test the effectiveness of the hot water treatment and to better understand the infection latency in grafted rootlings. Three groups of around 1000 grafted rootlings each were treated as followed: untreated control, 50°C x 45' and 52°C x 45'. After the hot bath, all the grafted rootlings were replanted in the nursery at the beginning of June and monitored over the vegetative season until their pulling out in winter. To avoid re-infections through vectors the nursery was localized away from vineyards or other grapevine nurseries and insecticide sprays were applied three times from June to the end of August. Chromotropic traps were distributed along the nursery rows and routinely inspected. No *Scaphoideus titanus* was found. Plants showing symptoms were labelled and leaf samples collected for RT-PCR analysis (Margaria *et al*., 2007). At the end of the season the grafted rootlings were pulled out and the % of first and second grade take was evaluated.

*Control of hot water treatment efficiency to eliminate BN*. Dormant woody cuttings collected in winter time from 7 adult vines of 5 different cultivars: Albarossa (1), Barbera (2), Chardonnay (1), Dolcetto (1) and Nebbiolo (2), which showed symptoms in the previous summer and resulted BN infected, were hot water treated at 52° x 45'. The trial was organized with a total of 140 treated cuttings and 137 untreated cuttings kept as a control. All the cuttings were rooted in pots and set under an insect proof screen house. Since the end of August, leaves were sampled from each pot and analysed by nested-PCR followed by a RFLP for FD and BN detection (Marzachì & Boarino, 2002).

## RESULTS AND DISCUSSION

*Effect of hot water treatment on nursery grafted rootlings*. In the second year of nursery (2007), several plants which looked healthy the previous year (2006) showed symptoms. In the control, 10 grafted rootlings out of 1395 planted were found symptomatic (table 1), 4 of them resulted 16SrV infected (FD), 3 16SrXII infected (BN), 1 was negative and 2 die before the sample was collected for PCR. In the 50°C x 45' plot only 1 plant resulted symptomatic and FD positive (table 1). No symptoms were shown in the rootlings treated at 52°C x 45'. The data confirmed the low rate of phytoplasma transmission through the propagation (0.7 % in the case of control), the possible latency of the infection until the second year of grafted rootling life (which would correspond to the first year after planting in commercial vineyards) and the efficacy of the hot water treatment to greatly reduce the risk of infection transmission. In terms of propagation material vitality, the 50°C x 45' treatment gave the best result in plant survival and nursery take with 75.4 % of first grade rootlings compared to 70.4 % of the control

and 61.9 % of the 52°C x 45' treatment (table 2). The results confirmed the reliability of HWT also when applied to grafted rootlings if done with proper equipment and using 50°C x 45' bath. On the contrary the treatment at 52°C x 45' induced as a side effect almost 10 % reduction of take and an increase of the 2° grade rootlings.

*Control of hot water treatment efficiency in BN eradication.* After potting, 116 rooted cuttings survived out of the originally 137 in the control as well as 105 survived out of 140 in the treated plot. The nested-PCR was carried out on all the potted plants. In the control 9 plants resulted BN positive whereas all were negative in the 52°C x 45' plot (table 3). It interesting to underline that all the infected potted plants derived from a single adult plant of Chardonnay and from two plants of Barbera originally infected in the field. Considering only these two cultivars the transmission through the cuttings resulted pretty high, particularly for the Chardonnay (35.3 %). No FD infections were detected, as expected. In our experiment the 52°C x 45' HWT showed to be highly efficient in preventing the transmission of BN confirming previous experiences (Mannini & Marzachì, 2007).

***Table1.*** Number of grafted rootlings dead or showing symptoms (and the resulting phytoplasma) out of the total planted for a 2th year in the nursery after hot water treatment. * 2 symptomatic plants die before PCR analysis and 1 resulted negative. The values followed by different letters are statistically different at p≤0,05.

| Rootlings (n.°) | Dead (n.°) | Symptom (n.°) | 16Sr V | 16Sr XII | 16Sr V+XII |
|---|---|---|---|---|---|
| TEST | | | | | |
| 1384 | 131 | 10* a | 4 | 3 | 0 |
| 50°C x 45' | | | | | |
| 1070 | 65 | 1 b | 1 | 0 | 0 |
| 52°C x 45' | | | | | |
| 1080 | 144 | 0 b | 0 | 0 | 0 |

***Table 2.*** Effect of different hot water treatments on grafted rootling vitality after one year nursery expressed as 1° and 2° grade grafted rootling take.

| Rootlings (n.°) | 1° rate (n.°) | 1° grade (%) | 2° grade (n.°) | 2° grade (%) | Dead (%) |
|---|---|---|---|---|---|
| TEST | | | | | |
| 1384 | 974 | 70.4 | 279 | 20.1 | 9.5 |
| 50°C x 45' | | | | | |
| 1070 | 794 | 75.4 | 211 | 18.7 | 5.9 |
| 52°C x 45' | | | | | |
| 1080 | 668 | 61.9 | 268 | 24.8 | 13.3 |

***Table 3.*** Number of potted cuttings from BN-infected vines after hot water treatment and the results of PCR.

| *Treatment* | *Test* | | | *52°C x 45'* | | |
|---|---|---|---|---|---|---|
| Cultivar | N.° total | N.° alive | 16Sr XII | N.° total | N.° alive | 16Sr XII |
| Albarossa | 12 | 7 | 0 | 12 | 3 | 0 |
| Barbera 1 | 29 | 21 | 1 | 25 | 22 | 0 |
| Barbera 2 | 8 | 8 | 2 | 8 | 6 | 0 |
| Dolcetto | 9 | 9 | 0 | 9 | 6 | 0 |
| Chardonnay | 17 | 17 | 6 | 20 | 16 | 0 |
| Nebbiolo 1 | 16 | 15 | 0 | 17 | 15 | 0 |
| Nebbiolo 2 | 46 | 39 | 0 | 49 | 37 | 0 |
| Total | 137 | 116 | 9 | 140 | 105 | 0 |

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# TOWARDS STRAIN DIFFERENTIATION AMONG GRAPEVINE BOIS NOIR PHYTOPLASMAS

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***Summary***

Surveys carried out to identify bois noir phytoplasmas in grapevine yellows disease outbreaks in vineyards located in Hungary, Serbia, and Italy were performed during summer 2008. Selected samples were studied to check for polymorphism presence in 16S and spacer region, *tuf* and *rpS3* genes. Using several restriction enzymes it was possible to identify different restriction profiles in some of the phytoplasma strains. Collective profiles of all bois noir strains studied were distinguishable from the three stolbur phytoplasmas used as reference strains and preliminary classified in the 16SrXII-A subgroup.

## INTRODUCTION

Over the last decade severe spreading of bois noir (BN) disease was described in several European grapevine-growing areas and the usefulness of *tuf* gene for epidemiological studies was shown (Langer and Maixner, 2004; Mori *et al*., 2008). However recent findings indicate that there is a molecular variability also inside the 16S gene of BN and other stolbur-related phytoplasmas (Quaglino *et al*., 2008; Duduk *et al*., 2009) that could be indicative for the presence of diverse strains, possibly relevant to study BN epidemic outbreaks. To investigate the presence of BN strains, selected samples were collected in Hungary, Serbia, and Italy in grapevine growing areas where the disease was spread.

## MATERIAL AND METHODS

During summer 2008, routine surveys carried out to verify identity of phytoplasmas associated with yellows symptoms in some grapevine growing areas in Hungary, Serbia, and Italy allow to identify BN phytoplasmas by RFLP analyses with *Tru*I on R16F2/R2 amplicons (Bertaccini *et al*., 1995). In Hungary six plants from two vineyards of variety Zweigelt both located in Sopron area (near to the Austrian border) were selected for further molecular characterization (Table 1). In Serbia eight plants were employed for BN characterization from Bela Crkva, Radmilovac, and Čoka (Table 1), representing some of the major viticultural areas of the Country. In Italy four samples collected in Veneto and Tuscany regions were studied (Table 1). Total nucleic acids were extracted from midribs and petioles, and amplicons produced with 4 primer pairs amplifiying 16S rDNA gene, spacer region and beginning of 23S gene were subjected to RFLP analyses with 7 restriction enzymes (Table 1). Further PCR/RFLP characterization was carried out on *tuf* gene (Langer and Maixner, 2004) and on *rpS3* gene (Martini *et al*., 2007). Reference strains employed maintained in periwinkle were STOL (from Serbia), STOLC and STOL-PO (from France). Reference strains plus seven samples showing polymorphisms in the 16S plus spacer region amplicons were sequenced in both directions using primers P1, P7 and M1 or P1A, B6 and M1 (Deng and Hiruki, 1991; Schneider *et al*., 1995; Padovan *et al*., 1995; Lee *et al*., 2004) after purification using Qiagen PCR Purification Kit with the BIG DYE sequencing terminator kit. The sequences were assembled using DNA STAR software, and compared with selected sequences of phytoplasmas in GenBank database using BLAST (version Blast N 2.2.18) at the National Center for Biotechnology Information. Virtual RFLP analyses on R16F2/R2 amplicons were carried out to compare results obtained in real RFLP analyses for sequenced strains adding *Hpy188*I to the enzymes used in regular RFLP analyses.

## RESULTS AND DISCUSSION

Tuf *and* rpS3 *genes characterization.* The amplification of *tuf* gene was achieved for all samples using nested-PCR procedures, and restriction with *Hpa*II enzyme showed that all the samples belong to tuf type II, enclosing the reference strains STOL, STOLC and STOL-PO (Langer and Maixner, 2004). RFLP analyses with *Ssp*I, *Hpy8*I and *Tai*I on samples positive for *rpS3* gene showed clear polymorphism in sample BC1 from Serbia (the only one amplified among Serbian samples), and possible polymorphisms in some of the strains from Hungary and Italy with *Ssp*I restriction enzyme.

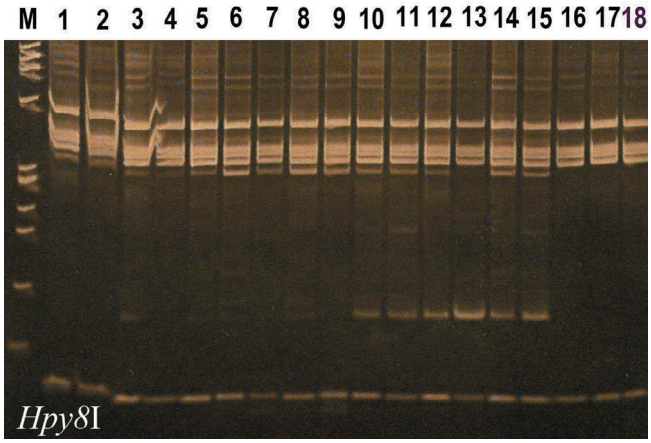


***Figure 1.*** Polyacrylamide gel showing some of the RFLP profiles of P1A/P7A products obtained in nested PCR on P1/P7 amplicons from BN phytoplasmas. 1-9, grapevine samples from Serbia; 10-15, grapevine samples from Hungary; 16, STOL; 17, STOLC; 18, STOL-PO. M, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

***Table 1.*** RFLP results on BN infected samples from Hungary, Serbia and Italy and from reference strains. Identical letter = identical profile; *, almost identical profiles; bold, phytoplasma strains sequenced; small characters, results of virtual RFLP.

| **Samples (Country) acronym** | **Primers P1A/P7A° (27-1,755/1,847 bp)** | | | **Primers F1/B6 (130-1,669/1,814 bp)** | | **Primers M1/B6 (758-1,669/1,814 bp)** | | | **Primers R16F2/R2 (149-1,397 bp)** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***Hpy8*I** | ***Mbo*I** | ***Mbo*II** | ***Tru*I** | ***Mbo*II** | ***Rsa*I** | ***Mbo*II** | ***Alu*I** | ***Tru*I** | ***Mbo*II** | ***Tsp509*I** | ***Hpy8*I** | ***Hpy188*I** |
| Langeveildeground (H) L1 | C | A | B | A | B | A | A | A | A | A | A | A | |
| **Langeveildeground (H) L2** | C | A | B | A | A | A | A | A | Aa | Aa | Aa | Aa | a |
| Brandmajor (H) B1 | C | B | C | A* | C | A | A | A | A | C | A | A | |
| Brandmajor (H) B2 | B | B | C | A | C | - | - | - | A | C | A | A | |
| Brandmajor (H) B3 | C | B | C | A | C | - | - | - | A | C | A | A | |
| **Brandmajor (H) B4** | C | - | C | A* | C | - | - | - | Aa | Ca | Aa | Aa | b |
| Bela Crkva (RS) BC1 | D | A | A | A | A | B | A | A | A | C | A | A | |
| **Bela Crkva (RS) BC2** | D | B | B | - | - | - | - | - | Aa | A*a | Aa | Aa | a |
| Bela Crkva (RS) BC3 | E | B | A | - | - | - | - | - | A | A | A | A | |
| Bela Crkva (RS) BC4 | D | B | C | - | - | - | - | - | A | A | A | A | |
| **Radmilovac (RS) R1** | D | B | B | - | - | - | - | - | Aa | Ac | Aa | Aa | a |
| Radmilovac (RS) R2 | D | B | B | - | - | - | - | - | A | A | A | A | |
| Radmilovac (RS) R3 | D | A | A | A | A | A | A | A | A | C | A | A | |
| **Čoka (RS) C1** | D | B | B | A | A | A | A | A | Aa | Aa | Aa | Aa | a |
| **Veneto (I) 39** | D | B | B | A | C | A* | B | B | Aa | Ca | Bb | Aa | a |
| Veneto (I) 41 | D | A | B | A | C | A* | B | B | A | C | A | A | |
| Tuscany (I) 2 | D | A | B | - | C | A* | A | A | A | C | A | A | |
| **Tuscany (I) 3** | D | A | A | - | C | A* | A | A | Aa | Ba | Aa | A | a |
| **STOL (RS)** | A | A | A | A* | B | B | A | A | Ab | Aa | Aa | Ab | a |
| **STOLC (F)** | A | A | B | - | B | A | B | B | Aa | Ba | Aa | Aa | a |
| **STOL-PO (F)** | A | A | A | - | - | A | A | A | Aa | Ba | Aa | Aa | a |

°, Martini, 2004

*16S ribosomal gene/spacer region characterization.* RFLP analyses showed different profiles in several of the examined samples, according with amplicon employed; overall restriction profiles indicate the possible presence of interoperon heterogeneity and/or of mixed phytoplasma population in same of the samples (Fig. 1, Table 1). Seven grapevine samples and the reference strains were sequenced on 16S ribosomal/spacer gene (about 1,500 bp), and virtual RFLP analyses carried out on R16F2/R2 amplicons showed in some cases differences between real and virtual RFLP profiles (Table 1). After Genbank search similar analyses on deposited BN strains showed identical *Hpy188*I profiles among sample B4 from Hungary and BN samples from Canada and Spain (EU086529 and AJ964960). A BN strain from Germany (X764281) showed unique *Bstu*I/*Hpy8*I profiles, and BN strain EU836649 from Lombardy shared *Btsu*I profile with STOL strain.

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# GENETIC DIVERSITY AMONG BOIS NOIR PHYTOPLASMA POPULATIONS IN ITALY: NEW 16Sr SUBGROUPS AND DISTINCT SNP GENETIC LINEAGES

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***Summary***

Extensive intra-species diversity among Bois noir phytoplasma populations in Italian vineyards was revealed through analyses of molecular markers in 16S rRNA gene sequences. Three 16Sr subgroups were recognized and multiple SNP lineages were identified. The findings underscore the need for future studies on relationships between diverse phytoplasma strains and dynamics of BN disease epidemiology.

## INTRODUCTION

Bois noir (BN) is a grapevine yellows (GY) disease caused by phytoplasmas of the stolbur group in Europe, where it induces severe crop losses in almost all varieties used for wine production. Results from a recent survey underscored the prevalence of stolbur phytoplasmas in vineyards in Italy (Botti & Bertaccini, 2007), suggesting potential for heavy impacts on Italian viticulture. BN phytoplasma is transmitted from plant-to-plant by *Hyalesthes obsoletus* Signoret, but in wine-growing areas where *H. obsoletus* is absent, the presence of stolbur phytoplasma implies the existence of alternative vectors (Boudon-Padieu, 2000). The biological complexity of BN disease has stimulated research on molecular markers of grapevine-affecting stolbur phytoplasma genetic diversity. Analysis revealed that three *tuf* sequence variants (VK-I, VK-II and VK-III) of '*Ca.* Phytoplasma solani' were present in BN-diseased grapevines, as well as in certain weeds (Langer & Maixner, 2004). Intriguingly, VK-I and VK-II BN phytoplasma strains are differentially distributed in distinct geographic regions in Italy (Quaglino *et al.*, 2007). In the present study, an unexpectedly high degree of genetic heterogeneity among BN phytoplasma populations in northern and central Italy was revealed through virtual restriction fragment length polymorphism (RFLP) analysis and single nucleotide polymorphism (SNP) analysis of 16S rDNA. The breadth of BN phytolasma genetic diversity prompts in-depth studies of the distribution of BN phytoplasma strains in weeds, insect populations, and grapevines from different geographic areas.

## MATERIALS AND METHODS

Leaf samples were collected from 96 GY-symptomatic grapevine plants in 29 vineyards located in northern (Lombardy) and central (Abruzzi and Marche) Italian regions. Detection of stolbur phytoplasmas was carried out through 16SrI/XII group-specific nested PCR-amplifications of 16S rDNA followed by *Mse*I-RFLP assays, as in Lee *et al.* (1998). Genetic diversity among BN phytoplasma strains was studied through virtual RFLP analysis of F2nR2 fragments boarded by the annealing sites of primer pair R16F2n/R16R2 as described by Wei *et al.* (2007, 2008). Furthermore, nucleotide sequences were aligned using ClustalX (V1.83) and searched for SNPs.

## RESULTS AND DISCUSSION

*Identification of 'Ca. Phytoplasma solani'-related strains and characterization of new 16Sr subgroups:* DNA templates derived from all 96 samples yielded products in 16SrI/rXII group-specific nested PCRs; all amplicons exhibited *Mse*I-RFLP patterns indistinguishable from that characteristic of the reference strain STOL, indicating that the strains detected in the diseased grapevines were '*Ca.* Phytoplasma solani'-related strains. Virtual RFLP analyses of the nucleotide sequences of the 96 F2n/R2 fragments yielded 24 different collective RFLP patterns from use of 17 restriction endonucleases. One pattern, indistinguishable from that characteristic of strains classified in subgroup XII-A, was exhibited by amplicons from PCRs containing template DNA from 73 diseased grapevines. The 23 remaining virtual, collective RFLP patterns differed from one another and from the patterns characteristic of strains previously classified in group XII, opening the possibility for recognition of new subgroups in this group. However, in accordance with criteria by Wei *et al.* (2007, 2008), only two new subgroups (16SrXII-F and XII-G) (Fig. 1) were confirmed in grapevine plants from northern Italy. Key enzymes for distinguishing among 16SrXII subgroups were *Alu*I, *Bfa*I and *Bst*UI (Fig. 1). Other 21 potentially new subgroups, observed only once, were described as tentative sugroups (16SrXII-A1 to 16SrXIIA-21).

*Additional 16S rDNA SNP genetic lineages in BN phytoplasma populations.* Alignments of 16S rDNA nucleotide sequences have revealed SNPs only at three nucleotide positions (Tab. 1), none of which involved a recognition site for any of the 17 enzymes used to delineate 16SrXII subgroups. At least nine 16Sr lineages, characterized by SNPs at positions 43, 875 and 1219 were described (Tab. 1). Seven of these lineages were found among strains in both northern and central Italy; two lineages were identified only in northern Italy. The existence of the SNPs, within and outside of restriction sites in 16S rDNA, underscores the remarkable genetic diversity of BN phytoplasma populations, and affords molecular markers that should be useful for detailed investigation of the biology and epidemiology of grapevine BN and other stolbur phytoplasma-associated plant diseases.

***Table 1.*** Bois noir (BN) phytoplasma 16S rRNA gene SNP lineages found in diseased grapevines

| 16Sr lineage | Accession number | Number of BN phytoplasma strains | | 16Sr SNP position from start of F2nR2 fragment | | |
|---|---|---|---|---|---|---|
| | | North Italy | Central Italy | 43 | 875 | 1219 |
| 16Sr-α | EU836644 | 17 | 4 | T | A | T |
| 16Sr-β | EU836645 | 5 | 0 | C | A | T |
| 16Sr-γ | EU836646 | 10 | 4 | T | G | T |
| 16Sr-δ | EU836647 | 26 | 3 | C | G | T |
| 16Sr-ε | EU836648 | 3 | 0 | C | A | G |
| 16Sr-ζ | EU836649 | 3 | 1 | T | G | G |
| 16Sr-η | EU836650 | 4 | 1 | T | A | G |
| 16Sr-θ | EU836651 | 8 | 2 | C | G | G |
| 16Sr-ι | EU836652 | 3 | 2 | T | G | A |

*Genetic diversity and ecology of BN phytoplasma strains.* Identification of SNPs delineating no less than two new grapevine-associated 16SrXII subgroups and of other SNPs in the present study underscored that phytoplasma intra-species strain diversity is far more complex than previously understood. Twenty nine percent of BN phytoplasma strains from Lombardy and 10% from Marche and Abruzzi exhibited virtual RFLP patterns distinct from those of 16SrXII-A subgroup. As 'Ca. Phytoplasma solani'-related strains from central Italy were characterized by RFLP patterns not observed in northern Italy, strain composition of BN phytoplasma populations appears to differ between northern and central regions of Italy, suggesting possible influence of different ecological and/or climatic niches on phytoplasma population composition.

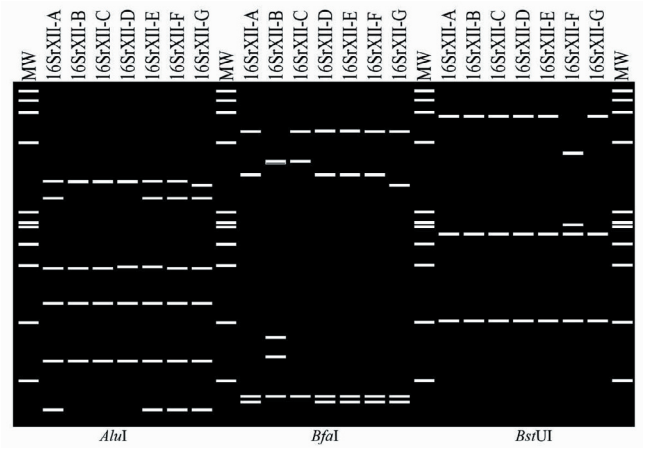


***Figure 1.*** Virtual F2nR2 RFLP patterns, obtained through digestion by key enzymes, of representative strains of 16SrXII confirmed subgroups.

This hypothesis is reminiscent of work by Cai *et al.* (2008), who found that genetic heterogeneity among cactus witches-broom (CaWB) phytoplasma strains in China was correlated with environmental conditions. Climatic and geographic features in vineyard ecosystems such as average altitude (211 M in Lombardy versus 407.6 M in central-eastern Italy), average annual temperature (12.1 ºC versus 13.6 ºC), average annual precipitation (919 mm versus 787 mm), and average annual humidity (77% versus 72%) may be significant, directly or indirectly, in determining the strain composition of stolbur phytoplasmas populations in different regions. For example, temperature as well as other climatic factors could strongly influence the life cycle and behaviour of the vector *H. obsoletus* Signoret (Johannesen *et al.* 2008), conceivably altering its host plant feeding preferences and selection of Stolbur phytoplasma-related strains; these factors could also influence the variety and number of weeds in and around the vineyard. Such extensive intra-species diversity, evidenced by 24 subgroups plus multiple SNP lineages, suggests complex population structure in stolbur phytoplasmas. Future studies should aid understanding of the dynamics of BN disease epidemiology in relation to stolbur phytoplasmas-related strain populations.

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# PRELIMINARY RESULTS ON THE VARIABILITY OF BOIS NOIR ISOLATES IN A VINEYARD SYSTEM IN SICILY

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***Summary***

Presence of bois noir phytoplasma in Sicily is known since 1996, but only recently data on its distribution in the region were collected. RFLP of partial *stol-1H10* gene, a Stolbur-specific putative membrane protein gene, was applied to evaluate the diversity of BN isolates among host plants (grapevines and weeds) and potential vectors collected in an organic Chardonnay vineyard, located in Sicily. All the collected samples produced PCR amplicons of the same size and RFLP analyses evidenced the prevalence of V1 profile. The V4, V9, V10 and V11 profiles were also present. BN was also identified in the weed species *Conyza bonariensis*, a new natural host of the phytoplasma in Sicily, and in several individuals of the cicadellid leafhopper species *Exitianus capicola*.

## INTRODUCTION

Grapevine bois noir (BN) phytoplasma (16SrXII-A) is widespread in Europe and is endemic in France and Italy. In Sicily, symptoms of grapevine yellows were associated with phytoplasmas belonging to 16SrXII group in Inzolia cv (Albanese *et al.*, 1996). A systematic survey for the evaluation of distribution of Grapevine yellows (GY) in Italy started in 2004 and the presence of BN phytoplasma in different grapevine cvs (Chardonnay, Nero D'Avola, Inzolia, Cabernet Sauvignon) was confirmed in Sicily. During the same survey the absence of Flavescence dorée phytoplasma and of its vector *Scaphoideus titanus* was also confirmed. The Sicilian BN isolates were firstly characterized by RFLP and SSCP analyses of the partial sequence of *tuf* gene and showed an identical *Hpa II* profile of VKII-type in all the considered isolates (La Rosa *et al.,* 2006). More recently the polymorphism of the Stolbur specific *stol-1H10* gene, encoding a putative membrane-exposed protein, of Sicilian BN affected grapevines confirmed their belonging to VKII group and identified three *stol-1H10* profiles: V2, V4, V9 (Pacifico *et al.*, 2009). In order to evaluate variability inside a complex pool of BN isolates, an organically-grown vineyard was chosen as "vineyard system". The results of the characterization of the BN phytoplasma isolates through RFLP analysis of the *stol1-H10* gene in this "vineyard system" (grapevine, weeds, insect vectors) in Sicily are here reported.

## MATERIAL AND METHODS

A 15 year old, organic Chardonnay vineyard, known to be affected by BN phytoplasmas was chosen in Catania province. Starting from late spring 2008, monthly field inspection was performed for the evaluation of phytoplasma-specific symptoms in grapevine and weeds. At each survey, the number of symptomatic grapevines and symptoms from grapevines and weeds were recorded. At each survey, potential phytoplasma vectors were also collected by netting in the canopy of grapevines and on wild vegetation. Total DNA was extracted starting from 1.5 g of foliar tissue of grapevine or weeds and from single insect of each species collected in the field. In order to ascertain the presence of BN phytoplasma, 2 µl of each DNA extracts was amplified by direct PCR with primers P1/P7 (Schneider *et al*., 1995), followed by nested-PCR with group-specific ribosomal primers R16S(I)F1/R1.The obtained amplicons were then digested with *Mse*I restriction enzyme and their restriction profiles were analyzed after electrophoretic separation on polyacrylamide gels. BN isolates were then characterized by PCR-RFLP of the *stol-1H10* non-ribosomal region by amplifying 2 µl of total DNA with H10F1/H10R1 primers, followed by a second PCR driven by H10F2/H10R2 primers. Restriction profiles of PCR products digested with *RsaI* and *AluI* were separated in 10% polyacrylamide gel (Pacifico *et al.,* 2009).

## RESULTS AND DISCUSSION

*Field inspection.* GY symptoms were observed on 208 out of 2000 Chardonnay grapevines. Phytoplasma-specific symptoms were observed on *Cynodon dactylon* (leaf yellowing and dwarfing), *Convolvolus arvensis* (yellowing and stunting) and on *Conyza bonariensis* (shortening of internodes). The insects collected during the inspections mainly belonged to the species *Exitianus capicola* and *Toya propinqua*. Only a single adult of *Hyalestes obsoletus* was collected.

*BN detection*. BN was detected in all the 20 symptomatic grapevine samples, in both symptomatic *C. bonariensis* samples, in 4/34 *Exitianus capicola* individuals, and in the only *H. obsoletus* collected sample. No amplification was obtained from DNAs of the symptomless samples of each vegetal species collected as control. These preliminary results exclude the involvement of *C. arvensis* in the specific pathosystem studied but evidenced *C. bonariensis*, a wide-spread weed in the Sicilian vineyards, as a new host of the BN phytoplasma. *H. obsoletus*, known vector of BN, confirmed its role in the epidemiology of the disease in Sicily, although the presence of the phytoplasma in *E. capicola*, already signaled as positive to other phytoplasmas (Weintraub *et al*., 2004) but never correlated to bois noir, allows to hypotize a role of this leafhopper in the considered BN system.

*Diversity of stol-1H10 gene.* Nested-PCR products with H10F2/H10R2 were obtained from all BN-infected samples. As expected, no amplification was obtained from the symptomless samples. The 27 obtained amplicons were identical and were 1820 bp long. The *RsaI* and *AluI* profiles evidenced the prevalence of V1 profile in all the species (20/27), followed by V9 (3/27), V4 (2/27). V11 and V10 were detected only in *V. vinifera* (1/27) and in *E. capicola* (1/27), respectively. The high variability of the *stol-1H10* gene was useful to differentiate the genetic diversity inside a specific but complex system of BN isolates. We have shown that one isolate (V1) accounts for more than 70 % of the BN variability in the Sicilian "vineyard system". Stol-1H10 V1 profile is wide-spread among BN isolates from several French viticulture areas and it is less represented among Italian BN isolates from various regions (Pacifico *et al*., 2009). The high incidence of this profile among the BN isolates in the Sicilian vineyard suggests a common geographic origin of the isolate. Chardonnay cv was introduced in Sicily about 30 years ago from France. An increase of the epidemic role of V1 isolate in the analyzed vineyard cannot be excluded. Further studies are required to explain the presence of this isolate in the *C. bonariensis* weed and in *E. capicola* individuals, since grapevine is considered as a dead-end host for BN phytoplasma.

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# INCIDENCE OF BOIS NOIR PHYTOPLASMA IN DIFFERENT VITICULTURE REGIONS OF SPAIN AND STOLBUR ISOLATES DISTRIBUTION IN PLANTS AND VECTORS

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***Summary***

The incidence of Bois Noir disease, the population of the vector, *Hyalesthes obsoletus* and the stolbur phytoplasma isolates distribution has been studied in five viticulture areas of Spain. The BN incidence in affected plots ranged from 3% to 70%. Individuals of *H. obsoletus* were identified in all sampled plots with lower populations, from 0.25 to 3 individuals per aspiration. A total of 24 individuals was the maximum number captured in a plot in 2007, after seven samplings. The percentage of stolbur-bearing *H.obsoletus* individuals ranged from 20 and 100%. The study of stol-1H-10 gene revealed the presence of three isolates in grapevine plants and two isolates in *H.obsoletus*.

## INTRODUCTION

Bois Noir disease was first identified in Spain in 1994, but in recent years there seems to be an increase of the incidence in different grapevine areas. For this reason, the disease incidence in several regions and in different grapevine varieties, the population of *Hyalesthes obsoletus* and the determination of stolbur phytoplasma isolates in plants and insects has been studied. The identification and characterization of isolates is an important tool to identify which are the vectors and the host plants implicated in the disease dissemination. The amplification of sequences of the gene of elongation Tu have allowed to distinguish two different stolbur isolates in grapevine and three in *Hyalesthes obsoletus*. Also studies of the stol-1 H10 gene, encoding a putative membrane protein showed genetic diversity of stolbur isolates (Pacifico *et al*., 2006).

## MATERIAL AND METHODS

The study was carried out in five viticulture areas, Álava, La Rioja, Navarra, Aragón and Catalonia. In each area different grapevine plots were sampled. The *H.obsoletus* population was followed for two years in two plots per region. During 2006 and 2007, from June to September, the insects were captured weekly with a D-vac aspirator over weeds within and at the edges of grapevine plots. Samples of grapevine plants as well as carrier insects of this phytoplasma have been analyzed. The PCR technique was used for phytoplasma detection in plants and insects.

The presence of different stolbur isolates in the different areas was evaluated using PCR-RFLP analyses with primers for the elongation gene *Tu* and primers *stol-1H 10*, for a gene encoding a membrane protein (Langer & Maixner, 2004, Pacifico *et al*., 2006).

***Table 1***. Percentage of infection in grapevine plots and in *H. obsoletus* and stolbur isolates distribution.

| **Region localisation** | **Incidence BN** | **No *Hyalesthes* (% positives)** | | **Stolbur isolates characterization** | | | |
|---|---|---|---|---|---|---|---|
| | | | | **Grapevine** | ***Hyalesthes*** | **Grapevine** | ***Hyalesthes*** |
| | | **2006** | **2007** | **Stol 1 H10** | | **Tuf AY** | |
| Aragón | | | | | | | |
| Vero 1 | 55% | 15 (75%) | n.a. | V1 / V4* | V4/V1 | II | II |
| Vero 2 | 53% | 4 (50%) | 4 (50%) | | | | |
| Estadilla/Salas | 53% | n.a. | 18 (60%) | V1/V3 | V1 | II | II |
| Navarra | | | | | | | |
| Saso | 10% | 3 (33%) | n.a. | V3 | V1 | | |
| Montitura | 70% | 10 (50%) | 4 (25%) | V1 | V1 | II | II |
| Monteagudo | 50% | n.a. | 21 (56%) | V1/V3 | V1 | II | II |
| Alava | | | | | | | |
| Carrabaños | 3-4% | 4 (33%) | 5 (80%) | | V1 | II | II |
| Morrolavieja | 1% | 5 (40%) | 2 (0%) | | V1 | II | II |
| La Rioja | | | | | | | |
| Manjarres | 70% | 5 (40%) | 6 (67%) | V3 | V1/V4 | I | II |
| Aranzana | 70% | 8 (44%) | 3 (67%) | V3 | V1 | I | II |
| Cardenas | 70% | 9 (42%) | 3 (100%) | V3 | V1 | I | II |
| Navarrete | 30% | 0 | 0 | V3/V1 | | I | |
| Catalonia | | | | | | | |
| Conca Barberà | 30% | 8 (40%) | 10 (50%) | V1 | V1 | II | II |
| Pla penedes | 10% | 0 | 2 (80%) | V1 | V4 | II | II |
| Bages | 10% | 5 (80%) | 7 (43%) | V1 | V4 | II | II |
| Terra Alta | 3% | 1 | 24 (25%) | V1 | V4 | II | II |

## RESULTS AND DISCUSSION

*Bois Noir Incidence :* The results of field samplings carried out during the last three years have shown a high incidence of the Bois Noir disease in some plots of La Rioja, Navarra and Aragón ranging from 10 to 70% (Table 1). The disease incidence was low in Catalonia (from 3% to 30%) and Álava (around 4%) (Table1). The main infected varieties were Chardonnay in Catalonia and Aragón and Grenache and Tempranillo in La Rioja, Álava and Navarra.

*Hyalesthes obsoletus population and percentage of individuals carriers of the phytoplasma :* The surveys of insects trapping showed that the main vector of BN, *Hyalesthes obsoletus* was present in all the geographical areas sampled. The highest number of individuals was recorded in Navarra, Aragón and La Rioja, where the maximum per aspiration ranged between 1.6 and 3 individuals, whereas in the plots of Álava, the population of *H.obsoletus* was lower, 0.75 individuals per aspiration (Figure 1). In Catalonia the number of *H.obsoletus* captured varied with the sampled area. The highest number of individuals was captured in Conca de Barberà (Poblet and Vallbona) and Terra Alta, meanwhile in Penedés they were either not captured or in a very low number. The peaks of population of *H. obsoletus* were attained at different dates, depending on the sampled zone, taking place between June 6th and July 14th (Figure 1). The percentage of infective *H. obsoletus* ranged from 0 % to 100 % (Table1). Results indicate a correlation between the presence of this species and BN disease incidence.

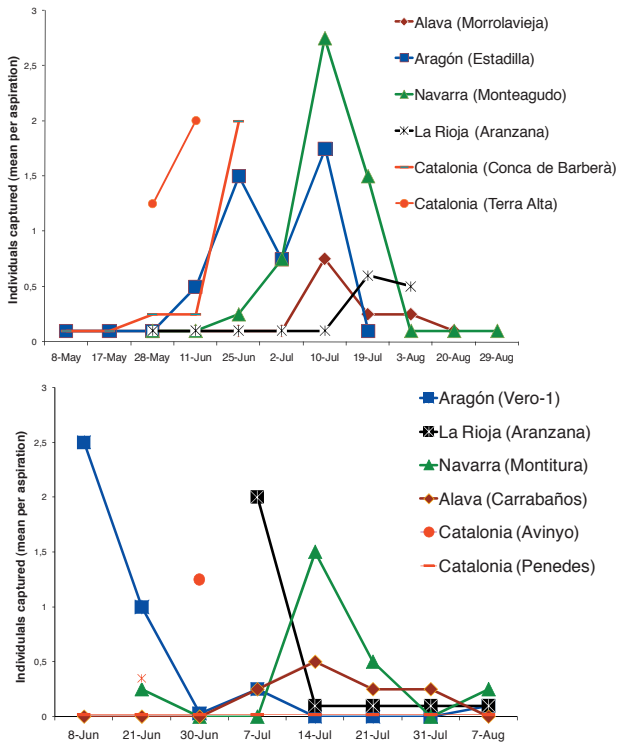


***Figure 1.*** Population evolution of *H.obsoletus* in different grapevine plots of Spain, in 2006 and 2007

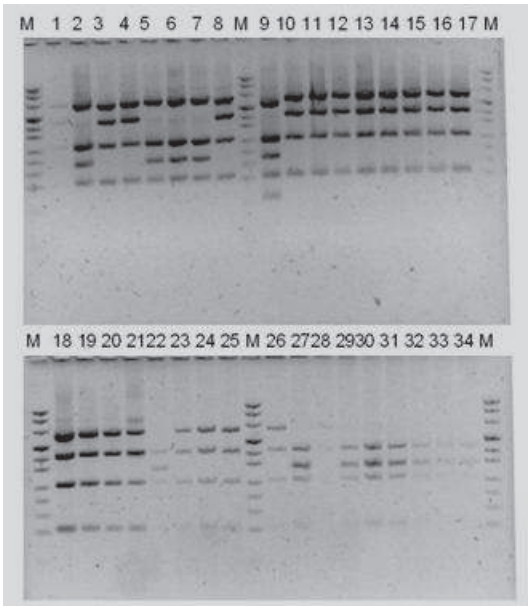


***Figure 2.*** RFLP-profiles of stolbur phytoplasma obtained after PCR with stol 1 H10 primers and digestion with enzyme R*sa* I Profiles: **V1** (Samples 1, 3, 4, 8,10,11,12, 13, 14, 15, 16, 17, 18,19,21, 23, 24, 25, 26), **V4** (2, 5, 6, 7, 9), **V3** (22, 27, 29, 30, 31, 32, 33, 34)

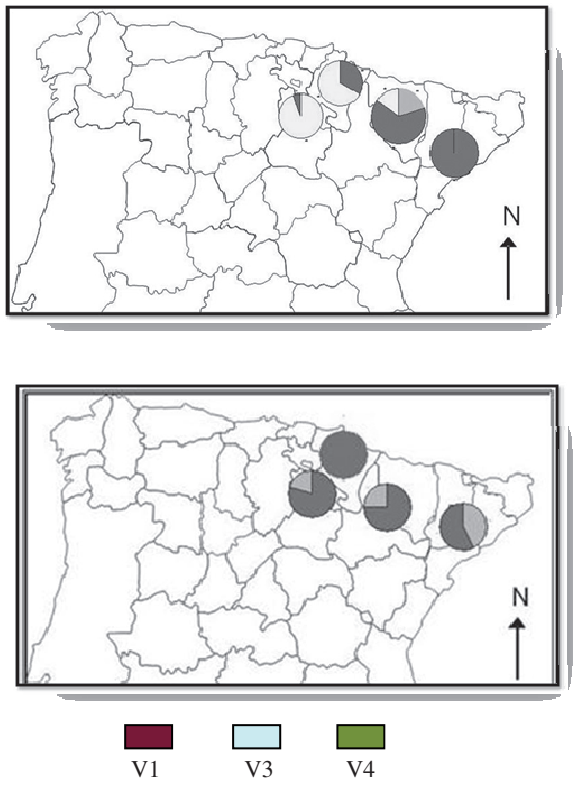


***Figure 3.*** Distribution of stolbur isolates in grapevine plants (upper) and in *H.obsoletus* (below) from the different sampled areas

*Molecular characterization of stolbur isolates :* The analyses of PCR-RFLP showed two profiles with primers Tuf (type I and type II) and three with stol 1-H10 primers (V1, V4 and V3) (Table 1, Figures 2 and 3). The isolated type II determined with the gene of elongation Tu and associated preferably to *Convolvulus arvensis* can be splitted in two isolates V1 and V4 by means of the study of

the gene stol-1 H10. This can be the explanation for the minor incidence of the disease in some specific locations of Catalonia (Terra Alta, Bages and Alt Penedes), where the grapevine plants are infected mainly with isolate V1 and the individuals of *Hyalesthes obsoletus* are carriers of the isolate V4. Nevertheless in Conca de Barberà (Poblet and Vallbona) where the incidence of the disease is slightly higher, both plants and insects are carriers of the isolate V1. The same correlation exists also in the plots of Aragón (Viñas del Vero and Estadilla) and in two out of three plots of Navarra where plants and insects are carriers of the V1 isolate. The isolate type I determined with the gene of elongation Tu and associated preferably to *Urtica dioica* seems to match with the profile V3 obtained after the study of the gene stol-1 H10. In Spain this isolate has been identified only in grapevine plants of La Rioja, in a plot of Navarra (Saso) and sporadically in some plants of a plot of Aragón (Estadilla), although no individuals of *H. obsoletus* have been identified yet as carriers of this isolate. In this area, other vectors might be involved in the spread of the disease. In previous studies different species of cicadellidae and fulgoridae had showed aptitude to transmit the disease under experimental conditions (Sabaté *et al*., 2006).

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# PHOTOSYNTHESIS AND TRANSPIRATION IN GRAPEVINES AFFECTED BY BOIS NOIR AND RECOVERED

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***Summary***

To study the physiological status of Primitivo grapevines (*Vitis vinifera* L.) affected by Bois noir, net photosynthesis and transpiration were measured on mature leaves of asymptomatic, symptomatic and recovered grapevines. Both parameters dramatically decreased in the second part of the season, after fruit set. This followed a particularly long dry season, from the end of June to September, which was characterized by very high temperatures. The recovered plant suffered the most in the middle of the summer, probably because the recovery inducing practice will have reduced the root volume (heavy root pruning from partial pulling in the previous year). The positive initial physiological status in the spring was masked by the middle of the dry season by the lower ability to absorb water. Finally, in September, the differences among the treatments were very limited.

## INTRODUCTION

Phytoplasma are widely spread plant pathogens that affect many plants and crops, and they are responsible for serious disease in orchards and vineyards. In grapevine, Flavescence dorée (FD) and Bois noir (BN) are the most recurrent grapevine yellows in Europe (Boudon-Padieu, 2003). Both diseases have similar symptoms, including growth reduction, leaf discolouration, downward rolling of leaves, and shrivelling of berry clusters, with reduced quality and quantity of yield. The symptoms are not uniform, and can appear on some or all shoots of infected vines. However, grapevines infected by phytoplasma can undergo spontaneous symptom remission, which is better known as recovery (Caudwell, 1961; Osler *et al*., 2003). This natural phenomenon has been seen in different varieties and viticultural regions (Osler *et al*., 2003; Garau *et al*., 2004; Maixner, 2006; Romanazzi *et al*., 2007), and it can also be promoted by exposing the grapevines to abiotic stress, such as transplantation (Osler *et al*., 1993) and partial uprooting or pulling (Romanazzi & Murolo, 2008), or through agronomical practices such as pruning or pollarding (Borgo & Angelini, 2002; Zorloni *et al*., 2002). The sanitary status of the plant is linked to physiological changes, and photosynthesis and transpiration are key processes. By monitoring these, it is possible to predict the general status of plants and their response to abiotic and biotic stress through the season (Bertamini & Nedumchezhian, 2001; Palliotti *et al.*, 2004; Silvestroni *et al.*, 2004; Petit *et al.*, 2006; Christen *et al.*, 2007). To further understand recovery in grapevines previously infected by BN, physiological changes were recorded, focusing mainly on the photosynthesis and transpiration

## MATERIAL AND METHODS

The study was performed on cv Primitivo grapevines (*Vitis vinifera* L.) planted in 1989, which were grafted onto Kober 5BB (*V. berlandieri* x *V. riparia*) rootstock, and trained as guyot. The experimental vineyard was located in Petritoli (AP, Italy) with an average annual temperature of around 14 °C and 700 mm annual precipitation. Net photosynthesis (A), stomatal conductance ($g_s$), and transpiration rate (E) were determined simultaneously on leaves, using a portable infrared gas analysis system (ADC, Maidstone, UK). The system was equipped with a clamp-on leaf cuvette that exposed 6.25 $cm^2$ of leaf area. Environmental conditions during the photosynthetic measurements were relatively stable: photosynthetic active radiation, ≥ 1,700 μmol $m^{-2}$ $s^{-1}$, relative humidity, 35 %, and chamber temperature, 30 °C. The climatic data (average weekly temperature and rainfall) of the area were kindly provided by weather stations of Agenzia Servizi Settore Agroalimentare (ASSAM), Marche region. Gas exchange measurements were conducted between 09:00 hours and 11:00 hours on two mature leaves of asymptomatic (4 plants), symptomatic (4 plants) and recovered (4 plants) grapevines, from June to September 2008. The recovered vines were obtained from previous trials, where parts of the plant roots had been broken using a small excavator (Romanazzi & Murolo, 2008). The sanitary status of the grapevines was verified using molecular tools (Lee *et al*., 1994).

## RESULTS AND DISCUSSION

From the historical climatic data from 1971, it can be seen that the mean annual temperatures have progressively increased; by the 2008 in particular, this increment has reached about 1.5 °C.

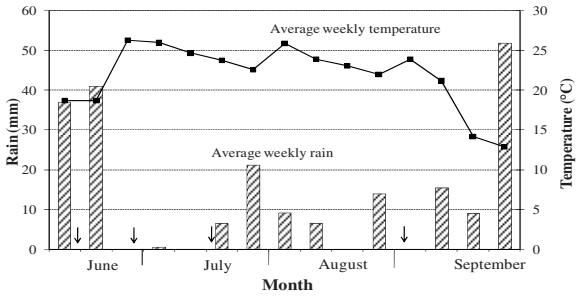


***Figure 1.*** Average weekly temperatures and rainfall recorded from June to September 2008 by the ASSAM weather station. The arrows show the days of survey (10/06, 24/06, 17/07 and 04/09).

The end of June and the first week of August were the hotest periods (Fig. 1), which were characterized by an increase of 4 to 7 °C and a 30 % decrease in rainfall, compared to the historical average climatic data. Under

these stress conditions in the summer following fruit set, common dramatic reductions in photosynthesis and transpiration were observed for all symptomatic, asymptomatic, and recovered vines. However in the middle of June, which was characterized by mild temperatures and heavy rainfall, the net photosynthesis was higher in asymptomatic as compared to the symptomatic and recovered grapevines (Fig. 2).

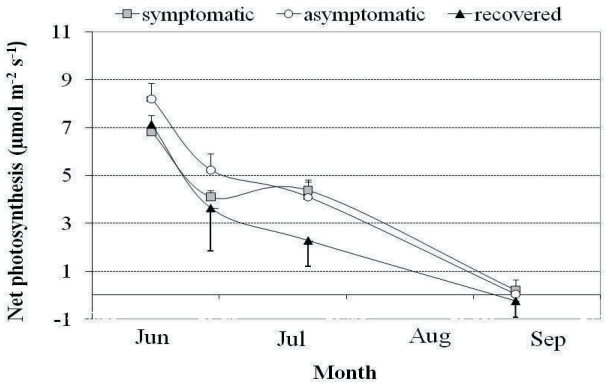


***Figure 2.*** Changes in the net photosynthesis (A) measured in leaves of symptomatic, asymptomatic and recovered grapevines from June to September, 2008. Bars represent standard errors.

From the end of June to the middle of July, the photosynthesis strongly decreased, with the recovered vines showing the lowest A values. In July, the behaviour of symptomatic and asymptomatic vines was very similar, with maintained good A values. In September, the photosynthesis was similarly reduced under all treatments, probably because the dry period and the high temperatures were excessive for any normal physiological activity. We can conclude from these data that recovered vines suffered the most because of water stress and high temperatures in the middle of the summer, while in September, all the vines reached the same stress status.

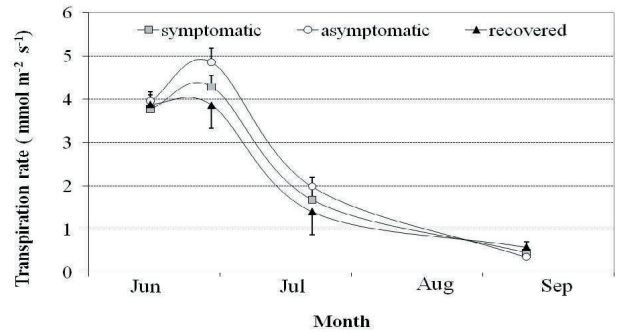


***Figure 3.*** Changes in transpiration rate measured in leaves of symptomatic, asymptomatic and recovered vines from June to September, 2008. Bars represent standard errors.

This behaviour can be ascribed to the partial uprooting used in the previous year to induce the recovery in BN infected vines. This involves the breaking of many absorbing roots and consequently a reduction in the root volume and the water uptake capacity in the recovered grapevines. To balance the water stress, the recovered vines reduced their transpiration rates (Fig. 3) earlier than was seen for the asymptomatic and symptomatic vines, progressively closing stomata from the end of June to September.

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# ABIOTIC STRESS AND TREATMENT WITH ELICITORS FOR THE CONTROL OF BOIS NOIR IN CHARDONNAY VINEYARDS

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***Summary***

To estimate the effectiveness of abiotic stress and field treatments with resistance inducers for the control of natural Bois noir (BN) infections, field trials were carried out in vineyards of cv Chardonnay located in the Marche and Sardinia regions, Italy. Partial uprooting was more efficient for recovery induction on cv Chardonnay grafted onto Kober 5BB than onto 420A rootstock. Field treatments with five commercial products based on chitosan, phosetyl-Al, two different mixtures of glutathione and oligosaccharines, and benzothiadiazole, promoted an increase in the incidence of recovered plants with respect to the controls. Even though the cv Chardonnay is considered one of the most susceptible varieties to BN infection, seemed to be induced to recovery status by abiotic stresses and treatments with resistance inducers.

## INTRODUCTION

Grapevine yellows (GY) are diseases that are caused by phytoplasma, which can result in severe loss of production and death of plants (Caudwell, 1961). After Flavescence dorée (FD), an A2 quarantine disease in the EPPO region, Bois noir (BN) is considered the most widespread disease in the main viticultural areas of the World (Boudon-Padieu, 2003). At present, there are no known effective means to reduce the incidence of phytoplasma diseases, and even preventive measures associated with treatments against vectors have effects that are not always clear for BN (Borgo & Angelini, 2002). Hence, the epidemiology and the control of BN infections are problematic. In the last decade, there have been several investigation into the mechanisms involved in the natural phenomenon of spontaneous symptom remission, known as "recovery". This event has been seen for different varieties and viticultural regions (Osler *et al*., 2003; Garau *et al*., 2004; Maixner, 2006; Romanazzi *et al*., 2007), and is related to climatic stress conditions (low rainfall and high temperatures) (Braccini & Nasca, 2008). Recovery can be induced by stressing the plants through their uprooting, followed by immediate transplanting (Osler *et al*., 1993; Romanazzi & Murolo, 2008). Agronomical practices such as pruning or pollarding can also promote this recovery (Borgo & Angelini, 2002; Zorloni *et al*., 2002; Credi *et al*., 2008). A further innovative possibility for the control of GY is spraying the plant canopy with resistance inducers (Romanazzi *et al*., 2009). Induced resistance is a non-specific form of disease resistance in plants that acts against a wide range of pathogens, and it can be activated by several non–specific inducers (elicitors). The aim of this study was to estimate the effectiveness of abiotic stress and field treatments with resistance inducers in the control of BN infections in the grapevine cv Chardonnay in two Italian regions, Marche and Sardinia.

## MATERIAL AND METHODS

*Abiotic stress.* In April 2006, grapevines cv Chardonnay grafted onto Kober 5BB (*Vitis berlandieri* x *V. riparia*) rootstock and onto 420A (*V. berlandieri* x *V. riparia*) rootstock symptomatically infected by BN, were subjected to partial uprooting, which was carried out by breaking parts of the roots using a small excavator. In April 2007, this treatment was performed on a new group of grapevines cv. Chardonnay grafted onto 420A rootstock. In each trial, five plants per treatment per year were analysed and five BN-infected plants were left as controls, to monitor for any eventual natural recovery.

*Field treatment with resistance inducers*. Five commercial products based on chitosan (ChitoPlant, ChiPro GmbH, Germany), phosetyl-Al (Aliette, Bayer Crop Science, Germany), two different mixture of glutathione and oligosaccharines (Kendal, Valagro, Atessa (CH), Italy and Olivis B2, Agrisystem, Lamezia Terme (CZ), Italy) and benzothiadiazole (Bion 50 WG, Syngenta Crop Protection, Switzerland), were dissolved in sterile deionized water, according to the manufacturer instructions (Tab. 1). These were sprayed on the canopies of selected BN infected grapevines cv Chardonnay. The treatments were carried out weekly from the beginning of June to the middle of July, 2007 (seven applications) and from the beginning of May to the beginning of August, 2008 (nine applications). Each treatment was applied to 35 plants in 2007 and about 15 plants in 2008. Both field trials were carried out in Marche and Sardinia, according to the same work plan. Moreover, the evaluation of disease symptoms was carried out at the end of July, August and September, 2006 and 2007, in both locations. The recovered plants were subjected to molecular analyses for the presence of phytoplasma (Lee *et al*., 1994).

## RESULTS AND DISCUSSION

Partial uprooting induced recovery in all of the five plants of cvs Chardonnay grafted onto the Kober 5BB rootstock. No disease symptoms were seen in these vines in the autumn of 2006 and 2007, whereas all of the control plants showed typical GY symptoms. No remission of

symptoms was seen in any of the BN-infected control plants. The same treatment applied to cv. Chardonnay grafted onto 420A rootstock in 2006 and 2007 proved to be less effective. Although both rootstocks are hybrid *V. berlandieri* x *V. riparia*, a possible interaction between the rootstocks and the effectiveness of this practice was seen, although the significance of this relationship remains to be clarified.

| Commercial product | Active ingredient | Preharvest interval (day) | Suggested application rates (kg or l/ha) | Suggested application time (day) | Registered for use on grapevine in Italy |
|---|---|---|---|---|---|
| Aliette | Phosetyl-Al | 40 | 2.5 kg/ha | 21 | Yes |
| Kendal | Glutathion + oligosaccharines | - | 2.5-3.0 l/ha | 5-10 | Yes* |
| ChitoPlant | Chitosan | - | 0.05-0.5 Kg/ha | n.a.[§] | No* |
| Bion | Acibenzolar-S-methyl | - | 0.15-0.2 Kg/ha | 7-14 | No* |
| Olivis B2 | Glutathion + oligosaccharines | - | 3-3.5 Kg/ha | 7-10 | Yes* |

***Table 1***. List and properties of the resistance inducers. Product not allowed as plant protectant in Italy; [§]information not available

In 2007, all of the treatments with resistance inducers increased the incidence of recovered plants with respect to the controls. In particular, during this year, which was characterized by sporadical rainfall from the end of winter until harvest with temperatures over the seasonal means, Bion, Olivis, Kendal and Aliette applications promoted the highest percentage of recovered plants, which were significantly ($P \leq 0.05$) different respect to untreated controls. An high rate of natural recovered vines was recorded (37.5%) as was seen by Braccini & Nasca (2008). In 2008, the only treatments to significantly ($P \leq 0.05$) reduce the incidence of symptomatic vines with respect to the untreated control were Kendal, Bion and Olivis, with a recovery rate more than 50%. In particular, Bion has also been shown to provide some protection and a delay in the syndrome on chrysanthemum infected by '*Candidatus* Phytoplasma asteris' (D'Amelio *et al*., 2007), and a protectant activity against X-disease phytoplasma and a reduced leafhopper survival when applied to *Arabidopsis thaliana* (Bressan & Purcell, 2005). In our trials, Phosetyl-Al and chitosan were not significantly different from each other across both of the years, differently from what was recorded in experiments carried out on phytoplasma infected *Catharanthus roseus* (Chiesa *et al*., 2007). Molecular diagnosis carried out on leaf veins from the recovered plants failed to find the phytoplasma, as also reported in BN infected cv. Chardonnay and Pinot noire after symptom remission (Osler *et al*., 2003; Morone *et al*., 2007) and in cv. Chardonnay, Sangiovese and Primitivo, vines recovered by partial uprooting and pulling (Romanazzi & Murolo, 2008). The data obtained over these study under field conditions and in two different locations (Marche and Sardinia) have allowed us to understand that even in cv Chardonnay, considered the most susceptible to BN infections, it is possible to induce recovery both by abiotic stress, where the rootstock can greatly affect the propensity to recover, and by treatment with resistance inducers, where the number and the period of applications appear to be fundamental.

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# REACTION OF DIFFERENT GRAPEVINE VARIETIES TO SUMMER PRUNING MEASURES FOR COMBATING BOIS NOIR

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***Summary***

Pruning measures in summer may be good tools for grapevines to recover from Boir Noir. But the effectivity of summer pruning measures depends on the variety and, by high infection pressure, makes only sense in combination with removing the host plants. Cabernet Dorsa needs to be totally pruned to recover successfully. On the other hand Chardonnay may have a chance to recover by using partial pruning. For Riesling and St. Laurent only a low number of test plants was available. Both varieties seem to recover better when totally pruned. The results presented in this report are in agreement with those of Stark-Urnau et al. (2008).

## INTRODUCTION

Since more than 25 years Boir noir (BN) disease is widespread in some areas of the German wine growing region 'Nahe', Rhineland-Palatinate. Here both types of the Stolbur phytoplasma (*tuf* type I and II) are found, sometimes in the same vineyard. In the wine growing region 'Palatinate' the BN disease is strikingly increasing since about four years. Here the Stolbur phytoplasma *tuf* type I is predominant. In the outer rows of infected vineyards the incidence rate of BN may reach up to 50 %.

At present only cultural techniques are possible for trying to combat BN. One is the elimination of diseased canes as soon as the first symptoms appear (summer pruning) to reduce or perhaps prevent the further spread of the bacteria. As a basis for advising recommendations pruning trials were started in 2006 and in 2007. The aim is to determine effects of different pruning measures on different grape varieties.

## MATERIAL AND METHODS

*Grapevine varieties*: Cabernet Dorsa 1 and 2 (Nahe region), Chardonnay, Riesling and St. Laurent (all in the Palatinate region), for details about rootstocks and planting year see table 1.

*Summer pruning measures*: Partial pruning: single shoots or parts of bows were eliminated if shoots at the end of the bow showed symptoms. Total pruning: the trunks of infested vines were cut back to about 30-40 cm height. The grapevines were cut as soon as the first symptoms appeared.

## RESULTS AND DISCUSSION

*Pruning experiments in the region 'Palatinate', variety Riesling:* The experiments started with 83 diseased grapevines which were until then only winter pruned (Figure 1). 22 % of these plants didn't show any symptoms in the following year without a special pruning measure. 77 % of the plants with symptoms in 2007 were pruned partially. 1 % was cut back totally to the trunk. 39 % of the plants without any symptoms in 2007 didn't show any symptoms in 2008 as well: the spontaneous "recovery" rate reached 8.4 %. But it has to be taken into account, that the plants were just 2 years without any symptoms. Actually 3 years are regarded as the time period for being cured. None of the plants completely pruned in 2007 showed any symptoms one year later. 9.4 % of the plants partially pruned in 2007 were without any symptoms in 2008. More than 81 % had to be partially pruned again in 2008 and 9.4 % to be pruned totally. In 2007 20 vines showed symptoms for the first time. 10 % of these plants were without any symptoms one year later and 90 % had to be pruned partially.

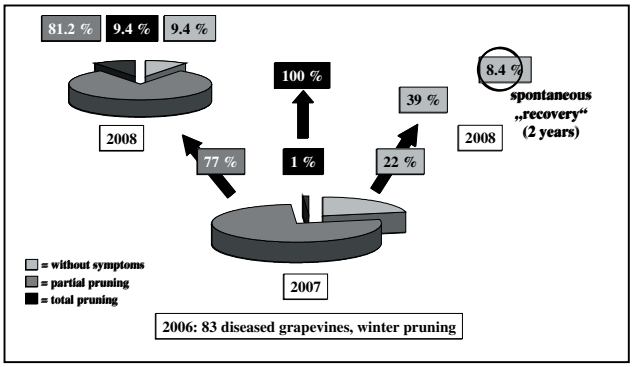


***Figure 1.*** Riesling, pruning results, date: 30.09.2008

*Pruning experiments in the region 'Palatinate', variety Chardonnay and St. Laurent:* The results obtained with Chardonnay and St. Laurent were similar to those with Riesling and are summarized in table 1. Chardonnay was the only variety which showed a relatively high recovery rate in both variants (partial pruning, total pruning).

Partial pruning was not very successful for the varieties Riesling and St. Laurent. For both total pruning seems to be more effective but it has to be considered, that only a very small number of both varieties could be tested. Compared to 2007 in all three varieties the rate of newly infected plants was relatively low in 2008. However in the adjacent areas of the Riesling- and Chardonnay-sites having a high incidence rate/infection pressure, the host plant stinging nettle was treated with herbicides. In the other vineyard no herbicides were applicated. By combining 'summer pruning' and 'use of herbicide' the incidence rate in the Chardonnay vineyard decreased from 26.4 % to 18.7 % (Table 1).

***Table 1.*** Summary of the pruning measure results with four different grape varieteis
* = number of healthy plants/number of pruned plants

| | Date: 30 September 2008 | | | | | | Date: 06 August 2008 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Riesling** | | **Chardonnay** | | **St. Laurent** | | **Cab. Dorsa 1** | | **Cab. Dorsa 2** | |
| **rootstock** | **SO4** | | **SO4** | | **SO4** | | **Binova** | | **125 AA** | |
| **year of planting** | **2002** | | **2000** | | **2002** | | **2002** | | **2000** | |
| | **number** | **%** | **number** | **%** | **number** | **%** | **number** | **%** | **number** | **%** |
| **number of plants** | **368** | | **235** | | **1236** | | **603** | | **1112** | |
| spontanous recovery (3 yrs without symptoms) | | | | | 6/44* | 13.6% | | | | |
| spontanous recovery (2 yrs without symptoms) | 7/83* | 8.4% | 3/38* | 7.9% | | | 5/17* | 29.4% | | |
| "Recovery" after **partial pruning** | 8/84* | 9.5% | 17/44* | 38.6% | 6/52* | 11.5% | 8/49* | 16.3% | 9/32* | 28.1% |
| "Recovery" after **total pruning** | 1/1* | 100% | 14/40* | 35.0% | 6/8* | 75% | 54/54* | 100% | 107/107* | 100% |
| | | | | | | | | | | |
| **newly infected plants** | | | | | | | | | | |
| 2006 | | | | | 9 | 0.7% | | | | |
| 2007 | 24 | 4.0% | 11 | 4.7% | 21 | 1.7% | 24 | 4.0% | | |
| 2008 | 4 | 1.1% | 4 | 1.7% | 6 | 0.5% | 30 | 5.0% | 259 | 23.3% |
| | | | | | | | | | | |
| **Incidence rate** | | | | | | | | | | |
| 2005 | | | | | | 3.6% | | | | |
| 2006 | | 22.6% | | 26.4% | | 3.7% | | 10.1% | | |
| 2007 | | 23.1% | | 25.5% | | 4.9% | | 8.9% | | 13.2% |
| 2008 | | 24.7% | | 18.7% | | 4.6% | | 7.1% | | 36.7% |

*Pruning experiments in the region 'Nahe':* The results concerning the variety 'Cabernet Dorsa' (2 vineyards) show that only total pruning to 30 cm trunk height offers a promising chance for the diseased plants to recover (Figure 2). All plants totally pruned (vineyard 1: 54 plants in 2006 and 2007; vineyard 2: 105 plants in 2007) were vigorously and without any symptoms up to September 2008. Unlike the plants partially pruned: 83.7 % of the plants pruned in 2006 and 2007 showed BN symptoms again in September 2008. 16.3 % of the pruned plants were without any symptoms up to September 2008. The results of the second Cabernet Dorsa-vineyard in the 'Nahe' region were similar to the results obtained in the first one, though the number of plants totally pruned was higher compared to the first one (Table 1).

Summarizing the results obtained with Cabernet Dorsa in the 'Nahe' region, it can be seen that partial pruning led to a low recovery rate (16 % - 28 %). Total pruning was very successful reaching a recovery of 100 %. For Cabernet Dorsa total pruning seems to be the only view of prospect. Alarming was the fact that in 2008 a very high level of newly infected plants was determined in the second Cabernet Dorsa vineyard: the rate reached more than 23 %. The infestation rate increased to more than 36 %. Due to the extremely high incidence rate this vineyard should be uprooted though it was planted only 5 years ago.

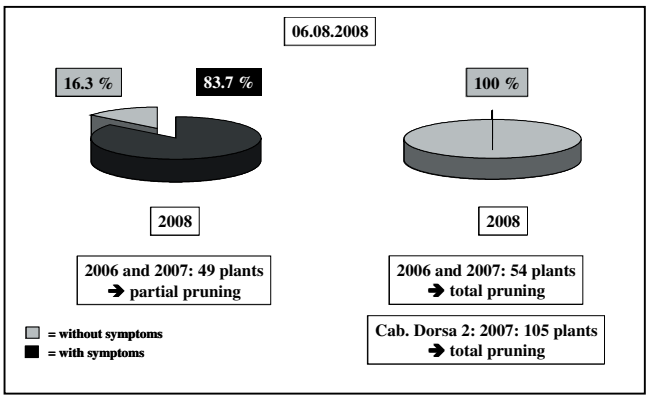


***Figure 2.*** Cabernet Dorsa, vineyard 1, pruning results, date: 06.09.2008

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# CAN PRUNING MEASURES COMBAT BOIS NOIR DISEASE OF GRAPEVINE ?

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## INTRODUCTION

During the last years inspections revealed a significant increase of Bois Noir in Austrian vineyards. Many vines show disease symptoms each year, but often spontaneous permanent recovery is observed (Osler *et al.,* 1993). Recovered vines carry normal bunches and produce satisfactory yields. Knowledge about the effect of pruning practices on recovery rates is essential for practical viticulture.

## MATERIAL AND METHODS

Bois noir infected vines (10 varieties, grafted on various rootstocks) were visually categorized into classes of symptoms according to severity. In the ensuing winter plants were either pollarded 15 cm above the graft union, or cane pruned or spur pruned. In the following three years the vines in all treatment groups were cane pruned and visually inspected. Data were evaluated by Chi squared test, Kruskal Wallis test and logistic regression analysis with the aid of the statistics programs SPSS 12.0 and Statgraphics Centurion Version XV.

## RESULTS AND DISCUSSION

Pollarding resulted in significantly higher recovery rates (yearly average 62-84%) in the following vegetation period than cane pruning (yearly average 29-49% in the proximate vegetation period). These results are widely in accordance with previously published experiments (Credi *et al.*, 2008, Ipach *et al.*, 2008, Stark-Urnau & Kast 2008). Reducing canes to spurs was statistically indistinguishable from cane pruning.

The number of vines that perished due to pollarding was negligible.

Pruning also significantly influenced recurrence rates of already recovered vines. In case of pollarding 86 % of these vines stayed free of Bois Noir symptoms during the next years, in case of cane pruning only 54%. The observed relations between recovery or recurrence and severity of Bois noir symptoms allow the conclusion that removal of most of the pathogens is the crucial point both for immediate and persistent success of pruning measures.

Disease development and recovery were related to the variety. This was also the case for pollarded vines although the majority of the vinifera-parts had been removed. Assumedly in some varieties spread of the pathogen to the rootstock proceeded faster than in others.

Plant age was a parameter influencing recovery rate of pollarded vines. It seems likely that spread of the pathogen proceeded faster in young plants.

Last but not least our analyses related recovery to the year of observation regardless of the pruning method.

From a practical point of view our study shows that pollarding of vines approximately 15 cm above the graft union can have an important share in sanitation of Bois noir infected vines. Apart from additional training and tying work, however, pollarding generates large pruning wounds. These might entail an increased rate of fungal infections, e.g. with Esca disease. Measures against such infections are obligatory in good viticultural practice.

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# *ANACERATAGALLIA RIBAUTI* (OSS., 1938) (HEMIPTERA, AUCHENORRHYNCHA, AGALLIINAE) TRANSMITS A STOLBUR TYPE PHYTOPLASMA

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## INTRODUCTION

Bois Noir disease induced by stolbur phytoplasma (tuf type II) is widespread in Austrian vineyards. Up to now only the Cixiidae *Hyalesthes obsoletus* (Hemiptera, Auchenorrhyn-cha) has been identified as field vector of Bois noir disease (Maixner *et al*., 1995, Sforza *et al*., 1998). In many parts of Austria, however, this species has not been found or found at very low population densities although simultaneously Bois Noir incidence is high (Riedle-Bauer *et al*., 2008). The aim of the present work was to study several Auchenorrhyncha species for their ability to transmit stolbur phytoplasma.

## MATERIAL AND METHODS

Adult insects belonging to 20 different Auchenorrhyncha species were collected alive in bois noir infected vineyards and transferred to *Vicia faba*, *Daucus carota* and *Convolvulus arvensis* seedlings or rooted grapevine cuttings in pot cages. Several insect species were reared in the laboratory (*Anaceratagallia ribauti*, *Dryodurgades reticulatus, Euscelis incisus, Laodelphax striatella, Macrosteles* spp.). Hatching nymphs were transferred to breeding cages containing potted *Vicia faba*, *Hordeum vulgare* and *Convolvulus arvensis* seedlings and stolbur-infected *Convolvulus arvensis* plants collected in the vineyard. Adults raised in these breeding cages were subjected to further pot cage experiments. All plants from pot cages and from breeding cages were inspected visually and analyzed by PCR.

## RESULTS AND DISCUSSION

In 5 out of 40 transmission trials with field captured *Anaceratagallia ribauti, Vicia faba* plants developed visible symptoms. These were rolling of leaves, yellowing and stunting of the plants. PCR with primers P1/P7 and STOLF/STOLR and analysis of the tuf-gene (Langer & Maixner 2004) confirmed the presence of stolbur phytoplasma. Laboratory reared *Anaceratagallia ribauti* leafhoppers transmitted stolbur phytoplasma to 7 out of 23 *Vicia faba* plants in the breeding cages. Laboratory raised *Anaceratagallia ribauti* adults transferred to healthy seedlings transmitted stolbur phytoplasma in 1 out of 13 experiments. No stolbur transmissions were observed during the experiments with any other Auchenorrhyncha species.

Our results demonstrate that not only Cixiidae planthopper species are involved in transmission of stolbur phytoplasma. To our knowledge this is the first time that an Agalliinae leafhopper was found to transmit this pathogen. Transmission efficiency of *Anaceratagallia ribauti* is low. Only in 4 out of 40 experiments with more than 400 field-trapped specimen transmission was ascertained.

Presently we have no successful transmission trials with crop species except *Vicia faba*. Experiments concerning the ability of *Anaceratagallia ribauti* to transmit Stolbur phytoplasma to field bindweed and grapevine are under way.

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# VECTOR–PHYTOPLASMA RELATIONSHIPS DURING NATURAL INFECTION OF *HYALESTHES OBSOLETUS*, *EUSCELIS LINEOLATUS*, *NEOALITURUS FENESTRATUS* AND *PSAMMOTETTIX ALIENUS* CAPTURED IN VINEYARD AGRO-ECOSYSTEMS IN THE MARCHE REGION (CENTRAL-EASTERN ITALY)

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***Summary***

Grapevine yellows (GY) associated with phytoplasma constitute a serious phytosanitary problem in many Italian regions. GY spread involves interactions between the vector and the reservoir plants of the phytoplasma. The objective of this study was to determine the role of the planthopper *Hyalesthes obsoletus* in the diffusion of Bois noir (BN) and other GY, as also for the leafhoppers *Euscelis lineolatus*, *Neoaliturus fenestratus* and *Psammotettix alienus*, which are widespread in the Marche Region and which are known to be, or are under investigation as, phytoplasma vectors. In 2007 and 2008, in vineyard agro-ecosystems affected by GY diseases, a total of 1,069 leafhopper and planthopper specimens were collected and analyzed. Each of the insect species was infected with phytoplasma. The phytoplasma infection levels varied according to the vineyard agro-ecosystem and the insect species, and they increased through the season. PCR-restriction fragment length polymorphism (RFLP) analysis detected phytoplasma belonging to subgroups 16SrI-B, 16SrI-C and 16SrXII-A (BN) in *E. lineolatus*, 16SrI-B in *P. alienus* and 16SrI-B and 16SrI-C in *N. fenestratus*. *H. obsoletus* showed only the 16SrXII-A subgroup. No X-disease and phytoplasma associated to the 16SrV group were detected in any of the specimens. The inoculation assays showed the ability of the insect species analyzed to inoculate the phytoplasma, detected in their bodies, into the artificial feeding medium.

## INTRODUCTION

Grapevine yellows (GY) are caused by phytoplasma and are found in many of the areas of the world where grapevines are grown. In Italy, GY constitute a serious phytosanitary problem. The phytoplasma originated from Gram-positive bacteria and are prokaryotes and obligate symbionts of plants and insects. They need both of their hosts for dispersal in nature. In Italy in particular, Bois noir (BN) and Flavescence dorée (FD) can cause severe economic damage to grapevine production. In central Italy, BN is spread throughout the viticulture areas, while the phytoplasma of the 16SrI, 16SrIII and 16SrV groups have been found sporadically in GY-diseased grapevines. The symptoms caused on the grapevines by different GY are similar, while they show considerable differences in epidemiology due to the different life histories of their respective vectors (Boudon-Padieu, 2003). The natural vector of BN (16SrXII-A) is *Hyalesthes obsoletus* Signoret (Hemiptera, Auchenorrhyncha, Fulgoromorpha, Cixiidae) (Maixner *et al*., 1995; Sforza *et al*., 1998), but other leafhopper and planthopper species might also have epidemiological implications in the spread of BN and other GY diseases. The epidemiology of a phytoplasma-induced disease is significantly influenced by the dispersal biology of their vectors and by the host spectrum of both vectors and phytoplasma (Weintraub & Beanland, 2006). The spread of a vector is affected by different environmental aspects, such as temperature, humidity, soil type, and presence of host plants (Boudon-Padieu & Maixner, 2007; Johannesen *et al*., 2008). Phytoplasma transmission by insect vectors is an active process, with several key events involved; indeed, detection of phytoplasma in insects does not necessarily indicate that the insect is inoculative. A prerequisite to transmission by the insect vector is the passage of the phytoplasma through the wall of the intestinal tract, its multiplication in the haemolymph, and its passage into the salivary glands, where it further multiplies before it is injected into the phloem tissue of the host plant (Ammar & Hogenhout, 2006). Biological transmission assays are the only way to ascertain phytoplasma transmissibility. The feeding medium inoculativity assay is an alternative to biological transmission assays, and it can be used on a large scale to determine insect inoculativity (Tanne *et al*., 2001). In this study, we have analyzed the role of *H. obsoletus* in GY diffusion, as well as the roles of other leafhoppers: *Euscelis lineolatus*, *Neoaliturus fenestratus* and *Psammotettix alienus* (Hemiptera, Auchenorrhyncha, Cicadomorpha, Cicadellidae). These are known to be, or are under investigation as, phytoplasma vectors (Weintraub & Beanland, 2006), and are widespread in vineyard agro-ecosystems in the Marche Region (central-eastern Italy).

## MATERIAL AND METHODS

The vector–phytoplasma relationship was studied in relation to the phytoplasma associated with BN and other GY. This study was carried out during 2007 and 2008 in four different vineyard agro-ecosystems in the Marche Region that were affected by GY diseases: Castelferretti (AN), Morrovalle (MC), Serrapetrona (MC) and Agugliano (AN). Adult insect species were captured in the rows and along the borders of the vineyards on the wild herbaceous plants, from the beginning of May to the beginning of September. With the aim of describing the passage of the phytoplasma through the insect body, inoculation assay tests were performed using an artificial feeding medium, according to the methods proposed by Tanne *et al*. (2001), with some minor modifications.

*DNA isolation.* Total DNA was obtained from single insect specimens according to the modified cetyl-trimethylammonium bromide (CTAB) method proposed by Murray and Thompson (1980). DNA was extracted from the Tris/EDTA (TE) sucrose feeding medium of the phytoplasma-positive insects following a procedure described by Zhang *et al*. (1998).

*Phytoplasma detection and identification* were performed using direct PCR amplification with the P1/P7 universal phytoplasma primer pair, followed by nested PCR (Lee *et al*., 1994), and if necessary, restriction length fragment polymorphism (RFLP) analysis, to detect and identify the phytoplasma associated with Stolbur (16SrXII-A subgroup), 16SrV, 16SrI and 16SrIII groups.

***Table 1*** Insect species captured in the Castelferretti agro-ecosystem vineyard in 2007 and PCR for phytoplasma detection in the bodies of the insects captured from early May to early September. T=Total captured; I= Infected percentage.

| | CASTELFERRETTI (AN) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *H. obsoletus* | | *E. lineolatus* | | *N. fenestratus* | | *P. alienus* | |
| | ***T*** | ***I*** | ***T*** | ***I*** | ***T*** | ***I*** | ***T*** | ***I*** |
| ***May 11*** | - | - | 23 | *4.3* | - | - | - | - |
| ***May 22*** | - | - | 30 | *6.7* | 11 | *9.1* | - | - |
| ***June 6*** | - | - | 29 | *3.4* | 1 | - | - | - |
| ***June 21*** | - | - | 19 | *15.8* | - | - | 18 | *11.1* |
| ***July 2*** | 6 | *16.7* | 16 | *37.5* | 24 | *16.7* | - | - |
| ***July 15*** | 11 | *45.5* | 9 | *55.5* | - | - | - | - |
| ***August 21*** | - | - | 6 | *0* | 15 | *6.7* | - | - |
| ***September 4*** | - | - | - | - | 22 | *18.2* | - | - |
| *TOTAL* | **17** | ***35.3*** | **132** | ***13.6*** | **73** | ***13.7*** | **18** | ***11.1*** |

## RESULTS AND DISCUSSION

A total of 1,069 leafhopper and planthopper specimens (525 *E. lineolatus*, 367 *H. obsoletus*, 96 *N. fenestratus* and 81 *P. alienus)* were collected and analyzed. Each insect species was infected with phytoplasma. PCR analysis of insect bodies showed mean phytoplasma detection of 26.7% for *E. lineolatus*, 17.9% for *H. obsoletus*, 13.5% for *N. fenestratus* and 6.2% for *P. alienus*. The infection levels changed according to the vineyard agro-ecosystems and the insect species, and they increased during the season (Table 1). PCR-RFLP analysis detected phytoplasmas belonging to subgroups 16SrI-B, 16SrI-C and 16SrXII-A in *E. lineolatus*, 16SrI-B in *P. alienus*, 16SrI-B and 16SrI-C in *N. fenestratus*. *H. obsoletus* showed only the 16SrXII-A subgroup (Figure 1). No X-disease and 16SrV group phytoplasma were detected in any of the analyzed specimens. *E. l*ineolatus was the more constantly present in the agro-ecosystems considered, but the phytoplasma infection that was also seen for *N. fenestratus* and *P. alienus* does not allow their exclusion from roles as vectors of phytoplasma disease. *E. lineolatus* showed an aspecific relationship with phytoplasma, indeed more subgroups of phytoplasma were detected in this species, and some specimens had mixed phytoplasma infection. In contrast, *H. obsoletus* showed a specific phytoplasma relationship: only the 16SrXII-A subgroup was identified in this species. Inoculation assays showed the ability of *H. obsoletus*, *E. lineolatus*, *N. fenestratus* and *P. alienus* to inoculate the phytoplasma detected in their bodies, to feeding medium. Moreover, the feeding medium assays showed different effects on survival of *E. lineolatus* and *H. obsoletus* according to the presence of phytoplasma in their bodies. The data presented in this study reveal the main roles in phytoplasma spreading and transmission to grapevine that these Auchenorrhyncha insect vectors appear to have in GY diseases. In particular, the epidemiology of these phytoplasma-induced diseases is significantly influenced by vector biology, ecology and host plants that inhabit the whole agro-ecosystem. This work contributes to improve knowledge of insect-vector and phytoplasma relationship useful in further epidemiological studies of GY in central-eastern Italy vineyard agro-ecosystems.

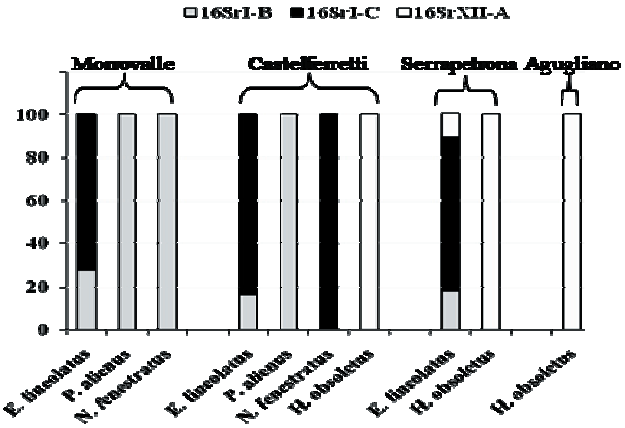


***Figure 1.*** Phytoplasma subgroup distribution (%) as identified in 2007 by PCR-RFLP analysis in insect bodies according to the monitored localities.

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# FURTHER DATA ON OCCURRENCE OF GRAPEVINE YELLOWS-ASSOCIATED PHYTOPLASMAS IN VINEYARDS OF VENETO REGION (NORTH-EASTERN ITALY)

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***Summary***

Results from a preliminary survey on grapevine yellows diseases in twelve vineyards in Veneto region (north-eastern Italy) underscored by PCR-RFLP based analyses the prevalence (64%) of infections by Bois noir-associated phytoplasmas. Data suggested the necessity of investigating more accurately the intriguing epidemiology of grapevine yellows diseases in Veneto region, in order to design appropriate control strategies.

## INTRODUCTION

Grapevine yellows (GY) diseases occur in widespread viticultural areas of the world, where they are characterized by similar symptoms in diseased plants and are attributed to infections by no less than five distinct phytoplasma species (Prince *et al.*, 1993; Davis *et al*. 1997). In Italy, two of these GY diseases are particularly spread: (i) Flavescence dorée (FD), associated with plant infection by strains of 16SrV phylogenetic group, classified in rDNA RFLP subgroup 16SrV-C and V-D; (ii) Bois noir (BN), attributed to stolbur phytoplasma strains, classified in rDNA RFLP subgroup 16SrXII-A, XII-F, and XII-G (Lee *et al*. 1998; Quaglino *et al*., 2009). The impressive biological complexity (insect vectors, alternative plant hosts) of putative GY causal agents has stimulated research on molecular markers for specific detection and identification, in order to develop appropriate control strategies. Recently, a large survey on phytoplasmal diseases affecting grapevines in Italy underscored through PCR-RFLP-based analyses the prevalence (72%) of '*Ca*. Phytoplasma solani' in affected vineyards (Botti & Bertaccini, 2007). In the present work, a preliminary study was carried out for evaluating the distribution and the impact of FD and BN diseases in twelve vineyards in Veneto region (north-eastern Italy), including three nurseries.

## MATERIALS AND METHODS

Field surveys on the incidence of GY disease were carried out from June to September of 2008 in 12 vineyards located in Veneto, north-eastern Italy. Leaf samples were collected from 62 GY-symptomatic grapevine plants randomly selected in vineyards. Detection of phytoplasmas associated with FD and BN diseases was carried out by PCR-RFLP analyses of 16S rDNA nucleotide sequences as previously described (Deng & Hiruki, 1991; Lee *et al.*, 1998; Davis & Dally, 2001). Determination of 16SrV subgroups and of stolbur (16SrXII) *tuf* (VK) genetic lineages were performed by using PCR-RFLP assays as described by Martini *et al.* (1999) and by Langer & Maixner (2004), respectively.

## RESULTS AND DISCUSSION

*Grapevine yellows-associated phytoplasmas in vineyards in north-eastern Italy:* PCR-based amplification allowed to detect the presence of phytoplasmas in 73% (45/62) of analyzed grapevine leaf samples. *MseI*- and *TaqI*-RFLP patterns, obtained by digestions of 16S rDNA amplicons, underscored the prevalence of '*Ca.* Phytoplasma solani' (16SrXII-A) among GY-associated phytoplasma isolates, here identified (64%, 29/45) (Table 1). These data are in agreement with a recent study reporting the wide prevalence of BN phytoplasmas in Veneto region (Botti & Bertaccini, 2007). Even BN phytoplasmas were prevalent in Veneto, FD-D (16SrV-D) phytoplasmas were detected in more grapevine plants in three vineyards in the provinces of Verona (Lazise) and Venezia [(Jesolo and San Donà in Piave (II)]. FD-C (16SrV-C) was identified only in three grapevine plants. Interestingly, '*Ca.* Phytoplasma asteris' (16SrI-B) infected two plants in a vineyard in the Verona province [(San Pietro di Lavagno (II)]; in the same vineyard, 16SrI-B phytoplasma subgroup was detected together with BN phytoplasma in one plant.

*Genetic diversity and distinct ecological niches of GY-associated phytoplasmas:* Three natural ecologies of BN phytoplasmas have been described: (1) the host system *C. arvensis* - *H. obsoletus* - *V. vinifera*, (2) the host system *U. dioica* - *H. obsoletus* - *V. vinifera*, and (3) the host system *C. sepium* - *H. obsoletus* - *V. vinifera* (Maixner *et al.*, 1995). In the present study, molecular characterization of BN phytoplasma isolates underscored the prevalence (19/29) of *tuf* genetic lineage VK-I; on the other hand, VK-II genetic lineage was prevalent in two vineyards in the province of Verona (Peschiera del Garda and Roncà) (Table 1). Since *tuf* lineages were consistently associated with different herbaceous hosts: VK-I with *Urtica dioica* L., VK-II with *Convolvulus arvensis* L. in other geographical region(s) (Langer & Maixner, 2004), these data raise the possibility that in Veneto BN epidemiology involves the co-existence of VK strain lineages in two ecological niches, one (prevalent in ten vineyards) involving the host plant *U. dioica*, and the other one (prevalent in two vineyards of Verona province) involving *C. arvensis*.

***Table 2.*** Occurrence of GY phytoplasmas in vineyards in Veneto region (north-eastern Italy).

| Province | Vineyard | Host | No. of samples | No. of PCR-positive samples | RFLP results[a] | |
|---|---|---|---|---|---|---|
| | | | | | 16S rDNA | *tuf* |
| Verona | Porto S. Pancrazio | rootstock 420A | 1 | - | - | - |
| | | rootstock 41B | 1 | - | - | - |
| | Lazise | Corvina | 5 | 5 | V-D | |
| | S. Pietro di Lavagno (I) | Chardonnay | 7 | 6 | XII-A | VK-I (5); VK-II (1) |
| | S. Pietro di Lavagno (II) | Chardonnay | 4 | 4 | XII-A | VK-I (4) |
| | | | 2 | 2 | I-B | AY (2) |
| | | | 1 | 1 | XII-A/I-B | VK-I/AY (1) |
| | Roncà | Chardonnay | 5 | 5 | XII-A | VK-II (4); VK-I (1) |
| | Peschiera del Garda | Trebbiano | 3 | 2 | XII-A | VK-II (2) |
| Venezia | Jesolo | Chardonnay | 1 | 1 | XII-A | VK-I (1) |
| | | Merlot | 1 | 1 | V-D | - |
| | | Pinot grigio | 1 | 1 | V-D | - |
| | San Donà in Piave (I)[b] | rootstock 420A | 2 | - | - | - |
| | San Donà in Piave (II)[c] | Merlot | 3 | 3 | V-D | - |
| | | Raboso Piave | 3 | 2 | V-D | - |
| | | | 2 | 2 | XII-A | VK-I (2) |
| | | Raboso Veronese | 1 | 1 | XII-A | VK-II (1) |
| Treviso | Breda di Piave | Pinot grigio | 4 | 2 | XII-A | VK-I (2) |
| | | | 1 | 1 | V-D | - |
| | Salgareda | Pinot nero | 10 | 2 | XII-A | VK-I (2) |
| | | | 1 | 1 | V-C | - |
| | | | 1 | 1 | XII-A/V-C | VK-I (1) |
| | Castello di Godega | Chardonnay | 1 | 1 | XII-A | VK-I (1) |
| | | | 1 | 1 | V-C | - |

[a]numbers of samples are given within parentheses; [b]rootstock mother vines; [c]mother vines

Interestingly, both 16SrV (-C and –D) and stolbur (16SrXII-A) phytoplasmas were identified in a nursery of mother vines (San Donà di Piave, Venezia), where the healthy sanitary status of plant propagation materials would be guaranteed. In fact, the presence of quarantine pathogens, such as FD phytoplasmas, in mother vines could determine strong epidemics of the disease in case insect vector populations are not controlled by insecticide treatments. Future studies will be focused on broad surveys on diffusion of GY diseases in Veneto regions. On the basis of encouraging preliminary studies (data not shown), accurate investigation of alternative host plants and of insect vectors will be carried out for underscoring the complex ecologies of BN and FD diseases in Veneto region.

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# SURVEY OF PHYTOPLASMA DIVERSITY IN HEAVILY GRAPEVINE YELLOWS - AFFECTED AREAS OF CROATIA

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***Summary***

In the period 2006-2008, six locations from Eastern Slavonia, Međimurje and Istria (Croatia) were assessed to be the most heavily affected by grapevine yellows and potentially under the most infective pressure from Flavescence Dorée phytoplasmas. Only Bois Noir (16SrXII-A) phytoplasmas were identified in 27 out of 49 symptomatic vines by PCR-RFLP and triplex real-time PCR assays. The neighboring spontaneous plants and entomofauna were sporadically tested and two *Clematis vitalba* plants from different regions were found infected by phytoplasmas from the 16SrV group.

## INTRODUCTION

Phytoplasmas of Flavescence Dorée (FD) and Bois Noir (BN) types are the most important Grapevine Yellows (GY) agents in Europe with the occasional findings of Aster Yellows (AY) phytoplasmas. Surveys of GY in Croatia are being conducted since 1997 (Šarić *et al.,* 1997) and only BN and AY phytoplasmas have been confirmed in GY affected vines. Since the finding of FD vector *Scaphoideus titanus* Ball (Budinščak *et al.,* 2005) and the 16SrV-C phytoplasma in the *Clematis vitalba* L. in the country (Filippin *et al.,* 2007), the awareness of the FD threat has been raised. Limited surveys of the vineyards assessed to be the most heavily affected by GY, the neighboring spontaneous plants and entomofauna were conducted with the aim of identifying areas under the most infective pressure by FD phytoplasmas.

## MATERIAL AND METHODS

In the period 2006-2008, leaves of 49 symptomatic vines were collected from locations in three regions (Istria, Međimurje, Eastern Slavonia) with the highest incidence of GY. In addition, 6 *C. vitalba*, 9 weed and 3 *S. titanus* samples were analyzed. Total nucleic acids were extracted following the procedures described in Mikec *et al.* (2006). The amplification of phytoplasma 16S rRNA gene was performed in a direct PCR using R16F1/R0 phytoplasma universal primers (Lee *et al.,* 1995). Nested PCRs were performed by using universal R16F2n/R2 (Lee *et al.,* 1993) as well as group-specific primers R16(I)F1/R1 and R16(V)F1/R1 (Lee *et al*., 1994) as described by Šeruga *et al*. (2000). Amplicons from all nested PCR assays were subjected to digestion with restriction endonuclease *Tru*9I for 16 h at 65°C. Obtained fragments were separated by electrophoresis through 5% polyacrylamide gel, stained with ethidium-bromide and their patterns compared with standard phytoplasma strains PO (16SrXII-A), EY-C (16SrV-A), FD70 (16SrV-C) and FD92 (16SrV-D) (Bertaccini, 2003). The *C. vitalba* sample from 2006, as well as all the samples from 2008 were simultaneously tested for the FD and BN phytoplasma presence by triplex real-time PCR procedure (Pelletier *et al.,* 2009).

## RESULTS AND DISCUSSION

*Identifying sampling areas:* The incidence of GY was visually monitored since 2002 and vineyard entomofauna since 2003. The county of Eastern Slavonia (Ilok, Kneževi Vinogradi) with the epidemiological GY spread identified earlier (Ćurković Perica *et al.,* 2003) was included in this survey, as well as Međimurje (Štrigova, Železna Gora) and Istria (Novigrad, Buje) primarily for the increased numbers of symptomatic vines in recent years. These locations also border with Serbian, Hungarian and Slovenian areas where FD phytoplasmas and/or *S. titanus* vectors were identified (Seljak, 1987; Duduk *et al*., 2004; Magud & Toševski 2004; Seljak & Orešek 2007; Dér *et al*., 2007; Kuzmanović *et al.,* 2008). The populations of *S. titanus* in Novigrad and Ilok were the most abundant in the monitored areas. The lack of insecticide treatment against the grape moths in these vineyards probably influenced the insect abundance in the survey period. For the above reasons, 6 denoted localities in 3 regions were chosen for phytoplasma diversity pilot study as potentially the most endangered by FD.

*Phytoplasma identification:* Over the whole 3-year period, out of 49 vines used for phytoplasma identification, 27 harbored BN phytoplasmas (16SrXII-A) with equal distribution of infected plants among the three regions. For the 2008 grapevine samples, in addition to the PCR-RFLP detection based on the 16S rDNA, the identity of these phytoplasmas was confirmed by sensitive triplex real-time PCR procedure able to detect the presence of BN-P and FD-P *map* genes (Pelletier *et al*., 2009). FD-P phytoplasmas (16SrV-C and -D) have not been detected in the Croatian grapevines, so far. No mixed infections of FD-P and BN-P have been detected either. All herbacious weeds tested here, including *Convolvulus arvensis* and *Calystegia sepium*, were found negative for the phytoplasma presence. Two out of six *Clematis* samples were found positive for the presence of FD-P phytoplasma both in the PCR-RFLP (Figure 1) and real-time PCR assays. One of the positive *C. vitalba* plant was sampled in Železna Gora (Međimurje) in 2008 and the other in Novigrad (Istria) in 2006. In Istria, the presence of 16SrV-C has already been reported in the same plant species as well as througout the northern Italy, Slovenian littoral and further to the south-east of the Balkans (Filippin *et al*., 2007). The insects from Novigrad

were sampled only sporadically and only 1 out of 3 *S. titanus* was found to carry phytoplasmas but of the AY type (16SrI-B).

Even though we report here rather limited survey results on the 16SrXII-A and 16SrV phytoplasma detection, it is apparent that the main phytoplasma associated with GY in all three most affected Croatian viticultural regions is still of BN type. This study has given no evidence that the colonization of vineyards by *S. titanus* in the Far West (Novigrad) and Far East (Ilok) of the country, has resulted yet in the epidemic transmission of FD-P phytoplasmas. However, the geographical occurrence of the vector infestation along the border with the epidemic FD regions in Serbia (Kuzmanović *et al*., 2007; 2008) calls for implementation of constant phytoplasma diversity monitoring in that region. In addition, identification of 16SrV group phytoplasmas in *C. vitalba* from heavily GY affected vineyards of Novigrad and Železna Gora, on the south-west and north-east Slovenian border, respectively, may be significant for determining 16SrV phytoplasma diversity and epidemiology (Arnaud *et al*., 2007). Further analyses of phytoplasmas detected in these two *Clematis* samples are needed in order to address these questions.

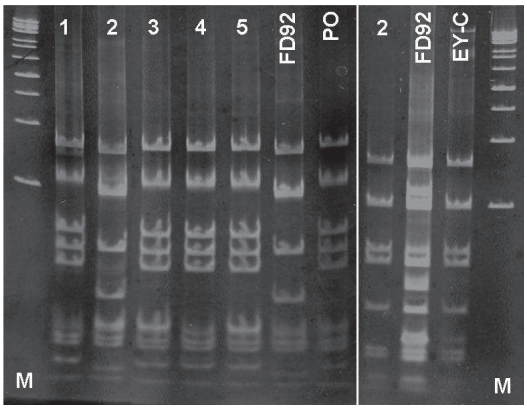


***Figure 1.*** RFLP profiles of R16F2n/R2 (left) and R16(V)F1/R1 amplicons (right) obtained by restriction with *Tru*9I. M-1kbp marker; 1, 3, 4, 5 – various grapevine samples; 2 - *Clematis vitalba* sample from Železna Gora; standard phytoplasma strains PO (16SrXII-A), EY-C (16SrV-A), and FD92 (16SrV-D).

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# IDENTIFICATION OF STOLBUR-RELATED PHYTOPLASMAS IN GRAPEVINE SHOWING DECLINE SYMPTOMS IN IRAN

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***Summary***

This study was carried out in the vineyards of North East of Iran in two separate locations during 2007-2008. Molecular analyses of nucleic acid extracted from leaf midribs and petioles of plants showing a decline syndrome allow the identification of phytoplasmas related to stolbur that could be differentiated from any other stolbur strain reported in grapevine or in other hosts by RFLP and sequencing analyses of 16Sr RNA gene. This is the first report of phytoplasmas infecting grapevine in Iran.

## INTRODUCTION

Phytoplasma associated diseases are reported in the majority of the viticulture areas worldwide where they are associated with yellows type symptoms (Boudon-Padieu, 2003). In this study two separate grapevine growing areas respectively in the North and in the South of the Khorassan province located in North East of Iran were inspected during 2007-2008 to verify etiology of a new emerging disease. Affected plants show first symptoms on leaves represented by a sudden drying and scalding of margin area. Scalded areas continue to expand towards the central area of leaf where in some cases reddening of the lamina is also observed (Fig. 1).

***Figure 1.*** Leaf of declining grapevine plant showing scalding at the lamina border and reddening inside (darker spots).

In spring, symptomatic plants are delayed in growth, and later in season, the plants show dwarfed canes and shrunken or dried small fruits. Infected plants may die within months or years; drought conditions and hot temperatures speed up symptom development. Symptoms and death of plants were observed sporadically in vineyards. Laboratory analyses carried out in the 2007 growing season on samples collected from several plants showing this symptoms in two vineyards revealed no culturable bacteria presence, and the improved ELISA procedure (Minsavage *et al*., 1994) showed no indications of *Xylella fastiodiosa* presence. Since the symptoms are physiologically similar to other conditions that disrupt the plant water movement, it was thought that perhaps drought, nutritional deficiencies and other possible pathogens could have combined action in the syndrome therefore more analyses were performed.

## MATERIAL AND METHODS

Samples were collected during August 2008 from leaf of four plants showing the above decribed symptoms. One plant (A) was from North Khorassan province while the other 3 (B, C, and D) were from the South of the same province. Nucleic acid was extracted following a CTAB-based procedure (Minsavage *et al*., 1994). Detection of phytoplasmas was done using 20 ng of total nucleic acid in direct PCR with phytoplasma universal primer pairs P1/P7, followed by F1/B6 in nested PCR (Duduk *et al*., 2004). A second nested PCR with R16F2/R2 (Lee *et al*., 1995) phytoplasma universal primer pair was then performed. Each 25 µl PCR reaction mix contained 2.5 µl 10X PCR buffer, 0.8 U of Subtherm DNA polymerase (Fisher, Hampton, NH, USA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 0.4 mM each primer. Thirty five PCR cycles were performed under the following conditions: 1 min (2 min for the first cycle) denaturation step at 94°C, 2 min for annealing at 50°C and 3 min (10 min for the last cycle) for primer extension at 72°C. PCR products were analyzed in 1% agarose gel, stained with ethidium bromide and visualized under UV transilluminator. Identification of detected phytoplasmas was done using RFLP analyses with *Tru*I, *Hha*I, *Hinf*I, *BstU*I, and *Mbo*II restriction enzymes on R16F2/R2 amplified DNA fragments. Products were analyzed in 5% polyacrylamide gel, stained with ethidium bromide and visualized under UV transilluminator. The F1/B6 amplified product from sample C was purified using Qiagen PCR Purification Kit (Qiagen GmbH, Hilden, Germany), sequenced in both directions with primers F1, M1 as forward primers, and B6 as reverse primer (Padovan *et al*., 1995), using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were assembled using DNA STAR software, and compared with

selected 16S ribosomal sequences of phytoplasmas in GenBank database using BLAST (version Blast N 2.2.18) at the National Center for Biotechnology Information. Virtual restriction analysis of R16F2/R2 amplicon from sequenced strain C was also employed to compare RFLP profiles of this strains with selected phytoplasma strains showing 99% homology in BLAST.

## RESULTS AND DISCUSSION

*Phytoplasma detection.* Direct PCR amplification did not produce positive results from any Iranian grapevine samples but sample C that was positive in first nested PCR assay with primers F1/B6. In nested PCR all samples provided amplification except sample B (data not shown).

*Phytoplasma identification.* Restriction fragment length polymorphism analyses with *Tru*I, *Hha*I, *Hinf*I, *BstU*I and *Mbo*II indicate that all positive samples were infected from phytoplasmas belonging to 16SrXII ribosomal group (stolbur phytoplasmas) (Fig. 2), however sample A shows different profiles from samples C and D with *Tru*I, *Hinf*I and *BstU*I. The latter two were indistinguishable from each others with all employed enzymes. These results indicates that two molecularly differentiable phytoplasma strains were present respectively in the North and in the South of the Khorassan province where the sampling was performed. The collective profiles of the phytoplasmas detected in the three grapevine samples from Iran were also distinguishable from a stolbur-related strains recently described in grapevine in China (Duduk *et al*., 2009).

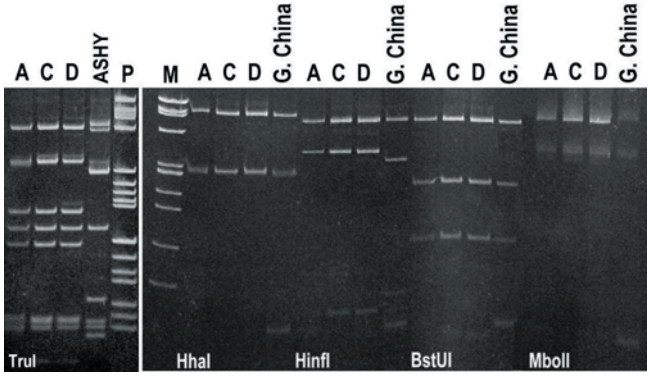


***Figure 2.*** Polyacrylamide gels showing the RFLP profiles of 16S rDNA amplified in nested PCR with primers R16F2/R2 from phytoplasma strains detected in grapevine samples from Iran. A phytoplasma strain from grapevine from China (G. China) and ASHY, Ash yellows in periwinkle are employed as reference strains. Restriction enzymes employed are at the bottom of gels. Labelling of phytoplasma strains from Iran is as reported in the text. P, marker pBR322 *Bsu*RI digested; fragment sizes in base pairs from top to bottom: 587; 540; 504; 458; 434; 267; 234; 213; 192; 184; 124; and 123; M, PhiX174, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Sequencing of F1/B6 amplicon of strain C resulted in a 1,532 bp sequence showing blast values of 99% with several phytoplasma strains related to stolbur from different geographical areas and different host plants. Virtual RFLP analyses were carried out with 7 restriction enzymes to verify the presence of restriction sites differences among representative of strains having 99% homology on R16F2/R2 amplicons (Fig. 3) and the C strain resulted clearly different form other strains either from grapevine (AF248959, AJ964960, X76428, EU086529, EU836649) in Europe and in Canada, potato (EU661607, EU344889) in Iran and in Russia respectively, corn in Serbia (DQ222972), and rhododendron in the Czech Republic (DQ160144).

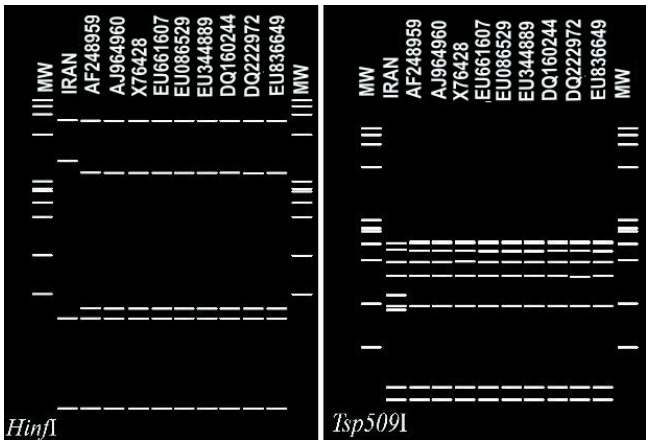


***Figure 3.*** Virtual polyacrylamide gels showing the RFLP profiles of 16S rDNA R16F2/R2 amplicons from phytoplasma strain C detected in grapevine from Iran and other strains from GenBank showing 99% homology. Restriction enzymes employed are at the bottom of gels. mw, PhiX174, marker ΦX174 *Hae*III digested.

The results indicate that grapevine stolbur phytoplasmas infecting vineyards in Khorassan province are distinguishable on 16S ribosomal gene from those reported in the major grape-growing areas worldwide and tentatively classified into the ribosomal subgroup 16SrXII-A.

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# NEW HIGH INCIDENCE OF AUSTRALIAN GRAPEVINE YELLOWS AND EVIDENCE ON PATHOGENESIS FOLLOWING NATURAL HEAT THERAPY IN SEVERELY INFECTED SOUTH AUSTRALIAN VINEYARDS.

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***Summary***

In summer 2008/09, grapegrowers at Nildottie, near Loxton, South Australia, reported much higher than normal levels of Australian Grapevine Yellows (AGY). Surveys of Riesling and other *cvs.* quantified what appears to be an Australian record-high incidence of AGY. Following extremely hot weather (40-46$^0$C), 'natural heat therapy' gave rise to regrowth of tips on severely affected shoots. PCR-tests confirmed the presence of AGY phytoplasma (AGYp) in affected shoot material discounting the idea of an incursion of a new yellows pathogen, and indicated AGYp in symptomless new growth. This challenges previous thinking on how AGYp causes disease and how natural heat therapy stimulates new growth. In addition, we bring first report of PCR-positive symptoms on the red *cv.* Sangiovese.

## INTRODUCTION

The incidence of AGY varies with locality and season (Magarey, 1986), but when high levels occur, usually between 30-50% vines/vineyard are affected. Natural heat therapy occurs when maximum temperature exceeds ~40$^0$C (Magarey et al, 1986). Badly affected shoots produce new growth from the tips 10-14 days later. This phenomenon and the use of hot-water treatment to restrict movement of other yellows diseases, *eg* Flavescence dorée (FD) (Caudwell *et al*, 1997), supported the theory that AGYp, the presumed pathogen of AGY, is heat labile. The assumption was that AGYp died or was denatured in extreme heat, allowing the phloem transport system to remobilise and shoot growth to recommence (Magarey PA, unpublished).

In summer 2008/09, extreme incidences of AGY noted at Nildottie, near Loxton, South Australia, in several *cv.* Riesling vineyards, queried an incursion of a new yellows pathogen. We report the result of vineyard surveys and PCR tests to investigate these events and review evidence on natural heat therapy that challenges our theories on the pathogenesis of AGY.

## MATERIAL AND METHODS

*Vineyard Surveys:* Casual observation of the incidence of AGY at Nildottie prior to season 2008/09 was followed in March 2009 (early autumn) by detailed survey of several cultivars (Table 1). The incidence of AGY was assessed by walking vineyard rows, scoring the vine canopy on each side of the row for presence or absence of AGY in at least three blocks of ≥ 50 vines/block, in each of (usually more than) three transect rows across each vineyard. A vine was considered symptomatic if typical symptoms of AGY were observed *ie.* yellowed, downward curled leaves, unlignified shoots and shrivelled bunches and/or berries.

*PCR-Tests:* On 4$^{th}$ March 2009, following a natural heat therapy event in Jan.-Feb. 2009, (usually) five shoots with typical AGY were selected at random from the cultivars listed in Table 2. Primary veins were sampled for PCR testing from: 1). Mature, yellowed leaves on AGY-affected shoots; and 2). Symptomless shoot-tip growth. Three replicate nested PCR's used primer pairs P1/P7 then nested primers R16F2n/m23SR (Constable *et al*. 2003). Specific AGYp detection used P1/P7 followed by AUSGY-F1 and AUSGYR2 giving a PCR product of 644 bp (Davis *et al*., 1997). The DNA samples were prepared using guanidine hydrochloride and silica to bind the DNA, essentially as described by Mackenzie *et al*. (1997).

## RESULTS AND DISCUSSION

*Vineyard Surveys:* Prior to season 2008/09, the incidence of AGY in the Riesling vineyards averaged ~3-5% vines/vineyard but in 2008/09 the detailed surveys revealed much higher levels (Table 1). In addition, an abnormally high incidence was recorded in the adjacent, and usually symptomless, red cultivars.

***Table 1.*** Incidence of AGY in vineyards of several cultivars, Nildottie, South Australia, March 2009.

| | Scores of AGY | | | | |
|---|---|---|---|---|---|
| **Vineyard** | **# Vines** | **% AGY** | **Vineyard** | **# Vines** | **% AGY** |
| Riesling 1 | 562 | 99.8% | Chardonnay 1 | 292 | 1.7% |
| Riesling 2 | 366 | 94.8% | Chardonnay 2 | 146 | Nil |
| Riesling 3 | 388 | 88.9% | Shiraz [1] | 150 | 3.3% |
| Sangiovese [1] | 150 | 19.3% | Sauv. Blanc | 150 | Nil |

[1] These are from conservative counts; actual incidence was likely higher.

Levels of AGY in the three Riesling vineyards were unprecedented in Australia as observed by the present authors. The previous maximum incidence on any cultivar was seen only once by the senior author *viz.* 86% at Renmark, SA, in 1978/79, also on Riesling. At Nildottie, the severity of disease was also extreme. Whereas usually AGY affects only 3-5 shoots/vine, in Riesling 1 where most vines were systemically infected, it was difficult to find an asymptomatic shoot. In effect, crop loss was complete.

*PCR-Tests:* The specific PCR tests confirmed the presence of AGYp in an high percentage of samples tested (Table 2). This negated the idea that the observed extreme incidence of AGY symptoms arose from an incursion producing disease levels similar to FD (Caudwell 1957), but didn't negate the question: Why the high levels of AGY?

At least two factors may be involved: 1). Severity. Seasonal variations in temperature in autumn, late winter and early spring 2008, may have increased the rate of multiplication of overwintering AGYp and thus its titre leading to higher severity of AGY in 2008/09. Average maximum/minimum temperatures in March 2008 at Loxton were warmer *viz.* 32.7$^0$C/12.5$^0$C respectively, compared with those of March 2009, *viz.* 29.6$^0$C/12.1$^0$C respectively. Surveys elsewhere in 2008/09 also showed higher severity but with few new infection sites (data not shown). 2). Incidence. The higher incidence only at Nildottie suggests a possible incursion of a more infective vector, or an increased activity of an initial vector(s) of AGYp. This remains unresolved though the latter seems the more likely.

***Table 2.*** PCR-tests of AGY-affected shoot material and of new tip-growth after a period of natural heat therapy in vineyards of several cultivars, Nildottie, South Australia, March 2009.

| | PCR-Tests for AGYp | | | | | |
|---|---|---|---|---|---|---|
| **Vineyard** [1] | **# Shoots AGYp** | **# Shoots Tested** | **% Shoots AGYp** | **# Shoot Tips AGYp** | **# Shoot Tips Tested** | **% Shoot-tips AGYp** |
| Riesling 1 | 4 | 5 | 80 | 4 | 4 | 100 |
| Riesling 2 | 5 | 7 | 71 | 4 | 6 | 67 |
| Riesling 3 | 4 | 6 | 67 | 1 | 5 | 20 |
| Sangiovese | 1 | 1 | 100 | - | - | - |
| Shiraz [2] | 1 | 1 | 100 | - | - | - |
| Sauv. Blanc | 0 | 1 | 0 | - | - | - |

[1] Vineyards are the same as cited in Table 1. [2] Weakly positive for AGYp.

The above PCR-tests appear to provide first record of AGYp in the red *cv.* Sangiovese. Symptoms were noted in previous seasons and on other red *cvs.* but with low incidence, 3-6% vines/vineyard. Our experience is that symptoms only appear in red cultivars where inoculum sources of AGY are close by, as evidenced by high levels of AGY in adjacent white grape vineyards. The higher levels in the red cultivars at Nildottie (Table 1) is consistent with this, suggesting the local source of AGY is located near the *cv*. Riesling there.

In a 12-day period of high temperature in Jan-Feb 2009 at Loxton, SA, there were 11 days with maximum T$^0$C $\geq$ 40$^0$C and five with maximum $\geq$ 43$^0$C. At Nildottie with similar conditions, new shoot growth appeared on affected vines 10-14 days later. PCR-tests of this symptomless foliage showed an high incidence of AGYp (Table 2), confirming similar tests at Loxton in earlier seasons. This suggests that 1). not all AGYp were killed or denatured by the heat; and 2). despite the presence of the supposed pathogen, the phloem transport system was significantly remobilised by the very hot weather.

Hot water treatment is used to reduce transmission rates of FD and other yellows pathogens. For efficacy, Caudwell *et al* (1997) showed this requires 45 minutes at 50$^0$C. Re-working their data, we translated this to mean delivering ~40$^0$C-hrs/treatment at 50$^0$C or ~200$^0$C-hrs at 45$^0$C.On five days in Jan.-Feb. 2009 at Nildottie, a range of 41-45$^0$C occurred for between 2 and 7 hrs, delivering 82-287$^0$C-hrs/day at $\geq$ 41$^0$C. On a sixth day, with maximum 46$^0$C, the vines were subject to ~246$^0$C-hrs at $\geq$ 41$^0$C and ~180$^0$C-hrs $\geq$ 45$^0$C. These naturally occurring heat treatments at $\geq$ 45$^0$C brought similar heat units to the vines as was required by Caudwell *et al*. (1997) for efficacy with FD while the temperatures at $\geq$ 41$^0$C were less than needed (data not shown). Previous supposition was that these temperatures were lethal to AGYp, and so triggered new shoot growth.

But by what mechanism does the natural heat therapy of AGY stimulate new growth? Symptoms of phytoplasma infection are associated with disturbed function of phloem cells, hormone imbalance and changed contents of phloem cells including deposition of callose in sieve cells (Lee *et al*. 2000). AGY-affected shoots are severely disrupted and die, either in summer or over winter, suggesting considerable dysfunction of phloem cells. Light microscopical investigation had also suggested callose accumulated at sieve plates (Magarey *et al.* 1986). If the symptoms were the result of that damage, the swift 'remission' of AGYp-infected and severely diseased shoots following natural heat events seems physically impossible. It raises conjecture as to the cause of AGY disease. Does high temperature change damaged phloem? Does heat unclog cells blocked with callose and/or AGYp?!

The new growth from the previously severely affected shoots tested positive for AGYp (Table 2). Does this, coupled with the supposed marginal efficacy of hot weather against yellows pathogens similar to AGYp, suggest that natural heat therapy significantly lowers but does not eliminate AGYp?

Of passing note, the efficiency of the PCR analyses to detect AGYp in the Riesling vineyards of Table 2 was similar to the trend in incidence of AGY in those vineyards (Table 1). Perhaps the more severely diseased vines had higher titre of AGYp and thus more readily tested positive in PCR analyses. The authors present these thoughts with view to foster discussion and progress in understanding the natural heat therapy phenomenon and the pathogenesis of AGY.

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### *ACKNOWLEDGEMENTS*

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# A UNIVERSAL VECTOR FOR EXPRESSION AND SILENCING IN PLANTS (INCLUDING GRAPEVINE)

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***Summary***

*Tomato yellow leaf curl virus* (TYLCV) has been disarmed. Twenty amino acids were deleted from its coat protein (CP) gene and most of the "replicase-associated gene" (rep) was removed. The rest of the TYLCV genome was inserted into bacterial plasmids (BlueScript, pDrive etc.). The resultant construct, termed IL-60, can be easily be propagated in bacteria. When injected into a plant, IL-60 replicates and spread throughout the plant. Any gene inserted into the multiple cloning site of the plasmid also replicates and spread and is expressed due to the TYLCV promoter in the virus's intergenic region (IR) which is placed upstream to the insertion. In addition, any gene placed downstream to IR (IR-X) is stabilized in the plant and remains, and replicates in the cells to which it entered for the duration of the life of those cells. However, IR-X by itself does not move and remains localized to these cells. A helper construct (native TYLCV; IL-60) engenders, in trans, IR-X movement and expression throughout the plant. Thus the system becomes inducible. IL-60 persists in the plant for the life-span of the plant, but does not integrate into the plant's genome and is not heritable. The IL-60 system was found active in every plant that has been tested, including wheat and woody plants. When a segment of a native gene is placed between two opposing IRs, the expression of the gene from the plant's genome is silenced. Biotechnological aspects and the revising of TYLCV functional genomic will be discussed.

## INTRODUCTION

We engineered a symptomless deletion clone of TYLCV and, as discussed further on, chose to insert a plasmid within the *rep* gene. Long (at least 5 kbp) inserts can thus be introduced into TYLCV-DNA. The virus-plasmid vector and the foreign gene inserted into it move systemically, and are expressed in plants. The engineered construct is easily introduced into the plant and it replicates in both plant and *Escherichia coli* cells. A target gene could be silenced by expressing ds RNA. In addition we engineered a series of artificial satellite DNAs activated to express or silence target genes following virus infection. By substituting the native virus with IL-60-BS, expression/silencing could be engendered without causing disease. We could also engender viral resistance by a unique approach for silencing (Peretz et al., 2007)..

## MATERIALS AND METHODS

See Peretz et al., 2007 for detailed procedures

## RESULTS AND DISCUSSION

We have deleted 60 bp of the CP gene of Israeli isolate of TYLCV and most of the viral *rep* gene. The construct (IL-60) was inserted into the plasmid BlueScript (or other bacterial plasmids), is termed IL-60-BS and can be propagated in bacteria. The chimeric TYLCV-plasmid construct spread and replicates normally following injection into plants. Genes inserted into the plasmid's MCS were expressed in all plant tissues.

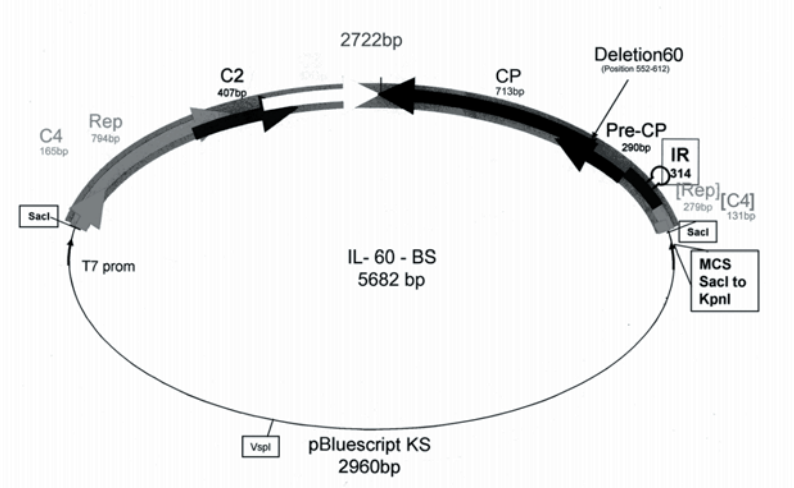


***Figure 1:*** Illustration of IL-60-BS

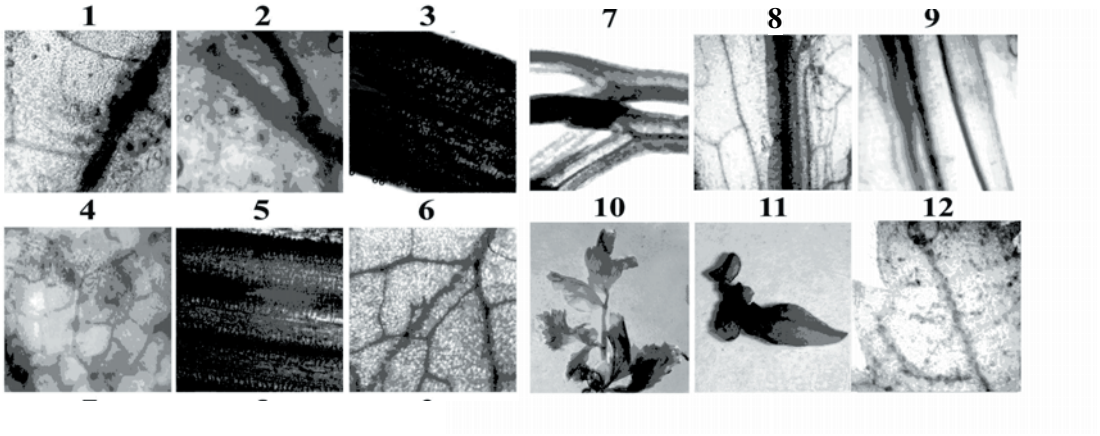


***Figure 2:*** Expression of GUS from IL-60 in various plants

Expression is transient and is not heritable. Every tested plant hitherto tested expressed reporter genes from the IL-60 system. Any gene that is fused to IR and injected to plants is stabilized and does not degrade. It multiples in the cells into which it had been introduced, but does not spread. *In trans* addition of IL-60 engenders it spread throughout the plant. Surprisingly, TYLCV's sense-oriented genes can induce spread as well and can replace IL-60.

IL-60 can accommodate and express long insert (the longest tested so far is ca. 7.5 bp; see Tanne et al., in this issue). It can accommodate and express an entire metabolic pathway. It expressed the PRN operon of *Pseudomonas* and the PRN-producing plants are resistant to several fungal and bacterial diseases.

***Figure 3:*** **Rizoctonia-resistant, PRN-expressing plants**

When a gene, or parts thereof, is placed between two opposing IRs and IL-60 is applied, that gene is silenced. We have silenced TYLCV genes *C2* and *C4* seemingly the silencing suppressors of the virus and engendered TYLCV-resistant tomatoes.

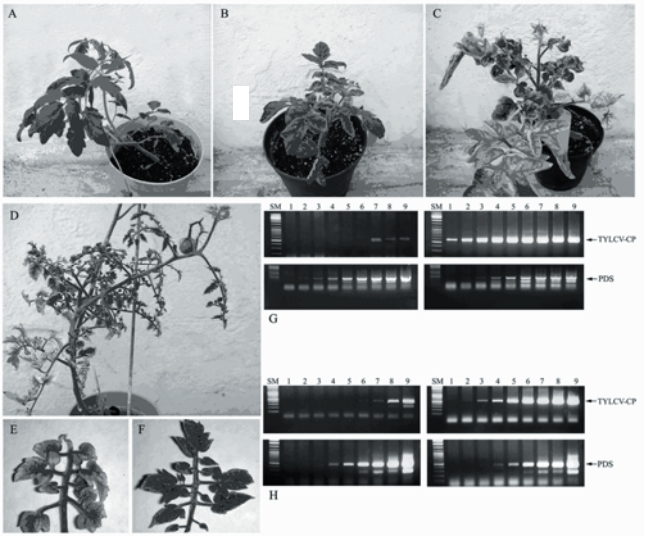


***Figure 4.*** Engendering TYLCV resistance in tomato.

## CONCLUSION

A durable expression/silencing vector has been developed from TYLCV. The construct does not integrate into the host genome but is stable throughout the life-span of the plant. It is expressing/silencing in any host plant, thus nit is universal. We have revised the functional genomics of TYLCV and shed light on the molecular biology of the viral infection.

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# EXPRESSION/SILENCING OF GVA COMPONENTS IN GRAPEVINE AND *NICOTIANA BENTHAMIANA* USING THE IL-60 SYSTEM

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Post-transcriptional gene silencing has been reported to play a role in the plant's reaction to infection and equilibrium between silencing and silencing suppression determines pathogenicity. We therefore attempted to engender non-transgenic resistance/tolerance by silencing the GVA silencing suppressor *p10*, thereby arresting the virus ability to exercise counter-silencing measures. The entire ORF 5 of GVA (coding for p10) was placed between two opposing IRs, producing IR-p10-RI. This construct was injected to *Nicotiana benthamiana* plants along with IL-60-BS. Five days later, the plants were inoculated with GVA.

Symptom appearance was monitored daily. While the control (un-injected) plants developed symptoms within a week, plants treated with IR-p10-RI + IL-60-BS were divided into two groups: Asymptomatic plants and plants with very mild symptoms (Fig. 1). Semi-quantitative RT-PCR indicated approximately 60-fold reduction of p10 expression in symptomless treated plants, as compared to untreated plants (Fig. 1C ) and about 30-fold reduction in plants exhibiting mild symptoms (data not shown). Thus, suppression of the viral silencing suppressor engendered plants which are GVA-resistant or tolerant.

A full-length GVA sequence was cut out from our infectious clone (Galiakparov *et al*., 1999; Galiakparov *et al*., 2003) and placed under the control of IR. The construct was injected to grapevines along with IL-60-BS. Figure 2 demonstrates that that the GVA has been expressed and spread **in the grapevine**.

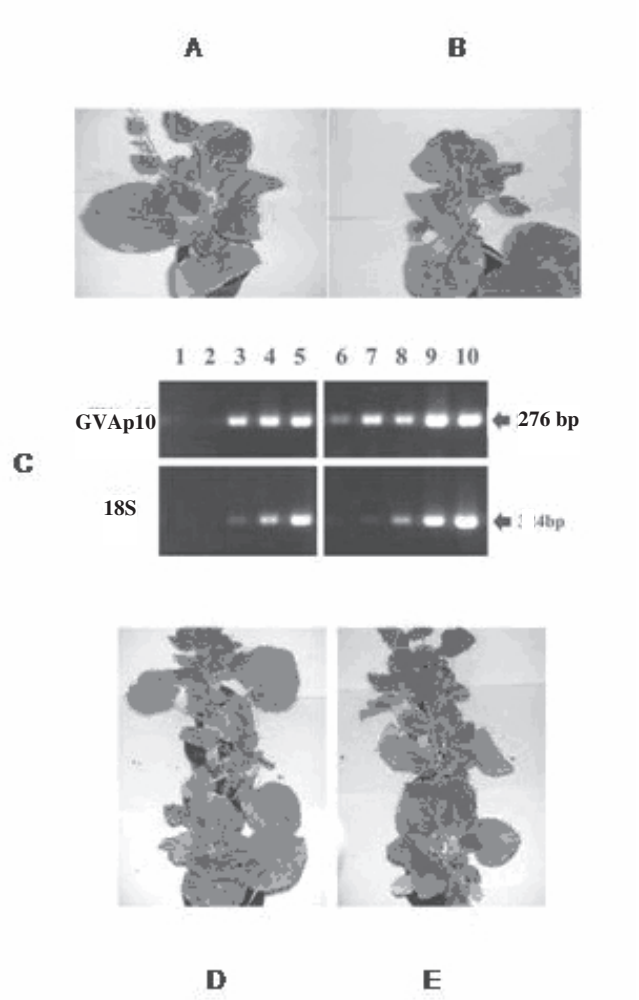


***Figure 1.*** Silencing of GVA-p10. A symptomless IR-p10-RI-treated, GVA-infected plant is shown in Frame A. A control, untreated, GVA-infected plant is shown in Frame B. Results of quantitative RT-PCR analysis of the plants shown in A and B are presented in C. Samples were withdrawn from PCR reactions at 3-cycle-intervals (cycles 18-30 for p10 and 12-24 for 18S-rRNA). Lanes 1-5: RNA was extracted from the plant depicted in frame A, pre-treated with IR- p10-RI and infected with GVA for 18 days. Lanes 6-10: RNA was similarly extracted from the plants depicted in frame B, untreated, and GVA-infected. Expression of 18S-rRNA, served as internal control, indicated that the same amount of RNA, extracted from both plants, was amplified. A general view of GVA-infected control, untreated plants (D) and IR-p10-RI-treated, resistant and tolerant plants (E).

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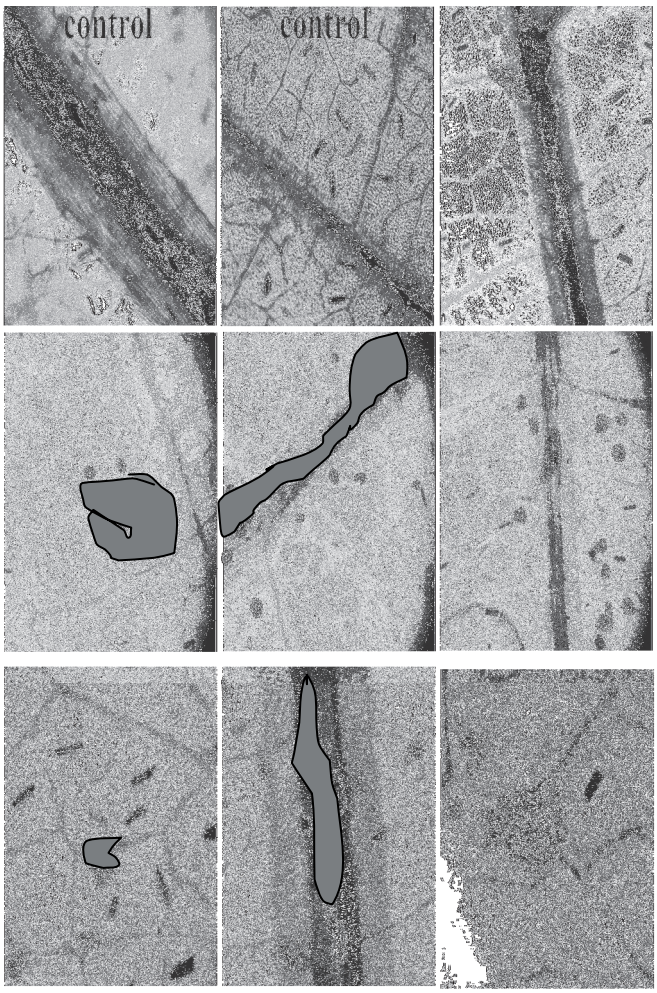


***Figure 2.*** *In situ* hybridization for detection of GVA nucleic acid in plants treated with IL-60-BS and pD-IR-GVA. Upper left two panels: untreated plants. All other panels: tissues from different locations on plants at least 3 leaves above the treatment site. The probe corresponded to ORF 5 of GVA. In this black and white figure some of the red-fluorescing areas were delineated and colored grey.

# VIROME OF A VINEYARD: ULTRA DEEP SEQUENCE ANALYSIS OF DISEASED GRAPEVINES

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***Summary***

The Illumina Genome Analyser was used to determine the sequence of the total viral population of a diseased vineyard that displayed typical grapevine leafroll symptoms and atypical virus symptoms. One gigabase of sequence information was generated from 50 ng of dsRNA, extracted from phloem tissue. These sequences were assembled into 179 scaffolds ranging in size from 301 nt to 8624 nt. GLRaV-3, GRSPaV, GVA and GVE were clearly identified, however 33% of sequences were shown to be unidentified ampeloviruses. A further 8% of sequences did not align to any known sequences or polypeptides in the NCBI databases.

## INTRODUCTION

Grapevine is subject to infection by more than 60 different viruses, the most known for any crop plant (Martelli & Boudon-Padieu, 2006). Grapevine virus diseases are often complex and diseased plants can be infected with up to nine different viruses (Prosser *et al.*, 2007) causing synergistic infections which are highly problematic and difficult to control. In South Africa, grapevine leafroll is regarded to be the most significant virus disease affecting grapevine. This disease has 10 different members of the family: *closteroviridae* associated with it. Although the industry recognises the importance of leafroll disease, there is debate on the importance of the individual viruses that are associated with leafroll. Presently only *grapevine leafroll-associated virus 3* is considered to be of significant importance. However, a project under the direction of Pietersen showed that many of the grapevine infecting viruses could not be reliably detected via PCR detection techniques. This raises important questions on the role that undetected viruses may play in leafroll disease and other virus-associated diseases (Pietersen, pers. comm.)

A new and powerful technique has become available that can be used to sequence viruses from environmental samples without the need for cloning or the availability of specific sequence data (Eisen, 2007). Further, this technique has proven to be most useful to identify new as yet undiscovered viruses in certain environments (Williamson *et al*., 2008; Edwards *et al*., 2006). A recent paper by Al Rwahnih *et al*. (2009) demonstrated the usefulness of deep sequencing by the discovery of a new virus in Californian Syrah decline grapes. The objective of this project is to determine the viruses associated with a severely diseased vineyard in South Africa and determine the prevalence and variability of different virus strains within the vineyard. The project may lead to the discovery of new, as yet undiscovered virus strains or the identification of viruses that have not previously been reported to infect grapevine.

## MATERIAL AND METHODS

A vineyard with a high disease incidence and known mixed infection status was identified for this study. Random vines in the vineyard were selected and marked to serve as source material and a logbook of symptoms observed in these vines was kept at each collection date. Petioles and phloem tissue were collected from each of the selected vines and dsRNA extracted according to a modification of Valverde *et al*. (1990). Reverse transcription PCR screening for the most prevalent viruses (GVA, GLRaV-3, GRSPaV) was performed to confirm the quality of the dsRNA.

In this study the Genome Analyzer by Illumina was used for sequencing. The sequencing technology is based on sequencing by synthesis, the detection of every fluorescently labelled nucleotide built into the growing DNA strand, rather than termination of the nucleotide chain with dideoxynucleotides, as in conventional Sanger sequencing (Guptra, 2008; Mardis, 2008).

The dsRNA was subjected to a heat denaturation step and prepared for sequencing using the Illumina mRNA Sequencing v2 kit. The denatured RNA was chemically fragmented, converted to cDNA, adapters ligated and 200 bp fragments selected for sequencing. PCR enrichment of the sample was quantified using qPCR to determine the optimal number of amplification cycles to prevent non-symetrical amplification of the viral genomic population. Enriched PCR fragments were attached to a glass flow cell using the Illumina Paired-end Cluster Generation Kit and 2.5pM DNA was sequenced in a paired-end sequence generation on the Illumina Genome Analyser II using the SBS Sequencing v3 kit.

Paired-end sequence data were assembled using the de novo assembler VELVET 0.7.31 (Zerbino & Birney, 2008) using a k-mer value of 23. Contigs larger than 300 bp from this assembly were then analysed using BLAST to identify relationships with known viral genome and protein sequences.

## RESULTS AND DISCUSSION

Good quality dsRNA was isolated from petiole and phloem tissue material using CF11 chromomatography. 50ng of dsRNA was subjected to denaturation and cDNA synthesis. Thirthy three cycles of PCR enrichment of the cDNA was conducted for sequence determination. In total, 13 million clusters were generated and approximately 1 gigabase of sequence data was obtained with a read length

of 86 bases in total from each 200 bp fragment. Twenty percent of these sequence reads were successfully assembled into contigs, which were further assembled into scaffolds, ranging in size from 301 nt to 8624 nt, using VELVET.

In total 179 scaffolds were obtained with the most prevelant reads to known viruses being: GLRaV-3 (17%) GRSPaV (7%), GVA (2%) and GVE (2%). Thirty three percent of sequences aligned to diverse members of the *Ampelovirus* genus. Eleven percent of sequence reads aligned to host chloroplast, mitochondrial, ribosomal or genome, 7% aligned to fungal genomes and 13% aligned to fungal infecting RNA viruses. A further 8% of sequences did not align to any known sequences within the NCBI databases and could be a reflection of as yet unknown and uncharacterised plant infecting viruses. This needs to be furher investigated.

***Table 1.*** Classification of sequence data as determined via BLASTn and BLASTx analyses. The table indicates the grouping of the sequences, the number of reads, contigs and scaffolds associated with the derived sequences.

| Sequence classification | No of reads | No of contigs | No of scaffolds |
|---|---|---|---|
| Ampelovirus | 1 366 329 | 33 | 21 |
| GLRaV-3 | 681 476 | 18 | 16 |
| GRSPaV | 296 352 | 1 | 1 |
| GVA | 76 770 | 40 | 4 |
| GVE | 71 678 | 7 | 2 |
| Fungal infecting viruses | 540 375 | 116 | 33 |
| Host genome | 463 895 | 55 | 29 |
| Fungal sequences | 264 853 | 46 | 18 |
| Unknown sequences | 309 274 | 138 | 55 |
| | **4 071 002** | **454** | **179** |

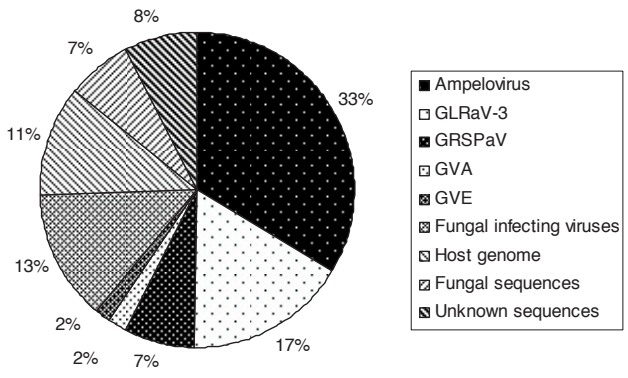


***Figure 1.*** Pie chart indicating the breakdown in percentages of sequence reads aligning to sequences within the NCBI database.

From the results presented in this abstract, it is apparent that GLRaV-3 is the most prevalent of the known viruses infecting grapevines in this vineyard. However, the high percentage of sequences aligning to members of the *Ampelovirus* genus, indicates that numerous viruses related to GLRaV-3 are also present in this vineyard and may play a more central role in the symptom diversity and severity of leafroll disease in South Africa. Due to the lack of full sequence data of most of these *Ampeloviruses* it is unclear whether the sequences obtained in this study are entirely new or are simply unsequenced portions of already identified *Ampeloviruses*.

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# DEEP SEQUENCING ANALYSIS OF VIRAL SHORT RNAS FROM PINOT NOIR CLONE ENTAV 115

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***Summary***

Short interfering RNAs (siRNAs) isolated from *Vitis vinifera* Pinot noir clone ENTAV 115 were sequenced by high throughput techniques. Viral (v)siRNAs of several grapevine viruses belonging to *Fovea-, Macula-, Marafi- and Nepoviruses* genera were identified. v-siRNAs were dominated by 21 and 22 nt species spanning the entire or being discontinuously distributed throughout *Grapevine rupestris stem-pitting associated virus* (GRSPaV) and *Grapevine fleck virus* (GFkV) genomic RNAs, respectively. v-siRNAs derived from positive and negative viral RNA strands for RSPaV or, abundantly, from negative viral RNA strands for GFkV.

## INTRODUCTION

In plants RNA silencing is a complex of related pathways which drives, among the other functions, the defense against viral parasites. This process relies on the activity of RNAse III Dicer enzymes (DCL) that cleave RNAs with double stranded (ds) features into 21 to 24 nt duplex siRNAs or microRNAs (miRNAs). Evidence from the model plant *Arabidopsis thaliana*, showed that RNA viruses are mainly targeted by DCL4, DCL2 and DCL3 to generate primary v-siRNAs of 21, 22 and 24 nt, respectively, which are followed, in a pathway controlled by host RNA-dependent RNA polymerase (RDR) enzymes, by secondarily generated v-siRNAs. v-siRNAs seem to derive either by genome-lenght dsRNAs or highly structured, single-stranded viral RNAs, which act as silencing trigger (Ding S.W. & Voinnet O., 2007).

Grapevine is affected by several viruses, some of which cause symptomless infections or express symptoms in specific genetic backgrounds or graft combinations (Martelli & Boudon-Padieu, 2006). This is the case of GRSPaV, a foveavirus associated with stem-pitting symptoms in *Vitis rupestris* and with vein necrosis in the rootstock 110R, and GFkV, a maculavirus which is latent in *V. vinifera* but induces specific foliar symptoms in *V. rupestris*. Both viruses, together with sequences related to the grapevine maculavirus Grapevine red globe virus (GRGV), the marafiviruses Grapevine rupestris vein feathering virus (GRVFV) and Grapevine asteroid mosaic associated virus (GAMaV), and *Grapevine fanleaf virus* (GFLV), were found in a subset of siRNAs obtained by deep sequencing total small RNAs from the grapevine clone ENTAV 115. The analysis of these data is the object of the present abstract.

## MATERIAL AND METHODS

*RNA isolation and RT-PCR detection.* Total RNA was extracted from leaf or phloem tissues with the silica capture protocol (Rott & Jelkmann, 2001). Two-step RT-PCR was carried out as previously described (Saldarelli *et al.,* 2006). PCR primers were from Al Rwahnih *et al*. (2009) and Abou Ghanem-Sabanadzovic *et al.* (2003).

*Cloning and sequencing of siRNAs.* Total RNA was extracted from leaves of the Pinot noir clone ENTAV 115, by guanidine thyocianate buffer (Rott & Jelkmann, 2001), followed by phenol/chloroform extractions. 19–24 nt small RNAs were isolated from 15% denaturing polyacrylamide gel and 15 µg were ligated to Solexa adaptors (Illumina Inc.). Short RNAs were converted to DNA by RT-PCR and the DNA was sequenced by a Solexa machine (Illumina Inc.).

*Analysis of vsiRNAs.* v-siRNAs reads were analyzed by BLASTN software against a viral genomic sequences database from NCBI (http://www.ncbi.hlm.nih.gov).

## RESULTS AND DISCUSSION

*Composition of the v-siRNA population in grapevine ENTAV clone 115.* Initial BLASTN analysis of the source library against the viral genomic (refseq_rna) database identified v-siRNAs having sequence homologies with several plant viruses (Table 1 refseq), and showed the largest reads for GFkV and GRSPaV. A further group contained several "non-grapevine" tymovirids belonging to the *Tymo-* and *Marafivirus* genera. Since genomic variations among grapevine tymovirids may account for these erroneus BLASTN identifications (Abou Ghanem-Sabanadzovic *et al.*, 2003), the original siRNAs data were filtered against a new database containg all the available sequences of GFkV, GRSPaV GAMaV, RGRV and GRVFV. This approach allowed the identification of v-siRNAs related to these new viruses whose presence, together to GFkV and GRSPaV, was ascertained by RT-PCR (Figure 1). Moreover, selected GRSPaV and GFkV viral genomic regions were sequenced from the original plant, confirming the trueness of the v-siRNAs sequences obtained.

***Table 1.*** BLASTN reads of v-siRNAs against a viral genomic sequences (refseq) and a selected database.

| Organism name | database | |
|---|---|---|
| | *refseq* | *selected* |
| *Grapevine fleck virus* | 3307 | - |
| *Grapevine rupestris stem-pitting associated virus* | 1125 | - |
| *Oat blue dwarf virus* | 74 | - |
| *Turnip yellow mosaic virus* | 55 | - |
| *Onions yellow mosaic virus* | 51 | - |
| *Okra mosaic virus* | 43 | - |
| *Maize rayado fino virus* | 28 | - |
| Citrus sudden death-associated virus | 16 | - |
| Grapevine red globe virus | - | 2856 |
| Grapevine asteroid mosaic associated virus | - | 33 |
| Grapevine rupestris vein feathering virus | - | 68 |
| *Grapevine fanleaf virus* | 27 | - |

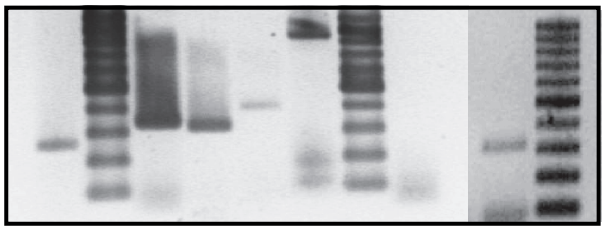


***Figure 1.*** Electrophoretic analysis of specific virus PCR products amplified from total RNAs extracted from *P. noir* ENTAV115. 1: GFkV; 2: GRSPaV; 3: GAMaV(nt); 4: RGRV(nt); 5: rRNAs control; 6: GSyV-1; 7:GFLV; L:DNA ladder.

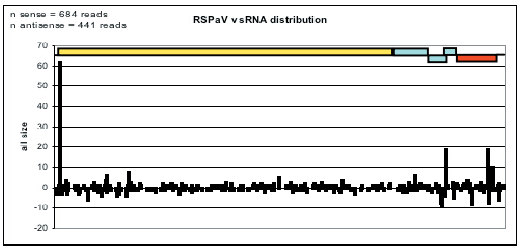


***Figure 2.*** Distribution of v-siRNA species along GRSPaV genome.

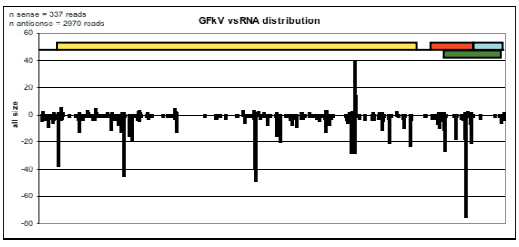


***Figure 3.*** Distribution of v-siRNA species along GFkV genome.

*Analysis of v-siRNA population in grapevine.* v-siRNAs size ranged between 18 and 26 nt, prevalent lengths being 21 and 22 nt. The majority of v-siRNAs were sequenced once or twice. When all v-siRNAs species were mapped on viral genomes two different pictures emerged for RSPaV and GFkV. GRSPaV v-siRNAs originated from sense (60,8%) and antisense (39,2%) orientations (Fig. 2), suggesting that both polarities had contributed v-siRNAs. Three hotspots were identified in the 5' and 3' genomic regions, being mainly composed of 21 nt species.

GFkV v-siRNAs had a different distribution (Fig. 3), as they were mainly of negative polarity (89.8%) and did not span the entire genome length, thus showing that several genomic regions did not contribute v-siRNA. Alignments of v-siRNA sequences at the identified hotspots showed that both 21 and 22 nt species overlapped and were shifted by one nucleotide. GFKV genome has a cytosine content (49.8%) higher than that of the type member of its family, *Turnip yellow mosaic virus* (TYMV) (Sabanadzovic *et al*., 2001), which would lead to a large proportion of unpaired cytosine residues in the viral RNA. According to Hellendoorn *et al.* (1996), TYMV RNA secondary structure consists of long cytosine-rich tracts interacting with the protein capsid and separated by weak stem-loop elements. By analogy, for GFkV, the complete lack of v-siRNAs from diverse genomic regions that have a relatively higher *C* content (75-80%), could be explained by the absence of secondary structures. Intriguing is the prevalence of negative-strand v-siRNAs originated from GFkV (and other related viruses), which does not find counterpart in the literature. Site (phloem tissue) and modality (vesicles originating from the invaginations of plastidial and/or mitochondrial bounding membranes) of GFkV replication differ from those of GRSPaV, and may have a bearing on the observed behaviour. As recently pointed out (Al Rwahnih *et al*., 2009), high throughput sequence analysis is a valuable tool for unraveling the complex interactions between the grapevine and viral pathogens in multiple infections. This information may assist in understanding the origin of mild or latent infections for a disease condition results from altered and/or synergic interactions between viral agents and the host.

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# HIGH-THROUGHPUT SEQUENCING ANALYSIS OF RNAS FROM A GRAPEVINE SHOWING SYRAH DECLINE SYMPTOMS REVEALS A MULTIPLE VIRUS INFECTION THAT INCLUDES A NOVEL VIRUS

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***Summary***

In a search for viruses associated with decline symptoms of Syrah grapevines, we have undertaken an analysis of total plant RNA sequences using Life Sciences 454 high-throughput sequencing. 67.5 megabases of sequence data were derived from reverse-transcribed cDNA fragments, and screened for sequences of viral or viroid origin. The data revealed that a vine showing decline symptoms supported a mixed infection that included seven different RNA genomes. Fragments identified as derived from viruses or viroids spanned a ~ten thousand fold range in relative prevalence, from 48,278 fragments derived from *Rupestris stem pitting-associated virus* to 4 fragments from *Australian grape-vine viroid*. 1,527 fragments were identified as derived from an unknown marafivirus. Its complete genome was sequenced and characterized, and an RT-PCR test was developed to analyze its field distribution and to demonstrate its presence in leafhoppers (vector for marafiviruses) collected from diseased vines. Initial surveys detected a limited presence of the virus in grape growing regions of California.

## INTRODUCTION

A "decline" of Syrah grapevines was first observed as an emerging disease in France (Renault-Spilmont *et al.,* 2004). More recently, a similar disease has appeared in California vineyards (Battany *et al.,* 2004). Symptoms include leaf reddening and scorching, swelling of the graft union, superficial cracking and pitting of woody tissue, stem necrosis, and eventual death of the vines.

The generation of sequence information representing the totality of the RNA from infected plants is now possible through the application of Life Sciences 454 sequencing (Rothberg & Leamon, 2008). Here we describe an application of this technique to a survey of viruses in diseased grapevine tissue, in an effort to identify and characterize causal agents of Syrah decline.

## MATERIALS AND METHODS

*Sample preparation*: Two grapevines from a UC Davis collection were used in this study. Syrah clone 6 (Syrah 6) showed severe decline symptoms (red leaves, swelling and wood necrosis at the graft union, stem pitting above the graft union), while clone Syrah 8 was asymptomatic. For each plant we compared two different sample preparation methods: in the first method dsRNA was extracted from 90 g of bark scraping. In the second method we used total nucleic acid (TNA) isolated from 1g of bark scraping using the RNeasy Plant minikit (Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized from both samples and amplified. The final DNA products were purified and quantified. Samples were subjected to 454 Life Sciences (Branford, CT, USA) high-throughput pyrosequencing, using the Genome Sequencer FLX. Bio-informatic analysis of the dataset used the GenomeQuest (Westborough, Mass.) High-Speed Sequence Search Suite algorithm

*Confirmation of candidate viruses and viroids:* Primers designed from published sequences were used for PCR amplification using the cDNA prepared from the Syrah 6 sample as template. PCR products were cloned, sequenced.

*Specific detection of GSyV-1 in planta and in insects:* Analysis for Grapevine syrah virus 1 (GSyV-1), as well as GRVFV, GAMaV, GFkV and RSPaV used specific primer sets for each virus. TNA was extracted from grapevine tissue and grapevine leafhoppers using Qiagene RNeasy mini kit, and analyzed for virus by RT-PCR (Rowhani *et al.,* 2000). Adult leafhoppers (*Erythroneura variabilis*) were collected during the growing season.

## RESULTS AND DISCUSSION

67.5 megabases of sequence information, from 351,590 fragment reads (each approximately 200 bases long) were initially produced in this study, derived from two source vines. Most of the sequences detected from Syrah 8 were identified as plant nucleic acids. Two viruses, RSPaV and GRVFV were detected in Syrah 8. The number of viral fragments detected for those viruses was less than one percent of that found in the extract of tissue from the symptomatic Syrah 6 vine.

The data from the two separate extraction procedures from the Syrah 6 vine were combined for the analyses described below. Initial BLASTN analysis of the data (Table 1) showed three categories of sub cellular parasites. Members of the first of these categories had homologies to known viruses or viroids from grapevine (Table 1A). Their presence was verified by PCR (Figure 1). A second category of viruses (Table 1B) had homologies to known viruses from grapevine, but the presence of the viruses in this category could not be verified in the plant by PCR detection. A third viral category of fragments carried sequences unknown among grapevine viruses. These fragments were identified as similar to members of the *Tymoviridae* (Table 1C).

***Table 1.*** Fragment counts for viral species identified in the BLASTN analysis of the total data set.

| Organism Name | Reads |
|---|---|
| *A* | |
| *Rupestris stem pitting-associated virus* | 46,029 |
| *Grapevine rupestris vein-feathering virus* | 9,791 |
| *Grapevine leafroll associated virus -9* | 16 |
| *Hop stunt viroid* | 13 |
| *Grapevine yellow speckle viroid* | 5 |
| *Australian grapevine viroid* | 4 |
| ***B*** | |
| *Grapevine asteroid mosaic-associated virus* | 113 |
| *Grapevine fleck virus* | 11 |
| ***C*** | |
| *Maize rayado fino virus* | 55 |
| *Citrus sudden death-associated virus* | 40 |
| *Oat blue dwarf virus* | 34 |
| *Okra mosaic virus* | 14 |
| *Kennedya yellow mosaic virus* | 2 |
| *Nemesia ring necrosis virus* | 2 |
| *Erysimum latent virus* | 1 |
| *Turnip yellow mosaic virus* | 1 |
| Total | 56,131 |

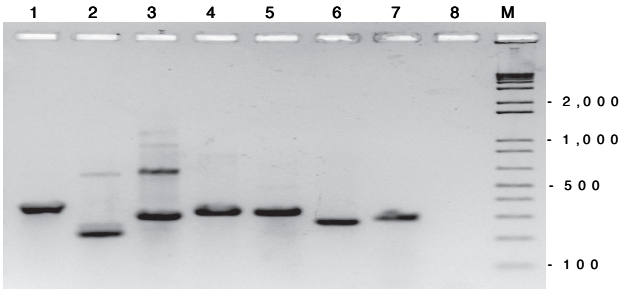


***Figure 1.*** Electrophoretic analysis of specific virus and viroid PCR products amplified from the extract of Syrah 6. 1: AGVd, 370 bp; 2: GYSVd, 220 bp; 3: HSVd, 300 bp; 4: RSPaV, 330 bp; 5: GRVFV, 328 bp; 6: GLRaV-9, 276 bp; 7: GSyV-1, 296 bp. 8: analysis for GSyV-1 in extracts of uninfected control ; M: size standards, labeled in base pair sizes.

Many of the fragments similar to viruses not detectable in the Syrah clone 6 vine (Table 1B, IC) showed only distant similarities, some as low as 40%, to their homologs in the database. We assembled large contiguous sequences from the combined unidentified fragments pool plus the viral annotated fragment pools. This approach identified Ctg.23 (2500 bp) and Ctg.75 (2183 bp), which incorporated sequences from the Table 1B or 1C viral categories; the presence of these contiguous sequences in grapevine was confirmed by PCR. Primers designed to bind within these contigs were used to fill in the gap in the sequence between them. The final 3' and 5' ends were sequenced by RACE PCR. 96% of the completed genomic sequence was found to encode a single, uninterrupted polyprotein reading frame unknown in the Genbank database. We have provisionally named this new virus Grapevine Syrah Virus-1 (GSyV-; Genbank accession number FJ436028).

The GSyV-1 genome was found to be 6,481 bases in length, and to include a 3-prime poly(A) tract. The virus was shown to be closely related to members of the genus *Marafivirus* based on coat protein amino acid homology and on the presence of a marafibox domain.

Using the specific PCR primers, GSyV-1 was detected in leafhoppers from plants showing Syrah decline symptoms. A field sampling survey of the endemicity of GSyV-1 in California revealed 19 % of declining vines positive for GSyV-1, placing the virus in three grape-growing counties (Napa, Sonoma, Yolo). Wider surveys for the virus are underway. No correlation could be drawn between the virus distribution and the decline symptoms.

Combination-of-viruses testing would address the possibility of the genesis of the Syrah decline through additive infective effects of multiple viral and viroid species. These tests will be a next step in our ongoing attempt to understand the etiology of this disease.

454 high throughput sequence analysis will facilitate that attempt. The multi virus infection revealed by deep sequencing in a Syrah vine may reflect complex interactions. Half of the identified species are represented by less than twenty five hits; do they play a role in Syrah decline even at low titers? Are those low titer viruses and viriods cryptic strains, suppressed infections, or recent inoculations? Will deeper sequencing reveal still lower titer subcellular parasites? The deep sequencing approach to pathogen census may well lead to greater understanding of the diversity of, synergies between, and host varietal interactions with strains and species of viruses and viroids in the vineyard setting. For more information see Al Rwahnih et al. (2009).

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# CHARACTERISATION OF THE GENOMIC AND SUBGENOMIC RNA OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* (GLRaV-3)

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***Summary***

*Grapevine leafroll-associated virus* 3 (GLRaV-3) is the type species for the ampeloviruses (*Closteroviridae*) and plays a major role in the economic important leafroll disease. The complete sequence of the South African isolate GP18 with a 5' untranslated region (5'UTR) 579 nt longer than previously published for GLRaV-3 (Maree *et al.* 2008, Ling *et al.* 2004) has been determined. The 5'-end of the genome was determined by RLM-RACE. RLM-RACE was also used to determine the 5'-ends of 8 putative sgRNAs. A GLRaV-3 mini-replicon was constructed to investigate sg-promoter activity.

## INTRODUCTION

*Grapevine leafroll-associated virus* 3 (GLRaV-3) is the type species of the genus *Ampelovirus* in the family *Closteroviridae* (Martelli *et al.* 2004). It is an economically important virus that is known to only infect *Vitis* spp. and has a negative impact on the wine and table grape industries worldwide.

To date, there have been three reports that claim the complete nucleotide sequence of GLRaV-3, isolate NY-1 (AF037268) by Ling *et al.* in 2004, isolate Cl-766 (EU344893) by Engel *et al.* in 2008 and GP18 (EU259806) by Maree *et al.* in 2008. The GP18 sequence is reported to have a 5'UTR of 737nt, 579nt longer than the NY-1 sequence. Apart from this sequence data, little information is available on the formation of sgRNAs by GLRaV-3 during replication.

In this paper we report on the first complete sequence of a South African GLRaV-3 isolate GP18 (Maree *et al.* 2008), the 5'-ends of 8 putative sgRNAs and the construction of a GLRaV-3 mini-replicon for the testing of sg-promoter activity.

## MATERIAL AND METHODS

*Sequencing of the GLRaV-3 genome:* Double-stranded RNA (dsRNA) was extracted from phloem tissue of GP18 infected grapevines that displayed disease symptoms, using an adapted cellulose (CF11) column method (Hu *et al.* 1990). RT-PCR was performed with primer sets designed to cover a large portion of the genome (nucleotides 1,835–17,905 of AF037268) in 10 overlapping clones. Amplicons were cloned and sequenced using standard molecular techniques.

*Poly(A) tailing:* Poly(A) tailing was performed on dsRNA in an attempt to determine the 5'- and 3'-ends of the genome as described by Meng *et al.* (2005).

*RLM-RACE:* To determine the 5'-ends of the GP18 genomic and subgenomic RNAs, total RNA was extracted from grapevine phloem tissue (White *et al.*, 2008) and subjected to RNA ligase-mediated rapid amplification of cDNA Ends (RLM-RACE) using the FirstChoice® RLM-RACE kit (Ambion, USA) as per the manufacturer's instructions. Nested reverse primers were designed at the 5' ends of ORFs 2-10 and 12 to search for sgRNA ends upstream of these ORFs. Nested reverse primers were also designed at the 5'-end of the genome. The amplicons generated, were cloned and sequenced.

*Construction of a GLRaV-3 mini-replicon:* RT-PCR was performed on total RNA extracted from grapevine phloem tissue. The mini-replicon, pBIN_LR3_MiniReplicon was assembled from cDNA fragments under the control of an enhanced cauliflower mosaic virus 35S promoter (Töpfer *et al.*, 1987) and a hammerhead ribozyme sequence (Shintaku *et al.*, 1996) in the plant expression binary vector pBIN_SN. The vector pBIN_LR3_MiniReplicon_GUSi was constructed and tested in *Nicotiana benthamiana* and *Vitis vinifera* plants by agrobacterium infiltration similar to procedures described earlier (Voinnet *et al.* 1998; Santos-Rosa *et al.* 2008).

## RESULTS AND DISCUSSION

*Sequencing of GLRaV-3 genome:* Sequences from the ten overlapping clones were aligned and a consensus sequence generated by using BioEdit (Hall, 1999). Primers were not included in the consensus sequence assembly of these overlapping sequences. The sequence results from the poly(A) tailing indicated that the 3'-end of GP18 is similar to that of the NY-1 isolate. However, we were unable to reach the reported 5'-end of the NY-1 isolate and consistently found amplicons that started at +50 nt. After further experimentation with PCR conditions, a range of amplicons were generated that extended beyond the NY-1 sequence's 5'-end. A possible explanation for this inconsistency, using poly(A) tailing, might be the high uracil content in the 5'-region upstream of the +1 and +50 sites, which could act as priming sites for the oligo(dT) primer and lead to fragments shorter than the true genomic size. The amplicon generated using RLM-RACE for the 5'-end of the GP18 genome was larger than expected and indicated that the 5'UTR extends beyond the sequence previously reported for the NY-1 isolate (Ling *et al.* 2004). The efficacy of RLM-RACE to determine the 5'-termini of multiple ssRNA viruses from total RNA in a single reaction was also investigated. RLM-RACE was performed on total RNA extracted from three grapevine plants (K1, K5, K6) that contained a mixture of viruses (GLRaV-2, -3 and grapevine rupestris stem pitting-associated virus (GRSPaV) to determine their genomic 5'-ends in the same reaction. Sequencing results indicated that GLRaV-2 (AY881628) and GRSPaV (AY881626) have the same 5'-ends as previously reported. However, GLRaV-3 showed the same extended 5'-end as was found for the GP18 isolate. The

absence of similar extended 5'-regions for GLRaV-2 and GRSPaV confirms that the GP18 extended 5'-end is not an artefact of the RLM-RACE reaction. The presence of this extended 5'UTR was present in all samples tested (data not shown). The 5'UTR was also found to be highly variable between previously identified divergent variant groups (Jooste & Goszczynski, 2005).

The complete genome sequence of the GLRaV-3 isolate GP18 is 18,498 nts long and shows a sequence identity of 93% with the NY-1 isolate covering nucleotides 580–18,498. The GP18 sequence is 579 nt longer than NY-1 at the 5'-end and reveals a 5'UTR of 737 nt. The extended 5'UTR has an adenine/uracil content of 68.4%, with a high uracil content of 48.5%. The 5'UTR of GLRaV-3 GP18 is much larger than the 5'UTRs of other members of the family Closteroviridae. This leaves one to speculate about the function of such a large 5'UTR, and its role in replication cannot be discounted and needs to be further investigated.

*RLM-RACE of subgenomic RNA 5'-ends:* The transcription initiation sites of the sgRNAs were also determined using RLM-RACE. All amplicons generated were cloned and at least 5 clones sequenced per amplicon. For the results of the predicted 5'ends see Table 1. ORFs 3 and 4 and ORFs 10 and 12 are predicted to be translated from the same sgRNAs.

***Table 1.*** RLM-RACE predicted transcription initiation sites of eight GLRaV-3 sgRNAs.

| ORF | Transcription Initiation Site * | sgRNA 5'UTR | # sgRNA | Size of sgRNA |
|---|---|---|---|---|
| 2 | **9001** | 286 | 1 | 9497 |
| 3 | **10477** | 32 | 2 | 8021 |
| 4 | **10477** | 188 | - | - |
| 5 | **12185** | 122 | 3 | 6313 |
| 6 | **13800** | 48 | 4 | 4698 |
| 7 | **14815** | 37 | 5 | 3683 |
| 8 | **16273** | 23 | 6 | 2225 |
| 9 | **16758** | 92 | 7 | 1740 |
| 10 | **17265** | 125 | 8 | 1233 |
| 11 | **ND** | - | - | - |
| 12 | **17265** | 774 | - | - |

* Nucleotide positions compared to GLRaV-3 (Isolate GP18, EU259806), ND- Not determined


***Figure 1.*** Schematic representation of the GLRaV-3 MiniReplicon inserted into in pBIN_SN with restriction enzymes SmaI and NotI. The restriction sites AsiSI and AflII can be used as insertion sites for test constructs containing the putative sg-promoter and GUSi as a reporter gene.

*Construction of GLRaV-3 mini-replicon:* The mini-replicon has been successfully assembled in a pBIN expression vector and agro-infiltrated into *N. benthamiana* and *V. vinifera*. Preliminary data indicate that the construct is replicating. The mini-replicon can potentially be used to evaluate GLRaV-3 sg-promoter activity in these plants plants by insertion of different putative sg-promoter sequences upstream of the GUSi reporter gene (Figure 1).

This study generated new information on the genomic RNA of GLRaV-3 and also reports the 5'-ends of 8 of the sgRNAs. We also report the construction of a GLRaV-3 mini-replicon that would assist in broadening our knowledge of GLRaV-3 replication.

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# *GRAPEVINE FANLEAF VIRUS* REPLICATION: ILLUMINATING THE WAY

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***Summary***

The replication of the nepovirus *Grapevine fanleaf virus* (GFLV) causes, in infected cells, massive proliferation of membranes that generally accumulate in the nuclear periphery to form a so called viral compartment. Previous studies have established that these membranes essentially originate from the ER. To investigate the viral and host determinants as well as the molecular mechanism responsible for the recruitment of theses ER vesicles, we constructed several recombinant viruses expressing EGFP- and/or TagRFP-tagged viral proteins.

## INTRODUCTION

Viral invasion of a plant is a complex process that requires the sequential accomplishment of key events such as viral genome replication and cell to cell movement. Plant RNA viruses have evolved the capacity to replicate in association with membranes originating from different sources such as the endomembrane system, chloroplasts, mitochondria, etc..

The replication of *Grapevine fanleaf virus* (GFLV) is not an exception to this rule since infected cells show a massive proliferation of membranes that generally accumulate in the nuclear periphery to form a so called viral compartment. Using tobacco BY-2 cells expressing the green fluorescent protein (GFP) targeted to different cell compartments as a marker, previous experiments have established that the membranes of the viral compartment originate essentially from the endoplasmic reticulum (ER). Immunoconfocal microscopy experiments further demonstrated this perinuclear compartment to be the site of GFLV replication as it contains double-stranded replicative forms, newly synthesized viral RNA, and the RNA1-encoded VPg (Ritzenthaler *et al.*, 2002). In addition, GFLV replication was shown to depend on *de novo* lipid synthesis and is sensitive to Brefeldin A. However, the viral and host proteins responsible for the recruitment of vesicles from the ER as well as the molecular mechanism that leads to this process remain to be characterized.

To understand how viral proteins hijack the cellular processes we tagged some viral products to the autofluorescent EGFP- and TagRFP and inoculated these recombinant viruses along with cellular markers.

## MATERIAL AND METHODS

*Construction* of recombinant EGFP coding *GFLV*: binary gateway vectors encoding EGFP fused to viral proteins were used as templates for PCR amplification of EGFP:1A, EGFP:1B, 2A:EGFP and EGFP:2B genes and subcloning into the full-length GFLV-F13 RNA1 and RNA2 cDNA clones (Viry *et al.*, 1993).

*Construction* of recombinant 2A:TagRFP coding *GFLV*: A unique X*ba*I restriction site was introduced by site-directed PCR mutagenesis into the RNA2 cDNA clone then the TagRFP gene (Merzlyak *et al.*, 2007) was introduced as an X*ba*I restricted PCR fragment.

*Inoculation of recombiannt viral RNAs*: Biological properties of recombinant viral RNAs were analyzed in protoplasts and *in planta* after inoculation of *in vitro* transcripts.

*Confocal imaging*: Laser Scanning Microscopy (LSM) was performed on a Zeiss LSM510 microscope using a 63X/1,2 water immersion objective.

## RESULTS AND DISCUSSION

To understand which proteins encoded by GFLV participate to the recruitement of endomembranes we constructed recombinant full-length cDNAs coding for a fluorescent protein fused to either of viral proteins 1A, 1B or 2A.

Protein 1A fused at its N-terminus to the EGFP gave rise to a fully infectious recombinant GFLV. Its subcellular localisation was in complete agreement with the localisation of the protein expressed ectopically from a binary vector. Protein 1B fused at its N-terminus to EGFP gave rise to a self replicating RNA1 when inoculated to either *Chenopodium quinoa* or Tobacco BY2 protoplats, but did not allow infection of plants inoculated by recombinant RNA1 along with wild type RNA2. Protein 2A was fused at its C-terminus to EGFP or TagRFP. Both fusions were compatible with a systemic infection on *Chenopodium quinoa, Nicotiana benthamiana* and *Arabidopsis thaliana* plants. Again, the localisation of the fused protein was compared to its behaviour when expressed ectopically.

Altogether these recombinant viruses confirmed that the fusions did not hinder the properties of the viral proteins as they fully supported GFLV replication. This important result allowed to compare the fused proteins either expressed ectopically or from the virus genome, pointing differences between their intrinsic properties and their fate in the viral context.

The combinaison of recombinant RNA1 encoding EGFP:1A and recombinant RNA encoding 2A:TagRFP yielded bicolored infection sites where the interplay between 1A and 2A became visible.

The last observations on co-visualization of viral proteins and cellular markers will be presented in the perspective of the recruitement of endomembranes for viral replication

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# VACUUM-AGROINFILTRATION OF DIFFERENT *V. VINIFERA* CULTIVARS AND APPLICATION OF VIGS IN THE CV. SULTANA

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***Summary***

The procedure of agroinfiltration is widely used to infect plants with viruses derived from full-length cDNA clones. For *Vitis vinifera* agroinfiltration procedures were described recently. The application and modification of these procedures was used to establish a successful transient expression protocol for the *V. vinifera* cultivars Sultana, Red Globe, Merlot and Shiraz and for the infection of the grapevine cv. Sultana with a *Grapevine virus A*-based VIGS vector via vacuum-agroinfiltration of leaves.

## INTRODUCTION

In recent years the genome sequence of several plant species were made available in public databases. For grapevine the genome of the cultivar Pinot Noir was determined (Jaillon *et al*., 2007; Velasco *et al*., 2007). Besides *in silico* analysis of the grapevine genome data which allows comparison to other sequenced plant genomes, the first tools for *in vivo* analysis in grapevine were described recently. These tools include transient expression protocols (Santos-Rosa *et al*., 2008) and virus-induced gene silencing (VIGS) systems (Muruganantham *et al*., 2009) for grapevine, which both include agroinfiltration procedures. By applying the method of agroinfiltration the infection of plants with plant viruses derived from cDNA clones can be achieved without the use of the natural virus vector. For several phloem restricted viruses, such as *Grapevine virus A* (GVA) in grapevine, agroinfiltration proved to be an efficient infection route as a mechanical inoculation is often not possible. We describe here a modified protocol for vacuum-agroinfiltration of *V. vinifera* leaves which is successfully used for GVA-based VIGS of the grapevine phytoene desaturase (VvPDS) gene.

## MATERIAL AND METHODS

*Plant Material*: Plantlets of *V. vinifera* cultivars Sultana, Red Globe, Merlot and Shiraz were grown and micropropagated *in vitro* in Perlite supplemented with plain Murashige and Skoog (MS) liquid medium. The plants were cultivated under artificial light at 23°C. After development of roots (~2-3 weeks), the plants were used in vacuum agroinfiltration experiments.

*DNA Constructs*: The intron containing GUS construct 35S:GUSi (Vaucheret *et al*., 1994) and the green fluorescent protein (GFP) containing 35S:GFP (Ghazala *et al.*, 2008) were described earlier. The T7-promter driven GVA118 expression vector (Haviv *et al*., 2006) was brought under control of an enhanced 35S promoter (e35S) leading to 35S-GVA118. For VIGS experiments, the 3'-terminal 363bp sequence of the VvPDS was introduced into the *NotI* multiple cloning site leading to 35S-GVA118-VvPDS.

*Agroinfiltration*: For agroinfiltrations the 35S-GVA118-VvPDS was cloned into a modified pBin19 vector and subsequently introduced into *Agrobacterium tumefaciens* C58C1 harbouring the helper plasmid pCH32 (Santos-Rosa *et al*., 2008). Before agroinfiltration, recombinant *A. tumefaciens* cells were incubated in 10mM MgSO4, 10mM MES and 100µM acetosyringone. Vacuum-agroinfiltration of *V. vinifera* was done on micropropagated plants. The upper 4-5 leaves of each plant were cut at multiple sites in the interveinal area using a scalpel. Small cuts were also made on the leaf midrib and the veins of the 2nd order near the petiole onset. The cut leaves were dipped for 2 seconds into a sonicating water bath before it was transferred into the recombinant *A. tumefaciens* suspension ($OD_{600}$=0.5). A strong vacuum (-90 kPa) was applied for 2x2 minutes by using a BioRad PDS1000 Gene Gun. Subsequently, plantlets were washed in sterile water and transferred into a container supplemented with perlite and water. Vacuum-agroinfiltrated *V. vinifera* were cultivated under artificial light at 23°C.

*GUS Staining and GFP Detection*: GUS staining was done 5-6 days after vacuum-agroinfiltration by vacuum infiltration of the staining solution (Jefferson, 1987) into detached leaves, as described above. GFP detection was performed by using a microscope with a GFP/dsRed filter combination.

## RESULTS AND DISCUSSION

*Vacuum agroinfiltration of V. vinifera*: The cultivars Sultana, Red Globe, Merlot and Shiraz were vacuum-agroinfiltrated with 35S:GUSi and 35S:GFP recombinant *A. tumefaciens* C58C1 (pCH32) in order to establish an efficient inoculation protocol. Strong GUS expression in the infiltrated leaves was observed. As reported earlier (Santos-Rosa *et al*., 2008) a difference in intensity of expression could be observed between infiltrated leaves of the same plant, showing highest GUS expression in the first fully expanded leaves. By using an inoculation suspension with an $OD_{600}$ of 0.5 we did not observe a striking contrast in GUS expression between the first and the second fully expanded leaves. We were also able to find larger patches of GUS expression in older leaves of the cultivar Sultana. In all four cultivars GUS expression could be observed in up to 80-90% of the leaf area (Fig. 1). The short incubation of plantlets in a sonicating water bath led to a higher and more uniform GUS expression in the cultivar Sultana (Fig. 1, E-F). Even if sonication led to leaf necrosis in some plants, this procedure was used for all vacuum-

agroinfiltrations of *V. vinifera*. All our attempts to use a GFP detection system, in order to circumvent the more expensive GUS staining procedure, failed. Whilst autofluorescence of the plant tissue was observed, we were unable to detect GFP-derived fluorescence in grapevine.

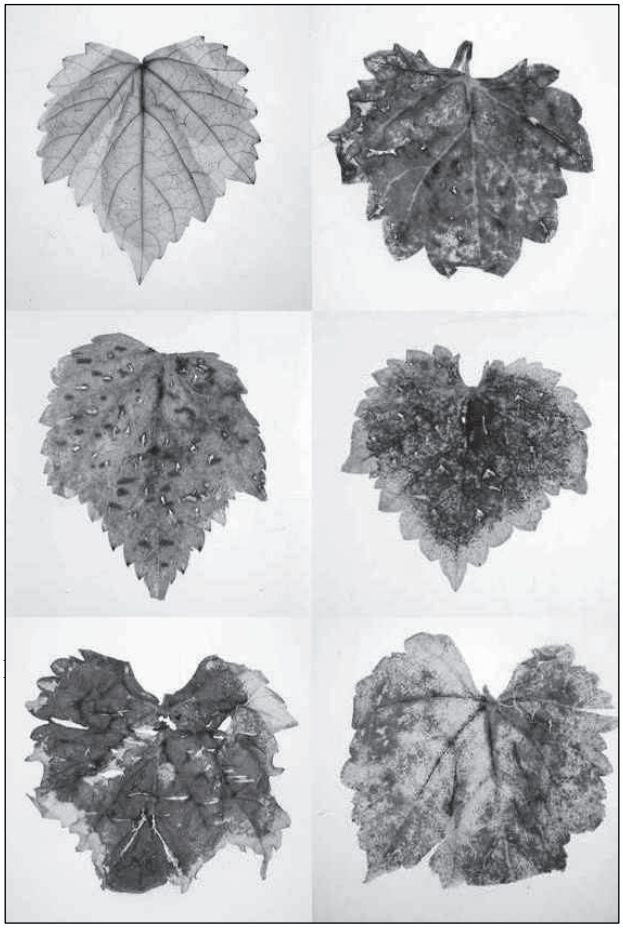

***Figure 1.*** GUS-expression in 35S-GUSi vacuum-agroinfiltrated leaves of the *V. vinifera* cv. Red Globe (B), Shiraz (C) and Merlot (D). GUS-expression with (E) and without (F) sonication of leaves of the cv. Sultana before vacuum-agroinfiltration. (A) 35S:GFP infiltrated leaf of cv. Sultana.

*GVA-based VIGS*: The vacuum-agroinfiltration procedure described above was used to agroinfect *V. vinifera* cv. Sultana with 35S-GVA118-VvPDS. The extensive infiltration of leaves with the agrobacterium suspension will allow successful agroinfection of phloem associated cells which might be crucial for systemic spread of the virus from the site of inoculation. As early as 14 dpi, clear photobleaching in leaves was observed, indicating successful PDS silencing. Systemic photobleaching was restricted to 2-3 leaves above the site of inoculation as described earlier for the cv. Prime (Muruganantham *et al*., 2009), but was restricted to the leaf midrib and veins of the 2$^{nd}$ and 3$^{rd}$ order in the cv. Sultana (Fig. 2). 35S-GVA118-VvPDS-derived virus was detected in those leaves by RT-PCR but not in newly developed leaves. The GVA phloem restriction reflected by defined photobleaching of the veins in the cv. Sultana might indicate a confined application possibility of GVA-based VIGS in some cultivars. In comparison the earlier described agrodrenching procedure of *V. vinifera* cv. Prime roots (Muruganantham *et al*., 2009), our results obtained so far showed that vacuum-agroinfiltration of leaves of the cv. Sultana led to comparable infection rates of 50%. For ongoing experiments we sequenced the 363bp VvPDS fragments from the cultivars Sultana, Red Globe, Merlot and Shiraz and found 99-100% nt identity. The 35S-GVA118-VvPDS can therefore also be used in the remaining three cultivars to compare the success of infection and the VvPDS VIGS phenotype.

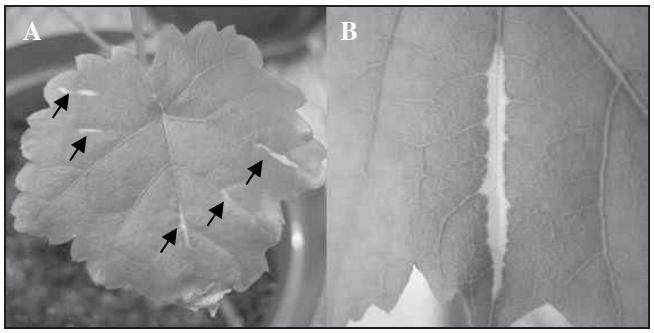


***Figure 2.*** Photobleaching symptoms (arrows) restricted to leaf veins (A) and closely adjacent tissues (B) on leaves of the cv. Sultana 3-4 weeks dpi with 35S-GVA118-VvPDS by vacuum-agroinfiltration.

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# TRANSGENIC ROOTSTOCKS EXPRESSING GFLV COAT PROTEIN GENE IN A THREE YEARS FIELD TRIAL; RESISTANCE ASSESSMENT, IMPACT ON GFLV DIVERSITY AND EXCHANGES BETWEEN ROOTSTOCK AND SCION

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***Summary***

Transgenic rootstock lines expressing the full-length translatable coat protein (CP) gene of *Grapevine fanleaf virus* strain F13 (GFLV-F13) are under evaluation in an open-field trial. Control plants were contaminated after nematode-mediated transmission of GFLV after three years. In contrast, viral infection of non-transgenic scion (*Vitis vinifera* cv. Pinot Meunier) was delayed in vines grafted onto transgenic rootstocks. Sequencing of CP genes from GFLV isolates from this field, suggests that CP gene-expressing transgenic rootstocks do not influence significantly natural viral biodiversity. Transgenic-derived expression products were detected in GM rootstocks. However, their systemic movement was not detected suggesting that the scions remain truly wild-type. All experiments were co-constructed and followed within a Local Steering Committee. Thus, this field trial is a unique support for the assessment of environmental impact of genetically-modified plants (GMPs) and a survey of public perception of GMPs.

## INTRODUCTION

The grapevine fanleaf degeneration, the major virus disease for the viticulture worldwide, is caused mainly by nepoviruses transmitted in a semi-persistant manner by longidorid ectoparasite nematodes. This disease is causing to French grapevine industry *ca.* 1 billion € losses per year with a prevalence of 60% of the total grapevine acreage (Fuchs *et al.*, 2006). *Grapevine fanleaf virus* (GFLV, genus *Nepovirus*, family *Comoviridae*) is the main aetiological agent and is transmitted by *Xiphinema index*. The control of fanleaf disease relies currently on prophylactic measures, cultural practices (fallows over ten years following devitalization of vines) and certification programs. Despite these measures and since the ban in Europe of some environmentally unfriendly agrochemicals, fanleaf disease remains an expanding pandemic leading to a technical deadlock in control strategies.

To date, no dominant nor recessive resistance genes toward GFLV have been found in grapevine. The implementation of genetic engineering opened up new strategies based on rootstock-mediated resistance, to develop virus-resistant grapevines (Laimer *et al.*, 2009). Transgenic rootstocks expressing the coat protein (CP) gene of GFLV-F13 strain have thus been obtained in the early 1990s (Mauro *et al.*, 1995). Their resistance in vineyard conditions has been assessed in an open-field trial in Champagne during 3 years (1996-1999). Facing strong public rejection, the trial had to be stopped. However, preliminary results suggested a lower incidence of GFLV infection in 3 transgenic lines, out of 18 tested (Vigne *et al.*, 2004a). Relying on an Interactive Technological Assessment approach (Joly & Rip, 2007), we designed experimental conditions for an open-field trial (2005-2009) with a Local Steering Committee (LSC) and met public acceptance so far in Europe (Masson, 2007).

The aim of this field experiment was firstly to evaluate the level of resistance toward GFLV infection in vineyard conditions and secondly to assess the potential environmental impact of transgenic CP gene-expressing rootstocks on molecular variability of the GFLV population. A key question addressed by the LSC concerned putative exchanges between genetically-modified (GM) rootstock and non-GM scion. Preliminary data about the long-distance movement of mRNAs and proteins will be presented.

## MATERIAL AND METHODS

*Transgenic lines and field trial conditions:* the gene encoding the full-length translatable CP of GFLV-F13 has been inserted in 41B rootstock hybrid (Mauro *et al.*, 1995), the *Neomycin phosphotransferase II* (NPTII) gene being used as a selection marker. The 5 transgenic lines G68, G77, G206, G219 and G240 were selected for the trial, subsequently green-grafted onto the wild-type Pinot Meunier cultivar. A total of 70 transgenic vines were used, 50 of them being planted in a *X. index*/GFLV infested soil that had been transferred from a fanleaf-infested vineyard into the experimental plot. During the whole experiment, inflorescences were systematically pruned and stored at -80°C for subsequent analyses. Resistance level was evaluated by DAS-ELISA on leaves of the scion in June each year.

*DAS-ELISA:* GFLV and NPTII proteins were detected respectively with specific anti-GFLV immunoglobulins from our laboratory and with commercial anti-NPTII antibodies (5Prime→3Prime, Inc.).

*IC-RT-PCR-RFLP:* GFLV particles of positive ELISA samples were immuno-trapped and the full-length CP gene

of viral RNA was subsequently amplified by RT-PCR as previously described (Vigne *et al.*, 2004b). The resulting fragments were characterized by RFLP using *Eco*RI and *Sty*I digestions.

*Cloning and sequence analyses:* PCR products with different RFLP patterns were selected and cloned using the pGEM-T vector system (Promega). Clones corresponding to putative variants were subsequently sequenced on both strands and further analyzed with Vector NTI software (Invitrogen). The phylogenetic tree was constructed with Mega4 software using the Neighbour-Joining algorithm.

*Transgene-expressed mRNAs detection*: Total RNAs were extracted from frozen leaves and inflorescences using RNeasy Plant Mini Kit (Qiagen). CP and NPTII mRNAs were detected by specific RT-PCR.

## RESULTS AND DISCUSSION

The progression of infection of vines, investigated by ELISA, was remarkable, from 3% positive vines in 2007 to 70% in 2008, for the non-transgenic controls, indicating a high nematode transmission efficiency of GFLV, though nematode-infested soil had been transported. Although all grapevines were asymptomatic in June 2008, DAS-ELISA performed on the leaves showed infection rates, ranging from 30% in the transgenic lines, to 70% in the controls. Though showing no resistance, our preliminary data suggest a delay in infection, in 2008. The ELISA results of June 2009 will be presented and discussed.

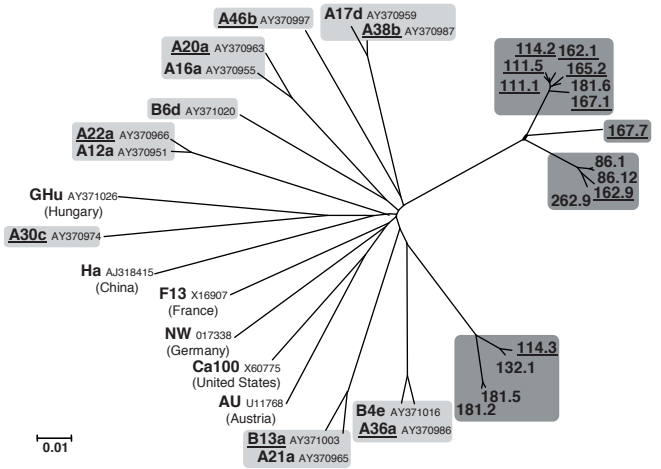


***Figure 1:*** unrooted phylogenetic tree reconstructed from the 1515 bp CP sequences of 35 GFLV variants from various geographic origins (including Champagne -light gray- and Colmar -dark gray- field experiments) and isolated from transgenic (underlined) and non-transgenic vines. Accession numbers are given for sequences retrieved from Genbank.

It has been shown previously that transgenic grapevine rootstocks expressing the GFLV CP gene did not favor the development of detectable GFLV recombinants or specific molecular variants from the Champagne trial (Vigne *et al.*, 2004b). In the present study, we confirm that transgenic rootstocks expressing CP do not promote the emergence of GFLV variants, after 3 years. Interestingly, the GFLV population structure from the Alsatian soil (this experiment) reveals, as preliminary results, two new clusters, and a genetic diversity different from that displayed by the Champagne isolates (Figure 1). The frequency of mixed infections seems to be higher than that of single ones, whatever the transgenic status of the vines. More data are nevertheless required to estimate the magnitude of the population structure and of the diversity of GFLV isolates in this field trial.

No translocation of the transgene-expressed products (CP and NPTII mRNAs and NPTII protein) has been detected between the GM rootstock and wild-type Pinot Meunier scion. This evidence is provided for the first time in a perennial crop in a long-term field trial. These original results are in accordance with those obtained for transgenic watermelon rootstock expressing the CP of *Cucumber green mottle mosaic virus* (*Tobamovirus* genus), where no translocation of CP mRNA or protein was detected in the scion (Youk *et al.*, 2009).

Through this field experiment, resistance toward GFLV, environmental impact of trangenic CP gene-expressing rootstocks, and systemic movement of transgenes products are being efficiently assessed. We proposed with the LSC to broaden our study on the impact of transgenic rootstocks on soil bacteria and GFLV genome using metagenomic approaches. An extension of duration of this trial aiming to address these scientific questions will be submitted. Our data, when confirmed at completion of the trial, may help refining guidelines for GMO release in Agriculture.

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# A CASE STUDY OF CONTROL OF GRAPEVINE LEAFROLL DISEASE SPREAD ON VERGELEGEN WINE ESTATE, SOUTH AFRICA, 2002-2008.

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***Summary***

Grapevine leafroll disease is a serious disease of grapevine worldwide associated with a number of viruses. In spite of a successful virus-elimination strategy and certification scheme in South Africa, it spreads rapidly to new vineyards. Recent local epidemiological studies of leafroll disease have shown that spread is mainly; 1) from a previously infected vineyard at a given site, 2) through infected planting material, 3) due to secondary spread within vineyards, and 4) from adjoining infected vineyards. Control strategies must therefore address each of these sources of infection. A collaborative project between Winetech, Vergelegen Wine Estate, Vititec and Citrus Research International to control and eradicate the disease from Vergelegen was initiated in 2002. Vineyards of Cabernet sauvignon, Merlot, Shiraz and Chardonney were established in 2002 on previously *Vitis*-free sites but surrounded by leafroll-infected vineyards. Implements and labourers were utilised in a manner to prevent spread of the disease from old infected vineyards to the new vineyards, and infected plants removed from surrounding vineyards where the incidence was less than 10% and in the new vineyards when they were detected. Replacement of old, highly infected vineyards have been in progress since 2006. The incidence of leafroll has been managed down since 2002 to only 0.08% in 2008 in the new and managed vineyards. The control strategies used have been very successful in curtailing the spread of leafroll disease, and serve as a model for control of this disease locally and internationally. It is in stark contrast to the general situation in the South African industry where the majority of producers do not exercise leafroll control and where leafroll spread is rampant.

## INTRODUCTION

Grapevine leafroll disease is a serious disease of grapevine worldwide, associated with a number of viruses. In South Africa grapevine leafroll associated virus type 3 (GLRaV-3) is the most important virus (Pietersen, *unpublished results*) as in other countries (Bovey *et. al.,* 1980), and is mealybug transmitted. In spite of a successful virus-elimination strategy and certification scheme in South Africa, it spreads rapidly to new vineyards. Recent local epidemiological studies of leafroll disease (Pietersen, 2006) have shown that spread is mainly; 1) from a previously infected vineyard at a given site, 2) through infected planting material, 3) due to secondary spread within vineyards, and 4) from adjoining infected vineyards. Control strategies must therefore include planting of virus-free certified material with systemic insecticide treatment at establishment and subsequent removal of infected material, prevention of secondary spread by rouging (Pietersen *et al.*, 2003), the use of fallow periods and removal of volunteer hosts, and sanitation and horticultural practices to prevent spread of viruliferous mealybugs on implements and labourers (Pietersen, 2004). Since 2002 a collaborative project to control and eradicate the disease from Vergelegen has bee implimented, in which the above strategies were employed in an integrated manner (Spreeth *et al.*, 2006). It is anticipated that Vergelegen serve as a model locally and internationally for control of leafroll.

## MATERIAL AND METHODS

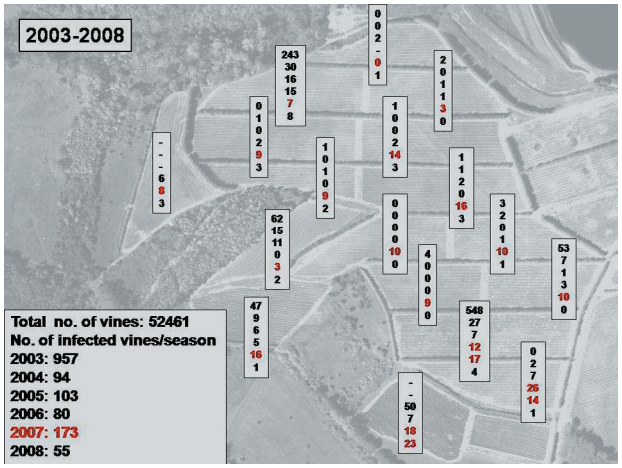
Vergelegen Wine Estate is situated in near Somerset West, South Africa. The eighteen vineyards, or 52461 vines, reported on in this presentation represent the first phase of a long term strategy of a leafroll eradication program on Vergelegen. These vineyards were all established on sites formerly planted with citrus, and had the advantage of being surrounded by fully-grown windbreaks. Furthermore, with one exception, they were separated by a wide road and verge from severely leafroll-infected vineyards, allowing a separation of usage of implements and labourers between the new vineyards and the old infected one's. Vineyards were of Cabernet Sauvignon, Chardonnay, Merlot and Shiraz and were established from certified planting material from Vititec a member of the South African Wine Grape Certification Scheme. Leafroll infection were monitored by TAS-ELISA annually during autumn by testing a pooled sample of two bays, and retesting individuals when the bay was positive, and also by visual assessment of symptoms in black-berried vineyard blocks. The spatial position (row number, plant position in row) of leafroll- infected plants was recorded for each year of assessment. For visual representation of infection spread, these were plotted in X-Y scatter-plots using the row and plant position as co-ordinates. Vines displaying leafroll symptoms, or testing positive for GLRaV-1, 2, or 3 were treated with insecticide and removed in late autumn, early winter of the same season, removing as much root material as possible. A variable number of surrounding vines (depending on the size of secondary spread foci detected in the vineyard) were treated with a soil drench of 100ml of a 1.5ml of 350g a.i./l suspension of imidachloprid (Confidor 350SC®, Bayer) in 1l water, and this was followed by 4 liters of water. Five vineyards had been established in 1999 prior to the leafroll control program and already contained levels of leafroll

varying between 0.46% and c.100%. The entire vineyard in three instances was treated in 2002 with imidachloprid as described above. The single vineyard with very high levels of leafroll was removed in 2005 and the other one, due to an oversight, was only monitored from 2006 onwards, but fortuitously had low infection levels. Three of the new vineyards, established in 2002, displayed high incidences of leafroll infected plants within one season, and both were therefore treated in their entirety with imidachloprid in 2003. Since 2004 *Planococcus ficus* specific mealybug pheromone capsules, yellow delta traps and sticky pads (Chempack, Cape Town) have been used to monitor mealybug levels and various contact insecticides applied when action thresholds (a bi-weekly trap count of 65 or more mealybug males) and mealybugs observed on vines necessitated it. Dormant vines were also treated preventatively annually in winter with chlorpyriphos (Dursban®, Dow AgroSciences) as per manufacturer instructions. A further three vineyards were established on old citrus orchards in 2006. Phase two has also been initiated, in which highly leafroll infected, old vineyards have been treated with herbicide (Glyphosate), removed, and left fallow for two seasons with removal of volunteers. New vineyards established since 2006 have been treated with imidachloprid, and are currently being monitored for leafroll infection.

## RESULTS AND DISCUSSION

Figure 1 illustrates the number of leafroll infected vines present in autumn of a given year, within each leafroll-managed vineyard block. The reduction in number of infected vines over time is self-evident, although a slight resurgence of infection in 2007 is discussed later. In most instances leafroll infected vines were removed the same season as detected, but a few exceptions occurred in the initial phases, due to systems not being fully in place. Success in halting, and reducing, high incidences of leafroll due to infected planting material is evident in three vineyards where relatively high levels of leafroll (12.2%, 1.9% and 5.75%) were detected in the first season post-establishment. Imidachloprid treatment was utilized on all vines in these vineyards and roguing conducted annually. By 2008 the incidence of leafroll in these three vineyards were 0.1%, 0%, and 0.2% respectively. Some spread of leafroll, probably from surrounding highly infected vineyards was noticed in 2006 and 2007, resulting in higher than expected numbers of leafroll infected vines in a few managed blocks. It is speculated that this may be due 1) to the presence of a highly infected block, not managed but on the same side of the road and hence sharing labourers and implements with the lower three vineyards in Figure 1, and 2) relatively long-distance wind dispersal of mealybugs, manifesting as increasing numbers of randomly spread single infected vines from 2007 onwards. The viruliferous mealybugs were probably from the highly infected vineyard blocks upwind and across the road of the new vineyards. The vineyards serving as the source of this infection were removed in 2006. It is anticipated that leafroll incidence on managed vineyards will decline significantly due to this. Initial phases of control of leafroll on Vergelegen relies heavily on insecticide usage. This is not desirable or sustainable in the long-term, and biological control of mealybugs will be utilized once leafroll infection is at negligible levels and few mealybugs are viruliferous. Clearly however the integrated use of certified planting material, roguing and judicious use of systemic and contact insecticides have allowed the establishment of healthy vineyards. Phase 2, the replacement of infected vineyards is likely to be more difficult due to the added leafroll reservoir presented by viruliferous mealybugs possibly surviving on leafroll infected root remnants.

***Figure 1.*** Number of leafroll infected vines per season in vineyards subjected to leafroll control strategies. Grey coloured values illustrate the increase in infected vines in 2007 presumably due to wind dispersal of viruliferous mealybugs from infected vineyards upwind of these.

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# TENTATIVE ANALYSIS OF THE ECONOMIC IMPACT OF GRAPEVINE LEAFROLL DISEASE IN THE VINEYARD OF VALAIS (SWITZERLAND)

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***Summary***

In a two-year study, the effects associated with GLRaV-1 and GLRaV-3 infections on the main parameters of must and wine were measured *in situ*, in 4 commercial vineyards in Valais. GLRaV-3 on Chasselas as well as on Pinot noir was associated with a strong detrimental effect on must composition, as a dramatic decrease of the sugar and an increase of the malic acid contents were observed. Furthermore, the phenolic compounds of Pinot noir wines were dramatically altered. The measured differences proved to be perceptible by sensory analysis. GLRaV-1 was associated with moderate effects on the composition of must and wines of Pinot noir that were not detected by tasting. These results are discussed in respect to the potential damage caused by GLRaVs in the vineyard, whereby a simple approach for estimating the economic impact is presented and some hypothesis discussed.

## INTRODUCTION

Viral diseases are reported to cause several detrimental effects on grapevine (Walter *et al.*, 1997). In particular, Grapevine leafroll disease (GLRD) was shown to affect yield, maturity of grapes, berry pigmentation and quality of wines. However, most of the studies dealing with agronomic vine performance due to GLRD were conducted on experimental plots with a few single clones. Experimental data from commercial vineyards are rare. Furthermore, data on the economic importance of grapevine viruses related to a whole vineyard or region are lacking. Such data would help to increase viticulturist's and decision-making person's awareness of the importance of viral diseases on grapevines. The aim of the present work was to investigate the effects of *Grapevine leafroll-associated virus 1* and *3* (GLRaV-1 and 3) on the quality of must and wine of Chasselas and Pinot noir in commercial plots in the vineyard of Valais. In a second step, the results obtained, coupled to epidemiological data (Besse *et al.*, 2009) will help us to propose a tentative analysis of the economic impact of GLRD in the vineyard of Valais.

## MATERIAL AND METHODS

Four commercial plots (2 of Chasselas and 2 of Pinot noir; grafted on 5BB) were selected in the area of Flanthey (Valais) based on homogeneity criteria in respect to the field work carried out by viticulturists in accordance with regional practice. Mature canes or leaf samples from symptomatic and apparently healthy vines were analysed by double-antibody-sandwich ELISA (DAS-ELISA) or by reverse transcriptase polymerase chain reaction (RT-PCR). DAS-ELISA was performed according to Gugerli (1986) with reference monoclonal antibodies from Agroscope ACW or commercial kits from BIOREBA AG (Reinach, Switzerland). The following viruses were assessed by ELISA: *Grapevine leafroll-associated virus 1* to *9* (GLRaV-1 to -9) except GLRaV-8, *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV), *Strawberry latent ringspot virus* (SLRSV), *Raspberry ringspot virus*-grapevine strain (RpRSV-g) and *Grapevine fleck virus* (GFkV). According to ELISA results, groups of 10 to 30 individual vines with a similar sanitary status were built up. Then, some selected samples (7 to 9 plants from each group) were further analysed by RT-PCR for *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus D* (GVD) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV). RNA extraction procedure, RT-PCR setup and thermal conditions as well as primer sequences were described by Rütsche (2008). Grapes from each group were harvested separately in the growing seasons 2006 and 2007. The main agronomic parameters (yield, pruning wood weight) and the composition of must, respectively of wine were assessed for each group and year. Statistical inference was performed by means of the free software R (R Development Core Team, 2007). A one-sample *t*-test was performed on the differences measured between the GLRaV group and the corresponding healthy one in two plots over 2 years ($H_0 : \mu = 0$ ; $H_A : \mu \neq 0$). The wines were further compared by sensory analysis and the results evaluated by means of the software FIZZ (Biosystemes, Couternon, France).

## RESULTS AND DISCUSSION

*Constitution of experimental groups on commercial plots.* According to the results obtained by ELISA, 10 groups of 10 to 30 individual vines were built up in the four commercial plots: groups of single plants infected by GLRaV-1, respectively by GLRaV-3 were compared to a control group of healthy grapevine in each plot of Pinot noir. A group of GLRaV-3 infected vines and a control group were constituted in each plot of Chasselas. Vines of GLRaV infected groups as well as healthy groups were mostly shown to be further infected by GFkV and GRSPaV in similar proportions. Thus, the experimental layout approached an *in situ* evaluation of GLRaV-1 and 3 effects on a background of other common viruses present in the vineyard.

*Agronomic parameters.* Lower yields and wood pruning weights were mostly observed for GLRaV infected groups in comparison to the healthy controls. However, only one significant difference was measured for the yield of GLRaV-3 groups on Pinot noir (Table 1). Yield was usually around 2 kg/ $m^2$.

*Must and wine composition.* The presence of GLRaV-3 was associated with considerable modifications of the main organoleptic features of must and wine of both Chasselas and Pinot noir (Table 1). In particular, a

significant decrease of the sugar and an increase of the malic acid contents were observed in the must. Furthermore, the phenolic composition of Pinot noir wine was dramatically altered in quantity as well as in quality (Table 1). The effects observed with GLRaV-1 infected vines were very different from those obtained with GLRaV-3 (Table 1). GLRaV-1 was positively associated with higher sugar content ($p<0.05$) and higher Formol number. The malic acid content of must was however significantly higher and the phenolic compounds were moderately affected..

***Table 1.*** Differences measured between GLRaV infected groups and healthy one on yield, must composition and phenolic compound of wine from 2 plots of Pinot noir and 2 plots of Chasselas over 2 years. Sugar content was calculated by refractometric index; total acidity was expressed as $C_4H_6O_6$ equivalent; TPCI: total phenolic compound index ($OD_{280}$); CI: colour intensity ($OD_{520} + OD_{420}$); Tint in degrees: *Arctan* ($OD_{520} - OD_{420}$). One sample *t*-test: *, ** and *** means statistically significant at $p<0.1$, $p<0.05$ and $p<0.01$ respectively.

| Variety | Virus | Yield ($kg/m^2$) | Must composition | | | | | | Phenolic compounds of wine | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Sugar content (Brix°) | Total acidity (g/L) | Malic acid (g/L) | Tartaric acid (g/L) | pH | Formol number | TPCI | Anthocyanins (mg/l) | CI | Tint (degrees) |
| Pinot noir | GLRaV-1 | -0.149 | 0.7** | 0.22 | 0.40* | -0.23 | -0.013 | 0.47 | -0.2 | -12.7 | 0.03** | -1.77* |
| Pinot noir | GLRaV-3 | -0.162*** | -2.11*** | 0.45 | 0.23* | 0.2 | -0.048 | -3.85*** | -1.4* | -21.2** | -0.24*** | -0.85 |
| Chasselas | GLRaV-3 | -0.065 | -1.13*** | -0.03 | 0.18** | -0.25** | 0.015** | -0.28 | | | | |

*Sensory analysis*. Only wines from GLRaV-3 groups were discriminated from their corresponding control wines. The colour was mostly perceived as significantly more intense in the healthy control wine (also in one vintage of Chasselas). The quality and fineness of tannins was also evaluated as much better in healthy control as in GRLaV-3 groups of Pinot noir wines. Chassleas wines were differently appreciated according to the year. In 2006, the healthy controls appeared more stressed whereas they were evaluated significantly finer in 2007.

All these results corroborate previous investigations that showed a negative impact on must and wine associated with the presence of GLRaV-3, especially in relationship with the polyphenol metabolism of red varieties. The present study further demonstrated that differences are also clearly associated to GLRaV-3 *in situ*, in commercial plots with a background of commonly found viruses. Furthermore, sensory analysis indicated clearly that measured differences in wine may be perceived by the consumer and therefore may depreciate those products. The lack of significant differences for wines of GLRaV-1 groups and the moderate effects on wine and must composition of Pinot noir associated with GLRaV-1 may be explained by the weak symptoms observed by GLRaV-1 infections on Pinot noir. In one plot, about ¼ of the GLRaV-1 positive vines were asymptomatic (late summer). This observation suggests that the negative impact associated with GLRaVs may correspond to the strength of the symptoms and reflect the intensity of the metabolism's perturbation.

*Tentative analysis of the economic impact of grapevine leafroll disease in Valais.* A simple theoretical approach to consider the economic impact of Grapevine leafroll disease in a given vineyard could be resumed as following: damage x incidence, whereby damage is expressed as economic loss per unit. Incidence of GLRD in Valais could be calculated directly from an extensive study about this thematic (Besse *et al.*, 2009). In the simplest model, we assume homogeneity (vineyard parameters are considered as unchanged throughout the region) and we consider mean values for all calculation (as the incidence was estimated as class of percentage, we may so consider mean values for each interval). Based on these hypotheses, the incidence of GLRD over the entire vineyard could be estimated to 14%. We also assume linearity for damage (the damage observed for a plot with 10% of infected plants corresponds to 10% of the damage estimated for a plot with 100% of infected plants). Using statistics on the vineyard of Valais (CVA, 2007), this simple approach enables the discussion of diverse scenarios on the economic impact of GLRD. For example, to consider a loss of 1.- CHF for a 7 dl bottle of wine made from GLRaV infected vines (represents 5 to 10% of the value of the bottle) leads to an economic impact that represents about 5% of the annual gross yield of the viticulture in Valais.

This simple model may be improved to be more specific. Other aspects and parameters may be considered, such as differential impact according to the varieties or non-linearity of damage (minimal threshold for damage). We may also consider that GLRaVs have different effects on the grape as suggested by the present study and may discriminate among variably detrimental GLRaVs or GLRaV complexes.

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### *ACKNOWLEDGEMENTS*

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# FIELD PERFORMANCES AND WINE QUALITY MODIFICATION IN A CLONE OF DOLCETTO (*VITIS VINIFERA* L.) AFTER GLRAV-3 ELIMINATION

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***Summary***

The effect of GLRaV-3 eradication was studied comparing in the vineyard conditions the healthy and the infected lines of the same clone of the wine cultivar 'Dolcetto'. The results confirm the advantages of using healthy vines in terms of field performances (vigour, yield, leaf photosynthesis, juice soluble solids), however the advantages in the wine quality were questionable.

## INTRODUCTION

Leafroll (LR), one of the most spread virus disease in grapevine, is due to single or mixed infections of different *Ampelovirus* and *Closterovirus*. At present, three of them are considered the most significant: *Grapevine leafroll-associated virus 1*, *2* and *3* (GLRaV-1, GLRaV-2 and GLRaV-3). According to the limited literature available, LR would penalize grape juice soluble solids and other qualitative parameters, such as the amount of anthocyanins in red grape, more than vegetative vigour and yield (Walter *et al*., 1996; Guidoni *et al*., 1997, Mannini, 2003). The aim of this study was to evaluate the effect of LR, associated to GLRaV-3, on the agronomic and technological parameters of a clone of 'Dolcetto' (*Vitis vinifera* L.), one of the most important red wine cultivar of Piedmont (North-west Italy).

## MATERIAL AND METHODS

A clone of 'Dolcetto' formerly infected by GLRaV-3 and tested free from GFLV, GLRaV-1, GLRaV-2, GVA, GVB and GFkV, was heat-treated obtaining the eradication of GLRaV-3. Buds collected from both GLRaV-3 originally infected mother plant and from the healthy line of the clone were propagated by grafting on healthy Kober 5BB rootstock. In 2000, a row of 80 infected vines was then planted side by side with a row of healthy vines in the middle of a commercial vineyard in a typical area for 'Dolcetto' cultivation. Both rows were vertically trained and single-cane pruned. The plantation density was 5000 vines per hectare. When the vineyard reached full production, the main agronomic parameters and the juice composition were assessed for two years (2005-07) on 4 replicates of 6 plants each along the rows. The 2006 vintage was lost due to summer hail. In 2007, a mid August leaf photosynthetic activity, transpiration rate and stomatal conductance were measured by mean of a portable gas-analyser. In 2005 and 2007 vintages, around 60 kilos of grapes from both infected and healthy vines were collected for small scale winemaking. Wines were bottled six months after harvest. Chemical and sensory evaluation were then performed on wines after a few months of rest in the cellar. Sensory evaluations were carried out by a 'three-way' tasting test (i.e. the panel must pick out the two identical wines among a group of three) followed by a ranking test (Ubigli, 2004). A characterization test was also used on 2007 wines to investigate the intensity of the different components of colour, bouquet and taste.

## RESULTS AND DISCUSSION

The vigour of healthy vines, expressed as winter pruning wood weight, was definitely higher than the one of GLRaV-3 infected plants as well as the yield in all the two years controlled (table 1). The superior production was due to both bigger bunches and higher fertility (expressed as n.° of clusters per vine at harvest). Despite the higher yield, the berry juice of healthy plants was characterized by an higher soluble solids concentration compared to the infected plants while titratable acidity was similar. The improved ripening was associated to better leaf photosynthetic efficiency registered by the mid summer gas analyser measurements (figure 1) and by the larger average leaf surface (183 vs. 158 $cm^2$).

Wine chemical composition in 2005 and 2007 vintages indicates an higher alcool degree for the 2007 wine obtained by virus-free plants (table 2). The phenolic content, however, was lower in both the vintages with some implications on wine colour whose intensity and tonality were slightly lighter. A trained panel of 15 tasters was statistically unable to correctly paired the same wines in the 'three-way' test (only 10 right responses over 15) meaning the differences between wines were quite low. Also the results of ranking test have no statistically significance and the preferences of tasters were shared fairly on both the wines (figures 2 and 3).

Where other tests failed, the characterization test may help to better understand the differences between the wines (figure 4). According to the described sensory characters, the 2007 wines from healthy vines statistically showed a less intense violet nuances in the colour, a richer bouquet (fruity flavours), a lighter body but also a lower astringency and a higher taste softness. In other words the wines obtained from virus-free plants were a little less structured but more 'ready to drink' than the wines from infected plants.

Summarizing the results of the trial, it can be said the GLRaV-3 eradication from a clone of 'Dolcetto' resulted in an overall improvement of field performances (higher vigour, yield, bunch size, leaf photosynthetic activity, juice soluble solids, etc.), however it did not result in a tangible

improvement of wine quality as it was expected. The wines obtained from healthy plants were richer in flavours and smoother in taste but lighter in colour and body. These characters, appreciable when the wine is consumed 'young', let say one year time from bottling, could become a weakness in case the wine is kept for longer in the cellar or on a market shelf. To check the wine evolution over the time, tastings will be repeated again two years after bottling.

In conclusion the results confirm the advantages of using healthy vines in terms of field performances however, at least in our experimental conditions, the advantages become more questionable considering the enological quality of the grapes. The complexity of factors concurring to influence the wine quality, such as vintage, cultivar, environmental conditions of cultivation, training systems, etc., makes the understanding of the virus infection/wine quality interaction quite complex and it requires further investigations.

***Table 1.*** Field performances of healthy or GLRaV3 infected vines of the same clone of 'Dolcetto', averages 2005-07 (* p≤0,05)

| Data | Healthy | GLRaV-3 |
|---|---|---|
| Yield (kg/vine) | 2,0* | 1,5* |
| Bunch wt (g) | 228* | 181* |
| Bunch/vine (n°) | 9,0* | 8,3* |
| Pruning wood wt (g/vine) | 382* | 242* |
| Solubile solids (g/l) | 229 | 215 |
| Titratable acidity (g/l) | 6,69 | 6,54 |
| pH | 3,12 | 3,11 |
| Tartaric acid (g/l) | 6,03 | 6,35 |
| Malic acid (g/l) | 1,49 | 1,46 |

***Table 2.*** Composition of wines obtained in two vintages from healthy or GLRaV3 infected vines of the same clone of 'Dolcetto'.

| Data | 2005 | | 2007 | |
|---|---|---|---|---|
| | Healthy | GLR3 | Healthy | GLR3 |
| Alcohol (%vol) | 14,80 | 14,76 | 12,43 | 11,89 |
| Dry extract (g/l) | 23,9 | 23,7 | 22,0 | 21,9 |
| Titratable acidity (g/l) | 5,92 | 5,44 | 5,77 | 6,15 |
| pH | 3,35 | 3,45 | 3,39 | 3,33 |
| Tartaric acid (g/l) | 1,68 | 1,49 | 1,64 | 2,03 |
| Lactic acid (g/l) | 1,11 | 0,45 | 1,63 | 1,56 |
| Potassium (mg/l) | 637 | 808 | 750 | 703 |
| Ash (g/l) | 1,86 | 2,02 | 2,01 | 1,90 |
| Total phenols (mg/l) | 1800 | 2050 | 2130 | 2450 |
| Total anthocyanins (mg/l) | 274 | 323 | 217 | 211 |
| Colour intensity (A420+520+620) | 16,50 | 16,80 | 8,42 | 8,56 |
| Colour hue (A420/520) | 0,56 | 0,55 | 0,67 | 0,60 |

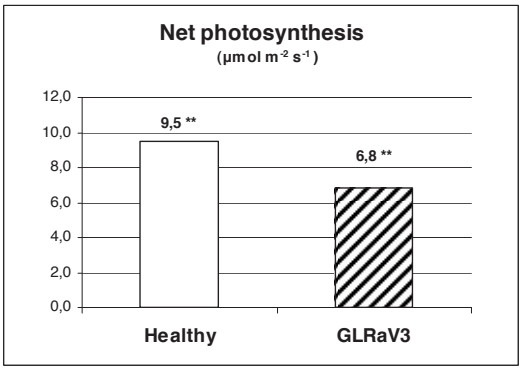


***Figure 1.*** Leaf photosynthesis registered in healthy or GLRaV3 infected vines of the same clone of 'Dolcetto', 2007 (** p≤0,01).

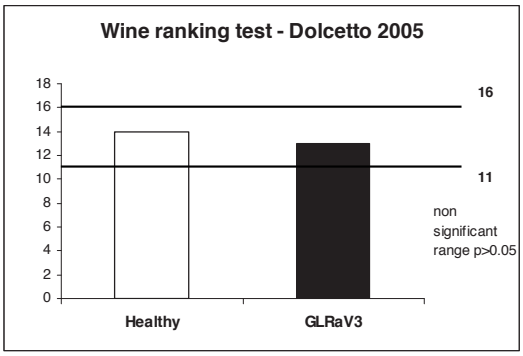


***Figure 2.*** Ranking test on 2005 wines from healthy or GLRaV3 infected vines of the same clone of 'Dolcetto'.

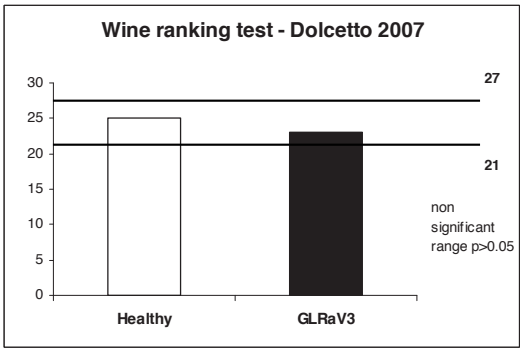


***Figure 3.*** Ranking test on 2007 wines from healthy or GLRaV3 infected vines of the same clone of 'Dolcetto'.

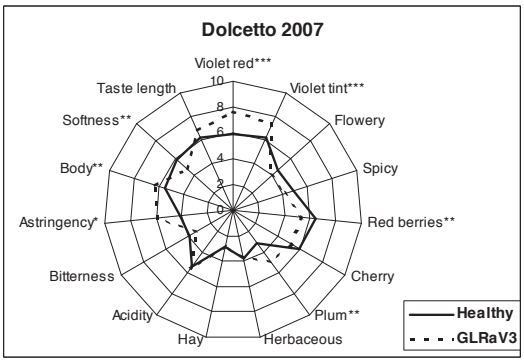


***Figure 4.*** Characterization test on 2007 wines from healthy or GLRaV3 infected vines of the same clone of 'Dolcetto' (* p≤ 0,05 ** p≤0,01 *** p≤0,001).

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# A MILD STRAIN OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* IS PRESENT IN DESIRABLE CLONES OF CRIMSON SEEDLESS TABLE GRAPES IN WESTERN AUSTRALIA

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***Summary***

We have obtained superior clones of Crimson Seedless (CS) table grapes in Western Australia (WA) by introducing combinations of the following viruses into the plants: a mild strain of *Grapevine leafroll-associated virus 3* (GLRaV-3), GLRaV-9 and -5 and *Grapevine virus A*. We detected two strains of GLRaV-3 in different clones of Crimson Seedless. One strain (GLRaV-3-s) produces a severe phenotype, it spreads naturally and is associated with poor quality grape berries. The other strain (GLRaV-3-m) gives a mild phenotype and is associated with larger, crisper and heavier berries as compared to the virus-free CS clone 8210. The nucleotide sequences of the coat protein genes of the mild and severe strains were compared and revealed a high degree of homology. Further sequencing analysis is in progress. A molecular assay is now available to differentiate between these two strains of GLRaV-3 and this will enable us to discard inferior plants infected with the severe strain.

## INTRODUCTION

Grapevine viruses are associated with yield loss, graft incompatibility, vine decline and inferior wine quality, due to an increased acidity and a lower sugar content of grape berries (Walter & Martelli, 1997). The severity of negative effects of viruses is related to the grapevine cultivar and the virus strains present. Antcliff *et al*. (1979) suggested that clones of *Vitis vinifera* cv. Sultana (syn. Thompson Seedless) infected with a mild leafroll virus may be better suited for the table grape industry as these clones had larger berries, fewer bunches and fewer berries per bunch.

Table grape research and clonal selection in Western Australia (WA) commenced in the early 1980's with the observation that local clones of *V. vinifera* cv Emperor with a mild leafroll disease produced crisper and bigger berries than 5 symptomless Emperor clones imported from California (Cameron, 1984). The work presented here summarizes the performance of selected Crimson Seedless (CS) clones following the introduction of certain viruses present in various local Emperor clones in WA. We also report on the diagnosis of the viruses associated with the selected CS clones and the development of a molecular assay for distinguishing between a mild and a severe strain of GLRaV-3.

## MATERIALS AND METHODS

*Plant material.* A virus-free clone of Crimson Seedless (CS), named 8210, was imported from California in 1996 and maintained in private vineyards in WA. Buds from local virus-infected Emperor clones was grafted onto CS clone 8120 and the grafted vines were planted at various sites in WA. Subsequent graftings and clonal selections were carried out for 10 years (Cameron, 2007). This resulted in the production of new clones whose virus status was determined by Waite Diagnostics. Lignified wood of CS clones and other varieties from different sites have been sent since 2004 at 6 month intervals to Waite Diagnostics for a continuous evaluation of virus infection status.

*Nucleic acid extractions and RT-PCR assay.* Total nucleic acids were extracted from wood shavings according to Mackenzie *et al*. (1997). dsRNA was obtained according to Ballija *et al*. (2008), except that a chloroform extraction step was included. For routine RT-PCR assay the forward [GATTTAAGCGCGTTTTTCAGGAC] and reverse [CGGCACGATCGTACTTTCTAA] primers on the P55 gene of GLRaV-3 were used (Mackenzie *et al*., 1997). Complete sequence of the CP gene was obtained following a single step RT-PCR using dsRNA as template and the following primer pairs: CP3U: [ATGGCATTTGAACTGAAATTAGGGC] and CP3D-942: [CTACTTCTTTTGCAATAGTTGGA]. For the RT-PCR detection of GVA, GLRaV-9 and GLRaV-5, the protocol described by Habili & Randles (2002) was followed. Sequencing of DNA fragments was carried out using the ABI Prism Big Dye Cycle Sequencing Ready Reaction kit by the Australian Genome Research Facility (AGRF), Brisbane, Australia.

An ELISA kit (Bioreba, Switzerland) handled according to the manufacturer's protocol was also used for the detection of GLRaV-3.

## RESULTS AND DISCUSSION

*Selected clones of Crimson Seedless and their viruses.* Local clones of virus infected Emperor showing desirable yield and quality criteria (Cameron, 2007) were grafted to virus-free clone 8210 of Crimson Seedless (CS). Three CS

clones were selected and their virus profiles are shown in Table 1.The desirable traits of the berries of each clone, i.e. superior berry colour, crispiness, size and weight have already been reported (Cameron, 2007). Two of these clones, CS-306 and CS-314, were infected with a mild strain of GLRaV-3 (Table 1). When the virus free clone (CS-8210) was inoculated with the bud from CS-314 a significant increase in the berry weight was observed (Table 2). In the same vineyard, scattered vines with inferior fruit quality were also observed (unpublished). When these vines were tested they were positive for the severe strain of GLRaV-3.

***Table 1.*** Selected clones of Crimson Seedless and their virus profile.

| Clone | GVA | GLRaV-3[1] (mild strain) | GLRaV -9 | GLRaV-5 |
|---|---|---|---|---|
| CS 8210 | no | no | no | no |
| CS 306 | yes | yes | yes | yes |
| CS 314 | yes | yes | yes | no |
| CS 311 | yes | no | yes | no |

[1] The mild strain of GLRaV-3 was only detectable by ELISA.

***Table 2***. Average berry weight in virus-free and virus infected Crimson Seedless clones.

| Site/ Clone | CS 8210 | CS 8210 top grafted with CS- 314 |
|---|---|---|
| Site A | 6.3 | 7.5 |
| Site B | 6.9 | 7.4 |
| Site C | 6.2 | 7.2 |
| Site D | 5.9 | 6.8 |
| Site E | 5.5 | 6.5 |

The severe strain of GLRaV-3 (GLRaV-3-s) was always detected in vines with undesirable berry traits. These vines were characterized by severe leaf rolling, reddening and green unripe berries. This strain was detectable both by ELISA and by PCR, while the mild strain (GLRaV-3-m) showed a mild leaf colouring in autumn with no leafrolling. This strain was only detectable by ELISA under our experimental conditions (at 56° C annealing). Since GLRaV-3 spreads in WA (Habili, 2006), and the coat protein can play a role in virus transmission (Bol, 2008) we attempted to sequence this gene in both GLRaV-3 strains. A PCR fragment comprising the full length CP was obtained only when a low annealing temperature of 40° C was employed. A preliminary sequence analysis indicated that the CP of both strains had ~99% nucleotide sequence homology with GLRaV-3 in GenBank.

Grapevine varieties show a range of susceptibility to viruses. While *V. vinifera* cv. Shiraz in Australia is severely affected by GVA, being associated with a disorder called Australian Shiraz Disease, (Habili & Randles, 2004), Cabernet Sauvignon is tolerant to this virus. It has been observed that table grapes are generally more tolerant to viruses than wine grapes. For example, in the Middle East, where old plantations of table grape varieties are prevalent, certain viruses like GVA, GLRaV-1 and 9 are very common (Habili *et al*., 2003; Mahfoudhi *et al*., 2007). Over the centuries table grapes have been selected for quality with no knowledge of the viruses present. However, contemporary breeders select for absence of these viruses during the breeding process. In these newly bred varieties the berry colour is darker and this is a less desirable trait in some markets (Cameron, 2007). By a controlled introduction of "beneficial" viruses into these varieties it may be possible to modify certain quality characteristics of table grapes.

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# DETECTION AND ELIMINATION OF *GRAPEVINE FANLEAF VIRUS* IN CALLUS, SOMATIC EMBRYOS AND REGENERATED PLANTLETS OF GRAPEVINE

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***Summary***

Indirect somatic embryogenesis was tested as a method for eradication of *Grapevine fanleaf virus* from grapevine. RT-PCR for GFLV detection was performed on tissues sampled at various steps of the embryogenic process: flower explants, embryogenic and non-embryogenic calli, single somatic embryos and regenerated plants. *In situ* hybridization using digoxygenin-labelled oligonucleotide probes was used for virus detection in calli. Four months after culture initiation 69 % of tested calli were infected. Only one sample out of thirty four regenerated plantlets was positive to the assay. Although GFLV showed to be able to invade embryogenic calli and embryo-derived plantlets, in our experimental conditions GFLV eradication was obtained by somatic embryogenesis alone with a success percentage close to 100 %.

## INTRODUCTION

*Grapevine fanleaf virus* (GFLV), a soil-borne nepovirus, is the main causal agent of grapevine fanleaf disease, one of the most damaging and widespread viral diseases affecting grapevine. In sensitive cultivars this nepovirus can cause rapid death of young plants or a progressive decline over several years (Martelli, 1993). GFLV is spread both via propagating material and the nematode vector *Xiphinema index*. Establishment of vineyards free of GFLV as well as of other dangerous viruses is an important control measure. Several methods have been used to eliminate viruses from infected grapevine clones. Meristem tip culture is particularly effective in eliminating phloem-limited viruses, while thermotherapy is normally required for the elimination of other viruses such as nepoviruses, that readily invade plant meristems. Somatic embryogenesis, usually adopted to regenerate plantlets in biotechnological breeding programs, was used to eradicate several phloem-limited viruses from grapevine (Goussard *et al*., 1991; Gambino *et al*., 2006). The mechanism whereby regenerated somatic embryos are freed of some viruses is not clear, though it has been noted that there was no translocation of phloem-limited viruses from infected tissue to somatic embryos (Goussard *et al*., 1991). An attempt to eradicate GFLV through somatic embryogenesis was reported by Goussard & Wiid (1992) who obtained the elimination of the virus only if somatic embryogenesis was combined with thermotherapy of the explants.

In the present work we investigated the presence of GFLV at the various stages of grapevine somatic embryogenesis: flower explants, embryogenic and non-embryogenic calli, somatic embryos and embryo-derived plantlets.

## MATERIAL AND METHODS

Embryogenic cultures were initiated from flower explants of Provinè (an Italian cultivar of *Vitis vinifera* L.) infected by GFLV. Immature anthers and ovaries were isolated from inflorescences collected in vineyard and indirect somatic embryogenesis was induced as previously described (Gambino *et al.*, 2006). Embryo-derived plantlets were micropropagated by culturing apical cuttings on a plant growth regulator (PGR)-free medium, thus giving rise to individual lines. In spring the plants were acclimatized and transferred to a cool greenhouse. Anthers and ovaries, embryogenic and non-embryogenic calli collected 4 months after culture initiation, somatic embryos and plants were tested by RT-PCR for presence of GFLV (Gambino *et al*., 2009). Regenerated plantlets were sampled during micropropagation and in the greenhouse. RNA extraction and RT-PCR for GFLV detection were carried out as previously described (Gambino *et al*., 2009).

For *in situ* hybridization, calli collected 4 and 6 months after culture initiation were embedded in 3 % agar, then fixed in 4 % paraformaldehyde and wax-embedded (Paraplast plus). Two GFLV-specific oligonucleotides were designed on conserved regions of coat protein and RNA-dependent RNA polymerase genes of the virus. The oligonucleotides were used as probes and labelled by tailing with the DIG Oligonucleotide Tailing Kit (Roche) according to the the manufacturer's instructions. Sections of 8 µm were transferred to poly-L-lysine-pretreated slides, deparaffinized in xylene and rehydrated through an ethanol series. Prehybridization was performed as described in the Roche protocol. Hybridization was performed at 37°C after dropping 40 µl of *in situ* hybridization buffer (2X SSC, 1X Denhardt's solution, 10% dextran sulfate, 50 mM phosphate buffer pH 7.0, 50 mM DTT, 250 µg/ml yeast tRNA, 100 µg/ml polyadenylic acid, 500 µg/ml denatured and sheared salmon sperm DNA, 47% formamide) containing a total of 225 ng of mixed oligonucleotide probes per ml, onto the slides. Posthybridization washes were carried out following the Roche protocol. The probes detection was performed using the chromogenic substrate BCIP/NPT resulting in a blue color precipitate that was viewed under a light microscope. Sections were then dehydrated through an ethanol-bioclear (Bio-Optica) series and mounted in Bio-mount (Bio-Optica).

## RESULTS AND DISCUSSION

Calli were obtained from both anther and ovary cultures and 4 months after culture initiation high percentages (69 %) of GFLV infection were found in calli (Table 1). These results are similar to those obtained previously (Gambino *et al*., 2006) for phloem-limited viruses. The percentages of infected calli varied according to their origin and it is possible that the larger size of ovules, compared to anthers, may have entailed a higher initial viral inoculum with consequently more frequent virus detection in ovary-derived calli. GFLV was detected in some somatic embryos and in one sample out of thirty

four plantlets regenerated from somatic embryos. Additional RT-PCR analyses were performed on embryo-derived plants cultivated in greenhouse: GFLV was never detected in any of the samples, thus confirming the results of the initial assays.

***Table 1.*** Results of RT-PCR on calli, somatic embryos and plants originated from anthers and ovaries of Provinè originally infected by GFLV.

| Explants | N° of infected samples/ tested |
|---|---|
| Non-embryogenic calli from anthers | 6/11 |
| Non-embryogenic calli from ovaries | 10/10 |
| Embryogenic calli from anthers | 2/6 |
| Embryogenic calli from ovaries | 2/2 |
| Somatic embryos | 2/9 |
| Embryo-derived plants | 1/34 |

*In situ* hybridization was performed to better understand the distribution of GFLV in calli and possibly the mechanism(s) underlying the differentiation of uninfected plantlets. Before the usual wax-embedding of samples, the friable calli were embedded in agar to preserve the original morphology of the calli and the cell arrangement within the samples. In calli originated from ovaries, after 4 months of culture the ovary tissues showed high infection levels (Fig. 1A) and several sectors of the calli as well as some somatic embryos were infected. In anther-derived calli, GFLV was less abundant (Fig. 1B), confirming the results of RT-PCR analyses. As reported by other authors (Scagliusi *et al*., 2002), the calli originating from virus-infected plants are usually a mosaic of infected and uninfected cells, with high concentrations of virus in some groups of cells. In our histological studies no vascular connection between neighbouring embryoids or between grapevine somatic embryos and the parent tissue was observed. However a translocation of GFLV from infected tissues to some somatic embryos (Fig. 1A) and to proembryogenic tissues (Fig. 1C) occurred. The hybridization analysis carried out 6 months after culture initiation showed a different situation, only a few cells of the calli being infected by GFLV and almost no somatic embryos showing hybridization signals (Fig. 1D). These observations are in agreement with previous results on phloem-limited viruses (Gambino *et al*., 2006) and with the results of the RT-PCR analyses performed on embryo-derived plants (Table 1). The average percentage of infected plants regenerated from GFLV-infected explants was 3%. These data differ from those of Goussard & Wiid (1992) who did not obtain any sanitated plant unless the anther cultures were previously heat-treated. There are important differences between the two methods, particularly in the composition of culture media. In our protocol PGRs (BAP, NOA and IAA) were used at high concentrations for prolonged periods of time. There are indications that treatments with cytokinins have a detrimental effects on some viruses, as well as high concentrations of IAA (Clarke *et al*., 1998). According to our preliminary observations, the GFLV-infected sectors of the calli and the early infected embryos seem to show limited proliferation. The rapidly proliferating, young virus-free cells and the somatic embryos regenerated from these may escape virus invasion. More ultrastructural and cytopathological studies of infected calli could provide clear explanations.

This work reports the elimination of GFLV through somatic embryogenesis. Somatic embryogenesis is more technically difficult compared to other sanitation techniques and is largely genotype-dependant, but has proved very effective in elimination of the most important grapevine viruses.

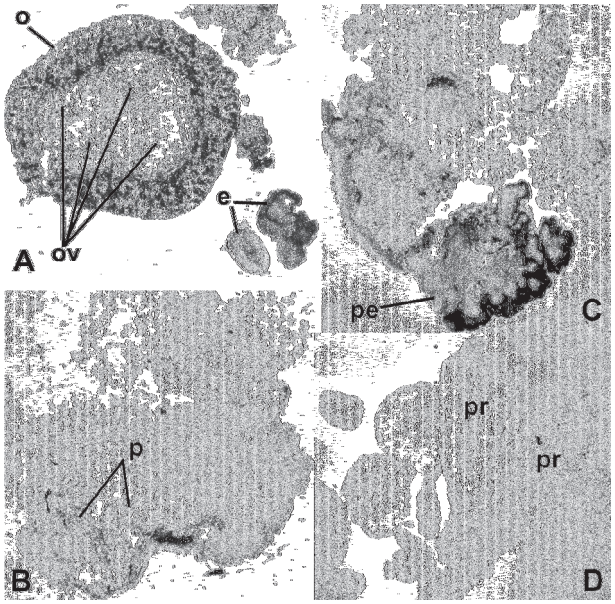


***Figure 1.*** *In situ* hybridization experiments on paraffin sections. Embryogenic calli from ovary (A) and anther (B, C) 4 months after culture initiation, and somatic embryos (D) 6 months after culture initiation. e, infected embryos; o, infected ovary; ov, ovules; p, pollen sacs; pe, infected proembryogenic tissues; pr, procambium.

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# VIRUS ELIMINATION IN INFECTED CLONES OF FOUR GREEK GRAPEVINE WINE CULTIVARS (RODITIS, XINOMAVRO, BAFTRA AND SAVATIANO) USING *IN VITRO* THERMOTHERAPY AND TIP MERISTEM CULTURE

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***Summary***

Four indigenous grapevine wine varieties infected by one or more grapevine viruses were processed through in *vitro* thermotherapy to establish rules effectively adapted in local conditions. Sanitation rate by in *vitro* thermotherapy for the Greek cultivars averaged 96%. The differences found in adaptation to the applied technique between the four grapevine cultivars may reflect the biodiversity of native wine cultivars.

## INTRODUCTION

Grapevine viruses are widespread in most viticultural areas of Greece. The establishment of vineyards with plant material free from some dangerous viruses of grapevine, is a control measure of great importance. Worldwide the *in vitro* thermotherapy combined with *in vitro* shoot tip culture, has been shown to be highly effective in the elimination of grapevine viruses belonging to *Nepovirus*, *Ampelovirus*, *Maculavirus*, and *Vitivirus* genus. In Greece, although many institutions are able to diagnose grapevine virus and virus-like diseases, there is not a national programme to recover and maintain healthy planting material. Furthermore, the available sanitation techniques have not been applied on indigenous varieties and there is not knowledge about their behaviour. In this work, four local grapevine wine varieties infected by one or more grapevine viruses were processed in a sanitation procedure to establish rules effectively adapted in our conditions.

## MATERIALS AND METHODS

The present study deals with the virus elimination using *in vitro* thermotherapy and meristem culture in single plants of vegetatively propagated clones of the Greek wine cultivars Roditis, Xinomavro, Vaftra and Savatiano. All plants were previously assayed by ELISA for the following viruses: Grapevine fanleaf nepovirus (GFLV), Grapevine leafroll-associated Closterovirus 1, 3 and 7 (GLRaV-1, GLRaV-3, GLRaV-7), Grapevine A Vitivirus (GVA), Grapevine B Vitivirus (GVB) and Grapevine fleck Marafivirus (GFkV). Variable periods of *in vitro* heat therapy at 36.5±0.5$^{o}$ C on plantlets *in vitro* (Zlenko *et al*., 1995) were tried for 55-102 days. Meristem tips of 0.4-0.6 mm in size were excised from *in vitro*-grown explants and plated on the grown medium (Murashige & Skoog, 1962; Berthelot, 1934). Regenerated plantlets were checked by using ELISA for virus presence starting from the fifth month (*in vitro*) and two years after *in vivo* acclimatizing.

## RESULTS AND DISCUSSION

Viability rate of *in vitro* thermotherapy for the four Greek cultivars averaged 58% with percentage varying from 42 and 86% mainly depended on vine clone (Table 1). Also the regeneration rate of meristem tips varied widely (5.4 to 58%) depending on cultivar and clone.

Sanitation rate combining *in vitro* thermotherapy and meristem tip explants reached 96%. The period of heat therapy between 75 and 102 days although decreased about 50% the number of treated *in vitro* plantlets, resulted in 100% sanitation. Among the four cultivars, Xinomavro was less adapted to the applied methodology (only the 83.4% of regenerated plantlets were sanitized), while the rates for Roditis, Vaftra and Savatiano were 92.5, 98.5 and 94%, respectively.

The confirmation of eliminating dangerous viruses from the 425 sanitized vines ( plants two years old in pots were checked by ELISA three times) indicate the practical usefulness of *in vitro* thermotherapy (Bottalico *et al.* 2003, Mannini, 2003). The differences in productiveness and adaptation between the four grapevine cultivars to the technique applied may reflect the biodiversity of native wine cultivars. It is worth to notice that plants arising from the healthy mother plants have been planted in pilots vineyards for agronomical and enological evaluation.

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***Table 1.*** Results of sanitation in virus infected clones of four greek grapevine cvs through *in vitro* thermotherapy and tip meristem culture.

| Variety | Clones | Phytosanitary status | Viability (%) | | Number of regenerated plantlets | Results of ELISA tests (infected/tested) after | | Healthy plants |
|---|---|---|---|---|---|---|---|---|
| | | | *In vitro* plantlets | Meristem tips | | 5 months (*in vitro*) | 2 years (in pots) | |
| Savatiano | V9 | GLRaV-2, GLRaV-6 | 66 (39/59) | 26 (10/39) | 41 | 2[1]/33 | 0/27 | 27 |
| | V10 | GLRaV-3, GVA | 86 (69/80) | 16 (11/69) | 40 | 0/32 | 0/26 | 26 |
| | V12 | GLRaV-3, GVA | 66 (31/47) | 29 (9/31) | 16 | 0/12 | 0/6 | 6 |
| Roditis | VD | GLRaV-3, GVA, GVB | 45 (13/29) | 45 (6/13) | 12 | 0/12 | 0/9 | 9 |
| | VE | GLRaV-3, GFLV, GFkV | 55 (37/67) | 57 (21/37) | 49 | 4[2]/36 | 0/31 | 30 |
| | VJ | GLRaV-3, GLRaV-7, GVA | 56 (28/50) | 14 (4/28) | 10 | 2[3]/9 | 0/7 | 7 |
| | V3 | GLRaV-2, GLRaV-3, GVA | 63 (36/57) | 22 (8/36) | 8 | 0/2 | 0/2 | 2 |
| Xinomavro | VB | GLRaV-6, GLRaV-7, GFLV | 42 (30/71) | 10 (3/30) | 12 | 0/12 | 0/11 | 11 |
| | VG | GLRaV-2, GLRaV-3, GFLV, GVA | 48 (37/77) | 5.4 (2/37) | 17 | 8[4]/17 | 0/8 | 8 |
| | VH | GLRaV-2, GLRaV,3, GFLV, GVA | 71 (26/37) | 23 (6/26) | 19 | 0/16 | 0/12 | 12 |
| Vaftra | Ba34 | GVA | 45 (27/45) | 58 (28/48) | 312 | 5[5]/289 | 0/236 | 233 |
| | Ba42 | GVA | 50 (30/60) | 11 (5/47) | 67 | 0/61 | 0/56 | 54 |

1= GLRaV-2 +GLRaV-6, 2= GLRaV-3+GFkV
3= GLRaV-7, 4= GFLV (six plants) and GFLV+GLRaV-2+GLRaV-3+GVA (two plants),
5= GVA

# VIRUS EFFECTS ON VINE GROWTH AND FRUIT COMPONENTS OF THREE CALIFORNIA 'HERITAGE' CLONES OF CABERNET SAUVIGNON

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***Summary***

Three Cabernet Sauvignon clones that were selected from highly regarded vineyards in Napa Valley and treated by meristem shoot tip culture to eliminate viruses, were planted in a randomized vineyard block with their original virus-infected parent selections to compare vine growth and fruit components. All three parent selections were infected with *Grapevine leafroll associated virus*-3 (GLRaV-3), *Grapevine virus B* (GVB), and *Grapevine fleck virus* (GFkV). All three original virus-infected selections had a significantly lower sugar content than their treated progeny, with an average –4.0 Brix. Virus effects on yield, pruning weight, and titratable acidity varied with clone. Two original selections, VIS 29 and VIS 31, had significantly lower yield (average -37.5%) and pruning weight (average -37.1%) than their treated progeny, FPS 29 and FPS 31. One original selection, VIS 31, had a significantly higher titratable acidity than its treated progeny (FPS 31). The variable responses may be due to differences between clones and/or virus strains. Different strains of each species of GLRaV would be expected by plant virologists to demonstrate variation in symptom severity. This data provides some evidence for that hypothesis.

## INTRODUCTION

Vine growth and fruit components of three California 'Heritage' Cabernet Sauvignon clones were compared with their original virus-infected parent selections. The heritage clones were field selections from highly regarded vineyards in Napa Valley known to have virus problems.

Clone FPS 29, the Niebaum-Coppola clone, was from old plantings near the Niebaum-Coppola winery which provided premium quality grapes. Clone FPS 30, the Disney Silverado clone, was from an old vineyard off Silverado trail believed to be planted with the See clone of Cabernet. Clone 31, the Mondavi clone, was collected from 50-year old vines in the To-Kalon vineyard. All three original virus-infected parent selections were infected with *Grapevine leafroll associated virus -3* (GLRaV-3), *Grapevine virus B* (GVB), and *Grapevine fleck virus* (GFkV). Each clone was treated by meristem shoot tip culture to eliminate virus (Golino et *al.,* 2001).

## MATERIAL AND METHODS

Wood for each Heritage clone came from the Foundation vineyard at Foundation Plant Services, UC Davis. For each new clone, wood from the original virus-infected source (VIS) was collected from the UC Davis Grapevine Virus Collection (Golino, 1992). The three pairs of virus-infected parent selections with their healthy progeny clones totaled six different treatments and were: VIS 29 & FPS 29 (Niebaum-Coppola); VIS 30 & FPS 30 (Disney Silverado); and VIS 31 & FPS 31(Mondavi).

The trial was established at the UC Oakville Station in Napa Valley with 1.5 x 2 meter (vine x row) spacing in rows oriented North-South. Budwood was field budded onto certified 101-14 Mgt in spring, 2002, in a randomized complete block design with 5 replications and 12 vines/ treatment/ replication for a total of 60 vines per clone or selection. Vines were trained to a bilateral cordon on a vertical shoot positioned (VSP) trellis, and spur-pruned.

Each plant was observed for leafroll symptoms in the fall. Selected vines were virus tested to confirm infection and monitor possible spread. Initial virus testing was done by ELISA and RT-PCR on both petiole and cambium tissue (Rowhani, 1992, Rowhani *et al*., 2000) Subsequent testing was done using real-time Taqman® RT-PCR (Osman *et al*., 2008). Vine yield components (cluster number, cluster weight, clusters per shoot, berries per cluster and berry weight), fruit composition (Brix, pH, titratable acidity) and vegetative growth parameters (pruning weight and shoot weight) were measured for two years when vines were three and four years old before they were fully mature. Subsequently, the virus-infected selections were removed due to concern that leafroll disease might spread to adjacent clones and trials.

## RESULTS AND DISCUSSION

*Symptom Observations and Virus Testing:* All plants of the original virus-infected selections exhibited typical leafroll disease symptoms consisting of interveinal red color and rolling down of the leaf margins in the fall. Virus test results on a subsample of each original virus-infected selection were positive for GLRaV-3, GFkV and GVB. No leafroll symptoms were observed in the treated clones. Virus testing on a subsample of each treated clone was initially negative. After two years in the vineyard, a few plants of one clone, FPS29, tested positive for GFkV. Testing on seven year old vines showed that FPS29 was infected with GFkV and GVB but not GLRaV-3.

*Vine Growth:* In general, the virus-infected selections required more replanting and took longer to establish trunks and cordons than healthy clones. Vine growth, as indicated by pruning weight, was significantly reduced by virus infection in two clones (Figure 1). Notably, VIS 29 was severely stunted; it had 58% less pruning weight compared to FPS 29. Selection VIS 31 had 24% less pruning weight compared to FPS 31.

*Yield:* Yield was significantly reduced by virus infection in two clones; VIS 29 had a 45% yield reduction

compared to FPS 29; VIS 31 had a 30% yield reduction compared to FPS 31. Also, yield between healthy clones was significantly different; yield of FPS 29 and FPS 30 was almost double that of FPS 31 (3.1, 3.0, 1.7 kg/vine, respectively) (Figure 2). The number of clusters/vine was significantly reduced by virus infection in one clone (28.8, 20.5 for FPS 29, VIS 29, respectively).

*Fruit composition:* Sugar content was significantly lower in all virus-infected selections by an average of 4.0 Brix (P< 0.0001). There was also a small but statistically significant difference in sugar content between healthy clones; FPS 29 had lower sugar content than FPS 30 and 31 (24.2, 24.8, 24.8 Brix, respectively) (Figure 3). Titratable acidity (TA) was significantly higher in virus-infected vines compared to healthy in one clone (7.7, 6.3 g/L for VIS 31, FPS 31, respectively) (Figure 4).

*Discussion:* Sugar content was significantly lower in all virus-infected selections, by an average of 4.0 Brix. In all other parameters, virus effects varied dependent on clone. Yield and pruning weight were reduced in the virus-infected selections in two pairs, VIS29/ FPS29 and VIS31/FPS31, but not VIS30/FPS30. Titratable acidity was increased in the virus-infected selection in one pair, VIS31/FPS31, but not the other two. This may be a result of clonal difference in virus response or strain difference between viruses. Although all three Cabernet Sauvignon selections were infected with the same virus species (GLRaV-3, GVB, and GFkV), they came from diverse sources. There may be strain differences between those species affecting the severity of symptoms. Different strains of each species of GLRaV would be expected by plant virologists to demonstrate variation in symptom severity. This data provides some evidence for that hypothesis.

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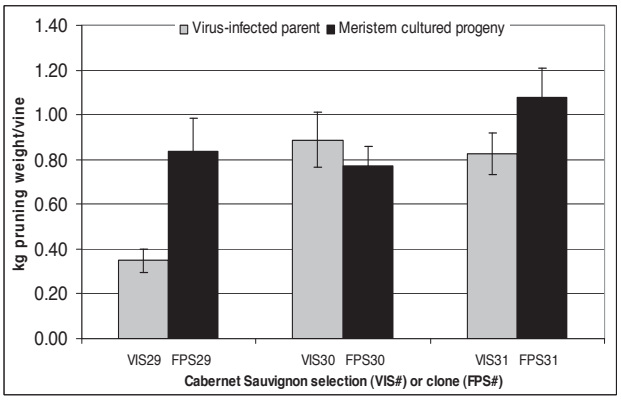


***Figure 1.*** Pruning weight, averaged over two years, for virus-infected parent Cabernet Sauvignon selections (VIS#) and meristem cultured progeny clones (FPS#) with standard error.

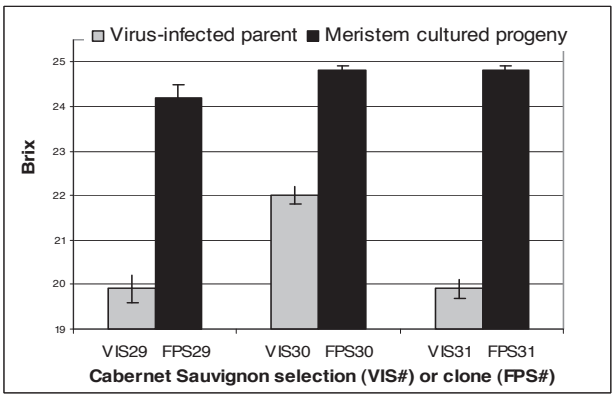


***Figure 3.*** Fruit sugar content (Brix), averaged over two years, for virus-infected parent Cabernet Sauvignon selections (VIS#) and meristem cultured progeny clones (FPS#) with standard error.

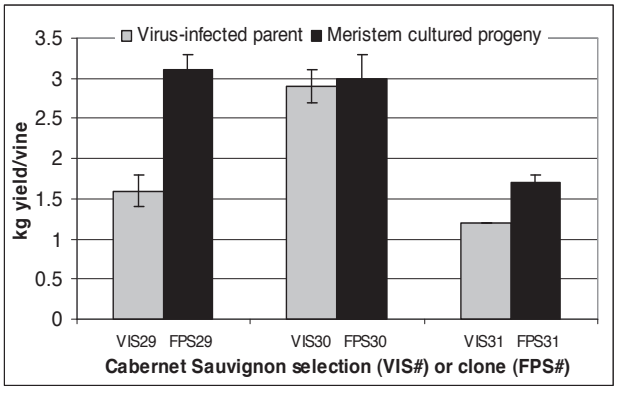


***Figure 2.*** Yield, averaged over two years, for virus-infected parent Cabernet Sauvignon selections (VIS#) and meristem cultured progeny clones (FPS#) with standard error.

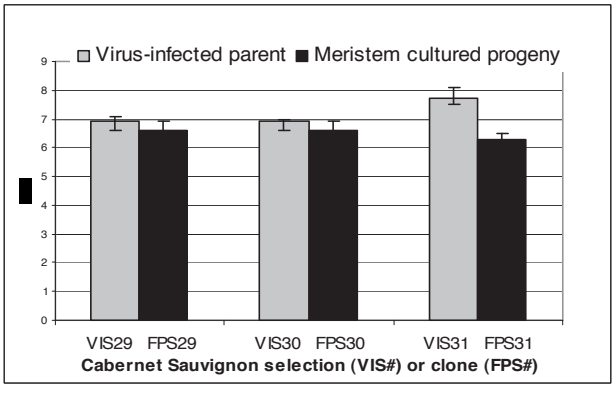


***Figure 4.*** Titratable acidity (TA) , averaged over two years, for virus-infected parent Cabernet Sauvignon selections (VIS#) and meristem cultured progeny clones (FPS #) with standard error.

# VIRUS EFFECTS ON VINE GROWTH AND FRUIT COMPONENTS OF CABERNET SAUVIGNON ON SIX ROOTSTOCKS

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***Summary***

A research block was planted to provide data on the effects of *Grapevine leafroll associated viruses* (GLRaVs), *Grapevine virus B* (GVB), *Grapevine fleck virus* (GFkV) and *Rupestris stem pitting associated virus* (RSPaV), in single and mixed infections, on vine growth and fruit components of Cabernet Sauvignon FPS5 grafted on six rootstocks. Rootstocks included: Couderc 3309, Kober 5BB, Paulsen 1103, Richter 110, Millardet et de Grasset 101-14, and Rupestris St. George. Vines on all six rootstocks had a significant reduction in fruit sugar, by an average of 1.75 Brix, when infected with leafroll associated viruses. There was no significant difference in fruit sugar between healthy and RSPaV-infected vines on any rootstock. Other growth and fruit components were variably affected, dependent on rootstock and virus. Vines grafted onto Kober 5BB and 101-14 also exhibited a significant reduction in yield and pruning weight when infected by leafroll associated viruses. Vines grafted onto 110R and 3309C exhibited a significant reduction in yield when infected by RSPaV but not the leafroll associated viruses; further investigation is needed to confirm these results. Vines grafted onto St. George and 1103P did not exhibit any yield or pruning weight effect of any virus in this study indicating that these two rootstocks were fairly tolerant to these viruses. Leaf symptoms in one virus treatment were markedly different on different rootstocks, possibly indicating different virus titer of and tolerance to different viruses in certain rootstocks.

## INTRODUCTION

A research block was planted to provide data on the effect(s) of *Grapevine leafroll associated viruses* (GLRaVs), *Grapevine virus B* (GVB), *Grapevine fleck virus* (GFkV) and *Rupestris stem pitting associated virus* (RSPaV), in single and mixed infections, on vine growth and fruit components of Cabernet Sauvignon FPS5 grafted on six rootstocks.

## MATERIAL AND METHODS

Vine growth and fruit components of Cabernet Sauvignon clone FPS5, with and without viruses, and grafted onto six rootstocks, was evaluated. Rootstocks included: Couderc 3309 (3309C), Kober 5BB (5BB), Paulsen 1103 (1103P), Richter 110 (110R), Millardet et de Grasset 101-14 (101-14), and Rupestris St. George (St Geo). Rootstocks were selected as representative of commercial rootstocks for the region with the exception of Rupestris St. George which was selected for its virus tolerance.

The trial was established at the UC Oakville Station in Napa Valley with 1.5 x 2 meter (vine x row) spacing in rows oriented north-south. Budwood was field budded onto certified rootstock in spring, 2002, in a randomized complete block design with 5 replications and 5 – 6 vines/ treatment/ replication. Vines were trained to a bilateral cordon on a vertical shoot positioned (VSP) trellis, and spur-pruned.

Virus-infected budwood was collected from the Davis Grapevine Virus Collection (Golino, 1992). Healthy budwood was collected from Foundation Plant Services (FPS). Virus treatments included: LR101- a single infection of GLRaV-3; LR109 - a mixed infection of GLRaV-2, GLRaV-3, and GFkV; RSP115 - a single infection of RSPaV, and LR102 - a mixed infection of GLRaV-1, GLRaV-2, GLRaV-5 and GVB. Thirty-one vines infected with virus accession LR102 were included in an edge row for observation purposes only, due to concerns that vines infected with this virus would not produce usable fruit.

Each plant was observed for symptoms and RT-PCR and/or ELISA tested to confirm virus infection (Rowhani, 1992, Rowhani *et al.*, 2000). Vine yield components (cluster number, cluster weight, clusters per shoot, berries per cluster and berry weight), fruit composition (Brix, pH, titratable acidity) and vegetative growth parameters (pruning weight and shoot weight) were measured for two years when vines were three and four years old before they were fully mature. The vineyard was subsequently removed due to concern that the viruses may spread to neighboring vineyards (Golino *et al.*, 2008).

## RESULTS AND DISCUSSION

*Virus infection:* All vines were successfully inoculated with the virus treatment based on laboratory testing and symptoms. No healthy controls were found to have virus with the exception of RSPaV. RSPaV was detected in 30% of the plants on Millardet et de Grasset 101-14, Kober 5BB, and Rupestris St. George, which we suspect came from the rootstock source. No RSPaV was detected in the healthy treatment plants of 1103P, 110R or 3309C.

*Yield:* Fruit yield was highly dependent on both virus and rootstock (Figure 1). Kober 5BB was most severely affected. The LR109 treatment yield was 12% of what was harvested on the healthy treatment (0.33, 2.71 kg/vine respectively). Some LR109-infected vines on Kober 5BB had no fruit at all. On the rootstock Millardet et de Grasset

101-14, yield was significantly lower in the leafroll infected treatments, LR109 and LR101, but not the RSPaV treatment, as compared to healthy (1.16, 1.58, 2.02, 2.21 kg/vine, respectively). On rootstocks Richter 110 and Couderc 3309, there was a significant difference in yield between the RSP115 treatment and healthy, but not the LR101 or LR109 treatments. This is the first time that RSPaV has been documented to affect yield. More years of data are necessary to confirm this and to see if this pattern continued as the plants matured. On rootstock Paulsen 1103, there was no significant difference in yield between any virus treatment and healthy (2.40 ± 0.29 kg/vine average for all treatments) although yield on the LR 109 treatment was lower than healthy. On St. George, as expected, there was also no significant difference in yield between any virus treatment and healthy (1.69 ± 0.19 kg/vine average for all treatments).

*Fruit Components*: Brix levels on all rootstocks were significantly lower when infected with LR 109 and LR101, but not RSP115 as compared to healthy (average 23.8, 25.1, 26.1, and 26.2 Brix, respectively) (Figure 2). Titratable acidity was significantly affected by viruses in all rootstocks except Millardet et de Grasset 101-14 (Figure 3).

*Discussion:* Vines on all six rootstocks had a significant reduction in fruit sugar, by an average of -1.75 Brix, when infected with leafroll associated viruses. There was no significant difference in fruit sugar between healthy and RSPaV-infected vines on any rootstock. Other growth and fruit components were variably affected, dependent on rootstock and virus. Vines grafted onto Kober 5BB and Millardet et de Grasset 101-14 also exhibited a significant reduction in yield and pruning weight when infected by leafroll associated viruses. Vines grafted onto Richter 110 and Couderc 3309 exhibited a significant reduction in yield when infected by RSPaV but not the leafroll associated viruses; further investigation is needed to confirm these results. Vines grafted onto Rupestris St. George and Paulsen 1103 did not exhibit any yield or pruning weight effect of any virus in this study, indicating that these two rootstocks are fairly tolerant to these viruses. Leaf symptoms in LR102- infected vines were different on different rootstocks, possibly indicating different virus titer and variable tolerance to different viruses, notably, GVB in certain rootstocks. The original goal of making wine from these vines to more clearly establish the effects of virus-infection on wine quality could not be completed due to concern about the rapid field spread of leafroll disease in this area (Golino *et al.*, 2008).

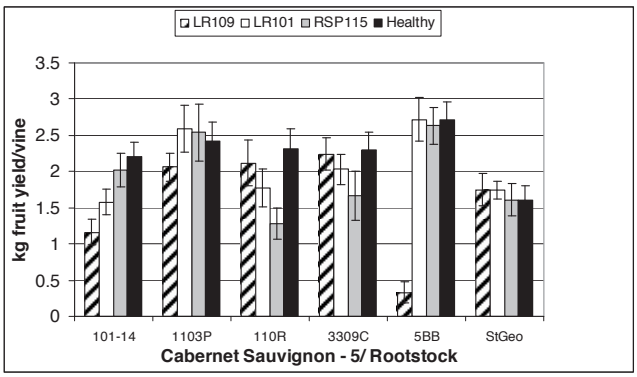


***Figure 1.*** Fruit yield per vine, averaged over two years, for Cabernet Sauvignon FPS5 grafted to six rootstocks.

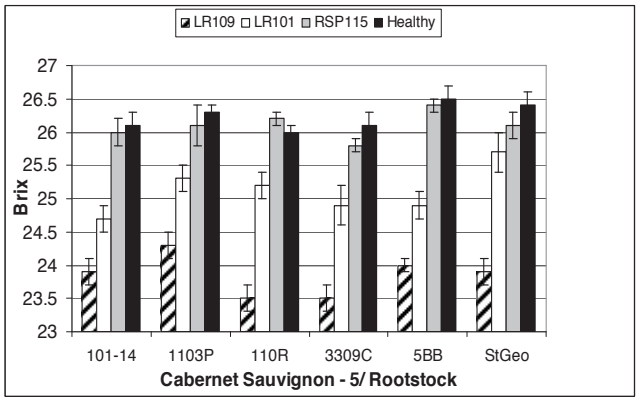


***Figure 2.*** Sugar content (Brix), averaged over two years, for Cabernet Sauvignon FPS5 grafted to six rootstocks.

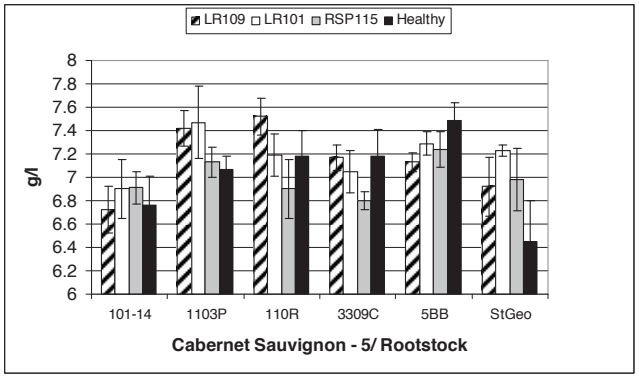


***Figure 3.*** Titratable acidity (TA), averaged over two years, for Cabernet Sauvignon FPS5 grafted to six rootstocks.

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# COMPARATIVE STUDY OF *IN VITRO* BEHAVIOUR OF GRAPEVINE (*V. VINIFERA* L, FETEASCĂ NEAGRĂ CV.) UNDER THE INFLUENCE OF VARIOUS VIRUS INFECTIONS

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***Summary***

*In vitro* studies accomplished with apex explants collected from healthy and GFLV, GLRaV1+3 or GFkV infected vines Fetească neagră cv. emphasized the influence of virus presence on the quantitative and qualitative characteristics of the culture. Significant differences between healthy and virus-infected material according to multiplication rate have been recorded. On the contrary, the elongation of the shoots and the rooting capacity were not significantly influenced by virus infection. The use of biochemical compounds for studying the *in vitro* behaviour of infected grapevine is not recommended. The behaviour of Fetească neagră variety did not differ widely on the virus type. This study provides evidence that Fetească neagră genotype has a tolerant response to various virus infection on *in vitro* conditions.

## INTRODUCTION

Taking into consideration the place of Fetească neagră cv. in the Romanian viticulture patrimony, the necessity of obtaining the grapevine plants, rapidly and at growers' demand quantities, a good sanitary status of the variety is recommended. The use of *in vitro* culture of micro shoots seems to be the best solution for rapid propagation of grapevine. In the same time, the *in vitro* culture represents an easy tool for investigation the behaviour of the plants under the influence of the virus in uniform conditions (Barba et al., 1993; Abracheva et al., 1994; Vişoiu et al., 2000; Buciumeanu and Vişoiu, 2001).

The paper deals with the *in vitro* investigation of Feteasca neagra variety under the influence of simple infections with fanleaf virus (GFLV), leafroll associated virus serotypes 1+3 (GLRaV1+3) and fleck virus (GFkV), some of the most dangerous viruses of grapevine.

## MATERIAL AND METHODS

*V. vinifera* L. plants belonging to Fetească neagră variety showing virus diseases symptoms, identified by ELISA tests (SEDIAG kits) to be infected with one virus (GFLV, GLRaV 1+3 or GFKV), are mentained in the grapevine virus-infected collection and represent the source of inoculum (apex) for *in vitro* investigation. The basic medium used in *in vitro* culture has been represented by M&S (1962) medium, supplemented with 6.6µM BAP and 1.1 µM AIA.

In order to achieve the objective of the work, different investigations have been done, concerning comparative behaviour of virus-infected and healthy biological material, quantitative and qualitative characteristics of *in vitro* culture (multiplication and rooting rates, shoots elongation, abnormal cuttings, necrosis and vitrification phenomena), during 90 days (3 sub-cultures). Biochemical analysis were concerned with the determination of the content of polyphenols, soluble carbohydrates, assimilating pigments (chlorophylls, carotenoids) and dray matter in leaves of regenerated grapevine plantlets. The polyphenols and soluble carbohydrates concentration were expressed in mg % dry weight (d.w.); the pigments concentration was expressed in mg/g fresh weight (f.w.).

Statistical significance of differences between virus-infected variants compared with the control (healthy) was analyzed by SPSS 10 for Windows, taking $P<0,05$ as significant according to one-way ANOVA.

## RESULTS AND DISCUSSION

*Quantitative aspects of the culture.* During the period of the culture, the biological material registered significant differences of the multiplication rate after each sub-culture. In the end of the Ist and the IInd sub-cultures, the GFLV and GFkV infected material registered apparently superior multiplication rates comparatively with the healthy one, due the abundant multiplication of adventive buds and primordia, specific to the viral infection (Table 1).

***Table 1.*** The influence of virus presence on the multiplication rate to grapevine, Fetească neagră cv., over three sub-cultures (I, II, III). Values are mean ± s.d. based on 3 determinations and letters represent statistical signification of differences as compared to control at $P<0,05$

| Sanitary status | Multiplication rate (X) | | |
|---|---|---|---|
| | I | II | III |
| GFLV infected | 1.50±0.02 d | 7.5±0.02 d | 0.51±0.01c |
| GLRaV 1+3 infected | 1.6±0.01 c | 2.7±0.01c | 1.00±0.03 a |
| GFkV infected | 3.0±0.02 b | 5.40±0.01 b | 0.47±0.02 b |
| Healthy (control) | 0.8±0.02 a | 4.75±0.01a | 1.00±0.01 a |

The rooting capacity was 100% both for healthy and viruses-infected cuttings. The elongation of the shoots, the number and the length of the rootlets were not significantly influenced by the presence of virus infection (Table 2).

***Table 2.*** Effects of virus infection on *in vitro* growth and rooting to grapevine, Fetească neagră cv. Values are mean ± s.d. based on 10 determinations and „a" represents statistical signification of differences as compared to control at P<0,05.

| Phytosanitary status | Shoots (cm) | Rootlets | |
|---|---|---|---|
| | | No | Length (cm) |
| GFLV infected | 3.75±2.41a | 3.70±2.79 a | 2.56±1.64 a |
| GLRaV 1+3 infected | 6.00±2.16 a | 4.45±2.21a | 2.86±1.70 a |
| GFkV infected | 5.07±2.92 a | 3.54±2.16 a | 2.83±1.52 a |
| Healthy (control) | 6.22±3.50 a | 3.33±2.18 a | 3.47±1.81 a |

*Qualitative aspects of the culture.* The qualitative analysis of *in vitro* multiplied material emphasized in the end of the culture, especially in the case of GFLV infection, the developing of compact groups of adventive buds (grown together and flat), without multiplication, with abnormal leaves, necroses and vitrification phenomena. The influence of GLRaV1+3 infection produced the multiple roots and abundant anthocyanic coloration of the leaves followed by the necrosis that led to the premature aging of the culture. Not significant differences have been observed on GFkV-infected material. The healthy material did not show vitrification or abnormal micro shoots during the multiplication period.

*Biochemical analysis of regenerated plantlets*. The biochemical content of plantlets indicated significant differences between healthy and virus-infected material for each analyzed parameter (Table 3). However, the differences have not occurred by the accumulation of polyphenols (as stress compounds) under the influence of virus infection; on the contrary, unexpected lower amounts of polyphenols in all infected samples have been registered. On the other hand, lower amounts of carbohydrates have been found in the healthy material comparatively to the infected one. The higher level of carotenoids in GLRaV1+3 infected plantlets have been correlated with the anthocyanic coloration of the leaves. Considering the virus presence, i*n vitro* biochemical tests were not correlated with the results on mature vines (Guță et al., 2004).

The behaviour of grapevine belonging to Fetească neagră variety did not differ widely on the virus type during the culture. This study emphasized that this genotype has a tolerant response to various virus infection on *in vitro* conditions.

***Table 3.*** Effects of virus infection on biochemical parameters of *in vitro* rooted grapevine plantlets, Fetească neagră cv. Values are mean ± s.d. based on 5 determinations and letters represent statistical signification of differences as compared to control at P<0,05.

| Biochemical parameters | Phytosanitary status | | | |
|---|---|---|---|---|
| | GFLV infected | GLRaV 1+3 infected | GFkV infected | Healthy (Control) |
| Dry matter (%) | 8.57±2.55 E-02 d | 10.42±2.49 E-02 c | 8.70±1.14 E-02 b | 9.954±1.51 E-02 a |
| Soluble carbohydrates (mg% d.w.) | 4.80±01.48 E-02 d | 6.16±1.304 E-02 c | 4.64±2.302 E-02 b | 2.01±1.00 E-02 a |
| Polyphenols (mg% d.w.) | 4.33±8.36 E-03 d | 3.68±1.92 E-02 c | 2.40±1.58 E-02 b | 6.26±1.51 E-02 a |
| Chlorophyll a (mg/g f.w.) | 0.604±8.366 E-04 d | 0.664±1.581 E-03 c | 0.546±1.402 E-03 b | 0.570±1.5811 E-03 a |
| Chlorophyll b (mg/g f.w.) | 0.233±4.03 E-03 d | 0.274±1.14 E-03 c | 0.211±1.78 E-03 b | 0.206±1.14 E-03 a |
| Chlorophyll a /b | 2.553±1.923 E-03 d | 2.429±1.516 E-03 c | 2.590±1.667 E-02 b | 2.768±2.30 E-03 a |
| Chlorophyll a+b (mg/g f.w.) | 0.840±1.92 E-03d | 0.939±1.14 E-03 c | 0.759±1.00 E-03 b | 0.874±8.36 E-04 a |
| Carotenoids (mg/g f.w). | 0.414±1.51 E-03 d | 0.453±1.30 E-03c | 0.357±1.40 E-03 b | 0.380±1.14 E-03 a |

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# A STUDY ON LN33 GRAPEVINE INFECTED WITH FIVE VIRUSES

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***Summary***

LN33 plants were artificially infected with *Grapevine leafroll-associated virus 1*, *Grapevine virus A*, *Grapevine virus B*, *Rupestris stem pitting-associated virus* and an unclassified tymovirus. Symptoms of leafroll were observed in infected plants. Corky bark symptoms were never observed. Using of dormant canes just before beginning of vegetation period was proved as the most suitable method for detection of grapevine viruses. For detection of *Grapevine leafroll-associated virus-1*, using ELISA was more reliable than RT-PCR. For detection of *Grapevine virus A*, RT-PCR was more reliable than ELISA. ELISA and RT-CPR gave identical results in detection of *Grapevine virus B*. *Rupestris stem pitting-associated virus* (RSPaV) and an unclassified tymovirus were distributed in infected grapevines different in individual shoots from the same plant.

## INTRODUCTION

Grapevine LN33 was graft-inoculated with five viruses: *Grapevine leafroll-associated virus-1* (GLRaV-1), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Rupestris stem pitting-associated virus* (RSPaV) and an unclassified tymovirus. The effect of this infection on growth of grapevine plants, as well as a distribution of individual viruses in infected plants was evaluated.

## MATERIAL AND METHODS

Inoculum from grapevine cv. Traminer, containing five viruses, was grafted onto young LN33 plants using budwoods in the beginning of vegetation period. Grapevines were further cultivated in a screenhouse. They were trained with short trunk and short horizontal arm of old wood, new shoots were positioned vertically.

Symptoms on leaves and plant habit were evaluated and compared with healthy plants.

Four years after infection the distribution of the five viruses in infected plants was evaluated. Viruses were detected in dormant canes using both ELISA (when available) and RT-PCR. From infected LN 33 plant, two random shoots were taken. They were divided into four sections of about 0.5 m long, described from A (lowest part of the shoot) to D (top part of the shoot). One shoot of grapevine plant cv. Traminer grown in the same conditions, was evaluated as a virus source. One shoot from a healthy LN33 was taken as a negative control.

Antibodies for ELISA detection of GLRaV-1, GVA and GVB were purchased from Agritest Valenzano, Bari, Italy and used according to manufacturers instructions.

Isolation of RNA from dormant shoots was done using RNeasy Plant Mini Kit (Qiagen). RT-PCR was done using OneStep RT-PCR Kit (Qiagen). Primers used for RT-PCR were as follows:

GLRaV-1 two pair of primers:
first according to Habili *et al*. (2003):
5´ TGGCATCGTTGCTAAATTGAG 3´, /
5´ AATCCTATGCGTCAGTATGC 3´, product 175 bp;
second pair of primers (Komínek *et al*. (2005):
5´ TCGGACAGCGTTTAAGTTCC 3´,
5´ CAGGCGTCGTTTGTACTGTG 3´, product 540 bp.

GVA primers (Goszczynski & Jooste, 2003):
5´ TCGAACATAACCTGTGGCTC 3´,
5´ GAGGTAGATATAGTAGGACCT 3´, product 271 bp.

GVB primers (Minafra & Hadidi, 1994):
5´ GTGCTAAGAACGTCTTCACAGC 3´,
5´ AGTAGCCCTTCGTTTAGCCGC 3´, product 158 bp.

RSPaV (this work):
5´ GGTTTCTTAAAGATCCCTTCTTTG 3´
5´ CACAGGCATTTGCACAGAATC 3´, product 432 bp.

Tymovirus primers (Sabanadzovic *et al*., 2000):
5´ CATGCANGTSAGRGGRCCRAA 3´
5´ CYCARCAYAARGTVAACGA 3´, product 386 bp.

## RESULTS AND DISCUSSION

*Symptoms on infected plants :* Several weeks after graft-inoculation, shock symptoms appeared on infected LN33 plants. Leaves were smaller and bronze comparing to a healthy plant, showing symptoms of deformations and shape changes. Growth of infected plants was reduced. A recovery was observed on the plant since second season, symptoms became milder.

Although LN33 grapevines were infected with GVB, corky bark symptoms were never observed, although LN33 is used as indicator of GVB presence. GVB is also considered to have a severe effect on infected grapevines. Rosa (2007) observed that LN33 plants infected with GVB showed extensive necrosis and a complete vine decline a few years after planting.

In our experiment, the only symptom observed in LN33 was a leafroll caused probably by GLRaV-1. One of possible explanations of missing severe symptoms is an occurrence of a milder strain of GVB. Other possible explanation was given by Shi *et al*. (2004), who also did not observed corky bark symptoms on LN33 infected with Australian isolates of GVB and concluded, that the corky bark disease is probably a complex disease caused by a mixture of viruses.

***Table 1.*** Distribution of viruses in grapevine plants

| Virus | | GLRaV-1 | | | GVA | | GVB | | RSPaV | tymovirus |
|---|---|---|---|---|---|---|---|---|---|---|
| Grapevine | Sample | RT-PCR 1 | RT-PCR 2 | ELISA | RT-PCR | ELISA | RT-PCR | ELISA | RT-PCR | RT-PCR |
| LN 33 | Shoot 1, A | + | + | + | + | + | + | + | + | + |
| | Shoot 1, B | - | + | + | + | + | + | + | + | + |
| | Shoot 1, C | - | + | + | + | + | + | + | + | + |
| | Shoot 1, D | + | + | + | + | + | + | + | + | + |
| | Shoot 2, A | - | + | + | + | - | - | - | + | + |
| | Shoot 2, B | - | + | + | + | - | + | + | - | - |
| | Shoot 2, C | - | + | + | + | - | + | + | - | - |
| | Shoot 2, D | - | + | + | + | - | + | + | - | - |
| Traminer | A | - | - | + | + | + | + | + | + | + |
| | B | + | - | + | + | + | + | + | + | + |
| | C | - | + | + | + | + | + | + | - | - |
| | D | + | - | + | + | + | + | + | + | + |

A to D = parts of tested shoot, from A = bottom, to D = top
RT-PCR 1 = primers according to Komínek *et al*. (2005)
RT-PCR 2 = primers according to Habili *et al*. (2003)
- negative; + positive

*Distribution of viruses :* Results of detection of GLRaV-1, GVA, GVB, RSPaV and a tymovirus are presented in Table 1.

*GLRaV-1.* Using ELISA, the virus was detected in all 8 samples from two shoots of LN33 and all 4 samples from Traminer. It seems that virus is present in all parts of dormant grapevine canes, which makes this tissue suitable for virus detection based on ELISA. RT-PCR method was not successfull to detect GLRaV-1 in all parts of tested plants. Primers according to Komínek *et al*. (2005) failed to detect GLRaV-1 in all 4 samples from shoot 2 of LN33. However, mentioned primers were not designed as detection primers. Primers according to Habili *et al*. (2003) consistently detected GLRaV-1 in all 8 samples from LN33, but were not so reliable in samples from Traminer. High specificity of RT-PCR detection decrease reliability of this method probably because of a presence of several different GLRaV-1 strains in one grapevine as reported in our previous work (Komínek *et al*., 2005).

*GVA.* Using RT-PCR, GVA was detected in all 8 samples from two shoots of LN33 and all 4 samples from Traminer. It seems that virus is present in all parts of dormant grapevine canes, which makes this tissue suitable for virus detection based on RT-PCR with primers according to Goszczynski & Jooste (2003). ELISA failed to detect GVA in all samples from second shoot of LN33, probably because of a lower virus concentration there.

*GVB.* Concerning results of GVB detection, we observed absolute agreement of both ELISA and RT-PCR using primers according to Minafra & Hadidi (1994).

*RSPaV and a tymovirus.* The only method to detect RSPaV and a tymovirus was RT-PCR because of unavailability of antisera for ELISA. Very similar results were obtained for the two viruses. One shoot from LN33 was competely positive, while in a second shoot only a sample from the lowest part was positive. The second shoot may has lower concentration of viruses, because the samples were negative not only for RSPaV and a tymovirus using RT-PCR, but also for GVA in ELISA.

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# EFFECT OF *GRAPEVINE FANLEAF VIRUS* (GFLV) AND *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* (GLRaV-3) ON RED WINE QUALITY

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The effect of grapevine viruses in the yield and composition of fruit is well stablished. *Grapevine leafroll-associated virus 3* (GLRaV-3, genus *Ampelovirus*, family *Closteroviridae*) is known to decrease fruit sugar content and titratable acidity (Cabaleiro & Segura, 1996; Borgo & Angelini, 2002). Sugar is stochiometrically converted to alcohol during fermentation, in such a way that final alcoholic degree of dry wines can accurately be predicted from fruit sugar content. Fixed acids remain mostly unaltered during white wine production, but red wine usually withstands a malo-lactic fermentation that decreases the malic acid content. Phenolic compounds, instead, are extracted from grape skins and seeds by the alcohol produced during fermentation. Anthocyanins and tannins, responsible for red colour and long-term stability, respectively, are among them. Moreover, while sugar and acidity measurements in fruit and/or must are standarized (Anonymous, 2006), this is not the case for phenolics. This way, colour intensity, a critical parameter of red wine quality, cannot be reliabily predicted from fruit analyses.

As a result of a previous survey on grapevine viruses, plants infected by either *Grapevine fanleaf virus* (GFLV, genus *Nepovirus,* family *Comoviridae*) or *Grapevine leafroll-associated virus 3* (GLRaV-3, genus *Ampelovirus*, family *Closteroviridae*) were marked in several vineyards of the red variety Tempranillo in the Rioja appellation (Basque Country and Navarre, Northern Spain). Over three years, separate microvinifications were done from infected and uninfected plants. Although this approach introduces more variables in the system, increasing the experimental error, will more reliably reflect the extraction of polyphenols during wine making.

The effect of virus infection on wine quality was smaller than that of the vineyard or the vintage, but still statistically significant. GFLV-infected plants suffered from severe fruit yield loss, mediated by virus-induced poor set. Like any other factor limiting vine vigour, this decrease in yield was counterbalanced by a higher sugar content. It resulted in a more concentrated wine than that from healthy vines, with higher alcoholic degree, titratable acidity and colour intensity. However, this counterbalancing didn't completely compensate for the overall yield loss due to the virus, and resulted in less alcohol, tartaric acid and anthocyanins produced per hectare.

GLRaV-3 was found to decrease final alcohol content by half a degree and seriously diminish colour intensity. Contrarily to the expected, no increase in wine acidity was detected. Borgo and Angelini (2002) already reported that the acidity increase due to this virus was mediated by malic acid. Upon malo-lactic fermentation, this would dissapear and, with it, the acidity difference. The fall of malic acid content is a physiological marker of grape maturity. A higher malic acid content in GLRaV-3 affected vines wolud be indicative of this virus inducing a delayed maturity.

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# LEAFROLL SYMPTOMS CAUSED BY GLRaV-3 ARE ASSOCIATED WITH AN INCREASE OF RESVERATROL IN GRAPEVINE LEAVES

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***Summary***

Biochemical and transcriptional responses induced by Grapevine Leafroll associated Virus-3 (GLRaV-3) were studied in cv. Merlot. At selected leaf developmental stages, stilbenes were quantified by HPLC and the stilbene syntase gene expression monitored by real time PCR. Concentration of piceid was generally higher in the leaves from infected plants than in uninfected ones, although differences were not statistically significant. In September, GLRaV-3-infected basal leaves showed a higher concentration of resveratrol, however no significant increase in the expression of the stilbene syntase gene I was recorded.

## INTRODUCTION

The Grapevine Leafroll-associated Virus 3 (GLRaV-3), one of the most widespread viruses in vineyards, affects the vegetative organs in grapevine, inducing the typical symptoms of foliar deformation and colour alteration.

The most frequently observed defence mechanism in grapevine is the accumulation of molecules belonging to the stilbene family and the synthesis of PR-proteins. Specifically, resveratrol and its derivatives piceid and viniferins have been found in response to biotic or abiotic stress (Jeandet *et al.,* 2002; Bavaresco & Fregoni, 2001). However, most viruses establish a compatible interaction without a resistance response, thus ensuring their replication and propagation.

The aim of this work was to investigate if GLRaV-3 induced changes in the content of resveratrol and piceid, at specific leaf developmental stages and during the onset of virus-induced symptom appearance.

## MATERIAL AND METHODS

In 2006 visual observation were conducted in an experimental vineyard located in Treviso and planted with cv. Merlot. ELISA and RT-PCR analyses were carried out for the diagnosis of leafroll and other viruses. In 2007, four plants (two GLRaV-3 uninfected and two infected) were chosen for the analyses. Basal, medial and apical leaves were collected at four defined developmental stages from May to September and tested for: i) GLRaV-3 presence; ii) expression of stilbene syntase I (*StSI*) gene; iii) concentration of piceid and resveratrol.

Total RNA was isolated from fresh leaves according to MacKenzie *et al.* (1997) and reverse transcribed. The diagnosis of the presence of GLRaV-3 was performed with PCR using virus specific primer pairs. The expression of *StSI* gene was evaluated by real time PCR using the primer pair developed by Aziz *et al.* (2003) and targeting *StSI* gene, and a primer pair developed in our laboratory and targeting the constitutive 26S rRNA gene of grapevine. The relative difference in the gene expression was evaluated with the methods of the ΔΔCt, which takes in account the differences in the threshold cycles (Ct) between the target and the endogenous constitutive gene.

Stilbenes were extracted from fresh leaves powder with 4 ml of methanol. The extract was evaporated to dryness, $10^{-3}$ M HCl and 0.5 g NaCl added to the residue and the resulting solution extracted with ethyl acetate. After completion of evaporation, the residue was dissolved in a methanol/formic acid solution and analyzed by HPLC using previously reported methods (Di Stefano & Flamini, 2008).

## RESULTS AND DISCUSSION

*Analyses of viruses.* ELISA and PCR analyses confirmed the presence of GLRaV-3 in the symptomatic plants, while other viral infections (GLRaV-1, GLRaV-2 and Grapevine Virus A) were excluded. Asymptomatic plants did not show the presence of any of the above mentioned viruses. PCR analyses detected GLRaV-3 in basal leaves at the end of May, although visual symptoms of leaf disease were still not apparent. The upper leaves only tested PCR positive for GLRaV-3 from mid-June. The leafroll associated viruses are known to first infect the basal parts of the branches, later moving towards the upper zones (Cohen *et al.,* 2003). From the beginning of July until September, foliar rolling and alterations in leaf colour became more evident, especially in the oldest basal leaves.

*Presence of stilbenes. Trans*-resveratrol was not detected in the basal leaves in either virus-infected or virus-uninfected samples at the beginning of vegetative growth (mid-June), in spite of the presence of its glycoside (*trans*- and *cis*-piceid) (Tab. 1). A similar result was obtained in mid-July from medial leaves. Both analyses showed a higher level of piceid in the infected leaves, however the differences were not statistically significant ($p>0.05$). Basal leaves were then analysed in September. Interestingly, highly symptomatic leaves were found to contain *trans*-resveratrol, together with piceid, while very little *trans*-resveratrol was detected in virus-uninfected basal leaves. Resveratrol, as well as its derivatives, is known to be induced in leaves by biotic or abiotic stress conditions

***Table 1.*** Stilbene content (mg/kg) and difference in expression level of *StSI* gene in GLRaV-3 infected and uninfected leaves from cv. Merlot collected at four developmental stages in 2007. All values are expressed as mean ± standard deviation. Nd: concentration below the limit of detection.

| Sampling date | Position of leaf | Virus-infected | | | Virus-uninfected | | | *StSI* fold change |
|---|---|---|---|---|---|---|---|---|
| | | Stilbene content (mg/kg) | | | Stilbene content (mg/kg) | | | |
| | | *trans*-resveratrol | *trans*-piceid | *cis*-piceid | *trans*-resveratrol | *trans*-piceid | *cis*-piceid | |
| 31 May | apical | | | | | | | 1.90±0.89 |
| 14 June | basal | nd | 123.2±9.5 | 79.5±0.6 | nd | 114.1±18.2 | 117.5±61.8 | |
| 12 July | apical | | | | | | | 1.12±0.78 |
| | medial | nd | 80.4±22.0 | 87.3±17.5 | nd | 37.4±12.1 | 41.8±11.3 | 0.50±0.37 |
| 5 Sept | apical | | | | | | | 0.50±0.33 |
| | basal | 14.1±14.0 | 102.3±50.1 | 95.7±31.4 | 1.38±0.30 | 37.8±24.1 | 60.3±26.5 | 1.70±0.88 |

(Bavaresco & Fregoni, 2001). In grapevine leaves the presence of *trans*-resveratrol, together with α-, ε-viniferins and *trans*-pterostilbene has been reported to be induced by *Plasmopara viticola* and *Botrytis cinerea* attacks (Poutaraud *et al.,* 2007; Pezet *et al.,* 2004a,b; Pezet *et al.,*2003; Bézier *et al.,* 2002). In contrast, grapevine leaves infected with GLRaV-3 behaved similarly to senescent tissues and increased transcript levels of some ROS (reactive oxygen species)-scavenger enzymes (Espinoza *et al.,* 2007). Therefore, it is tempting to speculate that in September the symptomatic virus-infected basal leaves of cv. Merlot showed resveratrol because of a virus-induced oxidative perturbation.

*Expression of StSI gene.* The expression of the *StSI* gene was studied in GLRaV-3-infected and uninfected leaves collected from May to September. The values varied between 0.5 and 1.9; however, considering a 2-fold cut-off, there were no significant differences, which means that in the tissues and in the developmental stages analysed the expression of the gene did not seem to depend on the presence of the virus. This was surprising, as both the piceid and resveratrol were present in the leaves in absence of a higher expression of the *StSI* gene. A possible reason for this result could be that, as the stilbene syntases are encoded by a multigenic family, it is possible that the stilbenes produced in response to the viral infection were produced by a *StS* gene different from the *StSI* analysed in this work.

*Conclusions.* This work is a preliminary investigation about the possible induction of the synthesis of stilbene by GLRaV-3 in grapevine leaves from cv. Merlot. Throughout the season, the concentration of piceid was generally higher in the infected plants than in uninfected ones, although the differences were not statistically significant. In September, at the end of plant vegetative growth, the virus induced the synthesis of *trans*-resveratrol at low levels in only the basal symptomatic leaves. A possible 'virus-induced' effect in such basal symptomatic leaves may be postulated. As the *StSI* gene did not seem to be responsible for the increase of resveratrol, other genes involved in the synthesis/ transformation of resveratrol will be investigated. Further analyses will be performed for a second year, using a larger number of plants and also investigating the content of viniferins.

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# EFFECTS OF GVA ELIMINATION ON PHYSIOLOGICAL, AGRONOMIC AND OENOLOGICAL CHARACTERISTICS OF A *V. VINIFERA* MARZEMINO CLONE

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***Summary***

In this work we compared, during two years in two vineyards (Trentino, Northeastern Italy), some physiological, agronomic and oenological characteristics of a *V. vinifera* cv. Marzemino clone before and after elimination of GVA. The best effects of sanitation on leaf parameters resulted in higher values of Fv/Fm at veraison, and dry matter at harvest. By the other hands, at harvest, weight of petioles and blades resulted higher in GVA vines. Sanitation affect significantly bud fertility whereas yield parameters did not resulted modified. In our environment, GVA vines are more vigorous than sanitised vines. Oenological parameters were did not significantly affected by GVA elimination.

## INTRODUCTION

The present work continue the evaluations of main characteristics on a Marzemino clone before and after GVA elimination as previously described (Malossini *et al*., 2003; 2006). Eradication of virus (both GLRaV-1 and GVA) from clone SMA 9 was performed by means of thermotherapy *in vitro* and meristem culture (Gribaudo *et al.*, 2003). Controls by ELISA test were carried out from 1998 till today, on different lines.

## MATERIAL AND METHODS

GVA infected and sanitated vines (cv. Marzemino, clone SMA 9) were compared in two vineyards during two years (2007 and 2008). Single vines samples of the control (infected vines = GVA) and of the healthy *ex-vitro* material (GVA-free) were tested from 2000 to 2008 by ELISA for ArMV, GFLV, GFkV, GLRaV-1, 2, 3 and GVA, using commercial kit (Agritest, Valenzano-Bari, Italy). GVA and GVA-free self rooted vines were stored in greenhouse and in field located at San Michele all'Adige (year 2001, Guyot trained). A second vineyard (year 2005, simple pergola trained) was planted at Rovereto, in the same cool climate condition than the first one, with vines of both materials propagated onto GLRaV-1 and GVA-free rootstock (Kober 5BB). In both experimental vineyards was planted two blocks with 4-20 vine for each treatment. During 2 years (2007 and 2008), 20 to 30 leaves from GVA and GVA-free plants were sampled at veraison and at harvest. Physical and chemical analyses of blades and petioles were carried out, i.e. nitrogen (by Kjeldahl) and mineral elements' content (by ICP-OES), SPAD-index (by SPAD 502 Chlorophyll Meter, Minolta; Porro *et al*., 2000) and chlorophyll fluorescence (PAM-2000 fluorometer; Walz, Effeltrich, Germany). Amount of chlorophyll (Chl), carotenoids (Car) and total soluble proteins were spectrophotometrically determined (Lichtenthaler, 1987). Fertility of buds, yield (grape and wood production) and characteristics of grape measured on 15 bunch for each treatment (weight of bunch, berry, and stem cluster, soluble solids, total acidity, pH, anthocyanins and polyphenols) were evaluated. For the comparison of treatments Student's t test has been used. All statistical analyses were performed with Statistica software (StatSoft, Tulsa, USA).

## RESULTS AND DISCUSSION

*Sanitary conditions.* Results of ELISA test confirm the GVA elimination (Table 1); however, 27.4 % of tested GVA originary infected resulted negative or false negative.

***Table 1.*** Results of ELISA test on samples of a clone of cv. Marzemino. Samples (leaf or wood) were gathered (from 2000 to 2008) in field or in greenhouse from single vine

| Original labelled clone | Conventional label | N of tested samples | % of positive or doubt samples | |
|---|---|---|---|---|
| | | | GLRaV 1 | GVA |
| SMA 9 | GVA | 134 | 0.0 | 72.6 |
| | GVA-free | 311 | 0.3 | 0.0 |

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*Leaf parameters.* Mineral content, SPAD Index and pigment content (Chl a, Chl b, Chl a+b, Carotenoids) of leaves were not affected by GVA sanitation both at veraison and at harvest (Table 2). Only the ratio between Chl and Carotenoids resulted significantly higher on GVA-free at veraison. The best effects of sanitation on leaf parameters resulted in higher values of Fv/Fm ratio (Chl fluorescence) at veraison, and dry matter at harvest. By the other hands, at harvest, weight of petioles and blades resulted higher in GVA vines.

*Agronomic and oenological parameters.* GVA sanitation mainly affects some agronomic characteristics, in particular bud fertility (both real and potential) resulted 23 % higher in GVA-free respect to GVA vines. Yield parameters did not significantly differ with the exception of vine vigour which resulted higher in GVA vines. Bunch analyses revealed significantly differences only on stem cluster weight (higher on GVA). Remaining parameters (weight of bunch and berry, soluble solids, total acidity, pH, anthocyanins and polyphenols) did not result affected by sanitation.

***Table 2.*** Effect of GVA sanitation on some parameters in leaves of cv. Marzemino – clone SMA 9, collected at veraison and harvest in two vineyards in years 2007 and 2008 (means ± std. error).

| | Treatment | | sig. |
|---|---|---|---|
| | GVA | GVA-free | |
| **VERAISON** | | | |
| Blade weight (g) | 5.65 ± 0.183 | 5.14 ± 0.176 | n.s. |
| Petiole weight (g) | 1.83 ± 0.088 | 1.74 ± 0.098 | n.s. |
| Leaf area ($cm^2$) | 289.4 ± 10.89 | 267.0 ± 10.25 | n.s. |
| Leaf dry matter (%) | 30.5 ± 0.554 | 29.2 ± 0.653 | n.s. |
| SPAD Index | 36.5 ± 0.300 | 36.3 ± 0.291 | n.s. |
| Fv/Fm | 0.733 ± 0.017 | 0.776 ± 0.011 | ** |
| Chl a + chl b | 1.47 ± 0.064 | 1.49 ± 0.058 | n.s. |
| **HARVEST** | | | |
| Blade weight (g) | 6.24 ± 0.228 | 5.44 ± 0.206 | * |
| Petiole weight (g) | 2.21 ± 0.133 | 1.85 ± 0.091 | * |
| Leaf area ($cm^2$) | 323.5 ± 19.08 | 282.9 ± 15.84 | n.s. |
| Leaf dry matter (%) | 31.1 ± 0.722 | 32.5 ± 0.384 | * |
| SPAD Index | 37.9 ± 0.332 | 38.1 ± 0.328 | n.s. |
| Fv/Fm | 0.720 ± 0.007 | 0.719 ± 0.007 | n.s. |
| Chl a + chl b | 1.45 ± 0.083 | 1.37 ± 0.108 | n.s. |

** = significant ($p < 0.01$), * = significant ($p < 0.05$), n.s. = not significant

***Table 3.*** Effect of GVA sanitation on some agronomic and oenological parameters of cv. Marzemino - clone SMA 9, in two vineyards in years 2007 and 2008 (means ± std. error).

| | Treatment | | sig. |
|---|---|---|---|
| | GVA | GVA-free | |
| Number of buds/vine | 16.5 ± 0.858 | 18.2 ± 1.127 | * |
| Budbreak (%) | 67.9 ± 1.72 | 69.5 ± 2.14 | n.s. |
| Real fertility | 0.70 ± 0.038 | 0.86 ± 0.041 | ** |
| Potential fertility | 1.01 ± 0.043 | 1.24 ± 0.043 | *** |
| Vigour (kg wood/vine) | 1.32 ± 0.156 | 1.11 ± 0.124 | * |
| Number of bunches/vine | 9.8 ± 0.945 | 11.5 ± 0.923 | n.s. |
| Yield (kg) | 2.92 ± 0.374 | 3.55 ± 0.421 | n.s. |
| Bunch weigth (g) | 298 ± 21.2 | 312 ± 23.8 | n.s. |
| Berry weight (g) | 2.43 ± 0.074 | 2.57 ± 0.091 | n.s. |
| Stem cluster weight(g) | 18.9 ± 1.73 | 16.2 ± 1.65 | * |
| Soluble solids (°Brix) | 19.8 ± 0.316 | 19.8 ± 0.478 | n.s. |
| Total acidity (g/L) | 6.53 ± 0.430 | 6.22 ± 0.464 | n.s. |
| pH | 3.29 ± 0.033 | 3.31 ± 0.042 | n.s. |
| Anthocyanins (mg/Kg berries) | 979.4 ± 87.3 | 855.2 ± 15.84 | n.s. |
| Polyphenols (mg/Kg berries) | 1061.9 ± 87.7 | 936.2 ± 29.7 | n.s. |

*** = significant ($p<0.001$), ** = significant ($p < 0.01$), * = significant ($p < 0.05$), n.s. = not significant

In conclusion, the sanitation of the GVA infected clone significantly increased only bud fertility. This result is however of practical interesting due to the natural low fertility of cv. Marzemino. In any case yield parameters did not result significantly affected.

On the contrary of previous results obtained by the sanitation from both GLRaV-1 and GVA (Malossini *et al*., 2003; 2006), in this work potential photosynthetic activity and pigment content did not result different, with the exception of chlorophyll fluorescence (Fv/Fm ratio) at veraison.

In the environmental conditions of trial (temperate climate without idric stress) GVA vines resulted most vigour (kg wood/vine), with bigger and heavy leaves at harvest respect to GVA-free vines.

Regarding the oenological characteristics all the analysed parameters were not affected by GVA sanitation.

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# EFFECTS OF GLRaV 1 ELIMINATION ON PHYSIOLOGICAL, AGRONOMIC AND OENOLOGICAL CHARACTERISTICS OF TWO CV. MARZEMINO CLONES

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***Summary***

In this work we compared, during two years in two vineyards (Trentino, North-East Italy), some physiological, agronomic and oenological characteristics of two *V. vinifera* cv. Marzemino clones, before and after GLRaV 1 elimination. The leafroll sanitation confirms the data previously obtained, resulting in an increased leaf dimension and efficiency (SPAD index, pigment content) at veraison and, especially, at harvest. On the other hand, the positive effects of leafroll sanitation on agronomic characteristics resulted only in a significant increase of bunch and stem cluster weight, while the fertility of buds, yield and vigour of vine seem to be not influenced. Leafroll sanitation of the two clones did not significantly change most of the oenological parameters analysed and the results of wine tasting.

## INTRODUCTION

The present work continues and specifies the evaluations reported in previous trials (Malossini *et al*., 2003; 2006), performed with only one clone (SMA 9) affected by double virus infection (GVA and GLRaV 1). In this paper a single virus sanitation (GLRaV 1) on two Marzemino clones (SMA 18 and SMA 9) was evaluated. Clone SMA 18 is confirmed to be affected only by GLRaV 1. Eradication of virus was carried out by means of thermotherapy *in vitro* and meristem culture (Gribaudo *et al.*, 2003). Controls by ELISA test were carried out from 1998 till today, on different lines.

## MATERIAL AND METHODS

GLRaV 1 infected (=LR1) and sanitated (=LR1-free) cv. Marzemino clones (SMA 9 and SMA 18) vines were compared in two vineyards during two years (2007 and 2008). Single vines samples of the control (infected vines = LR1) and of the healthy *ex-vitro* material (LR1-free) were tested from 2000 to 2008 by ELISA for ArMV, GFLV, GFkV, GLRaV-1, 2, 3 and GVA using commercial kit (Agritest, Valenzano-Bari, Italy). LR1 and LR1-free self rooted vines of two clones were stored in greenhouse and in field located at San Michele all'Adige (year 2001, Guyot trained). A second vineyard (year 2005, simple pergola trained) was planted in Rovereto, in the same cool climate conditions as the first one, with vines of both materials propagated onto GLRaV-1 and GVA-free rootstock (Kober 5BB). In both experimental vineyards two blocks with 4-20 vine for each treatment and clone were planted. During 2 years (2007 and 2008), 20 to 30 leaves from LR1 and LR1-free plants were sampled at veraison and at harvest. Physical and chemical analyses of blades and petioles were carried out, i.e. nitrogen (by Kjeldahl) and mineral elements' content (by ICP-OES), SPAD-index (by SPAD 502 Chlorophyll Meter, Minolta; Porro *et al*., 2000) and chlorophyll fluorescence (PAM-2000 fluorometer; Walz, Effeltrich, Germany). Amount of chlorophyll (Chl), carotenoids (Car) and total soluble proteins were spectrophotometrically determined (Lichtenthaler, 1987). Fertility of buds, yield (grape and wood production) and characteristics of grape (weight of bunch, berry, and stem cluster, soluble solids, total acidity, pH, anthocyanins and polyphenols) measured on 15 bunch for each treatment were evaluated. The grapes (120 kg/sample) from one vineyard (at Rovereto) were processed according to the following main steps: crushing-destemming, 50 mg/L SO2, inoculum with selected dry yeast (30 g/hL), punching the cup twice a day, 7-day skin-contact, soft pressing of pomace with total blending of free run and press wine, malolactic fermentation using selected lactic bacteria, sulfiting, sterile bottling. Paired-preference test of the wines (control *vs* LR1-free) were carried out with 64 judges. For the comparison of treatments, Student's t test has been used. All statistical analyses were performed with Statistica software (StatSoft, Tulsa, USA)

## RESULTS AND DISCUSSION

*Sanitary conditions.* Results of ELISA test confirm the GLRaV 1 elimination (Table 1); the materials of LR1-free SMA 9 clone confirm, with 72.6 % of samples, GVA originary infection.

***Table 1.*** Results of ELISA test on samples from single vine of two clones of Marzemino.

| Original labelled clone | Conventional label | N of tested samples | % of positive or doubt samples | |
|---|---|---|---|---|
| | | | GLRaV 1 | GVA |
| SMA 9 | LR1 | 123 | 96.6 | 98.0 |
| | LR1-free | 134 | 0.0 | 72.6 |
| SMA 18 | LR1 | 110 | 96.6 | 0.0 |
| | LR1-free | 209 | 0.2 | 0.0 |

Samples (leaf or wood) were gathered (from 2000 to 2008) in field or in greenhouse.

*Leaf parameters.* Pigment content (Chl a, Chl b, Carotenoids) and SPAD index of leaves (Table 2) were highly affected by GLRaV 1 sanitation both at veraison and harvest: LR1-free samples showed higher values. Fv/Fm ratio (chlorophyll fluorescence) revealed the same values on treatments both at veraison and harvest. Leaves of LR1-free samples showed only at harvest a significant increasing in weight of petioles and blades as well as in leaf area.

Some mineral elements resulted significantly different between treatments (data not show). At veraison in LR1-free blades S, Ca and Mg resulted higher than in LR1 blades; on the contrary, K was higher in LR1 blades. In LR1-free petioles both Zn and Ca resulted higher. At harvest only S in blades resulted affected by sanitation with higher values on LR1-free samples.

***Table 2.*** Effect of GLRaV-1 sanitation (LR1 *vs* LR1-free) on some parameters in leaves of two cv. Marzemino clones, collected at veraison and harvest in two vineyards in years 2007 and 2008 (means ± std. error).

| Parameter | Treatment | | sig. |
|---|---|---|---|
| | LR1 | LR1-free | |
| **VERAISON** | | | |
| Blade weight (g) | 5.06 ± 0.145 | 5.12 ± 0.149 | n.s. |
| Petiole weight (g) | 1.55 ± 0.066 | 1.62 ± 0.061 | n.s. |
| Leaf area ($cm^2$) | 261.3 ± 8.65 | 263.6 ± 8.25 | n.s. |
| SPAD Index | 33.6 ± 0.202 | 37.3 ± 0.210 | *** |
| Fv/Fm | 0.767 ± 0.008 | 0.758 ± 0.011 | n.s. |
| Chl a | 0.960 ± 0.038 | 1.100 ± 0.033 | ** |
| Chl b | 0.357 ± 0.015 | 0.404 ± 0.011 | * |
| Car | 0.318 ± 0.009 | 0.376 ± 0.088 | *** |
| **HARVEST** | | | |
| Blade weight (g) | 4.79 ± 0.133 | 5.40 ± 0.176 | ** |
| Petiole weight (g) | 1.48 ± 0.056 | 1.87 ± 0.086 | *** |
| Leaf area ($cm^2$) | 236.4 ± 7.71 | 276.2 ± 12.4 | ** |
| SPAD Index | 35.4 ± 0.280 | 38.4 ± 0.226 | *** |
| Fv/Fm | 0.733 ± 0.005 | 0.721 ± 0.005 | n.s. |
| Chl a | 0.832 ± 0.033 | 1.044 ± 0.046 | *** |
| Chl b | 0.315 ± 0.014 | 0.416 ± 0.022 | *** |
| Car | 0.315 ± 0.009 | 0.377 ± 0.012 | *** |

*** = significant ($p < 0.001$), ** = significant ($p < 0.01$), * = significant ($p < 0.05$), n.s. = not significant

*Agronomic and oenological parameters.* The virus elimination effects in yield resulted significant only in bunch and stem cluster weight, wich showed higher values in LR1-free samples (Table 3). Vigour of vines (Kg wood/vine) resulted in the same range. Fertility of buds was not significantly different among treatments, but LR1 revealed higher values of total and break buds per vine. Must analyses revealed slight differences only in pH and K content, with higher values in LR1. No significant differences between treatments emerged by means of wine tasting.

In conclusion, the effects of GLRaV 1 sanitation confirms the previous information, resulting in an increased leaf dimension and efficiency (SPAD index, pigment content) at veraison and, especially, at harvest. On the other hand, the positive effects of leafroll sanitation on agronomic characteristics resulted only in a significant increase of bunch and stem cluster weight, while the fertility of buds, yield and vigour of vine seem to be not influenced. Moreover, leafroll sanitation of the two clones did not change significantly most of the oenological parameters analysed and the results of wine tasting.

Similar studies will be continued also in other conditions, for a better knowledge of the interactions between virus and grapevine clones.

***Table 3.*** Effect of GLRaV-1 sanitation (LR1 *vs* LR1-free) on some agronomic and analytical parameters of two clones cv. Marzemino, in two vineyards in years 2007 and 2008 (means ± std. error).

| Parameter | Treatment | | sig. |
|---|---|---|---|
| | LR1 | LR1-free | |
| Number of buds/vine | 19.6 ± 0.862 | 15.9 ± 0.591 | *** |
| Budbreak (%) | 70.8 ± 0.015 | 69.9 ± 0.011 | n.s. |
| Real fertility | 0.81 ± 0.034 | 0.78 ± 0.028 | n.s. |
| Potential fertility | 1.13 ± 0.361 | 1.10 ± 0.032 | n.s. |
| Vigour (Kg wood/vine) | 1.13 ± 0.077 | 1.18 ± 0.097 | n.s. |
| Number of bunches/vine | 11.1 ± 1.132 | 11.3 ± 0.692 | n.s. |
| Yield (kg) | 2.81 ± 0.283 | 3.38 ± 0.297 | n.s. |
| Bunch weigth (g) | 259 ± 14.4 | 303 ± 13.9 | * |
| Berry weight (g) | 2.28 ± 0.089 | 2.34 ± 0.059 | n.s. |
| Stem cluster weight (g) | 14.7 ± 1.34 | 19.31 ± 1.61 | ** |
| Soluble solids (°Brix) | 19.8 ± 0.342 | 19.6 ± 0.284 | n.s. |
| Total acidity (g/L) | 6.27 ± 0.302 | 6.26 ± 0.258 | n.s. |
| pH | 3.32 ± 0.025 | 3.29 ± 0.020 | * |
| Anthocyanins (mg/Kg berries) | 952.3 ± 36.8 | 977.2 ± 47.7 | n.s. |
| Polyphenols (mg/Kg berries) | 1029.5 ± 31.0 | 1039.8 ± 48.3 | n.s. |

*** = significant ($p < 0.001$), ** = significant ($p < 0.01$), * = significant ($p < 0.05$), n.s. = not significant

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# CONTROL OF GRAPE BERRY MECHANICAL PROPERTIES MODIFICATIONS DUE TO GFLV BY MEAN OF TEXTURE ANALYSIS

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***Summary***

Healthy and GFLV+GFkV infected lines of the same 'Nebbiolo' clone were compared in a vineyard in La Morra (North-west Italy) to investigate the influence on grape quantitative parameters and juice qualitative composition due to virus infection. In 2008, the observations were focused to evaluate the main mechanical properties of the berry skin and of the whole berry by mean of several Texture Analysis tests. The results showed some significant differences in the berry parameters which may have practical effect on wine quality.

## INTRODUCTION

The soilborne nepovirus *Grapevine fanleaf virus* (GFLV) is the casual agent of fanleaf degeneration, one of the main viral diseases worldwide spread in vineyards. The symptoms, ranging from leaf and cane malformations to chrome-yellow discoloration, are reported very often associated with vegetative vigour and yield reduction (Mannini *et al*., 2000; Mannini, 2003). The *Grape fleck virus* (GFkV) is associated to fleck, a widespread viral disease which induce latent infection in *Vitis vinifera* (Credi & Babini, 1997*).* 'Nebbiolo', the grape cultivar which gives origin to some of the most renowned red wines of Piedmont (North-west Italy), is highly sensitive to GFLV and a reduction of vegetative vigour and of crop quantity due to the virus is reported (Mannini *et al.*, 1999).

In red wines, and particularly in 'Nebbiolo' wines, the content of phenols is considered crucial to determine the quality affecting colour, body, taste, stability and aging. The texture analysis is an excellent analytical technique to verify skin hardness measurements, parameters able to estimate the extractability of phenol with adequate reliability (Rolle *et al.*, 2008b). Furthermore, the value of some mechanical parameters such as the ease of detachment of the pedicel, the hardness and thickness of the skin, is directly related to shattering and to resistance against splitting and berry rot (Gabler *et al*., 2003; Lang & During, 1990).

The aim of this study was to investigate the effect of *Grapevine fanleaf virus* (in mixed infection with *Grapevine fleck virus*) on the berry mechanical properties in a Nebbiolo clone.

## MATERIAL AND METHODS

Vines of healthy and GFLV+GFkV infected lines of the same clone of 'Nebbiolo', grafted on certified '420 A' rootstocks, were established side by side in a vineyard in La Morra (North-west Italy)**.** The sanitary status of every single vine was confirmed by (DAS) or (DASI)-ELISA performed on dormant cane samples collected in winter 2007-08 and using commercial kits (Agritest Srl, Valenzano, Ba, Italy) according to the manufacturer's instructions. At the harvest (1/10/2008), observations were carried out on around 20 single vines of both healthy and infected lines for yield parameters determinations. In addition two samples of around 100 berries each were collected from a three vines parcels replicated three times along the rows. Berries were randomly picked with attached pedicels from both sides of each cluster on the three vines of each parcel. Half berry samples were weighted and then pressed for juice composition analysis and the remaining half utilized for Texture Analysis tests. The juice analytical parameters of grapes (soluble solids content, total acidity, pH) were estimated with EEC methods (1990). Tartaric and malic acid content was evaluated by HPLC (Schneider *et al*., 1987). The measurements for the appraisal of grape mechanical properties were made on sub-samples of 30 berries taken out of the 100 berries of each replicate. The tests were performed the same day the berries were picked in order to avoid alterations. Before the test, the berries, arranged on a single layer, were thermally conditioned at 20 ° C in thermostat. A Universal Testing Machine TAxT2i Texture Analyzer (Stable Micro Systems - SMS, Surrey, UK) equipped with a HDP/90 platform (perforated or not) and a 5 kg load cell was used. All the acquisitions were made at 400 Hz; data were evaluated using the Texture Expert Exceed software package (vers. 2.54 in Windows 2000). Skin hardness was evaluated by puncture test (Letaief *et al*., 2008a) while for whole berry characteristic a texture profile analysis (TPA) test was used. Skin thickness was calculated as the distance between the point corresponding to the probe contact with the berry skin (trigger) and the platform base during a compression test (Letaief *et al*., 2008b).

## RESULTS AND DISCUSSION

Although GFLV symptoms on the infected vines were rather limited, field parameters such vigour and yield resulted in favour of healthy vines, confirming the results of previous experiences (table 1). Also the berries were bigger as shown by the higher weight and the lager diameter (table 3). Juice composition indicates a slightly better ripeness of the grapes harvested on infected vines (higher soluble solids, lower acidity, higher pH) compared to the ones from the more yielding healthy vines (table 2).

The mechanical characteristics of berries at the harvest are reported in table 3. In general terms, several parameters determined by the TPA resulted different among healthy or infected vines showing a different firmness of the whole berry according to the virological status. This become particularly evident with normalized data (i.e. referred to the respective berry diameters).

***Table 1.*** Field performances of healthy or GFLV (+GFkV) infected vines of the same clone of 'Nebbiolo', 2008.
*=p< 0.05, **= p< 0.01, ns=not significant.

| Data | Healthy | GFLV | Sign. |
|---|---|---|---|
| Yield (kg/vine) | 2.41 | 1.76 | ** |
| Bunch wt (g) | 350 | 296 | ns |
| Berry wt (g) | 1.59 | 1.48 | ** |
| Pruning wood wt (g/vine) | 798 | 648 | * |

***Table 2.*** Juice composition of healthy or GFLV (+GFkV) infected vines of the same clone of 'Nebbiolo', 2008.

| Data | Healthy | GFLV |
|---|---|---|
| Solubile solids (g/l) | 230 | 235 |
| Titratable acidity (g/l) | 7.07 | 6.80 |
| pH | 3.02 | 3.06 |
| Tartaric acid (g/l) | 6.03 | 5.65 |
| Malic acid (g/l) | 0.91 | 0.84 |

***Table 3*** Mechanical parameters of healthy or GFLV (+GFkV) infected vines of the same clone of 'Nebbiolo', Stand = parameter referred to berry diameter.
*= p≤0.05, **= p≤ 0.01, *** =p≤ 0.001, ns=not significant

| Data | Healthy | GFLV | Sign. |
|---|---|---|---|
| Berry diameter (mm) | 12.88±0.42 | 12.30±0.66 | *** |
| Skin break force (N) | 0.52±0.09 | 0.51±0.10 | ns |
| Skin break energy (mJ) | 0.31±0.11 | 0.28±0.09 | ns |
| Young's Modulus (N/mm) | 0.42±0.07 | 0.45±0.07 | * |
| Skin thickness (µm) | 199±22 | 217±24 | ** |
| Berry Hardness (N) | 4.53±0.46 | 4.68±0.56 | ns |
| Berry Cohesiveness (-) | 0.73±0.02 | 0.72±0.02 | ns |
| Berry Gumminess (N) | 3.28±0.26 | 3.35±0.36 | ns |
| Berry Springiness (mm) | 2.15±0.08 | 2.04±0.11 | *** |
| Berry Chewiness (mJ) | 7.05±0.64 | 6.86±0.98 | ns |
| Berry Resilience (-) | 0.40±0.02 | 0.40±0.02 | ns |
| Berry Hardness stand. | 0.35±0.04 | 0.38±0.04 | ** |
| Berry Gumminess stand. | 0.25±0.02 | 0.27±0.02 | ** |
| Berry Springiness stand. | 0.17±0.002 | 0.17±0.001 | ns |
| Berry Chewiness stand. | 0.04±0.004 | 0.05±0.004 | ** |

The most relevant differences are related to skin thickness parameters which resulted higher in the berries of infected vines.

Berry skin thickness, together with the skin break force, is an important technological parameter for the red grapes as related to the extractability of the phenolic substances from the skin (Rolle *et al.,* 2008a; Rolle *et al*., 2008b). An interaction of effect between skin break force and stages of ripening is reported for the extraction of the anthocyanins (Rolle *et al*., 2009). Harder skins lead to a more complete extraction of anthocyanins, but with slower extraction kinetics. Indeed, break skin force and skin thickness are directly related to cell permeability index (EA%) (Rio Segade *et al*., 2008), which provides information on the ease of transfer of these compounds. As more EA% values are higher, as slower is the dissolution of anthocyanins in the must (Cagnasso *et al*., 2008).

In the case of 'Nebbiolo', being a cultivar rich of di-substituted anthocyanins whose stability is low, a slower dissolution of these pigments could be considered a technological advantage in term of wine colour.

In conclusion GFLV infection in vines seems to significantly interfere with physical and mechanical features of berries. Further investigations however have to be carried out to better understand the practical implication of these modifications on winemaking and wine quality.

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# 2-AMINO-6-MERCAPTOPURINE: A PRELIMINARY STUDY OF A NOVEL CHEMICAL GROUP FOR GRAPEVINE ANTIVIRAL THERAPY

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***Summary***

Chemicals that can inhibit other sensible point of purine biosynthesis than inosine monophosphate dehydrogenase are considered as potential antiviral drugs. Among them, mercaptopurine and its derivates are immunosuppressive drugs widely used in medical therapy, which mechanism of action can be partially associated to inhibition of purine biosynthesis. Aim of this research is a preliminary study regarding the antiviral action of 2-amino-6-mercaptopurine against Grapevine Leafroll-associated virus-1 and -3 in grapevine plants. The drugs sems to be effective on virus replication but any eradication of virus was registered. These findings suggest further steps of treatments and/or higher dosage of drug, making mercaptopurines interesting as group of chemicals for searching novel antiviral drugs for grapevine chemotherapy.

## INTRODUCTION

Chemical procedures are considered potential tool for restoring plants infected by viruses, even if few chemicals have been found to eliminate or substantially reduce replication of phytoviruses (Griffiths *et al*., 1990) as compared to the broad range of therapeutic chemicals available against human viruses. Anyway plant research can get useful information from medical studies, considering chemicals which ability to interfere to virus replication in animal tissue could be potentially transferred to plant systems. This approach had lead to investigate effect of ribavirin against many viruses (i.e. Shepard, 1977; De Fazio & Vicente, 1991; Nascimento *et al*., 2003; Panattoni *et al*., 2007a), tiazofurin (De Fazio & Vincente, 1991; Panattoni *et al*., 2007b), or mycophenolic acid (D'Anna, 2006). These chemicals, considering medical research, are involved in inhibition of the inosine monophosphate dehydrogenase (IMPDH), a key point of purine biosynthesis, even if others way of action can be linked to final effect in virus elimination.

Considering IMPDH as a starting point for investigate novel chemicals in plant application, we focus our attention in drugs able to interfere not only against that enzyme, but chemicals that can inhibit other sensible point of purine biosynthesis. Among them, mercaptopurine and its derivates are immunosuppressive drugs widely used in medical therapy, which mechanism of action can be partially associated to inhibition of purine biosynthesis. 6-mercaptopurine can be converted into thioinosinic acid (TIMP), an intercellular nucleotide able to inhibit various chemical reaction referred to inosine monophospahte (IMP), as its conversion in xanthosine mophosphate, acting as IMPDH alternative substrate (Gearry & Barclay, 2005). Moreover TIMP and its derivate 6-methilthiosinate (MTIMP) are able to inhibit de novo synthesis of purine. In fact, this metabolic process need production of phosphoribosylamine catalyzed by glutamine-5-phosphoribosyl-1-pyrophosphate (PRPP) amidotransferase (Zrenner *et al*., 2006). TIMP and MTIMP are inhibitors of PRPP amidotransferase (Koeningsknecht et al., 2007) reducing IMP pool, with final effect in available purine for virus synthesis. Another action point of TIMP is its competitive action against guanosine for hypoxanthine-guanine phosphoribosyl transferase (Gearry & Barclay, 2005).

This mechanism of action is potentially replicable in plants, with interesting results obtained by 1- and 2-amino-mercaptopurine against Cucumber Mosaic Virus in tobacco plants (Luvisi *et al*., data not published). Considering that, aim of this research is a preliminary study regarding the antiviral action of 2-amino-6-mercaptopurine (2-AMP) against Grapevine Leafroll-associated virus-1 and -3 (GLRaV-1 and -3) in grapevine plants (*Vitis vinifera* L. cv. Sagrantino).

## MATERIAL AND METHODS

*Source of in vitro-material : In vitro* grapevine explants were obtained from field-grown *Vitis vinifera* L. cv. Sagrantino naturally infected by GLRaV-1 and -3. All explants were maintained in a controlled environment chamber and transferred to proliferating medium (Quorin & Lepoivre, 1977) at 30 day intervals. After an acclimatization period, the sanitary condition of each plantlet was confirmed by the ELISA test and RT-PCR. Therapeutic drug was added separately to the proliferation medium for treated replicates. At the same time, untreated explants were maintained on drug-free proliferation medium.

*Antiviral drug :* 2-AMP was purchased from Sigma–Aldrich (St. Louis, MO). Drug was hydrated in stock solution and, immediately prior to use, ultra-filtrated and added to proliferation medium. The experimental design involved drug administration for three consecutive subcultures, for a total treatment duration of 90 days. A preliminary screening on healthy explants submitted to 30 days of administration at several concentrations (from 0.10 to 0.30 mM) was carried out to determine drug-induced phytotoxicity. To define the phytotoxic levels, the number of dead explants out of total treated explants was counted at the end of the first subculture for each dose.

*Virus detection by ELISA :* The ELISA test was performed according to the method described by Clark and Adams (1977). Tissue samples from healthy and infected explants were used as negative and positive controls, respectively. Polyclonal antibodies to GLRaV-1 and -3 (Agritest, Italy) were used in ELISA tests. After each

treatment (30 days), surviving explants were counted. The apical portion (0.5-1.0 cm) was then transferred to fresh supplemented medium, and the residue was assayed with the ELISA test. Absorbance at OD405 nm was recorded by photometry (Titertek multiskan). Readings were normalized as R value (OD sample / OD healthy), identifying the R = 2 threshold which distinguishes the positive response versus the negative response (Monette, 1983). Virus RNA was extracted from grapevine explant using components of the Plant RNeasy kit (Qiagen, Germany). RNAs were detected using a one step RT-PCR procedure based on the Qiagen One Step RT-PCR kit.

## RESULTS AND DISCUSSION

*Drug toxicity on explants :* No mortality on untreated healthy control explants was observed due to the micro propagation technique. No toxic effect on 2-AMP-treated healthy explants was detected up to 0,30 mM concentration.

*Effects of chemotherapy on GLRaV-1 and -3 :* ELISA readings (R) of treated and untreated infected explants were examined at the end of each therapeutic period. Registered R values of treated explants showed a progressive decrement after each treatment, suggesting a virus static-like effect of 2-AMP against GLRaV-1 (Fig. 1) and -3 (Fig. 2). Anyway, 3 times treatment, no virus eradications from explants were registered by RT-PCR.

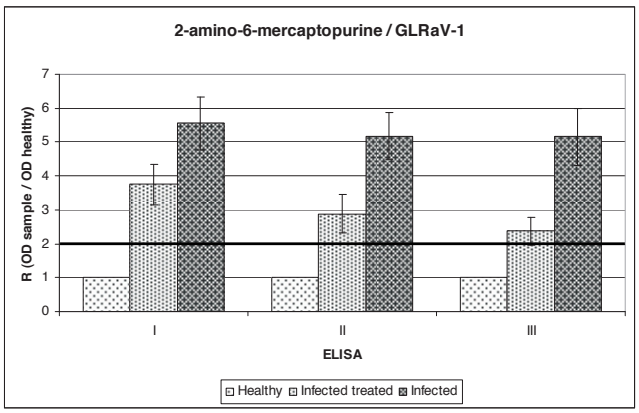


***Figure 1.*** R values in treated and untreated infected plants by GLRaV-1 compared to healthy plants.

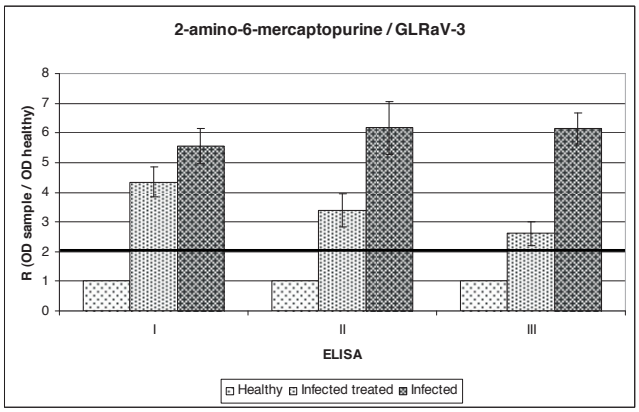


***Figure 2.*** R values in treated and untreated infected plants by GLRaV-3 compared to healthy plants.

The genomic similarity between some phytoviruses and some animal viruses has prompted the suggestion that compounds which have shown efficient results against animal viruses should also be screened for efficacy against plant viruses. 2-AMP belong to the class of purine biosynthesis inhibitors that include drugs currently being investigated in medical antiviral research programs, and some preliminary results shown a potential antiviral effect. Our results showed no toxic effects on grapevine explants after 2-AMP administration up to 0,30 mM, and a 3 times treatment showed a reduction in R values in treated infected plants compared untreated ones, causing a virus static-like effect against GLRaV-1 and -3. Conversely, RT-PCR analysis confirm any eradication of viruses in explant, suggesting further steps of treatments and/or higher dosage of drug. These results made mercaptopurines interesting as group of chemicals useful for searching novel antiviral drugs for grapevine chemotherapy.

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# ADVANCES ON THE ERADICATION OF *GRAPEVINE RUPESTRIS STEM PITTING ASSOCIATED VIRUS* (GRSPaV) FROM *Vitis Vinifera* EXPLANTS

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***Summary***

Elimination studies of *Grapevine rupestris stem pitting associated virus* (GRSPaV) were carried out in various grapevine cultivars using *in vitro* chemotherapy and a combination of *in vitro* thermotherapy with meristem (≤ 0.2 mm) or shoot tip culture (≤ 0.5 cm). Data obtained herein indicate that GRSPaV elimination depends on the genotype of grapevine though in most cases high eradication percentages were achieved. The application of antiviral compounds, which were tested here for the first time against GRSPaV, gave promising results. Likewise the cultivation of larger plant tissue parts (shoot tips) combined with an appropriate thermotherapy profile *in vitro* has led to high elimination rates.

## INTRODUCTION

GRSPaV, a member of the genus *Foveavirus*, is implicated in the development of two economically important grapevine viral diseases namely Rugose Wood (RW) syndrome and Grapevine Vein Necrosis (GVN) (Martelli, 1993; Zhang *et al*., 1998; Bouyahia *et al*., 2005). According to the European and Mediterranean Plant Protection Organization (EPPO), the certification scheme for grapevine should include tests for many virus and virus-like diseases, among which for GVN (Roy, 2003).

GRSPaV is reported to be particularly difficult to eliminate using meristem tip culture, *in vivo* and *in vitro* thermotherapy (Minafra & Boscia, 2003; Skiada *et al*., 2009). Somatic embryogenesis seems to be so far the most promising method for its elimination (Gribaudo *et al*., 2006) however it is technically more difficult, time consuming and jeopardizes for mutations.

In this study two different approaches were evaluated for the elimination of GRSPaV. More specifically, treatment with antiviral compounds of the inosine monophosphate dehydrogenase (IMPDH) group, which gave promising results against other grapevine viruses (GLRaV-3, GVA), and a combination of *in vitro* thermotherapy with meristem or shoot tip culture were applied on various GRSPaV-infected grapevine cultivars.

## MATERIAL AND METHODS

*In vitro* established grapevine explants of the Agiorgitiko, Mantilaria, Prevezaniko and Malagouzia cultivars naturally infected with GRSPaV were used. Agiorgitiko and Malagouzia were culured in a modified WPM and a GAL medium respectivelly (Skiada *et al*., 2009), whereas Mantilaria and Prevezaniko were cultured in a modified QL medium (unpublished data). Heat treatment was carried out in a growth chamber for 6 weeks, where 16 h photoperiod and cool white fluorescent light (40 μM $m^{-2}$ $s^{-1}$) were set. The initial temperature was 26 °C for the day and 23 °C for the night. These temperatures were gradually increased by 3 degrees per week and the final temperatures, which were maintained for one week, were 40 °C for the day and 37 °C for the night except for the case of Malagouzia where the maximum tolerable temperature applied was 36 °C. When the heat treatment period ended, meristems (0.1-0.2 mm) and shoot tips (0.5 cm) were excised from the apical bud of the plantlets and further cultivated in the appropriate for each cultivar medium.

The antiviral compounds Tiazofurin (Tr), Ribavirin (Rb) and Mycophenolic acid (MPA) were also used for the elimination of GRSPaV in Agiorgitiko. Tr and Rb were hydrated in stock solution while MPA was diluted in 100% ethanol and, immediately prior to use, ultra-filtrated and added to the proliferation medium. Concentrations of 1, 5, 10, 15, 20, 30, 40, 60 and 80 μg/ml from each drug were administered to the explants. A control medium without any antiviral compounds was also included. The duration of the treatment was 80 days and it was divided into two subcultures. Drug-induced phytotoxicity was defined by the number of dead explants out of total treated explants and was estimated at the end of the first subculture for each drug dose (40 days of culture).

GRSPaV diagnosis was based on the generic nested RT-PCR developed for the detection of viti- and foveaviruses (Dovas & Katis, 2003). Virus testing was conducted every six months in *in vitro* and *ex vitro* conditions in the case of heat treated plantlets and at the end of 80 days of chemotherapy.

## RESULTS AND DISCUSSION

*Survival rates and GRSPaV elimination post thermotherapy*: The combination of *in vitro* thermotherapy with shoot tip culture was effective for the elimination of GRSPaV, as shown by the nested RT-PCR assays. In all cases almost half of the explants subjected to thermotherapy survived the heat stress (Table 1). The majority of the survived plantlets showed no shoot tip or other kind of necrosis. The highest percentage of explants originating from shoot tips of the four cultivars, survived and regenerated while a great portion of these were also found GRSPaV-free (Table 1). The virus eradication was remarkably high in Prevezaniko (92.85%) followed by Agiorgitiko (50.81%). The application of meristem culture after the thermotherapy step was also successfully applied in the case of Agiorgitiko and Malagouzia cultivars (Table 1). These results were consistently confirmed by all the nested RT-PCR assays conducted both in *in vitro* and *ex vitro* conditions.

***Table 1.*** Survival rates and virus elimination post thermotherapy.

| Grapevine cultivar | Number of explants survived post thermotherapy | MERISTEMS | | SHOOT TIPS | |
|---|---|---|---|---|---|
| | | Number of regenerated plantlets provided by meristems | GRSPaV elimination | Number of regenerated plantlets provided by shoot tips | GRSPaV elimination |
| **Agiorgitiko** | **137/260 (52.60%)** | **34/61 (55.70%)** | **23/34 (67.64%)** | **61/76 (80.30%)** | **31/61 (50.81%)** |
| Malagouzia | 179/350 (51.14%) | 30/100 (30%) | 7/30 (23.30%) | 41/79 (51.89%) | 12/41 (29.26%) |
| Mantilaria | 95/160 (59.37%) | 2/40 (5%) | 0/2 (0%) | 53/55 (96.36%) | 21/53 (39.62%) |
| Prevezaniko | 72/172 (41.86%) | 0/36 (0%) | - | 28/36 (77.77%) | 26/28 (92.85%) |

***Table 2.*** Elimination of GRSPaV in Agiorgitiko explants, 80 days post- treatment with Tr, Rb and MPA.

| Antiviral | Concentration (µg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 5 | 10 | 15 | 20 | 30 | 40 | 60 | 80 |
| Tr | 0/60 | 6/44 | 14/34 | 8/10 | 0/2 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| | 0% | 13.6% | 41% | 80% | 0% | - | - | - | - | - |
| Rb | 0/60 | 10/58 | 12/52 | 12/46 | 10/32 | 8/20 | 10/16 | 0/0 | 0/0 | 0/0 |
| | 0% | 17.2% | 20% | 26% | 31.2% | 40% | 62.5% | - | - | - |
| **MPA** | 0/60 | 10/60 | 22/36 | 22/30 | 16/22 | 12/14 | 10/12 | 8/10 | 6/8 | 2/4 |
| | 0% | 16.6% | 61.1% | 73.3% | 72.7% | 85.7% | 83.3% | 80% | 75% | 50% |

*Phytotoxicity induced by antivirals and GRSPaV eradication*: At the end of 40 days of culture all the explants derived from the control media survived whereas, all antivirals caused plant mortality at high concentrations. Drug toxicity was more severe in the case of Tr, while side effects caused by MPA were milder. In concentrations 20 µg/ml Tr and 40 µg/ml Rb and higher total mortality of the Agiorgitiko explants occurred. At the end of the antiviral treatment virus-free plantlets were detected. Higher elimination rates were achieved at concentrations of 10 µg/ml Tr, 30 µg/ml Rb and 20 µg/ml MPA, respectively (Table 2).

Even though much work has been done on the eradication of GRSPaV using conventional techniques (Gribaudo *et al*., 2006) there is still space for further experimentation in order to evaluate the efficacy of each method and define the most suitable technique.

Overall, the results of our study indicated that both the combination of *in vitro* thermotherapy with meristem or shoot tip culture and the chemotherapy with Tr, Rb or MPA can adequately eliminate GRSPaV in grapevine. Virus elimination was easier in some of the cultivars (Prevezaniko, Agiorgitiko) which underwent thermotherapy, thus supporting the conclusion that the success of this process depends on the specific interaction between GRSPaV and grapevine genotype. The fact that GRSPaV eradication was feasible with larger plant tissue parts (shoot tips), in combination with *in vitro* thermotherapy, is particularly important. The application of a proper temperature profile in combination with the culture of a large number of shoot tips can lead to an adequate number of healthy plantlets, thus avoiding the problems of meristem culture even for viruses which are difficult to eradicate such as GRSPaV. To our knowledge, this is the first report on the use of antiviral drugs for the production of GRSPaV-free *Vitis vinifera* plants. The elimination rates varied according to the type of antiviral compound. Further experimentation is needed for the evaluation of the antiviral efficacy of these or other compounds in other grapevine cultivars as well.

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