LIST OF POSTER PRESENTATIONS

THE FLIGHT ACTIVITY OF *SCAPHOIDEUS TITANUS* BALL (HOMOPTERA CICADELLIDAE): SPATIAL DISPERSAL AND DAILY MOVEMENT

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Introduction

Flavescence dorée, which belongs to the 16Sr-V (subgroups –C and –D) or Elm yellows group (EY) (Boudon-Padieu, 2003), is at present the most threatening among grapevine yellows in Italy (Alma & Conti, 2002). It is transmitted by the nearctic leafhopper *Scaphoideus titanus* Ball, which was introduced in Europe in the 1950s': although in the US it was found on many other trees and shrubs, in Europe this leafhopper can feed and reproduce only on grapevine (Alma, 2004). Since a compulsory pest management is foreseen against this species, the knowledge of its flight behaviour is important to improve its control: this research investigates the spatial dispersal patterns and the daily flight activity of *S. titanus* in northwestern Italy.

Materials and methods

Studies have been conducted during 2001 and 2002 in an experimental vineyard and in a farm vineyard, using yellow sticky traps. The first vineyard was divided in to two, the first half having a standard layout of planting (standard plant density) and the second with many gaps within (low plant density): sticky traps were placed in both parts and externally to determine the trend of *S. titanus* density. For three consecutive weeks between July and August, traps were checked every three hours from 8.00 AM to 9.00 PM to study the leafhopper's daily movement. In the second vineyard, traps were placed both vertically within the canopy and horizontally at a height of 2.40 m from the ground using wooden poles, to investigate the capability of *S. titanus* of flying inside and above the vine canopy.

Results and discussion

Captures of *S. titanus* were statistically higher in standard than in low plant density conditions, and very few individuals were captured outside the vineyard, showing that the leafhopper has a poor horizontal movement. Captures on traps placed vertically above the canopy were very low compared to those on vine rows. Many more males than females were captured (Tab. 1). The flight activity was greater between 9.00 PM and 8.00 AM, suggesting a crepuscular behaviour of this leafhopper (Fig. 1).

Table 1. Horizontal and vertical spatial distribution of *Scaphoideus titanus* Ball. Different letters (capital for males, small for females) indicate no statistical difference between trap position (One Way ANOVA, $P<0.05$); differences in vertical movement were so evident that no statistical analysis was performed.

		Horizontal movement			Vertical movement	
		Standard plant density	Low plant density	External	Within canopy	Above canopy
2001	Males	38.7 ± 10.1 A	5.6 ± 2.9 B	0.2 ± 0.2 C	191.0 ± 20.4	3.4 ± 0.3
	Females	10.9 ± 4.4 a	2.6 ± 1.4 a	0.0 ± 0.0 b	42.1 ± 5.5	1.0 ± 0.5
2002	Males	62.5 ± 7.3 A	9.3 ± 5.3 B	0.5 ± 0.3 C	68.0 ± 11.3	0.1 ± 0.1
	Females	17.0 ± 2.4 b	2.8 ± 1.5 b	0.2 ± 0.2 b	26.9 ± 1.9	0.4 ± 0.2

The flight activity of *S. titanus* seems to be strongly restricted to its host plant, although a few individuals were captured above the canopy. It is likely that this species is a smaller-tree and bush dweller, having therefore a flight boundary layer of less than 2 m from the ground, the same as other leafhoppers such as *Alebra* sp, *Fagocyba* sp. and *Oncopsis* sp. (Lessio & Alma, 2004a). On the other hand, the specimens captured above the canopy are likely to have been transported by the wind. However, since movement occurs mainly during night time and early morning, this leafhopper is not so likely to rely on atmospheric lift for dispersal (Lessio & Alma, 2004b). Although other leafhoppers, such as *Circulifer tenellus* (Baker) (Taylor, 1985) and *Macrosteles quadrilineatus* Forbes (Hoy *et al*., 1992), have a long-range migratory behaviour, this does not seem to be the case of *S. titanus*. Males seem to be more likely to fly than females: it is still to be proved if there is a different transmission efficiency between genders, as it happens in *M. quadrilineatus* (Beanland *et al.*, 1999), although it has been demonstrated that the fitness of females is reduced by the acquisition of phytoplasmas (Bressan *et al*., 2005). These results should add knowledge on the biology of this insect vector, and improve pest management strategies.

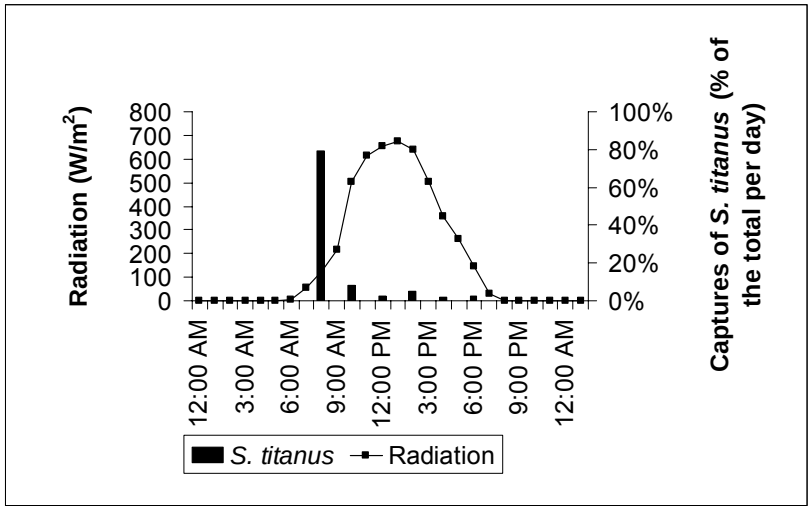

Figure 1. Daily flight activity of *Scaphoideus titanus* Ball.

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MOLECULAR CHARACTERIZATION OF A DIVERGENT STRAIN OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3

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Introduction

The genetic variability of grapevine leafroll-associated virus 3 (GLRaV-3) has been extensively studied in recent years. The GLRaV-3 strain NY1 has been sequenced completely (Ling *et al.*, 1998; Ling *et al*., 2004). Sequence information on RNA-dependent RNA polymerase (RdRp), coat protein (CP), heat shock protein 70 (HSP70) homologue and 55 KDa protein (p55) genes of several other isolates is available in Genebank. From available molecular data, GLRaV-3 seems to be a single undifferentiated population with low genetic diversity, as all strains characterized so far are very similar to each other and to the NY1 strain. Only one isolate identified in South Africa (Genebank accession number AY704412) seems to be quite different in 623 nucleotides of the 5'-end sequenced so far.

During a sanitary survey in the ISV laboratory, a new variant of GLRaV-3, here named GLRaV-3-Tempr, was found in a sample from a Spanish accession of cv Tempranillo, which reacted positively with an antiserum raised against GLRaV-3. The aims of this work were to provide the following: (1) partial nucleotide characterization of the new strain; (2) molecular comparison with other known GLRaV-3 strains; (3) a study of GLRaV-3-Tempr occurrence in grapevine.

Materials and Methods

The grapevine accession of cv Tempranillo came from a Spanish vineyard. It was used for RT-PCR assay and for nucleotide sequencing. The following four primer pairs for the detection of GLRaV-3, described elsewhere, were tested: c547/h229, LC1/LC2, CP3U/CP3D and c629/h330. A genomic portion including the intergenic fragment placed between ORF1b and ORF2, the whole RdRp and a part of the helicase genes was double-strand sequenced.

GLRaV-3-Tempr nucleotide sequence was aligned with replicase gene partial sequences from other GLRaV-3 isolates and from GLRaV-2, using CLUSTALW software. Cluster analyses were carried out using the UPGMA method, with the aim of comparing the new strain with other known isolates.

About 1300 grapevine samples collected in 2000-2005 were analyzed by ELISA test (Bioreba, Switzerland) for the detection of GLRaV-3 and by RT-PCR assay for the identification of the GLRaV-3-Tempr strain. Further sanitary checks were performed on another 33 vine accessions: 23 samples belonging to Spanish varieties, collected from the ISV ampelographic collection in Conegliano, and 10 samples from Spanish vineyards. The 33 accessions included 6 samples from cv Tempranillo.

Results and Discussion

A genomic fragment of 410 bp was obtained in the RdRp gene from the Tempranillo accession with primer pair c547/h229. The fragment was 70 bp longer than expected, as this primer pair usually amplifies a 340 bp fragment in GLRaV-3-infected samples (Minafra *et al.,* 1994). This finding was very useful as far as sanitary screening on grapevine samples is concerned, as the heavier band could be used as a molecular marker for the identification of the new GLRaV-3-Tempr strain. No amplification bands were obtained using the other three primer pairs, targeting the HSP70-like and CP genes and another portion of the RdRp gene.

A total of 2759 bp in the 3'-end of ORF1 was double-strand sequenced. The sequenced genomic fragment was 80.6% identical in nucleotide sequence to GLRaV-3-NY1 in 2703 nt overlap, while at the aminoacid translation level the identity was 88.5% in 852 aa. The highest diversity was found in the intergenic fragment placed between ORF1b and ORF2.

Dendrograms were built using sequencing data from other GLRaV-3 strains available in Genebank. The first comparison was performed in a 340 nt genomic portion including the intergenic fragment placed between ORF1b and ORF2 and the 3'-end of the RdRp gene, both at nucleotide and aminoacid level. The trees clearly showed that GLRaV-3-Tempr is a very divergent variant of GLRaV-3 (Fig. 1a, 1b). Its nucleotide sequence was about 50% identical to GLRaV-2, while it was 70% identical on average to the

other GLRaV-3 variants. The other GLRaV-3 strains formed quite a homogenous group: the Chinese and NY1 isolates were almost identical, followed by the Czech and Jordan strains, while the Brazilian variant was slightly different. The corresponding dendrogram built with the aminoacidic data showed the same topology, but GLRaV-3-Tempr was clearly closer to the other GLRaV-3 strains and further from GLRaV-2 compared to the previous tree constructed with the nucleotide data.

Another UPGMA dendrogram was built with nucleotide sequences obtained from a 653 nt internal fragment of the RdRp gene, included between primers P3U and P3D (Turturo *et al*., 2005). 15 GLRaV-3 isolates were compared (Fig. 2). The results confirmed that GLRaV-3-Tempr is very different from all other strains, with an average nucleotide difference of about 20%. As far as the other strains are concerned, the topology of the tree agrees with data from Turturo *et al.* (2005).

The survey carried out on 1300 vine accessions showed that 227 samples (17.5%) were infected with GLRaV-3. Among them, only another two samples, belonging to the cvs Tempranillo and Santa Paula, proved to contain the GLRaV-3-Tempr strain. All three GLRaV-3-Tempr-infected accessions were included in the 33 vine samples of Spanish origin. This finding suggests that the occurrence of this strain could be important in clonal selection and sanitary controls on Iberian grapevines.

Fig. 1a. UPGMA dendrogram obtained from sequencing data of the ORF1b 3'-end (340 nt) in 6 GLRaV-3 isolates together with GLRaV-2-Sem. GLRaV-3 strains: NY1, AF037268; Chinese, AY495340; Czech, AY424407; Jordan, AY628766; Brazilian, AF438411.

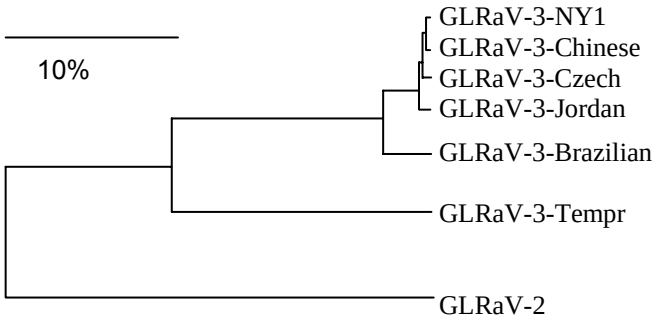


Fig. 1b. UPGMA dendrogram obtained from the deduced aminoacid sequences of the ORF1b 3'-end (69 aa) in 6 GLRaV-3 isolates together with GLRaV-2. GLRaV-3 strains: see Fig. 1a.

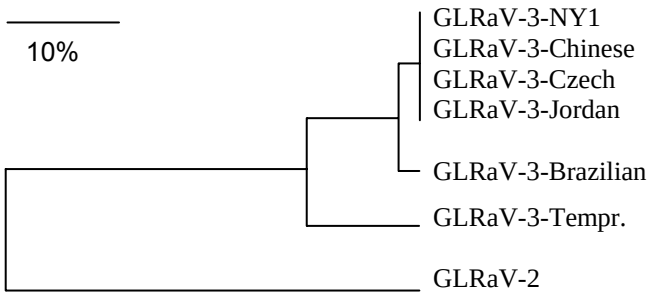
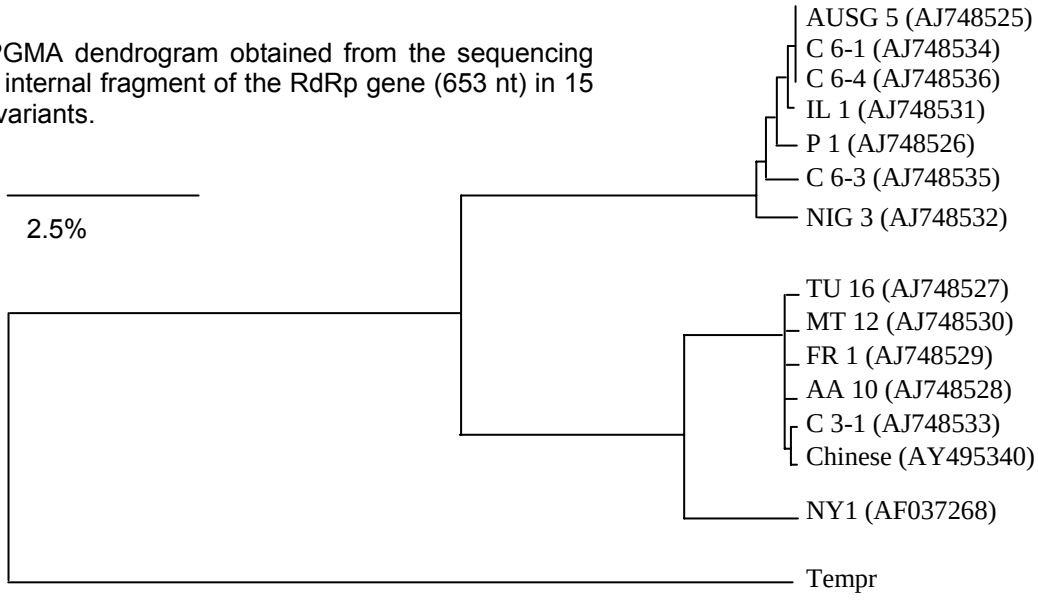


Fig. 2. UPGMA dendrogram obtained from the sequencing data of an internal fragment of the RdRp gene (653 nt) in 15 GLRaV-3 variants.



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SANITARY STATUS OF GRAPEVINE MOTHER PLANTS OF LOCAL VARIETIES IN CRETE, GREECE

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Introduction

The introduction and establishment of Phylloxera in 1978 has caused a gradual reduction of the viticultural area in the island of Crete (Greece). The total area cultivated with grapevine, about 50.000 hectares in the decade of 1970, was reduced to about 22.000 ha. The introduction of American rootstocks and the replacement of old vineyards of local table, raisin and wine grape varieties, with non certified material, created serious problems in the Cretan viticulture. Except the massive introduction of foreign, mainly French wine varieties, and the reduction of traditional viticultural areas, new virus and virus-like diseases were widely spread. Rugose wood and secondarily leaf roll disease, which were very rare in the old vineyards, have now a higher distribution in the island and new plantings using virus-free propagating material are required.

The aim of this study was to check the mother plots of native grape cultivars in order to establish virus-tested material.

Materials and Methods

Dormant canes collected from mother vine plantations for production of propagating material belonging to the two local main grape nurseries were tested twice by ELISA (Clark and Adams 1977) using diagnostic kits obtained from Agritest (Valenzano Italy). Grapevine cortical scrapings were macerated in extraction buffer at a dilution 1:15. Samples with absorbance readings (A_{405}) over three times the average of four wells/plate of healthy control were considered positive.

Testing was carried out on 414 plants from three red (Kotsifali, Mandilari, Liatiko) and four white wine varieties (Vilana, Vidiano, Plyto, Dafni). All samples were analysed for the presence of the following viruses: *Grapevine fanleaf Nepovirus* (GFLV), *Grapevine leafroll associated Closteroviruses* 1 and 3 (GLRaV-1 and GLRaV-3), *Grapevine Vitivirus A* (GVA) and *Grapevine fleck Maculavirus* (GFkV).

Results and Discussion

All the main grapevine wine varieties were highly infected with GVA and GLRaV-1 (54.8 and 51.5%, respectively). Incidence of GFkV and GLRaV-3 was relatively low (7 and 2.4%, respectively), whereas GFLV was not detected at all (Tab. 1). All mother plants of cvs Vidiano and Dafni were infected by GVA and GLRaV-1, whereas incidence was lower in cvs Kotsifali, Mandilari, Vilana and Plyto. Only one out of 119 mother plants of cv Liatiko was found virus-free, showing that the sanitary status is unsatisfactory.

Clonal and sanitary selection of the grapevine in the island of Crete was initiated about ten years ago and was based on visual inspection. Previous surveys on the sanitary status of vineyards in Crete revealed the significant presence of three Closteroviruses (GLRaV-1, -3 and -7), one Vitivirus (GVA), one Maculavirus (GFkV) and one Nepovirus (GFLV) (Avgelis *et al.* 1997, Avgelis and Rumbos 2000, Avgelis and Nikolantonakis 2003).

The present investigation – in accordance with the results of previous surveys - confirms that (1) the sanitary status of the Cretan's viticultural industry is too far from being satisfactory and (2) production of certified grapevine propagating material is urgent needed in order to improve the quality of yield and increasing the importance of the local wine industry.

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Table 1. Frequency of single or mixed virus infections detected by ELISA in 414 Cretan wine vine varieties.

	Grapevines		Frequency of virus infection (%)				
VARIETIES	Tested	Negatives	GFLV	GLRaV-1	GLRaV-3	GVA	GFkV
KOTSIFALII	139	60	0	38	5.8	37.4	10
MANDILARI	26	15	0	0	0	42.3	0
LIATIKO	119	1	0	96.6	0.8	98.3	9.2
VILANA	60	45	0	15	1	11.7	6.7
VIDIANO	20	0	0	75	1.7	100	0
PLYTO	30	25	0	3.3	0	0	13.3
DAFNI	20	0	0	100	0	100	0
TOTAL	**414**	**146**	**0**	**51.5**	**2.4**	**54.8**	**7**

ELIMINATION OF *GRAPEVINE ANGULAR MOSAIC VIRUS* (GAMV) BY HEAT TREATMENT AND MERISTEM SHOOT TIP CULTURE

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Introduction

In the 1990's a virus was isolated from BaresanaxBaresana *Vitis vinifera* hybrid vines established in the variety collection of the Grapevine Institute, National Agricultural Research Foundation, Athens. Diseased vines showed angular mosaic, malformed and small leaves, dropping off, few berries with smaller, wrinkled and no germinating seeds, stunting and gradual declining. Further investigations showed that the causal agent is a new putative Ilarvirus - distinct from Grapevine line pattern virus, which has been reported to occur in a few cultivars with very low incidence in Hungary (Lechoczky *et al.* 1990) - to which the provisional name Grapevine angular mosaic (GAMV) has been assigned (Girgis *et al.* 2003). As no information regarding sanitation of grapevines infected by Ilarvirus is available, studies on its elimination from infected vines were carried out and results are reported in this paper.

Materials and Methods

Dormant canes from five net house grown Baresana x Baresana hybrid vines infected by GAMV – as confirmed by ELISA - were collected. Large quantities of shoots were produced by bud forcing at 25^{o} C with the basal parts of canes immersed in water. Axillary buds 0.8-1.0 cm long were taken, washed in detergent and surface-sterilized in 70% ethanol for 30 sec followed by 10% calcium hypochlorite solution for 20 min. The explants were subsequently washed in three changes of sterile distilled water and were placed on glass tubes containing 10 ml of the Zlenco *et al.* (1995) medium supplemented with IBA (0.25 mg/l). Tubes were incubated at 25±0.5^{o} C with a 16 h light period of 3,500 Lux for 45 days. The micro-propagation was repeated to produce two groups of 15 and 30 plantlets from each hybrid vine differing in age: 116 and 55 days, respectively. The heat treatment period (36.5±0.5^{o} C and lighting of 4,000 lux for 16 hours) was 90 days.

Shoot tips 0.3 to 0.4 mm in size, including the apical dome and 1-3 leaf primordial, were taken from survived plants and placed in glass tubes containing a medium (Berthelot 1934, Murashige and Skoog 1962) supplemented with 2 mg/l 6-benzyl-aminopurine. Subsequently, at 3-week intervals, the meristem callus cultures transferred to fresh medium. On the fifth transfer, each callus culture was subdivided and placed on the same medium, supplemented with 0,45 mg/l 6-benzyl-aminopurine. Shoots about 1 cm in length were excised and transferred to glass tubes containing a modified medium of Zlenko et al. (1995), supplemented with IBA (0.25 mg/l) to enhance root formation. The complete regenerated plantlets were potted in peat-pots under glasshouse conditions for gradual adaptation and the survived plants were planted in pots containing a soil mixture of 15 l under a shade house.

The GAMV presence were carried out before adaptation and after a year in pots. Plants were tested by ELISA (Clark and Adams 1977) using a locally produced polyclonal antiserum. Wells were coated with 100 µl of γ-globulin diluted 1:1000 in coating buffer. Leaf extracts were prepared in a dilution of 1:15 in PBS-Tween and 100 µl of alkaline phosphatase conjugated γ-globulin was used (1:1000 in PBS-Tween). At final step, 100 µl of p-nitrophenyl phosphate diluted in substrate buffer to a final concentration of 1 mg/ml was added. Absorbance was recorded at 405 nm using a microplate reader and samples were considered positive when their values exceeded three times the negative (healthy) control value.

Results and Discussion

The behavior of plantlets submitted to thermotherapy was depended mainly on the age of the plantlets (Table 1). Only 13 out of 75 older plantlets survived and 13 shoot tips were taken for plant regeneration. On the contrary more young plantlets overcome better the heat shock (52% survival) and 78 meristems were excised and transferred to glass tubes. About 251 regenerated plants were produced and after glasshouse adaptation 133 vines were potted. The first test for GAMV showed that only 14 out of 251 (5.6%) plantlets were infected, while in the second ones the virus was detected in 4 out of 137 (≈ 3%) potted plants. Infected plants did not exhibited typical symptoms of GAMV infection.

The results showed that the combination of *in vitro* thermotherapy and *in vitro* apical culture is an effective method to eliminate GAMV from the infected hybrid vines Baresana x Baresana. The same methodology has been also effective for the elimination of other viruses such as *Prunus necrotic ring spot* (PNRSV) and *Prunus dwarf* (PDV) *Ilarviruses* from *Prunus* trees (Maganaris *et al.* 2003). This technique could be also applied successfully for the elimination of other viruses commonly found on grapevines.

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Table I. Results of elimination of *Grapevine angular mosaic virus* by heat treatment and meristem shoot tip culture of five hybrid vines Baresana x Baresana.

Hybrid vines	No of plantlets	Age (days)	No of survived plantlets	No of excised shoot tips	Regenerated plantlets	Potted plantlets	Healthy vines
B10	15	116	2	2	0	0	0
	30	55	16	16			
B11	15	116	3	3	128	54	54
	30	55	16	16			
B11b	15	116	1	1	115	76	72
	30	55	21	21			
B12	15	116	2	2	0	0	0
	30	55	10	10			
B13	15	116	5	5	8	7	7
	30	55	15	15			

NATIONAL ITALIAN RESEARCH PROJECT: "GRAPEVINE YELLOWS: A LIMITING FACTOR OF GRAPEVINE PRODUCTION": FIRST YEAR OF ACTIVITY

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Introduction

Flavescence dorée (FD) was observed in Italy at the end of '60 years in Oltrepo' pavese (Lombardia region, northern Italy). Later, FD occurred in other regions including Veneto, Liguria, Friuli Venezia Giulia, Piemonte, Emilia and Trentino where severe damage to the plants and serious crop losses for the production were recorded: also, in the same areas, high populations of *Scaphoideus titanus*, the vector of the disease, were observed.

The phytoplasmas detected in FD affected plants belong to elm yellows group (16SrV) and in particular to the subgroups 16SrV-C and 16SrV-D. Bois noir (BN), the less epidemic form of grapevine yellows, is widely distributed in Italy: the phytoplasma 16SrXII-A is generally associated with diseased grapevines. Seldom Aster yellows phytoplasmas have been found associated to diseased plants in different Italian regions.

Nowadays, in northern Italy, FD is present in almost all the areas were grapevine is cultivated, whereas BN largely occurs in all the Italian regions even if its importance is increasing.

To reduce the economic losses due to the spreading in vineyards of these different phytoplasmas the Italian Ministry of Agriculture adopted different actions, of which the most important is the publishing of a decree of the compulsory eradication of grapevines affect by FD (DM 31-05-2000).

The Ministry of Agriculture financed also a national research project *"Grapevine yellows: a limiting factor of grapevine production"* to harmonize all different studies carried out in research Institutes scattered in different Italian regions. The Project covers the following aspects:

- Define the diffusion in the open field of yellows caused by phytoplasmas; particular attention is given to the characterization of different strains of phytoplasmas and all units covering these aspects adopted common protocols for detection and characterization of the etiological agents.
- Define the diffusion of yellows specific vectors; also in this case vector population is monitored in infected vineyards, according to common previously defined protocols, and particular attention is given to the identification of new potential vectors of the disease.
- Control the disease through the i) individuation of natural reservoirs of phytoplasmas (weeds, forest trees), ii) the evaluation of efficacy of different pesticides to control vector population in the respect of integrated and/or organic production, iii) the study of the physiological basis of the recovery phenomenon.

The activity performed in the first year of the Project allowed to obtain interesting results:

Yellows field monitoring

About 250 vineyards were controlled in most of the Italian areas where grape is cultivated. About 500 samples were molecularly analyzed, following harmonized protocols. FD and BN were identified by direct PCR with universal primers P1/P7, followed by nested PCR with primers specific for ribosomal groups 16SrV and 16SrI/XII, and RFLP analyses with *Bfa*I and *Mse*I respectively. Samples positive to 16SrV group phytoplasmas were then amplified again, following the protocol described by Martini *et al.* (2002), in order to identify ribosomal subgroups.

About 80% of the vineyards inspected from the different teams participating into the Project resulted positive for the presence of yellows symptomatology. FD-C and FD-D are present in northern Italian regions and in some vineyards of Central Italy. FD-D seems to be more spread than FD-C, even if FD-C was identified also in some areas of Umbria and Tuscany. BN is widespread in Italy and it is associated with the presence of 16SrXII-A phytoplasmas. Occasionally phytoplasmas belonging to aster yellows (16SrI-B) and elm yellows (16SrV-A) were isolated from symptomatic grapevines. (Borgo *et al.* 2005).

Vectors monitoring

To evaluate the role of the fauna in the epidemiology of GY disease, an insect sampling was made from April to October in different vine growing areas of Italy (Piedmont, Aosta Valley, Friuli-VG, Tuscany and Latium).

Adults of *S. titanus*, *Hyalesthes obsoletus* and other species living in the vineyard ecosystem were captured inside the vineyards by yellow sticky traps and sweep-net samplings. The collected individuals were determined in the laboratory and submitted to molecular analysis to detect the presence of phytoplasmas.

The monitoring results confirmed the presence of *S. titanus* only in the vineyards of northern Italian areas (Piedmont, Aosta Valley, Friuli-VG, Tuscany), whereas *H. obsoletus* was found in all investigated areas on herbaceous weeds (nettle and bindweed) rather than on grapevine. Many other species of Auchenorrhynca were also found: the cixiids *H. luteipes* Fieber, *Reptalus panzeri* (Low), *R. quinquecostatus* (Dufour) and *Cixius* sp., the deltocephaline cicedellids *Euscelidus variegates* (Kirscbaum), *Euscelis incisus* (Kirschbaum), *Fieberiella florii* (Stal), *Anoplotettix fuscovenosus* (Ferrari), *A. putoni* Ribaut and *Macrosteles quadripunctulatus* (Kirschbaum) (Alma *et al*., 2005).

Control strategies against FD

The control of the vector is the main strategy to prevent the spreading of phytoplasmas from infected areas to healthy vineyards. The use of pesticides anyway, must take in consideration the respect of the environment and the fact that many vineyards are cultivated in the respect of integrated or biological protocols. To effort this aspect, different chemical control strategies are in progress to determine the most effective, also taking into consideration the entity of the population of *S. titanus* in the vineyards.

Some research groups are evaluating the role that infected propagative material plays in the diffusion of the disease. The identification of FD phytoplasmas or *S. titanus* in vineyards of central or southern Italy, where both of them normally do not occur, could be explained by the introduction in an healthy area of infected plants carrying on the vector or the FD phytoplasma.

Much attention is given also to the remission of symptoms observed in infected plants. Preliminary observations report on the stability of this phenomenon and on the absence of phytoplasmas in recovered plants. Studies on the parameters regulating recovery and its genetic and physiological bases are carrying out. (Pavan *et al*., 2005)

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MOLECULAR CHARACTERIZATION OF STOLBUR PHYTOPLASMA ISOLATES IN GRAPEVINES AND INSECT VECTORS IN SPAIN

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Methods for identification and characterization of phytoplasma isolates are important for epidemiological studies, to prevent the dissemination of diseases and to search for their vectors. Several reports on the diversity of phytoplasmas have previously been done, using PCR amplification and restriction analyses of 16S rDNA gene and the 16/23S spacer region. However, rDNA is highly conserved and its degree of polymorphism can occasionally be suited for differentiating phytoplasma strains within a given group.

The stolbur phytoplasma is present in a high number of plant families and an important number of insect species, belonging mainly to the cicadelidae and fulgoridae, has been identified as carriers of this phytoplasma. Therefore, the genetic variability of this phytoplasma could be important. Some papers have showed the genetic variability of this group with different methods as RFLP (4), southern blot hybridization (5), heteroduplex mobility analysis (HMA) (3) and SSCP (2). The utilization of primers for the amplification of the Tuf gene and later restriction analyses have allowed to distinguish two different stolbur isolates in grapevine and three in *Hyalesthes obsoletus* (4). This gene encoding the elongation factor Tu of several culturable mollicutes and primers designed from its sequences amplified most of the tuf gene from phytoplasmas of the aster yellows, stolbur and X-disease groups (6).

In an epidemiological study of the stolbur phytoplasma in different geographical areas of Spain, this phytoplasma has been identified in different crops and with a different degree of expression of symptoms. Likewise, the presence of potential vectors and their population levels also vary considerably with the area. The aim of this work has been to determine if there is genetic variability of this phytoplasma in the grapevine plots affected by Bois Noir disease and to determine the vectors involved. We have characterized stolbur isolates in different areas of Catalonia and La Rioja, using the method followed by Langer et al. 2003 and SSCP (2). Samples of grapevine plants from plots of both regions have been analyzed, as well as carrier insects of this phytoplasma, captured in the plots. The analyses of restriction with H*pa*II of the amplification product obtained with Tuf-AY primers, have showed that in both, grapevine samples from two plots of Catalonia and in individuals of *H. obsoletus* captured in the same plots, the profile obtained belonged to isolate B (Figure 1). In grapevine samples from plots of La Rioja, the isolate detected was isolate A (Figure 1), whereas in the insects captured in these plots isolate B has been detected in individuals of *Hyalesthes obsoletus*, *Aphrodes* sp and *Issus* sp. The profile of isolate A has been identified only in individuals of *Euscelidius variegatus* and *Euscelis obsoletus,* cited as possible vectors of the stolbur in previous studies (1). Both isolates are present in the affected plots of La Rioja, isolate A in plants and insects, isolate B in insects only. A more extensive study is necessary to determine the distribution of stolbur isolates in this region.

In transmission trials conducted using individuals of *H.obsoletus* captured in Catalonia and carried out with *in vitro* culture grapevines of the variety Chardonnay, the transmission was obtained, and isolate B was identified, both in insects and in plants. In transmission trials using insects belonging to different species captured in plots of La Rioja, isolate B was transmitted by *Issus* sp , *Aphrodes* sp. and *H.obsoletus*. Plants inoculated with *Agallia laevis* and individuals of other insect species captured in La Rioja plots showed profiles different from those indicated for stolbur.

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Figure 1.- (left) RFLP-profiles of stolbur phytoplasma obtained after PCR with Tuf AY primers and digestion with H*pa* II enzyme, from samples of grapevine plants from "La Rioja" (1 to 6) and from *Hyalesthes obsoletus* sampled in the same plots (7 and 8). (right) RFLP-profiles obtained from grapevine plants of Catalonia (1 and 2) and from *H. obsoletus* sampled in Catalonia (3 and 4).

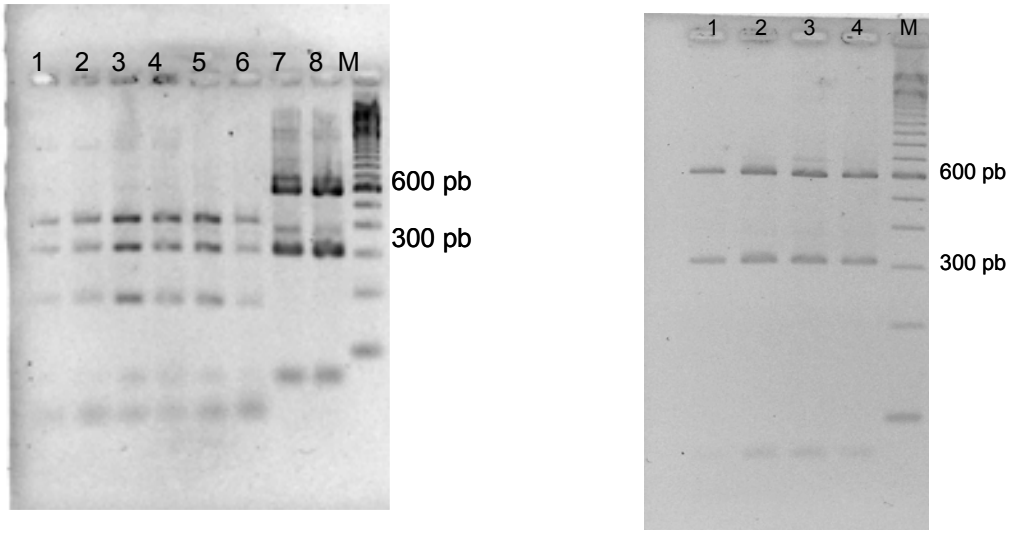
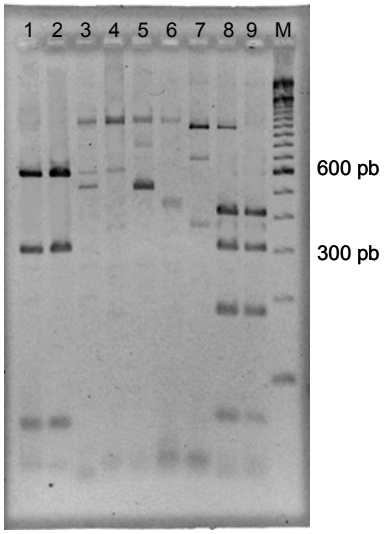


Figure 2.- RFLP-profiles obtained after PCR with Tuf AY primers and digestion with H*pa* II enzyme, from samples of grapevine plants incubated with different insect species captured in "La Rioja". 1 and 2 isolate B of stolbur from plants exposed to *Aphrodes* sp and *Issus* sp. 3, 4, 5 ,6 and 7 profile obtained from plants inoculated with Agallia laevis. 8 and 9 isolate A of stolbur from plants incubated with *E.variegatus* and *E.obsoletus*.



EPIDEMIOLOGICAL OBSERVATIONS ON 'BOIS NOIR' IN LAMBRUSCO GRAPEVINE GROWING AREAS OF ITALY

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Introduction

Bois Noir (BN) is a grapevine yellows (GY) associated with the presence of 16SrXII-A phytoplasma ('*Candidatus* Phytoplasma solani') of increasing economic importance in the Northern Italy provinces where Lambrusco is cultivated (Bertaccini *et al.*, 2003). BN phytoplasma is transmitted by *Hyalesthes obsoletus* Signoret (*Homoptera Cixiidae*) (Alma *et al*., 2002), and nettle (*Urtica dioica*) is considered its main host plant. Molecular identification of BN and Flavescence dorée (FD, ribosomal subgroup 16SrV-D, '*Candidatus* Phytoplasma vitis') phytoplasmas was achieved in routine testing in Modena and Reggio Emilia provinces since 2000 (Bertaccini *et al.*, 2001). Further monitoring carried out by Provincial Inspection Services from 2000 to 2004 demonstrated the widespread and increasing of grapevine plants showing yellows symptoms.

Materials and Methods

Insects and grapevine samples showing typical yellows symptoms were collected during routine inspections in the vineyards of Modena and Reggio Emilia provinces (Northern Italy). From a total of 1020 grapevine samples, nucleic acids were extracted using 1 g of leaf midribs following the protocol described by Prince *et al.* (1993). *H. obsoletus* samples were also extracted from batches of 1 to 4 following the protocol of Zhang *et al.* (1995). Nucleic acids were diluted to a final concentration of 20 ng/μl in sterile deionized water. One μl of this dilution was used in the amplification assays described below. Direct PCR was performed using universal primer pair P1/P7, both for plants and for insects, while nested PCR reactions on direct PCR products diluted 1:30 in sterile distilled water, were performed using primer pairs M1/B6 or R16F2/R2 (Martini *et al*., 1999; Lee *et al*., 1995). Each 25 μl PCR reaction mix contained 2,5 μl 10X PCR buffer, 0,8 U of *Taq* polymerase (Polymed, Florence, Italy), 0,2 mM dNTPs, 1,5 mM $MgCl_2$ and 0,4 μM each primer. Thirty-five PCR cycles were performed in an automated thermocycler (Biometra, Uno Thermoblock, Gottingen, Germany, EU) as described (Bertaccini *et al.,* 2003). Six μl of PCR products were analysed in a 1% agarose gel stained with ethidium bromide, and then visualized with an UV transilluminator. To differentiate among phytoplasmas, PCR products were digested with *Taq*I and *Tru*I restriction enzymes; the RFLP fragments were separated in a 5% polyacrylamide gel stained with ethidium bromide, and then visualized with an UV transilluminator.

During 2003 and 2004, in some of the most symptomatic vineyards monitoring of *H. obsoletus* populations was performed using entomological nets and then aspirators. This method resulted to be more efficient than the use of yellow chromotropic traps (Cavallini *et al*., 2003). Insect collection by using entomological nets was also carried out every week between the border grapevine rows and inside the drains nearby to the vineyards, among weed plants belonging to genera *Urtica*, *Convolvolus* and *Medicago*. Insects then were classified and tested by nested-PCR as described above to verify phytoplasma presence.

Survival trials of *H. obsoletus* are carried out in green house using some weed species resulted positive to the BN phytoplasma (Credi *et al.*, 2004) and cultivated in pots.

Results and Discussion

Molecular analysis (PCR/RFLP) carried out on symptomatic plants showed the striking prevalence of BN phytoplasma on FD. Out of 557 samples collected in the Reggio Emilia province, 55,7% were positive to BN whereas 14,4% of the tested plants resulted infected by FD. Regarding the 463 analyses on samples collected in the Modena province, 67,4% samples resulted positive to BN, while those positive to FD were 10,8%; the 2,3% of the Reggio Emilia collected samples appeared positive to both phytoplasmas. A number of symptomatic grapevines was negative: 27,3% in Reggio Emilia and 21% in Modena provinces.

High percentages of symptomatic plants were observed in the first years of BN occurrence at vineyards borders with a decreasing gradient to the center of the field as already reported (Credi *et al*., 2004); however this gradient was progressively disappearing over the years.

Data of *H. obsoletus* adults' flying period in BN affected areas indicate their presence from the middle of June to the end of August, with a peak in the middle of July. Surveys on adult's capture have statistically shown that they are more present along the perimeter of vineyards rather than in the centre. Insects distribution is correlated to the presence of nettle and other weed species in the areas near to vineyards. Molecular analysis carried out by PCR/RFLP revealed that in 2003 the 15,1% of field collected *H. obsoletus* adults were infected with BN phytoplasma, while in 2004 the 26,6% of specimens was positive to the same phytoplasma.

Green house studies were performed to verify the possibility that the vector is able to survive and complete its biological cycle on some perennial dicotyledon, chosen among the most widespread in our viticulture areas, and resulted positive to the BN phytoplasma. The tested species were: *Cirsium arvense*, *Convolvolus arvensis*, *Medicago sativa, Plantago major*, *Taraxacum officinale* and *U. dioica*. In our experimental conditions the insect has revealed good survival on *P. major*, capacity of laying eggs on all weeds, and to achieve nymphs development in *C. arvense*, *C. arvensis*, *M. sativa, P. major* and *U. dioica*. The cixiid survives only for a few days on grapevine.

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GRAPEVINE VIRUSES ASSOCIATED WITH RODITIS LEAF DISCOLOURATION

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Introduction

In 1981, an apparently new disease of grapevines of cv. Rodites was observed in central Greece. Field symptoms, i.e. yellow and/or red discolourations and deformation of the leaves and small-berried bunches were reproduced in cv Mission by grafting, thus the name "Roditis leaf discoloration" (RLD) was proposed (Rumbos *et al.,* 1989). From diseased vines, *Grapevine fanleaf virus* (GFLV) and *Carnation mottle virus* (CarMV) were consistently transmitted to herbaceous hosts (Avgelis *et al*., 1991). However, later studies showed that neither of these viruses was present in symptomatic vines from vineyards other that that where RLD had been first observed (Rumbos and Avgelis, 1993). Further investigations using ELISA and RT-PCR for virus identification were therefore undertaken.

Materials and Methods

Mature canes from apparently healthy and diseased cv Roditis vines collected in the last two decades, were rooted, potted, and maintained in an isolated shade-house in the experimental farm of N.AG.RE.F Heraklion (Crete). From 1999 to 2004, 89 diseased and 13 "healthy" vines were checked yearly by ELISA for the presence of four nepoviruses: GFLV, *Arabis mosaic virus* (ArMV), *Raspberry ringspot virus* (RRV) and *Tomato black ring virus* (TBRV), seven members of the family *Closteroviridae*: *Grapevine leafroll associated virus* 1, 2, 3, 4, 5 and 6 (GLRaV-1, GLRaV-2, GLRVa-3, GLRaV-4, GLRaV-5, GLRaV-6) and the unassigned species Grapevine leafroll associated virus 7 (GLRaV-7), two vitiviruses: *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB), *Grapevine fleck virus* (GFkV), and CarMV.

Home made and commercial ELISA kits (Agritest Italy, Bioreba Switzerland, Bio-Rad France) were used for all viruses but GLRaV-4, an antiserum to which was supplied from Dr. D. Gonsalves (Cornell University). Tips from spring shoots were used for detecting GFkV and nepoviruses whereas cortical scrapings from mature canes were used for closteroviruses and vitiviruses. Each vine was tested at least twice. Positive and negative controls were included in each plate.

Besides ELISA, 23 vines (11 symptomless and 12 symptomatic) were tested for GVB by RT-PCR as described by Turturo *et al.* (2005). Total nucleic acids were extracted from cortical scrapings by chromatography on silica particles, and random primed cDNA were amplified with GVB-specific primers Bh123 (5'**atagtaggggtaccatcaatatctc**) and Bc637 (5'**ctatatctcgacagactgctcaccc**). Amplified products were analyzed in 5% polyacrylamide gel electrophoresis.

Results and Discussion

Results of ELISA testing were highly consistent over time for GFkV, nepoviruses, GLRV-1, and GLRV-3, but were erratic for vitiviruses and other ampeloviruses. Thus, assays for these latter viruses were repeated several times (up to nine cheks). The overall results showed that the totality of 102 Roditis accessions were infected by one or more virus (Table 1). All contained GLRV-3, regardless of whether or not they showed RLD symptoms (Table 1). GVA was also highly represented in both symptomatic (84%) and symptomless (54%) vines. GVB and GLRV-7 were detected in the majority of symptomatic vines (62% and 60%, respectively), while GLRV-7 (38%) but not GVB was also present in those without symptoms. GFkV was highly represented in symptomatic and symptomless vines (43% and 30%, respectively). Among nepoviruses only GFLV was identified in a restricted number of symptomatic vines (9%), while CarMV was exclusively present in the only two surviving vines from the vineyard in which RLD was originally discovered.

The much deteriorated sanitary status of cv. Roditis does not allow to draw conclusions on the aetiology of RLD. None of the viruses that were encountered with a high incidence (30 to 100%), i.e. GLRaV-3, GLRaV-7, GVA, and GFkV, were in fact more or less equally represented in both diseased and symptomless plants. GVB constituted an exception for it was detected by ELISA in 61% of the symptomatic but in none of the symptomless accession. However, the meaning of this finding, which

was essentially confirmed by RT-PCR (Table 2) is still obscure. It represents an interesting hint that requires additional and more extensive investigations, now underway

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Table 1. ELISA detection in 13 symptomless and 89 RLD-affected vines of cv Roditis

Viruses	Symptomless vines	RLD affected vines	Viruses	Symptomless vines	RLD-affected vines
GFLV	0/13	8/89	GLRaV-5	1/9	0/89
ArMV	0/13	0/89	GLRaV-6	0/13	8/89
RRSV	0/13	0/89	GLRV-7	5/13 (38%)	54/89 (60%)
TBRV	0/13	0/89	GVA	7/13 (54%)	75/89 (84%)
GLRV-1	0/13	0/89	GVB	0/13	55/89 (62%)
GLRaV-2	2/13	9/89	GFkV	4/13 (30%)	38/89 (43%)
GLRV-3	13/13	89/89	CarMV	0/13	2/89
GLRaV-4	0/9	0/89			

Table 2. ELISA and PCR detection of GVB in 11 symptomless and 12 RLD-affected vines of cv Roditis

RLD-affected vines	GVB - ELISA	GVB - PCR	Symptomless vines	GVB - ELISA	GVB - PCR
No 49	+	+	No 7	-	-
No 14	+	+	No 12	-	-
No 52	-	-	No 6	-	-
No 27	+	+	No 1	-	-
No 4	+	+	No 9	-	-
No 78	-	+	No 4	-	+
No 5	+	+	No 11	-	-
No 76	-	+	No 13	-	-
No 71	-	-	No 8	-	-
No 72	-	-	No 5	-	-
No43	-	-	No 2	-	-
No 61	-	-			

FD-RELATED PHYTOPLASMAS AND THEIR ASSOCIATION WITH EPIDEMIC AND NON EPIDEMIC SITUATIONS IN TUSCANY (ITALY)

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Introduction

Flavescence dorée (FD) is a devastating disease of grapevine reported in several European countries such as France, Italy, Spain, Serbia, and recently Switzerland (Boudon Padieu *et al*., 2003; Duduk *et al*., 2004; Anonimous, 2004). In Italy the disease was reported as epidemic in Northern areas (Bertaccini, 2002) but, sporadically, it starts spreading also in central Italy, mainly in Tuscany (Bertaccini *et al*., 2003). In this region field inspections followed by molecular analyses were carried out since 1998 on a total of 370 grapevine samples collected all around Tuscany; this survey allowed identification of both Bois Noir (BN) and FD phytoplasmas. The latter were detected in 38 grapevine samples belonging to cultivars Sangiovese, Vermentino, Ciliegiolo and Albarola collected in different fields of two Tuscanian provinces [Massa Carrara (MS) and Florence (FI)]. Three DNA fragments (16S rDNA, SecY and rpS3) used as markers to determine FD population variability, and possible relationships between genetic polymorphism and epidemiology were employed to study FD strains from the areas of Tuscany where this phytoplasma was detected.

Material and Methods

Total nucleic acids were extracted from 1 g of grapevine mid-vein tissues, according to the DNA extraction protocol described by Prince *et al.* (1993). Nucleic acids were precipitated in ethanol and the pellets, suspended in 100 μl of TE buffer, were diluted to 20 ng/μl final concentration in sterile deionized water. For the amplification of 16S rDNA, one to 3 μl of this dilution were used in direct PCR with the universal primer pair P1/P7 followed by nested amplification with universal primer pair M1($16R_{758f}$)/B6 (Martini *et al.*, 1999). PCR reaction was performed in a total volume of 25 μl PCR mixture containing 2.5 μl of 10X PCR buffer, 200 μM of each dNTP, 0.4 μM of primers pair and 0.8 U of *Taq* polymerase (Polymed, Florence, Italy, EU). For both direct and nested PCR amplification 35 cycles were conducted under the described conditions (Martini *et al*., 1999). PCR products were analysed by electrophoresis in 1% agarose gel, stained with ethidium bromide, and then visualized under UV transilluminator. 100 to 200 ng of M1/B6 amplicons were digested with *Tru*I and *Taq*I at 65°C and restriction fragments were separated by electrophoresis in a 5% polyacrylamide gel in 1X TBE buffer, and visualized as above.

To further characterize the 38 strains two primer pairs [rp(V)F1/rpR1 followed by rp(V)F1A/R1A used in nested PCR] were employed to amplify the ribosomal protein operon containing the 3' end of *rpl22* gene and *rps3* gene (Lee *et al.,* 2004). RFLP analysis was performed on nested products with the enzymes *Tru*I, and *Tsp509*I. SecY gene was also amplified using the primer pair FD9f2/r in direct PCR and FD9f3/r2 in nested (Angelini *et al.*, 2001), and amplicons were digested with *Tru*I, *Taq*I, *Tsp509*I, and *Alu*I. The PCR reagents and the reaction conditions employed were as previously reported (Angelini *et al.*, 2001; Lee *et al.*, 2004). As controls were employed the French strains FD70 kindly provided by E. Boudon-Padieu (INRA, Dijon, France), a Serbian strain SERBIA (Duduk *et al.,* 2004), Italian strains PV259 and AL202 (Martini *et al.*, 2002) from Lombardy and Piedmont respectively, TV54 from Veneto, LIG and PG24 from Liguria (Botti and Bertaccini, 2003), and Umbria (Botti *et al*., unpublished).

Results and Discussion

PCR using universal primers P1/P7 and M1/B6 detected the presence of phytoplasmas in all the 38 grapevine samples as well as in the positive controls. RFLP analyses of the P1/P7 and M1/B6 products (about 1.8 kb and 1.6 kp respectively) with *Tru*I and *Taq*I indicated that all the FD phytoplasmas detected in Tuscany belong to 16SrV-C ribosomal subgroup, '*Candidatus* Phytoplasma vitis' (IRPCM, 2004). RFLP analyses with 4 enzymes on the SecY gene delineated 4 different groups within 16SrV-C subgoup. Semi-nested PCR using the primer pairs rpVF1A/rpR1A generated a DNA fragment (about 950 bp) from all the strains employed, and their RFLP analyses defined 2 different patterns. Comprehensive RFLP analyses showed that these conserved phytoplasma DNA sequences allowed to differentiate 5 phytoplasma variants inside the ribosomal subgroup 16SrV-C. These molecular data confirm that only some of the FD identified variants can be assumed as epidemic such as those detected in S. Casciano (FI), Podenzana (MS), Caprigliola-Aulla (MS) that are undistinguishable from those detected in the FD epidemic areas of Piedmont, Lombardy and Liguria. One of the two molecular variants detected in Fosdinovo (MS) resulted

undistinguishable from the strains from Serbia, Veneto (TV54), and Umbria (PG24), and don't appear to have epidemic ability; all the other strains detected in 2 samples from Fosdinovo (MS), and in 3 samples from Montignoso (MS) seem to be distinguishable from each others, and from all the strains described in this and in previous works (Martini *et al*., 2002; Botti and Bertaccini, 2003).

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MAP GENE AS A NEW NON RIBOSOMAL MARKER TO STUDY THE MOLECULAR VARIABILITY OF GRAPEVINE YELLOWS AND RELATED PHYTOPLASMAS FROM GROUP 16SRV IN EUROPE

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Introduction

The Flavescence dorée (FD) and Palatinate grapevine yellows (PGY) phytoplasmas belong to the phylogenetic group 16SrV, a group characterized by a high 16S-rDNA homology (> 97 %). European isolates in that group comprise phytoplasmas infecting not only grapevine but also alder, elm, rubus and spartium. Elm yellows phytoplasma isolates are now defined as a unique species *Candidatus* phytoplasma ulmi (Lee *et al*., 2004). The phylogeny and taxonomy of group 16SrV has been previously studied using three distinct genetic loci namely 16SrV (Davis *et al*., 2001), *secY* (Angelini *et al*.,2003) and *rpl22-rps3* (Martini *et al*., 2002). However these markers did not appear resolutive enough to clearly separate phytoplasmas isolated from different hosts especially in the case of FD, PGY and Alder yellows isolates. To better document the genetic variability of group 16SrV phytoplasmas, it is therefore important to study new variable markers which can be used in combination with previous ones to develop a multi-locus sequence typing (MLST) strategy. Here we describe the sequence variability of the *map* locus encoding a methionine aminopeptidase in group 16SrV phytoplasmas collected in Europe. We propose a classification of the group based on this marker.

Materials and Methods

The *map* gene was isolated and characterized from FD70 (Caudwell *et al*., 1970) isolate by chromosome walking in the vicinity of the *sec Y* locus (Angelini *et al*., 2003). A panel of 103 group V phytoplasma isolates was obtained from infected grapevines, alders, elms, spartium and rubus collected in Italy, Germany and France. Most of them were kindly provided by E. Angelini (Conegliano, Italy), A. Bertaccini (Bologna, Italy), L. Carraro (Udine, Italy), D. Clair (Dijon, France), G. Cloquemin (Colmar, France), F. Costard (Bordeaux, France), M. Maixner (Bernkastel, Germany), C. Marcone (Potenza, Italy) and C. Marzachi (Torino, Italy). The strains maintained in periwinkle or broadean were FD70 (A. Caudwell) and FD92 (E. Boudon-Padieu), ALY (C. Marcone), EY1 (WA Sinclair, NY USA), ULW (G. Morvan, Avignon, France), RuS (E. Seemüller, Germany), PGY-A and PGY-B (M. Maixner). After DNA extraction, the locus *map* was amplified by nested PCR with primers FD9-F5/MAP-R1 and FD9-R6/MAP-R2 designed for the whole 16SrV phytoplasma group. The 803 pb fragments were sequenced with primer MAP-R2. Sequences were aligned and compared on 700 pb length using the program ClustalW. Phylogenetic analyses were performed using MEGA ver. 2.1. Tree was constructed according to the maximum parsimony method. Variability between isolates was determined by counting the total number of nucleotide substitutions on *map* sequences, it was expressed as a mutation rate in %.

Results and Conclusion

The *map* marker displayed an important variability with a mutation rate up to 4.3% between isolates of group 16SrV, 2.6% between different PGY isolates, 1.86% between different FD isolates and 3.71% between FD (FD 70) and EY (EY1) isolates. As a comparison, *rpl22-rps3* marker reached 2.4% among strains of group 16SrV, 0.8% between FD strains and 1.9% between FD 70 and EY1 (9).

Table 1 shows results phylogenetic analyses and quotes information on identified vectors of isolates. Three main robust groups of isolates could be distinguished. The Rubus stunt group was very homogenous. It contained various isolates from France an Italy isolated from *Rubus* sp. which all shared 100% identity on *map* marker. The *Candidatus* Phytoplasma ulmi group was more variable (1.14%) and could be divided in two homogenous subgroups named EY1 and EY2. Both of them comprised elm isolates from different areas of France and Italy. The Grapevine and alder yellows phytoplasmas represented the third and most variable group (2.6%). It could be divided in three distinct subgroups of FD phytoplasmas isolated from vines: FD1 (=FD70), only described in France with 0.47% variability, FD 2 (=FD92=FD-D) very homogenous (no variability) and present in France and Italy, and FD3 (FD-C), the most variable (0.57%) and described in Italy only. A fourth subgroup named PGY-A contained isolates

from vines and alders from France, Germany and Italy. In addition to these four coherent groups, other isolates could not be precisely classified. They showed an important variability between them (2.6%). They were isolates from spartium (SpaWB) for which the vector is not known and different PGY and AldY isolated in France, Italy and Germany.

In conclusion, the *map* locus appears as a useful variability marker to allow a precise classification of group 16SrV phytoplasmas. However, some isolates still remain not phylogenetically well determined. Addition and combination of distinct variable markers in a MLST strategy should permit to better classify such isolates.

Grapevine and Alder yellows group (2.6 %)[1]						Rubus stunt group (0 %)[1] *M. fuscula*[2]	*Candidatus* Phytoplasma ulmi group (1.14 %)[1] *M. mendax*[2]	
Subgroup FD-2 (0%)[1] *S. titanus*[2]	Subgroup FD-3 (0.57 %)[1] *S. titanus*[2]	Subgroup PGY-A (0.43 %)[1] *O. Alni*[2]	Subgroup FD-1 (0.43 %)[1] *S. titanus*[2]	Imprecise Phylogenetic classification (0%)[1] Unknown [3]	Imprecise Phylogenetic Classification (2.6 %)[1] *O. Alni*[2]		Subgroup EY 1 (0.14 %)[1]	Subgroup EY 2 (0 %)[1]
Italy: 1 FD-D Veneto **Germany:** Not described **France:** 1 FD 92 Languedoc-Roussillon 19 FD Aquitaine 2 FD Bourgogne 5 FD Languedoc-Roussillon 2 FD Limousin 14 FD Midi-Pyrénées 4 FD PACA 1 FD Pays de Loire 4 FD Poitou-Charentes 6 FD Rhône-Alpes	**Italy**: 2 FD-C Piemonte 2 FD-C Veneto 2 FD Liguria 1 FD Toscana **Germany:** Not described **France:** Not described	**Italy:** 1 AldY Friuli **Germany:** 1 PGY-A Pfalz **France**: 1 AldY Languedoc-Roussillon	**Italy:** Not described **Germany:** Not described **France:** 1 FD-70 Midi-Pyrénées 4 FD Aquitaine 3 FD Midi-Pyrénées 4 FD Poitou-Charentes 1 FD Rhône-Alpes	**Italy:** 2 SpaWB Basilicata	**Italy:** 1 ALY 1 AldY Basilicata **Germany:** 1 PGY-C Pfalz 1 PGY-B Pfalz **France:** 1 GY Alsace	**Italy**: 1 RS 1 RuS Basilicata 2 RuS Friuli **Germany:** Not described **France:** 1 RuS Languedoc-Roussillon	**Italy**: 1 ElmY Friuli **Germany:** Not tested **France:** ULW 2 ElmY Languedoc-Roussillon 2 ElmY Limousin **(USA):** EY1	**Italy**: 1 ElmY Basilicata **Germany:** Not tested **France:** 1 ElmY Limousin 1 ElmY Loire-Atlantique

Table 1. Classification and geographic repartition of group 16Sr-V phytoplasma strains based on the sequence of the *map* locus.

1. Maximum mutation rate between strains, 2. Known insect vector, 3. Unknown insect vector. Isolates underlined were shown to be transmitted by the corresponding underlined vectors (2, 3, 5, 7, 8, 10, 11). Only FD and PGY isolates were obtained from yellows-diseased grapevines.

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INVESTIGATING VECTOR SPECIFICITY OF FLAVESCENCE DORÉE PHYTOPLASMA OF SOME HEMIPTERA SPECIES

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Introduction

Identification of vectors and potential vectors of Grapevine yellows (GY) diseases is crucial for epidemiology and control of these important diseases (Boudon-Padieu, 2005). Few information are reported in literature concerning the specificity of vector transmission of phytoplasmas. Some phytoplasma strains seem to be specifically transmitted, for example phytoplasmas in the Elm yellow group seem to be transmitted by only a few leafhopper species. Other strains have low vector specificity, such as those clustered in the Aster yellows group, which are transmitted by several leafhopper species. Flavescence dorée (FD) of the grapevine is associated with a phytoplasma (FDP) that seems to be specifically transmitted in the field by the leafhopper vector *Scaphoideus titanus* Ball (Hemiptera, Cicadomorpha, Cicadellidae) (Schvester *et al*., 1963; Vidano, 1964; Mori *et al*., 2002). However Caudwell *et al*. (1972) showed that in experimental conditions, *Euscelidius variegatus* Kirschbaum (Hemiptera, Cicadomorpha, Cicadellidae) was an efficient alternative vector of FDP to herbaceous hosts although it cannot feed-acquire FDP from infected grapevines. Hence the species of source and recipient plants are very important both in natural diffusion and experimental assays.

In the present work we studied the vector specificity of FDP by 15 Hemiptera Cicadomorpha and Fulgoromorpha species collected in European vineyards. To test vector transmission we adopted needle injection to deliver phytoplasma suspensions into the abdomen of candidate vector species (Purcell, 1996). After an incubation period we fed the injected insects on artificial diets through a Parafilm membrane (Zhang *et al*. 1998; Tanne *et al*. 2001) and tested the feeding medium for the presence of FDP DNA using PCR amplification of FDP DNA.

Materials and Methods

Healthy colonies of *E. variegatus* were maintained on maize inside cubical Plexiglas cages and used for feeding acquisition and transmission on broadbean to maintain phytoplasma strains (Caudwell and Larrue, 1977). Healthy nymphs or young adults were periodically collected for injection assays from the latter colonies.

Suspensions of FDP were obtained from FDP-infective *E. variegatus* according to Whitcomb and Coan (1982). Optimization of the concentration of viable phytoplasma extracts and latency in vectors were monitored by injecting healthy-reared *E. variegatus* leafhoppers. Then insects were injected using phytoplasma extracts that ensured the highest rate of FDP acquisition and transmission by *E. variegatus.* Transmission was attempted to an artificial diet through a Parafilm membrane about three weeks after insect injection.

Leafhopper and planthopper specimens were collected by using a D-vac or a sweep-net in viticulture areas located in the Mosel Region(D), Burgundy Region (F) and Veneto Region (I) or kindly provided by other laboratories. Insects were caged on suitable host plants. Trapped univoltine species were directly used for injection assays, while plurivoltine species were reared for at least one generation and the progeny was used in injection assays. All the insect species were maintained or reared in a climatic chamber (23 ± 1 ºC, L16:D8).

To confirm transmissibility of FDP by some tested species, insects were confined on FD-infected broadbeans or on healthy broadbean seedlings (as control) for an acquisition period of 15 days and then confined to healthy broadbean seedlings after an incubation period of about 35 days.

Detection of FDP in insects, in plants and in feeding medium (Zhang *et al*. 1998; Tanne *et al*. 2001) was done with PCR amplification of phytoplasma DNA using FDP-specific primers (Clair *et al*. 2003).

Results

Among the batches of FDP-injected insects belonging to 15 Hemiptera species, that were confined in cages and fed through a Parafilm membrane in the medium for a 4-5 days inoculation access period (IAP), FDP DNA was detected by PCR in the feeding medium inoculated by the leafhoppers (Cicadellidae) *Anoplotettix* fuscovenosus (Ferrari), *Aphrodes makarovi* Zachvatkin, *Euscelidius variegatus* Kirschbaum and *Euscelis incisus* Kirschbaum but not in the feeding medium where injected insects of the other 11 species were confined: the leafhoppers *Agallia consobrina* Curtis *Circulifer haematoceps* (Mulsant & Rey), *Fieberiella florii* (Stål), *Psammotettix* sp.; the spittlebug (Cercopidae) *Philaenus spumarius* (Linneus); the treehopper (Membracidae) *Stictocephala bisonia* (Kopp &Yonke); the Fulgoromorpha: *Agalmatium flavescens* (Olivier), *Hyalesthes obsoletus* Signoret, *Laodelphax striatellus* (Fallén), *Metcalfa pruinosa* (Say), and *Pentastiridius* sp.. Detection of FDP was positive in injected insects of all the Hemiptera species although band intensities in the agarose gels were positively associated with the transmissibility of FDP to artificial diets.

The ability of *E. variegatus* and *E. incisus* and the inability of *C. haematoceps* and *F. florii*, to transmit FDP, were confirmed by feeding the insects for acquisition on FDP-infected broadbeans then for transmission on healthy broadbean seedlings. FDP in inoculated broadbeans was confirmed by symptom expression and by PCR detection. *E. variegatus* and *E. incisus* that transmitted to feeding medium also inoculated broadbean; *C. haematoceps and F. florii* transmitted neither to feeding medium nor to broadbean.

Discussion

Injection technique is a potential useful tool for searching for insect vectors (Whitcomb and Coan, 1982) because it increases the rate of phytoplasma acquisition and reduces the latent period. Additionally it is possible to test insects with different feeding habits or different host plant preferences.

The use of feeding medium assays to test the inoculative potential of insects allows a significant reduction of time if compared with transmission to host plants. Also, it eliminates the effects of host plant-vector interactions in the inoculation process. In addition, Ge and Maixner (2003) found that the technique increased the efficiency of vector transmission when compared to feeding transmission to host plants.

The four insect species that transmitted FDP after injection belong to the family Cicadellidae, as well as the economic vector of FDP, *S. titanus*. The other Hemiptera species tested could not transmit FDP after abdominal injection of the phytoplasma suspension. Therefore we may assume that the latter species are not potential vectors of FDP or are extremely inefficient in transmitting the mollicute. Passage of plant pathogens from the haemocoel to the salivary glands and subsequent transmission is not enough by it self to recognize if one insect is a vector. Actually, in natural conditions, phytoplasma cells should overcome at least the two physical barriers that are the gut and the salivary glands (Lefol *et al*., 1994); Fletcher *et al*., 1998). Therefore other assays based on feeding acquisition from FDP-infected host plants are in progress to confirm the ability to transmit FDP by *A. fuscovenosus* and *A. makarovi*.

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LIMITED SUSCEPTIBILITY OF SYRAH CV. TO FLAVESCENCE DORÉE AND BOIS NOIR IN SOUTH OF FRANCE

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Introduction

Cultivation of Syrah cv. is historically important in south-east France, in the area of the Rhone river and has recently developed rapidly in that region (about 18,000 ha) and in other regions in south (Gard, Aude, Hérault) and south west (Tarn, Tarn et Garonne, Haute Garonne) France. However few if any cases of Syrah grapevines affected with Flavescence dorée (FD) or Bois noir (BN) have been reported since the first description of FD in France (Caudwell, 1957), in spite of a quick and severe diffusion of FD epidemics in almost all viticulture regions of south France (Boudon-Padieu, 2002). Similarly, although Bois noir (BN) is an endemic disease in all French viticulture, it has been detected only in a few occurrences on Syrah grapevines (unpublished data).

Most of the other grapevine cultivars used in France, including local varieties, are susceptible or very susceptible to GY diseases (Boudon-Padieu, 1996; Caudwell *et al*., 1987). Hence the situation of Syrah raises questions. Might Syrah grapevines be latently infected without symptoms and be nevertheless source plants for acquisition of phytoplasma by vectors ? If so, identification of FD-infected vines would be very important in order to set up control measures to prevent diffusion in the fields to susceptible cultivars. Alternatively, the absence of real incidence of GY phytoplasmas on Syrah would offer new research fields on its possible resistance to feeding by insect vectors and/or its possible resistance to phytoplasma multiplication. The present work was based on the recent detection of FD-diseased plants in a Syrah plot in the Drôme region where FD has occurred for the first time in 2002. It was set up to evaluate the susceptibility of Syrah to FD and BN (stolbur) phytoplasmas in comparison with the highly susceptible cultivar Grenache noir.

Material and Methods

Two vineyards planted with Syrah and adjacent plots planted with Grenache noir were used.

Vineyard A was in Rousset-les-Vignes (Drôme). One Syrah plant with symptoms of Grapevine yellows (GY) tested FD-positive with PCR in autumn 2003. In 2004, 7 other plants scattered in the same plot, showed symptoms and were also FD-positive. In autumn 2004, samples were taken on vines with no symptoms in the same plot. Each mixed sample consisted of leaves collected on 5 symptom less vines. Four mixed samples were constituted from leaves taken on vines surrounding each FD-infected plant : (i) 5 vines beneath and (ii) 5 vines beyond each diseased plant on the same row, (iii) 5 vines on one side and (iv) 5 vines on the other side of each diseased plant on the two neighboring rows. As a whole, 23 mixed samples (from 111 vines) were collected. Another series of 26 mixed samples were collected at sites randomly chosen in the Syrah plot and consisted each of leaves taken on 5 successive symptom less vines on a row. In addition, 10 similar samples were collected on symptom less plants of cultivar Grenache noir cultivated in an adjacent plot in which GY symptoms occurred for several years and both FD and BN phytoplasmas have been consistently detected.

Syrah vineyard B was in Séguret (Vaucluse) about 30 kms from the first plot. It was planted next to a Grenache noir plot where GY symptoms occurred for several years and both FD and BN phytoplasma have been regularly detected. Only one Syrah plant was suspected of GY infection in 2004. Forty randomly designed mixed samples similarly consisting each of leaves taken on 5 successive symptom less vines on a same row, were collected in the Syrah plot and 11 similarly designed samples were randomly collected in the Grenache noir plot. When the 5 vines in the Grenache noir plot were next to a GY-affected plant, the latter was not included in the mixed sample but it was separately tested.

The procedure for simultaneous routine detection of FD and BN (Stolbur) phytoplasmas developed by Clair *et al*. (2003) was used : Veins and petiole of one leaf of each of the 5 plants in one mixed sample were detached and cut into pieces. DNA was extracted from 1g aliquots, using the CTAB-buffer procedure. Purified DNA was submitted to a multiplex nested PCR assay using primers designed on the specific fragments FD9 and Stol11. Amplification products were submitted to agarose gel electrophoresis, stained with Ethidium bromide and visualised under UV light. Final FD9 (1160 bp) and Stol11 (720 bp) amplification procducts could be identified according to their size on the electrophoresis gel.

Results and Discussion

Incidence of symptoms of grapevine yellows in Syrah and Grenache noir vineyards. In vineyard A, 7 plants of Syrah out of 1963 (0.36%) showed GY symptoms and tested FD-positive. Conversely, in the adjacent Grenache noir plot 147 vines out of 3,368 (4,36%) showed symptoms of GY. The presence of both FD and BN phytoplasmas were confirmed in a selection of the latter 147 plants. In vineyard B, apart for one suspected Syrah plant that eventually tested phytoplasma negative, no other plant showed any GY symptoms out of 845 vines (0,00%). The Grenache noir vineyard that neighbored plot B showed 96 GY-affected out of 1500 plants (6.40%) and a second Grenache noir plot in the close vicinity showed a rate of GY-affected plants of 50 out of 2,800 plants (1,79%). These data strongly support that Syrah is much less susceptible to GY infection than Grenache noir.

Phytoplasma detection. Apart from the 7 plants of Syrah from vineyard A that showed symptoms in 2004 and tested FD-positive, none of the mixed sample collected in plot A revealed either FD or BN infection, either from the 23 mixed samples situated next to the 7 FD-infected vines, or from the other 10 mixed samples randomly selected in vineyard A. Symptom less vines from the adjacent Grenache noir vineyard also all tested negative either to FD or to BN.

A different situation was observed in Séguret. Here again, no mixed sample from Syrah grapevines in vineyard B tested positive for FD or BN phytoplasma. However, 2 mixed samples out of 11 collected in the Grenache noir plot tested BN-positive. Actually, the latter two samples were taken from symptom less grapevines situated close on the same row or on the next row, to BN-affected symptomatic vines. The latter result suggests that phytoplasma could be detected using nested-PCR in plant material incubating phytoplasma before the expression of symptoms. The PCR-positive plants will be checked in the following years for symptom expression.

As a conclusion, the present data are the first reported investigation on the susceptibility of Syrah cultivar to phytoplasma infection. Although more observations must be done, the situation in vineyard A, compared to detection results obtained in Grenache noir close to vineyard B, support that Syrah cultivar is not tolerant and/or healthy carrier of phytoplasmas. The 7 plants in vineyard A were systemically infected and showed generalized typical symptoms including lack of lignification of most of the shoots and canes, leaf discolouration and rolling and withering of flowers and berries. Actually it might be expected that neighbor plants of FD-diseased vines would be infected in turn, since vine-to-vine transmission of FD is frequent, due to feeding habits of the leafhopper vector *Scaphoideus titanus.* The procedure used has been shown to be sensitive enough to detect phytoplasma infection in one out of the 5 plants constituting a mixed sample (Clair *et al.*, 2003 and unpublished data). The absence or rarity of GY infection on Syrah must result from a low appetance of the variety to auchenorrhyncha vectors and/or a low inoculation efficiency or a reduced multiplication of phytoplasma in phloem tissue, leading to a reduced rate of infection of the cultivar.

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HYALESTHES OBSOLETUS : DISPERSAL FROM NETTLE AND TRANSMISSION EFFICIENCY OF STOLBUR PHYTOPLASMA TO GRAPEVINE

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Introduction

Hyalesthes obsoletus Signoret (Hemiptera, Fulgoromorpha, Cixiidae) is a European vector of Stolbur phytoplasma which is the agent of grapevine yellows called Schwarzholzkrankheit, Bois noir or Legno nero (Maixner 1994, Sforza *et al.* 1998, Alma *et al.* 2002).

The planthopper seems to be polyphagous to herbaceous host plants, although preference host plant species may vary according to different geographical areas (Alma *et al.* 1987, Maixner *et al.* 1995, Sforza *et al.* 1998, Sharon *et al.* 2005).

In Italy *Urtica dioica* L. is a principal host plant of *H. obsoletus* (Alma *et al.* 1987) and it may support high population of the vector. Additionally this host plant has been found to host Stolbur phytoplasma (Alma *et al.* 2002, Langer and Maixner 2004).

We took advantage of the presence of an important nettle band adjacent to a vineyard located in Veneto Region (North-East Italy) to study the vector activity of *H. obsoletus*, in particular (i) quantify vector dispersal and vector daily flight activity from the nettle band to the vines, (ii) quantify the time required by the vector to transmit Stolbur phytoplasma to grapevine, (iii) quantify incidence of Stolbur phytoplasma in the population of *H. obsoletus* living on the nettle band.

Material and Methods

For the study we considered a vineyard separated from a large nettle band with a 5m-large road. A high population of *H. obsoletus* occurred in the nettle band. The vineyard was planted with Merlot cultivar and cultivated with monocotyledons in the inter-rows (monocotyledons species seem not to be preferential host plants for the vector).

Transparent sticky traps (10.5 cm X 15 cm) were positioned (a) in the middle of nettles, (b) along the vineyard border (6m from the nettle band) and (c) inside the vineyard at distances of 16m, 26m and 36m from the nettle band. Insects were counted weekly during the flight activity.

To monitor the daily flight activity, 12 sticky traps were placed in a row along the nettle border and insects were counted every 2 hrs along the day. Counting was repeated on three different days.

Batches of 20 *H. obsoletus* were confined on grapevine cuttings (Chardonnay) for different inoculation access periods (iAP). Survival of insects was recorded. Cuttings were kept in an insect-proof greenhouse for symptom expression.

Individual insects and test plants were tested with PCR for the presence of stolbur phytoplasma according to Clair *et al*. (2003).

Results

Captures of *H. obsoletus* were much more numerous on traps positioned in the nettle band than on those positioned on the border or inside the vineyard. Insect captures on traps followed a gradient as shown in Figure 1. Planthopper daily flight activity showed a peak late in the evening before sunset (Figure 2).

We detected Stolbur phytoplasma in 33 out of 114 adults of *H. obsoletus* (29%) during 2003, and in 26 out of 115 adults of *H. obsoletus* (22%) in 2004.

Transmission of Stolbur phytoplasma to grapevine cuttings exposed for IAPs of 3, 6, 12, 24 and 48 hrs, revealed that minimum IAP ranged between 3 and 6 hrs (Figure 3). *H. obsoletus* survived poorly on grapevine cuttings (Figure 3).

Discussion

Insect vector activity depends upon several factors such as population vector density, transmission efficiency, vector infectivity and vector inter-plant movement.

Our data showed that populations of *H. obsoletus* might occur in very high density on nettle plants. The rate of stolbur phytoplasma infection in planthoppers ranged between 20 and 30% of insects tested, in accordance to previous reports (Sforza *et al.* 1998; Alma *et al.* 2002). Phytoplasma transmission may occur rapidly to grapevine, although dispersal of adults into the vineyard seemed to be limited in the observed conditions. The very low survival rate of the planthopper on grapevine cutting confirmed that grapevine is only an occasional host plant for the *H. obsoletus* (Maixner *et al.*, 1995; Sforza *et al.*, 1998).

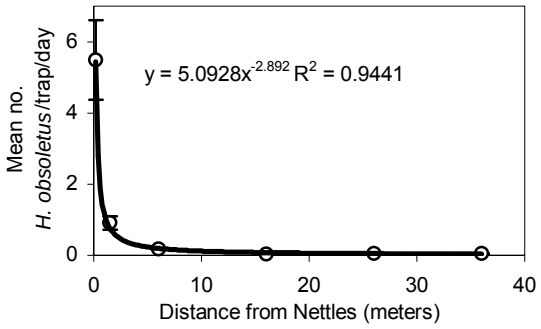

Figure 1
Dispersal pattern of *H. obsoletus*

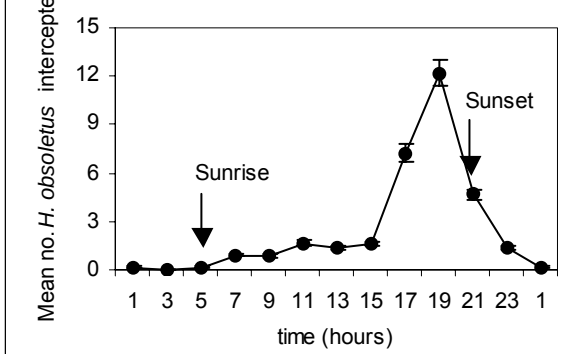

Figure 2
Daily flight activity of *H. obsoletus*

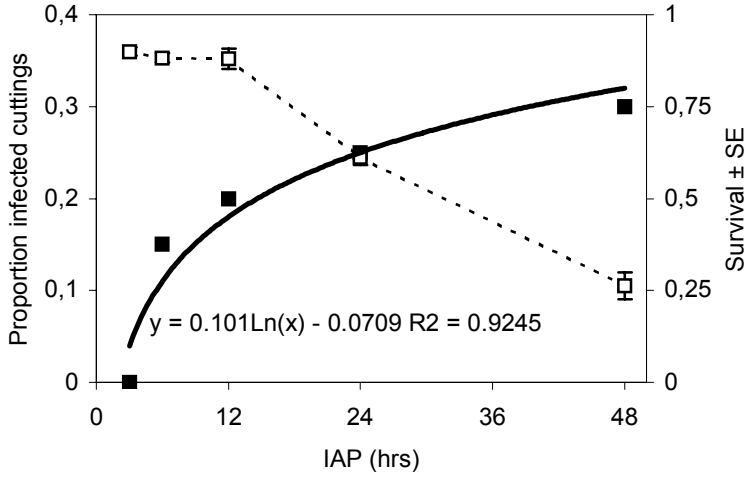

Figure 3
Transmission of Stolbur phytoplasma to grapevine by *H. obsoletus* and vector survival

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CHARACTERIZATION OF HSP70 GENE OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1*

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Introduction

Grapevine leafroll-associated virus 1 (GLRaV-1) is a worldwide distributed pathogen of grapevine (Zimmermann *et al*., 1990). Similarly, GLRaV-1 is of the most spread viruses of grapevine in the Czech Republic (Komínek and Holleinová, 2003).

During last years, an attempt was made in our laboratory to characterise the molecular variability of GLRaV-1. Especially a viral homologue of the HSP70 family of heat shock proteins, which is a unique feature of plant closteroviruses (Agranovsky *et al*., 1991), was targeted.

Material and methods

Dormant wood was sampled from grapevines in the Czech Republic (viticulture region Karlštejn), Slovakia and Turkey (Olur district of Erzurum city). Total RNAs were isolated from cortical scrapings using Qiagen RNeasy Plant Mini Kit. A primer pair was designed to amplify a 540 bp long portion of HSP70 gene of GLRaV-1. Primer sequences were as follows: "plazleft" (5´ caggcgtcgtttgtactgtg 3´, sense) and "plazright" (5´ tcggacagcgtttaagttcc 3´, antisense). Amplified RT-PCR products were cloned into pGEM-T Easy vector (Promega) and commercially sequenced. Obtained sequences were analyzed to estimate GLRaV-1 variability and compared to sequences available in GenBank database. For differentiation of GLRaV-1 groups, products of RT-PCR were subjected to digestion with restriction endonucleases *Bsi*WI (Roche) and *Sal*I (Promega).

Results and Discussion

RT-PCR with designed primers was successfully used for GLRaV-1 detection. The virus was detected by RT-PCR even in those grapevines, for which the DAS-ELISA was negative.

Obtained RT-PCR products from eight grapevines were cloned and several clones derived from each grapevine sample were individually sequenced to estimate inter- and intra-isolate variability. All sequenced clones were found to be GLRaV-1, indicating a high specificity of used primers and their suitability for diagnostic purposes.

Variability of obtained sequences comprising a 540 bp long fragment of HSP70 protein gene was evaluated. Results showed grouping of obtained sequences into two groups tentatively named A (as „American“ - according to similarity of this sequences to previous GLRaV-1 GenBank sequences originated from America) and E (as „European“, based on the similarity to partial sequences of an Italian and an Austrian isolates). Genetic divergence between A and E group reached 13.9% (Komínek *et al*., 2005)

Restriction enzymes distinguishing A and E groups were found. *Sal*I site is present only in A- type isolates, whereas *Bsi*WI cleaves E group only. Those enzymes were used for a large-scale testing of GLRaV-1 infected grapevine samples from Czech Republic and Turkey. Based on our tests, GLRaV-1 isolates of both A and E groups occurr on these countries. A mix infection of A and E group in a single grapevine was also found.

Interestingly, a shorter fragment was observed in two cases after RT-PCR. Sequence analysis of those fragments revealed deletion of 195 bp from HSP 70 gene of GLRaV-1.

Acknowledgements

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PRELIMINARY STUDIES ON GRAPEVINE LEAFROLL ASSOCIATED VIRUS 1 AND 3 DETECTION IN *V. VINIFERA* L., FETEASCA NEAGRA CV. BY PRIMER SPECIFIC GENE AMPLIFICATION

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Introduction

Our activity concerning the detection of grapevine viruses aims the improvement of diagnosis of the diseases and the elimination of pathogens from nursery stock as well, preventing the spread of the diseases into new vineyards (Buciumeanu et al., 2000; 2003).

Leafroll is the second only to fanleaf in economic importance and it is probably the most widespread virus disease of grapevine. Several viruses have been associated with the disease (Gugerli, 2003; Marteli, 2004).

The paper deals with the GLRaV 1+3 detection in *V. vinifera* L., Feteasca neagra cv. by RT-PCR. Until recently, biological indexing and serological methods have been used to detect leafroll disease. In the beginning a step of optimisation and standardization of a proper method for the detection of GLRaV1+3 by specific gene amplification has been developed. Also, the paper presents an increased interest due to the Feteasca neagra variety of red wine, reputedly indigenous to Romania.

Materials and Methods

Biologic material collected from mature twenty years old grapevines showing leafroll symptoms have been used in this study. The presence of GLRaV 1+3 was detected by dDAS-ELISA method, using commercial reagents (Bio-Rad). Virus infected grapevines showed perturbances of the growth consisting in down-rolling and reddning of the mature leaves on the low part of the bunches. No significant modifications of biochemical composition of infected leaves have been found. The quality of the grapes has occurred in correlation with the presence of the virus. Low amount of sugar in juice and higher acidity have been registered with virus infected grapes.

Double stranded RNA was extracted in two ways: (a) from 5-10 mg dormant wood ground in mortar with liquid nitrogen, then processed with AquaPure RNA isolation kit (Bio-Rad) according to the protocol for plant tissue. Briefly, this kit uses a proprietary modified salt precipitation procedure in combination with highly effective inhibitors of RNase activity, in which the cells are first lysed in the presence of a RNA preservative using an anionic detergent which solubilizes the cellular components. Contaminating DNA and proteins are then removed by salt precipitation. Total RNA is finally isolated by first precipitating with alcohol and then hydrated in RNase-free water. This approach was compared with plant extracts (b) prepared according a modification of the protocol described by Archeche et al., 1999: scraped bark tissue from dormant cannes was ground in liquid nitrogen, homogenized with 50 mM citrate buffer (pH 5.6, containing 2% PVP, and 20 mM DIECA) at a ratio of 5/1 (v/w), supplemented with carborundum, and centrifuged at 16,000 x g for 5 min. The supernatant was diluted to 40% in distilled water containing 1% Triton X-100, then incubated at 65ºC for 15 min.

The reverse transcription was performed (a) with iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The RT mix (consisting of 1 µl of iScript Reverse Transcriptase preblended with RNase inhibitor, 4 µl of RT buffer containing a unique blend of oligo (dT) and random hexamer primers, and 2 µl of isolated RNA), in a total reaction volume of 20 µl was subjected to the following thermal profile: 25°C for 5 min, 42° C for 30 min, 85° C for 5 min, 4° C hold. In the second approach (b), we used 5 µl of plant extract, and the same RT mix without iScript Reverse Transcriptase which was incubated at 100ºC for 2 minutes, on ice for 2 minutes, and at room temperature for 10 minutes. Then we added 1 µl of 1:5 diluted iScript Reverse Transcriptase (total reaction volume 25 µl) for the final incubation at 37ºC for 1 hour.

Two microliters of cDNA samples were amplified using a primer pair that targets a 942 bp fragment of GLRaV 1+3 coating protein gene: downstream primer-C50, 28 nucleotides, and upstream primer-H49, 25 nucleotides (Marrakchi, 1998-2004; Büchen-Osmond, 2003). A total of 50 µl amplification reaction mix

has the following formulation: PCR Buffer (1x PCR buffer for Bio-Rad hot-start iTaq, 20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.5 mM MgCl2, 200 µM dNTPs, 1.25 U iTaq™ DNA Polymerase (Bio-Rad), and 0.240 µM each primer. We applied several thermal profiles for amplification using MyCycler thermal cycler (Bio-Rad), starting with those proposed by Dar-Issa O. et al., 2002, several touch-down protocols, and finally we optimized the thermal profile with a 3 minute hot-start at 95°C, 15 cycles amplification (annealing step at 52°C), 20 cycles amplification (annealing step at 54°C), followed by a 10 minute final extension step.

Results and Discussion

The amplicons were resolved 1.5% agarose/TBE gel, stained with ethidium bromide, and examined under UV light, where a specific band 942 bp representing the coating protein gene fragment was observed, togeteher with some unspecific products (about 400, 500, and 1100 bp, respectively). Both pre-PCR approaches gave expected signal with the optimized thermal profile, but Bio-Rad reagents/protocols were easier to handle, yielded more stable cDNAs, being also more reproducible and generating higher signals in all ELISA positive samples. We intend to continue PCR-step optimizations in order to eliminate the unspecificities and increase PCR reaction efficiency and sensitivity.

The PCR method will be extended for the diagnostic of other grapevine viruses and will be used especially to complement or estabish the sanitary status of economicaly important grapevine cultivars and clones.

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DETECTION OF GRAPEVINE LEAFROLL VIRUSES USING DIRECT IMMUNO-PRINTING (DIP)-ELISA

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Introduction

Direct Immuno-printing (DIP), known also as Tissue Printing (TP), Tissue Blot Immunoassay (TBIA), Direct Tissue Blotting assay (DTBA) and other denominations, has been used for the detection of various viruses in several crops as an alternative to DAS-ELISA (Lin *et al.*, 1990; Hsu and Lawson, 1991); although DIP has not been used for grapevine viruses, several other closteroviruses (Citrus tristeza virus, Pineapple mealybug wilt – associated virus) are readily detected using DIP (Garnsey *et al.*, 1993; Sether *et al.,* 1998). Essentially, DIP consists in cutting the plant material and gently pressing the surface onto a nitrocellulose membrane, without any extraction process; the membrane is then blocked with proteins during a short period of time, and then incubated in specific antibodies conjugated with alkaline phosphatase; after washing in a saline buffer the membrane is incubated with the substrate which will react with the enzyme giving a blue-purple colour. Most authors found a good correlation between DAS and DIP ELISA for the detection of plant viruses and quite similar sensitivity (Whitworth *et al.*, 1993; Couceiro *et al.*, 2005). DAS-ELISA is quite time and labour consuming as need a previous extraction of the sample to be tested and several steps with long incubation periods; the cost of DIP-ELISA per sample is considerably lower than for DAS-ELISA (Couceiro *et al.*, 2005).

In epidemiological studies, when we want to correlate leafroll symptoms and damages with virus presence in grapevine, the best moment to take the samples is late summer. Given the number of leafroll viruses which could be implicated in the leafroll symptoms, the price of the reactives and plates, and the time required to perform DAS-ELISA, we decided to test DIP-ELISA for GLRaV-1, 2 and 3. DIP-ELISA could also be an interesting technique for the study of the distribution and movement of leafroll viruses along the plant.

Materials and Methods

DAS-ELISA: The antibodies were from Bioreba AG (Basel, Switzerland) and the tests were performed following the indications of the supplier concerning buffers and antibody dilution (1:1000) for GLRaV-1, 2 and 3. Leaf blades with part of the petiole were used.

DIP-Direct Immunoprinting. The samples were printed onto membranes of nitrocellulose with 0.45 µm pore size (Sartorius, Goettingen, Germany). The membranes were blocked in non-fat dried milk at 1% in extraction buffer for grapevine (the same as for ELISA) during one hour at room temperature or overnight at 4-6ºC. Membranes are then dipped directly in alkaline phosphatase conjugated antibody solution (in conjugated buffer as for ELISA) and incubated for 2-2.5 h at room temperature or overnight at 4-6ºC. After incubation the membranes are washed carefully for at least five minutes (stirring), three times, in a saline buffer (0.085% NaCl, 0.05% Tween 20 in distilled water). The membranes are then covered with BCIP-NBT ready-to-use liquid substrate (SIGMA B-1911). The purple colour develops after 10-15 minutes of incubation but occasionally it takes up to 30 minutes, therefore the reaction is not stopped (with tap water) until the membrane starts getting dark and the positive control is clear. In the case of leafroll associated viruses, a dissecting microscope must be used to observe the purple colour which develops only on area corresponding to the phloem vessels. One membrane per virus is necessary only when any of them is to be identified; in other cases the antibodies can be mixed and one membrane is enough to perform the test.

In order to optimize the protocol, some tests were carried out with our control plants to find the best point to make the cut and the imprints. The petioles and main ribs of several leaves from each plant were cut in at least 10 portions and any tip was printed onto the membranes (two times each). Petioles were also cut longitudinally. At the beginning of July, August and September a whole shoot was removed from any plant (Cabernet franc and Albariño with the same GLRaV-3 virus) and all leaves were tested in two points to find out the best leaf sample. Leafroll symptoms were recorded. DIP and DAS ELISA were compared. A total of 526 samples were collected in control plants which had been ELISA tested in previous years and in several experimental plots; after DAS-ELISA the same conjugated antibody solution was used for

DIP-ELISA. Agreement between DIP and DAS-ELISA was counted only when DIP readings and ELISA results matched sample to sample.

Results and Discussion

With some changes in the existing protocols for DIP, we managed to get quite good results for the viruses tested. The use of the grapevine ELISA extraction buffer instead of water or saline buffer for the blocking step was basic but also waiting a little bit more before the final reaction is stopped. The best point to make the cut was as close as possible to the leaf blade. The cuts must be as much clear and perpendicular to the petiole as possible to obtain many points getting the purple colour in case of virus presence. As in DAS-ELISA, tendrils, young stems or petioles from young leaves are not reliable samples. The leaves showing very clear leafroll symptoms gave poorer positive results than leaves with mild o no symptoms in the same infected plant, and most cultivars tested gave clearer positives in July than in August or September probably due to phloem degeneration. The comparative study DAS and DIP ELISA gave quite good results (about 90% coincidence sample to sample); when there is no coincidence, usually there are more positive samples using DIP than using DAS ELISA. With DIP, there are less doubtful results and less false positives providing enough imprints (2-4) better from two leaves are made for any plant. The technique has been used in several crops as a cheap assay for testing thousands of tubers, nursery plants or seedlings (Potato viruses, CTV-citrus, TSWV-tomato). It is also very useful for occasional tests in fields, when a single or very few samples are to be analyzed and when the results are needed as soon as possible.

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WILD HOST PLANTS OF STOLBUR PHYTOPLASMA AND ITS VECTOR, *HYALESTHES OBSOLETUS*, AT SITES OF GRAPEVINE BOIS NOIR OCCURRENCE IN EMILIA-ROMAGNA, ITALY

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Introduction

Stolbur phytoplasma (16SrXII-A) is a serious pathogen affecting a range of agronomic crops. On grapevine (*Vitis vinifera* L.) it causes bois noir (BN), an important disease which occurs with high incidence levels in most European viticultural regions. *Hylesthes obsoletus* Sign. (Homoptera, Cixiidae) has been identified as the main natural vector of stolbur (Fos *et al*., 1992); subsequently, the role of this planthopper and some alternate non-crop plant hosts of the phytoplasma in the spread of BN was demonstrated (Maixner *et al*., 1995; Sforza *et al*., 1998).

Research into the epidemiology of grapevine BN has recently been started in viticultural areas of Emilia-Romagna (northern Italy). Field surveys demonstrated a wide distribution of *H. obsoletus*; winged adults were present from the end of June to the end of August, planthopper populations peaked around the middle of July, up to 40% of captured adults and immature stages tested positive for stolbur by PCR and some naturally phytoplasma-infected weed species were found in vineyards with the disease (Credi *et al*., 2002). As in the case of other insect-transmitted plant pathogens, wild plants likely contribute to the spread of stolbur by serving as both a reservoir for phytoplasma infection and as reproductive hosts for the vector. Elimination of these plants may be an important part of an integrated control strategy for BN disease in vineyards. However, these aspects are still not well understood in our ecological conditions. This study was conducted to identify the putative species which could play such a role in grapevine BN epidemiology, through field collections of common weeds and shrubs.

Materials and Methods

Wild plant species, with or without symptoms, were collected randomly in different vineyards located in the Reggio-Emilia, Modena and Ravenna districts of the Emilia-Romagna region. These are important grapevine-growing locations with a known BN history and where high disease incidences have been recorded over the last few years. A systematic botanical inventory was established for all sites; sampling was done from June through September of 2003 and 2004. Total DNA was extracted from leaf veins and petioles. Samples were then screened using the universal primer pair P1/P7 in direct PCR, and the primer pair fStol/rStol to amplify in nested PCR a specific target sequence from the stolbur phytoplasma (Maixner *et al.,* 1995). Plant species were also surveyed from April to May 2005 to obtain information on the biology of *H. obsoletus*. Roots were examined for instar larvae (nymphs) and their presence was considered indicative that the plant would potentially support reproduction of the insect vector.

Results and Discussion

During the surveys, a total of 162 non-crop native plant samples, comprising 30 different species, were collected within and/or in the surroundings of vineyards where a high incidence of BN has been recorded. When PCR tested individually for stolbur, 78 (48.1%) samples of 20 species representing 15 families assayed positive. These included (numbers in parentheses represent number of plants infected/total tested): Amaranthaceae: *Amaranthus retroflexus* L. (3/4), Caryophyllaceae: *Silene alba* (Miller) Krause (2/4), Chenopodiaceae: *Chenopodium album* L. (3/6), Compositae: *Artemisia vulgaris* L. (2/5), *Cirsium arvense* (L) Scop. (5/7), *Picris echioides* L. (1/4), *Sonchus oleraceus* L. (2/2), *Taraxacum officinale* Wigg. (2/5), Convolvulaceae: *Calystegia sepium* (L.) R. Br. (2/2), *Convolvulus arvensis* L. (11/13), Labiateae: *Mentha arvensis* L. (2/7), Leguminosae: *Medicago sativa* L. (2/6), Malvaceae: *Malva sylvestris* L. (1/2), Plantaginaceae: *Plantago lanceolata* L. (1/6), Poaceae: *Setaria viridis* (L.) Beauv. (2/4), Rosaceae: *Potentilla reptans* L. (3/3), Salicaceae: *Salix alba* L. (3/5), Solanaceae: *Datura stramonium* L. (2/2), Ulmaceae: *Ulmus campestris* L. (5/6), and Urticaceae: *Urtica dioica* L. (24/41). Most of these species appear to be new recordings as hosts for the stolbur phytoplasma. Infected weeds included 5 annual, 1 biennial and 12 perennial species. The phytoplasma was also detected on 2 woody plant species: *S. alba* (white willow) and *U. campestris* (English elm). In general, symptoms on the tested plants consisted of

stunting, resetting, chlorosis, leaf malformation, little leaf, leaf yellowing, reddening and necrosis; some species were however symptomless, including *A*. *retroflexus* (redroot pigweed), *C. album* (lambsquarter) and *U. dioica* (stinging nettle). Among the locations sampled, stolbur was commonly found in *C*. *arvensis* (bindweed) and in *U*. *dioica*. The two plants are perennial in growth habit and frequently found throughout the survey locations, although their populations vary in abundance spatially. Populations of *C*. *arvensis* are usually very abundant both in and beside the vineyards; populations of *U*. *dioica* are instead more prevalent in the field borders, where plants may remain undisturbed for long periods.

Among the stolbur susceptible plant species, when evaluated in the field, only *U. dioica* and *C*. *arvensis* proved to harbour immature *H. obsoletus*, but were not equal in their ability to support reproducing populations of the insect. The mean proportion of its nymphs on the plant roots varied considerably, indicating a different epidemiological importance of the two weeds. High levels of *H. obsoletus* larval instars were found on *U. dioica*: 16 sites with nymphs over 18 sites surveyed, 80 plants showing nymphs over 103 examined, with a mean of 51 nymphs per plant. On the contrary, *C*. *arvensis* appeared to support a very low level of the planthopper nymphs: 1 site positive over 14 sites surveyed, only 1 plant with 2 immature insects over 228 observed. At a site in the Reggio-Emilia district, *Humulus lupulus* L. (common hop) was also found to be colonized in the roots by the insect nymphs (7 plants of 8 checked, mean of 4 nymphs per plant), but all the plants tested so far were not positive for stolbur.

The nested-PCR technique was used to identify stolbur reservoir hosts in some major grapevine-growing areas of Emilia-Romagna. Stolbur is a phytoplasma having a wide host range (Garnier 2000; Maixner *et al*., 1995; Sforza *et al*., 1998; Credi *et al*., 2002). Here we report additional wild plant hosts commonly found within and outside vineyards with BN infection. Including these, the phytoplasma now occurs in about 45 species. An understanding of stolbur-vector relationships with specific plant species is important in determining the main reservoir hosts. In the epidemiology of grapevine BN disease, *C*. *arvensis* has been shown to be a major reservoir for stolbur and *H. obsoletus* in Germany and France (Maixner *et al.,* 1995; Sforza *et al*., 1998). Although a high percentage of stolbur-infected *C*. *arvensis* plants were identified in our study, in effect the species was not associated with immature stages of the vector. Of the several plants inspected, only one plant was found harbouring just two nymphs on the roots. On the contrary, *U. dioica* was shown to be the major reservoir for stolbur and the planthopper vector. Hence, plant species that are susceptible to stolbur infection but are poor hosts for reproduction by *H. obsoletus* are less likely to be important epidemiologically than are stolbur-infected plants that support high levels of the planthopper reproduction. In conclusion, results from our surveys demonstrate that only the perennial *U. dioica* is host for both stolbur phytoplasma and its insect vector, confirming the results of Alma *et al*., (2002) in Piedmont. However, the real impact of this weed and other wild species in BN epidemics needs to be elucidated with further investigations.

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MECHANICAL TRANSMISSION AND IDENTIFICATION OF THREE VITIVIRUSES ASSOCIATED WITH GRAPEVINE RUGOSE WOOD

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Introduction

Grapevine rugose wood (RW) is a complex disease of viral nature with a worldwide distribution. It is transmissible by grafting and is biologically detected using proper selective indicators (Martelli, 1993). The causative agents of RW symptoms are attributed to members of the *Vitivirus* genus (Martelli *et al*., 1997). In particular, kober stem grooving (KSG) is closely associated with *Grapevine virus A* (GVA) and corky bark (CB) with the *Grapevine virus B* (GVB) infection (Garau *et al*., 1994; Bonavia *et al*., 1996). The association of *Grapevine virus D* (GVD) with wood abnormalities has also been reported (Abou-Ghanem *et al*., 1997), but its precise role on the expression of any of the different RW syndromes, including LN 33 stem grooving (LNSG), has not yet been conclusively identified (Boscia *et al*., 2001).

During a sanitary clonal selection program, several candidate clones of *Vitis vinifera* L. cultivars were found to be carriers of pathogenic agents inducing pitting and/or grooving of the woody cylinder. On the basis of the availability of these infected materials and the current knowledge on RW in grapevine, studies were set up to characterize the wood disorders (Credi, 1997). The experiments reported here were conducted to determine if possible disease-associated viruses could be transmitted from affected grapevines to herbaceous hosts. This communication reports on the mechanical sap transmission of GVA, GVB and GVD to *Nicotiana occidentalis* (Wheeler).

Materials and Methods

Field growing RW-affected grapevines of some *Vitis vinifera* L. cultivars were used in this study. These are part of a foundation vineyard of several clone selections which over time have been subjected to standard indexing procedures for virus and virus-like diseases (Credi, 1997). Separate samples of veins and petioles of old basal leaves and/or dormant cane bark scrapings were collected, ground with a pestle and mortar in liquid nitrogen then the inocula rub-inoculated onto a range of herbaceous indicators. Leaf-dip preparations from virus-infected plants and particle decoration tests (ISEM), carried out using polyclonal antisera (kindly supplied by Istituto di Virologia Vegetale del CNR, Torino and Bari, Italy) to GVA, GVB and GVD, were negatively stained with 2% aqueous uranyl acetate and the grids examined in a Philips CM10 electron microscope (Milne, 1993). Grapevine inoculum sources and infected herbaceous hosts were tested by enzyme-linked immunosorbent assay (ELISA) for grapevine viruses using commercial kits (Agritest, Valenzano, Italy). In addition they were analysed by reverse transcription-polymerase chain reaction (RT-PCR) with specific primers for GVA (GVA6591F/6862R), GVB (C410/H28) and GVD (GD1/GD2) synthesized according to published sequences (Goszczynski and Jooste, 2003; Minafra and Hadidi, 1994; Abou-Ghanem *et al*., 1997), and with other primers developed in our laboratory. Total RNA extraction was performed, with a few modifications, according to MacKenzie *et al*. (1997) and standard procedures were employed for reverse transcription and PCR amplification. PCR amplified products were analyzed by electrophoresis in 1.2% agarose gels and visualized by ethidium bromide staining.

Results and Discussion

Ten days after inoculation, necrotic lesions were observed on some of the *N. occidentalis* seedlings inoculated with infected grapevine tissues ground in 0.01 M sodium phosphate buffer pH 7.0 containing 3% nicotine plus 0.01 M cysteine HCl (Boscia *et al*., 1993). Local symptoms were followed by systemic chlorosis of the smaller veins, yellowing of the internerveinal tissues, leaf deformation, rolling and extended necrosis. The original virus isolates were then transmitted to *N. benthamiana* (Domin.), except the isolate designated AR obtained from cv. Albarossa. Inoculated *N. benthamiana* plants reacted with chlorotic/necrotic local lesions, systemic vein clearing and leaf curling symptoms. In leaf-dip preparations the virus particles were approximately 800 nm in length, 12 nm in width with striations 3.5 nm in pitch, fitting the size range of the members of the genus *Vitivirus* (Martelli *et al*., 1997). Infection with GVA (isolate designated LGR 7, transmitted from a cv. Lambrusco Grasparossa clone), GVB (isolate AR, transmitted from a cv. Albarossa clone) and GVD (isolates LGR 4A, LGR 4B, LGR 5, LGR 7 and RSS 6; transmitted from clones of the cvs. Lambrusco Grasparossa and Rossiola) were first confirmed with ISEM tests and ELISA.

In infected plants of *N. occidentalis*, *N. benthamiana* and the original grapevine inoculum sources, the detection and identification of the viruses were definitively obtained by RT-PCR. Fragments of the expected size of 271 bp (for GVA), 450 bp (for GVB) and 700 bp (for GVD) were amplified (Goszczynski and Jooste, 2003; Minafra and Hadidi, 1994; Abou-Ghanem *et al*., 1997). Among the oligonucleotides designed in our laboratory, two primer pairs, GVBCPF1/CPR3 (forward: 5'-CCC ATG TAC TTC AGG ACC C-3', reverse: 5'-CTA CCA CAA CAG TAA CAA CG-3') and GVDORF2F1/ORF2R1 (forward: 5'-TAA TAG GGC CTA AGT C-3', reverse: 5'-GGG CGT TGA ATA CAC CTT TAG C-3'), were chosen for a reliable detection of GVB and GVD. RT-PCR assay consistently produced strong products with the expected molecular weights of 609 bp and 371 bp respectively. The results obtained indicate that the use of these new primers greatly improves the diagnosis of these viruses in grapevine. To characterize further the virus isolates, amplicons generated with GVA6591F/6862R, GVBCPF1/CPR3 and GVDORF2F1/ORF2R1 primer pairs were cloned, but the clones are still in the process of sequencing.

Mechanical transmissions of GVA, GVB and GVD directly from tissue sap of RW-affected grapevines to herbaceous hosts were successfully achieved, confirming the few cases so far reported (Martelli *et al*., 1997). The viruses could be clearly differentiated from one another serologically and molecularly. The transmission of GVD, five isolates obtained from different clones of two local (Emilia-Romagna region) *V. vinifera* cultivars, appears to be the first occurrence of this virus in northern Italy. Our findings will facilitate future studies on its etiological role.

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INFLUENCE OF VIRUSES ON THE PERFORMANCE AND QUALITY OF CV. CRIMSON SEEDLESS

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Introduction

To widen the offer for an increasingly demanding market, a number of new table grape varieties, among which the cv. Crimson Seedless, have been introduced in the last years in Apulia, the leading table grape growing region in Italy, and in Metropolitana IV, V and VI Chile regions. In many areas young vines of Crimson developed a disease condition that caused much concern to the growers. Affected vines delayed bud break by one-two weeks and exhibited extensive bud failure. The vegetation was less vigorous than normal and crop visibly reduced. Leaves displayed a light early reddening and rolling of the blades and bunches were pale-coloured, failing to ripen properly.

The severity of the symptoms suggested to investigate the disorder in more detail to ascertain its nature and the agents involved.

Materials and methods

In autumn 2004 some Crimson vineyards were selected in Apulia and Chilean regions and vines with and without symptoms chosen. Differences in productivity and berry quality between symptomatic and symptomless vines were determined and the viruses present in both types of plants were identified by ELISA.

Tests were made on cortical scrapings extracts by DAS-ELISA (GFLV, GLRaV-1, GLRaV-2 and GLRaV-3), DASI-ELISA (GFkV), and protein A-DAS ELISA (GVA) (Boscia *et al.,* 1996). Polyclonal antisera and monoclonal antibodies raised at University of Bari and from Agritest, Valenzano-Bari (Italy), were used as reagents.

Results and discussion

As shown in Tab 1, the presence of symptoms was associated with a remarkable reduction of the yield (from 20 to 44%, in two years of observation) mainly due to the reduced number of fertile buds, thus of bunches per vine. Other significant detrimental effects were the lower sugar content (from 13 to 26%) and the irregular and scarce coloration of the berries (Tab. 2). Because of the pale colouring many of the bunches were unmarketable, the economic loss for the grower was much higher than that caused by the simple reduction in the weight and number of clusters.

Table 1. Influence of sanitary status on vegetative characteristics and bud fruitfulness of Crimson Seedless grapevines in Italian vineyards.

*	Year	Bud load/ vine (n)	Shoots/vine (n)	Clusters/vine (n.)	Clusters per vine/ Buds per vine	Clusters per vine/ Shoots per vine	Yield/ vine (kg)	Yield/hectare (ton)
S	2004	84.67 aA	39.53 aA	25.07 bB	0.29 bB	0.62 bB	11.48 bB	18.37 bB
H		86.93 Aa	44.87 aA	37.67 aA	0.47 aA	0.86 aA	20.37 aA	32.59 aA
S	2005	46.87 aA	27.07 aA	14.93 aA	0.32 aA	0.52 aA	6.54 aA	10.46 aA
H		51.6 aA	29.4 aA	17.33 aA	0.35 aA	0.62 aA	8.18 aA	13.09 aA

* S = symptomatic; H = symptomless;
Values followed by different letters differ at $P \leq 0,05$ (small letters) e per $P \leq 0,01$ (capital letters)

Table 2. Influence of sanitary status on the cluster and colour characteristics on Crimson Seedless at harvest.

*	Year	Vineyard	Cluster Weight (g)	Berries/ Cluster (n.)	Berry weight (g)	Total soluble Solids (°Brix)	Brightness** L
S	2004	1	457,97 bA	100,93 bA	4,53 aA	12,70 bB	38,49 aA
H	2004	1	540,80 aA	115,77 aA	4,70 aA	17,23 aA	31,17 bB
S	2004	2	604, 8 aA	144,90 aA	4,20 bB	14,81 bB	40,14 aA
H	2004	2	573,17 aA	114,14 bB	4,96 aA	20,10 aA	30,15 bB
S	2005	1	437,72 aA	134,50 aA	3,22 bA	14,13 bB	29,80 aA
H	2005	1	472,03 aA	118,65 aA	3,92 aA	16,20 aA	20,68 bB

* S = symptomatic; H = symptomless; **this parameter is strictly correlated with the colour and to the anthocians content. Values followed by different letters differ at $P \leq 0,05$ (small letters) e per $P \leq 0,01$ (capital letters).

All six viruses tested were detected, to a different extent, in the totality of the 166 vines examined (Table 3). Symptomatic vines had an higher infection rate (94%) than symptomless vines (69%). The surprisingly high infection rate of symptomless vines was mainly due to GLRaV-2 (63%), which was detected in all the Italian Crimson vines tested, and to GFkV (24%). In symptomatic vines the most common viruses were: GLRaV-2 (80%), GFkV (72%), GLRaV-3 (67%), GVA (64%) and GLRaV-1 (35%). GFLV was detected only in 4 vines in Chile, therefore its presence was considered only occasional and unrelated with the disease. GLRaV-1, GLRaV-3 and GVA were only sporadic or completely absent in symptomless vines.

Table 3. Results of ELISA tests on Chilean (Ch) and Italian (It) vines of cv. Crimson.

	*	Total vines (n.)	Infected vines (%)	GVA (%)	GLRaV-1 (%)	GLRaV-2 (%)	GLRaV-3 (%)	GFkV (%)	GFLV (%)
Ch	S	43	86	35	60	56	37	42	9
	H	27	19	15	-	-	-	4	-
It	S	51	100	88	14	100	92	98	-
It	H	45	100	7	-	100	9	36	-
Tot	S	94	94	64	35	80	67	72	4
Tot	H	72	69	10	-	63	6	24	-

* S = symptomatic; H = symptomless

A comparative analysis of virus incidence in diseased and symptomless vines (Tab. 3) suggests that: (i) the syndrome under study has a clear-cut viral origin since, in general, symptomatic vines in both countries had the worst sanitary condition. In Chile 86% of symptomatic vines were infected, versus 19% of the symptomless ones, and in Italy, if GLRaV-2 is excluded because of its presence in all vines tested, 100% of symptomatic vines were infected, versus 40% of the symptomless ones; (ii) excepted for GLRaV-2, whose presence was consistent in both symptomatic and symptomless vines (in particular in Italian vineyards), the other viruses looked for, i.e. GLRaV-1, GLRaV-3, GVA and GFkV, were detected at a significant higher rate in symptomatic than in symptomless vines; (iii) rather than by the infection by single specific viruses, as the widespread presence of GLRaV-2 also in symptomless vines seems to indicate, the diseased status seems to be determined and enhanced by mixed infections by two or more viruses; (iv) viruses of the *Closteroviridae* family seem to have a major role in eliciting leafroll and its detrimental effects, as suggested by their highly frequent detection in most of the Italian and Chilean symptomatic vines; (v) GVA and GFkV, which were especially found in symptomatic Italian vines, seem to influence the severity of the symptoms.

The origin of such a heavy presence of filamentous viruses is unknown, but it is likely that it came from budwood and/or rootstocks rather than from mealybug vectors. If so, the present case represent a further example of the devastating consequences of the introduction of new varieties in an area without previous evaluation of their susceptibility to the prevailing viruses, and of the use of sanitary uncontrolled propagation material.

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A PRELIMINARY SURVEY OF GRAPEVINE VIRUSES IN SYRIA

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Introduction

With a surface of 54,300 Ha (FAO Statistics, 2004), grapevine ranks second among the fruit crops of Syria, after olive. Vineyards are distributed all over the country, with an higher concentration in the south and in the center. Table grape varieties are by far the most widely grown, with the local varieties Hellwany, Balady, Betamony, Zainy and Shamy prevailing by and large. Among wine grapes, cvs Ashory and Salty are the most important and are mainly cultivated in the south.

The sanitary status of Syrian viticulture is little known. Records exist for *Grapevine leafroll associated virus-1* (GLRaV-1), *Grapevine leafroll associated virus-3* (GLRaV-3), and *Grapevine fanleaf virus* (GFLV), that were found in about 31% of the accessions tested (Al Chaabi *et al.*, 2000) and to *Grapevine fleck virus* (GFkV) and *Grapevine virus A* (GVA) that were detected in vines showing graft-incompatibility disorders (Dawood *et al.,* 1991). Given the scarcity of information available an investigation was initiated, the preliminary results of which are hereafter reported.

Materials and Methods

Surveys were conducted in October 2004 and May 2005. Mature canes were collected at random from 836 individual vines (736 *Vitis vinifera* and 100 rootstocks) in 58 commercial vineyards and 4 nurseries distributed in seven different regions (Aleppo, Dara, As Suwayda, Al Qunaytirah, Homs, Hamah, Tartous). All samples were brought to Bari to be analysed. To this aim, leaf extracts of forced cuttings were mechanically inoculated to a standard series of herbaceous hosts from 225 samples of native varieties chosen at random.

The presence of virus-like diseases was checked by indexing a certain number of vines onto appropriate indicators. Grafting onto 110R was specifically done to verify the reported correlation between vein necrosis disease and *Grapevine rupestris stem pitting-associated virus* GRSPaV (Bouyahia *et al*., 2005)

ELISA was used for identification of the following viruses: GFLV*, Arabis mosaic virus* (ArMV), *Grapevine fleck virus* (GFkV), GVA, *Grapevine virus B* (GVB), GLRaV-1, *Grapevine leafroll-associated virus 2* (GLRaV-2) and GLRaV-3. Tests were made using cortical scraping extracts by DAS-ELISA (GFLV, ArMV, GLRaV-1, GLRaV-2, and GLRaV-3), DASI-ELISA (GFkV), direct binding-ELISA (GVB) and protein A-DAS ELISA (GVA) (Boscia *et al.*, 1997). Polyclonal antisera and monoclonal antibodies, raised at the University of Bari or from Agritest, Valenzano-Bari (Italy), were used as reagents.

RT-PCR was used for ELISA-negative samples. GRSPaV was searched for in 135 vines with specific primers (A. Rowhani, personal communication) whereas degenerate primers (Tian *et al*., 1996) were used for ampelovirus detection species in 72 vines.

Results and Discussion

Symptoms of leafroll and rugose wood were more frequent than fanleaf (yellow mosaic form, in particular).

Viruses recovered by mechanical transmission were GFLV, ArMV, and GVA. Vein necrosis developed in *c.* 53% of graft-inoculated 110R indicators and vein mosaic in *Vitis riparia* inoculated with cv. Corna Alegra.

Serological assays were more informative (Tab. 1). A total of 71% of the ELISA-tested *V. vinifera* plants (520 out of 736) were infected by one (14.8%) or more (55.8%) viruses. GVA was the most frequent (54,7%), followed by GLRaV-1 (47.3%), GFkV (29.7%) and GLRaV-3 (23.9%). The other viruses were only scarcely represented, i.e. GLRaV-2 (9.0%), GFLV (0.8 %) and ArMV (0.1%), or not present (GVB).

The most important Syrian grapevine varieties, i.e. Hellwany, Salty, Balady, Aswad Kary, and Zeiny, had average infection rates that ranged between 90.6% and 42.2%.

The highest incidence of infections was observed in the south (As Suwayda, 78%); it was around 75% at Aleppo in the north and Tartous on the coast. Rootstocks were in much better sanitary conditions (25% infection). GFkV (22.0%) was the prevalent virus whilst negligible was the presence of GLRaV-3 (3.0%), GLRaV-1 and GFLV (1.0%).

GRSPaV was detected in 72.3% of the samples by RT-PCR. Comparative analysis of PCR results with those of indexing onto 110R indicator plants confirmed the strict correlation of GRSPaV with vein necrosis (Bouyahia *et al*., 2005) because symptoms were shown by 80% of the GRSPaV-positive vines and by none of the GRSPaV-negative.

The amplification from numerous samples (20 of 72) of genomic fragments when degenerate primers for closteroviruses were used suggests that other such viruses, whose identification remains to be ascertained, may infect Syrian grapevines.

Given the very high infection level in several local varieties, the implementation of a sanitation programme seems highly desirable to improve the sanitary status of Syrian viticulture.

Table 1. Incidence of eight different viruses in Syrian grapevine varieties and rootstocks

Virus	*V. vinifera* cvs. (736 samples)		Rootstocks (100 samples)	
	Infected samples	%	Infected samples	%
GVA	420	54.7	0	0
GVB	0	0	0	0
GLRaV-1	348	47.3	1	1.0
GLRaV-2	66	9.0	0	0
GLRaV-3	176	23.9	3	3.0
GFLV	6	0.8	1	1.0
ArMV	1	0.1	0	0
GFkV	218	29.7	22	22.0
Total	520	70.7	25	25.0

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GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 TRANSMISSION EFFICIENCY OF *PLANOCOCCUS FICUS* AND *PSEUDOCOCCUS LONGISPINUS* (HEMIPTERA: PSEUDOCCIDAE)

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The vectors of grapevine leafroll-associated virus-3 (GLRaV-3) are mealybugs (Pseudococcidae) and scale insects (Coccidae) (1). The pseudococcid *Planococcus ficus* (Signoret) is considered the most important vector of GLRaV-3 in South Africa (2). However, another pseudococcid grapevine pest, the longtailed mealybug *Pseudococcus longispinus* (Targioni Tozzetti), has also been shown to transmit GLRaV-3 (3). *Planococcus ficus* is the most abundant mealybug in vineyards in the Western Cape region of South Africa. Studies on the life-cycle of the two species have shown *P. ficus* to be better adapted to survival on grapevine than *P. longispinus*, an observation that might in part explain why the former generally maintains higher population levels in South African vineyards. This phenomenon also suggests that *P. ficus* is a more efficient vector of GLRaV-3 than *P. longispinus*. However, no study has thus far determined the efficiency of GLRaV-3 transmission by these species.

The objective of the study was to examine the relationship between the number of first- to second-instar nymphs per plant of both *P. ficus* and *P. longispinus* and the infection rate of healthy grapevine plants in order to determine transmission efficiency.

First- to second-instar nymphs of *P. ficus* and *P. longispinus* reared on *Cucurbita moschata* Duchesne (Cucurbitaceae) and *Alocasia macrorrhizos* L. (Araceae), respectively, were used. Mealybugs were allowed acquisition access periods (AAPs) on virus source plants (LN33) for 5 days and then groups of 1, 5, 10, 20 and 40 nymphs of each mealybug species were transferred to separate indicator plants (Cabernet franc). Ten plants per species and group were used. Mealybugs and plants were tested for GLRaV-3 using RT-nested-spot-PCR (4). After completion of the transmission experiments sub-samples of each mealybug species were tested for GLRaV-3 and the indicator plants were tested from 8 weeks after transmission for GLRaV-3.

All controls performed as expected; mealybugs from the non-virus hosts and LN33 tested negative and positive for GLRaV-3, respectively; all Cabernet franc plants repeatedly tested negative before the experiments and the LN33 plants tested positive for GLRaV-3. Infestation of *P. ficus* and *P. longispinus* nymphs was similar between groups within and between species, except for the '40-nymph group' of *P. longispinus*, which had a higher number of infested nymphs (Fig. 1). Likewise, the number of plants infested with GLRaV-3 was similar for *P. ficus* (38-78%) and *P. longispinus* (20-71%), except for *P. ficus* for the '10-nymph group' per plant where all plants tested negative (Fig.2).

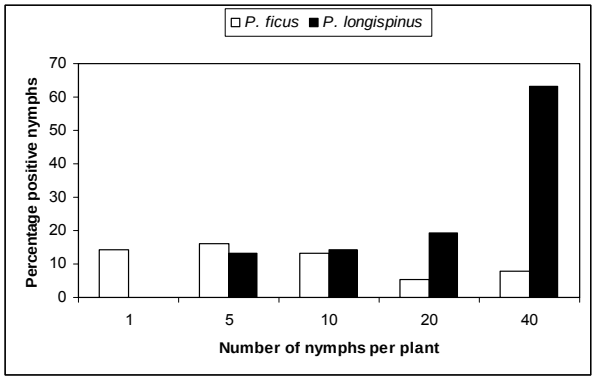

Fig. 1. Percentage of GLRaV-3 positive *P. ficus* and *P. longispinus* nymphs after transmission experiments.

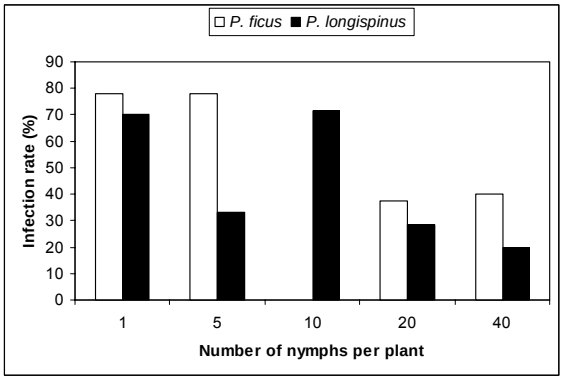

Fig. 2. Infection rate of healthy plants by different numbers of *P. ficus* and *P. longispinus* nymphs.

The present study has shown that *P. longispinus* is as efficient a vector as *P. ficus* and that a single individual of either *P. ficus* or *P. longispinus* is capable of transmitting GLRaV-3, thus highlighting the importance of sanitary measures in vineyards to reduce virus spread.

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STATUS OF GRAPEVINE YELLOWS IN SERBIA

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Introduction

Grapevine yellows are grapevine diseases associated with phytoplasmas. Until now phytoplasmas from 7 different ribosomal groups were found on grapevine in the world (Varga *et al.,* 2000; Boudon-Padieu, 2003; Gajardo *et al.*, 2003).

In Serbia, after the first detection of '*Candidatus* Phytoplasma vitis' (Flavescence dorée phytoplasma, FD) (IRPCM, 2004) on grapevine in 2003 (Duduk *et al*., 2003), two other phytoplasmas, '*Candidatus* Phytoplasma solani' and '*Candidatus* Phytoplasma prunorum' (IRPCM) were found in 2004 (Duduk *et al*., 2004). The aim of this work was to investigate the presence of FD and of other phytoplasmas associated with grapevine yellows in the most important grapevine growing regions of Serbia.

Materials and Methods

More then 150 samples of symptomatic grapevines were collected from 2002 to 2005 in 15 different localities from the main grapevine growing regions of Serbia. Total nucleic acids were extracted from leaf midribs and bark tissues following the protocol described by Prince *et al*.(1993).

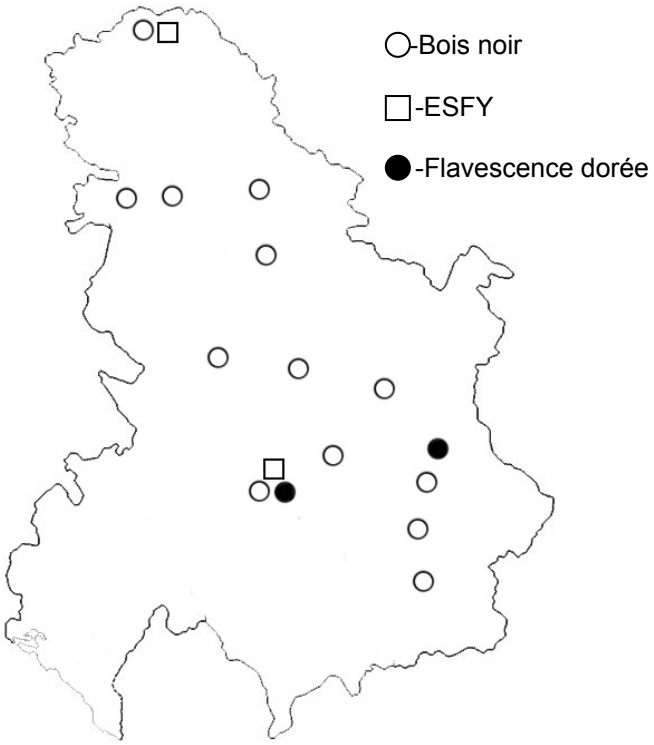
Detection of phytoplasmas was done using nested PCR with phytoplasma universal primer pairs P1/P7 in direct PCR, followed by F1/B6 in nested PCR (Duduk *et al*., 2004). Negative samples were then subjected to a second nested PCR with $16R_{738f}$/B6 (Martini *et al*., 1999) phytoplasma universal primer pair. Selected samples were subjected to another second nested PCR using R16(X)F1/R1 (Lee *et al*., 1995) primer pair specific for phytoplasmas belonging to apple proliferation 16SrX group. Each 25 µl PCR reaction mix contained 2.5 µl 10X PCR buffer, 0.8 U of *Taq* polymerase (Polymed, Italy, EU), 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 with *Tru*I, *Taq*I, *Rsa* and *Ssp*I restriction enzymes on amplified DNA fragments. RFLP products were analyzed in 5% polyacrylamide gel, stained with ethidium bromide and visualized under UV transilluminator.

Results and Discussion

As described in Fig. 1 FD was found in 2 localities: Aleksandrovac, and Vrelo-Jasenovik; all detected '*Ca*. P. vitis' were molecularly identical, and belonged to 16SrV-C ribosomal subgroup. '*Ca*. P. solani' was found in all surveyed localities except Vrelo-Jasenovik, and no molecular variability was found either on 16S or on tuf genes. '*Ca*. P. prunorum' was found in two samples, both times in mixed infection; one sample was from Palić where this phytoplasma was detected in mixed infection with BN, and second sample was from Aleksandrovac where it was detected in mixed infection with FD. FD appears not spread from the localities where it was already reported (Duduk *et al*., 2004, Kuzmanovic *et al*., 2004), while BN is associated with yellows in all the other localities surveyed (Table 1).

In several cases the presence of symptomatic plants was just scattered, while in some localities such as Radmilovac, the intensity of BN disease appears to be more severe and spreading over the years. The role of '*Ca*. P. prunorum' is still not clear since its presence was detected in erratic way and only on two samples but it can be correlate with the presence of European plum cultivations in many viticultural areas of Serbia. Beside the *Scaphoideus titanus* presence in many of the grape growing regions of Serbia, the presence of other known and/or potential phytoplasma vectors is under investigation.

Fig 1. Map of distribution of phytoplasmas in grapevine in Serbia



Locality	Phytoplasma detected
Palić	BN, BN+ESFY
Sremski Karlovci	BN
Erdevik	BN
Vršac	BN
Radmilovac	BN
Kragujevac	BN
Topola	BN
Koceljeva	BN
Aleksandrovac	FD, BN, FD+ESFY
Kruševac	BN
Paraćin	BN
Niš	BN
Vrelo-Jasenovik	FD
Leskovac	BN
Vranje	BN

Table 1. List of localities surveyed for phytoplasma presence

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DETECTION OF A PHYTOPLASMA BELONGING TO GROUP I IN DECLINING SYRAH

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Introduction

Syrah is one of the most important grape varieties cultivated in southern French vineyards. Since the 1990s, specific symptoms were described on this variety. This disorder, so-called "Syrah decline", is characterized by grooving and cracking at the graft union and leaf-reddening during autumn. Several studies have been initiated on physiological and pathological aspects (5,6). This set of experiments did not allow to identify the Syrah decline origin so far. However, some of the previously described symptoms (leaf-reddening and problems of wood maturation) appear similar to some of the physiological disorders classically observed in grapevine yellows. Consequently, we have decided to research a potential phytoplasma in Syrah declining plants.

Materials and Methods

Grapevine samples were collected in 2003 from a vineyard of Syrah located in Languedoc-Roussillon. Numerous samples, made of petioles/veins and flowers/clusters, were taken away on each plant in order to increase the possibility of detecting this pathogen agent. They were collected in May, July and October: 1g of each sample was frozen until analysis. They were taken away from plants showing different degrees of Syrah decline: vines without any symptom; vines showing only swelling and cracking at the graft union without leaf reddening; and vines expressing both cracking at the graft union and foliar reddening

DNA of the various samples was extracted using the phytoplasma enrichment procedure described by Daire *et al.* (1) with some minor modifications. A nested PCR procedure was used with combination of two phytoplasma-universal primer pairs P1/P7 followed by U5/U3 (2, 3, 4). In order to identify and determine the phytoplasma group, some PCR products were submitted to sequencing. The sequences obtained were analysed with the Blast software available on NCBI (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/blast/). They were aligned to other 16SrRNA sequences available in the databank and pairwise comparisons were realised to calculate percentage sequence similarities. Three of them, chosen to be representative of these sequences, were used to establish a phylogenetic tree.

Results and Discussion

32 of the 130 samples collected from Syrah plants from May to October 2003 showed one band at the expected size of 880 bp after the nested PCR. Positive samples were found in both symptomatic and symptomless plants in the following proportions:

- 19% (8/42) of the samples taken from vines without any symptom
- 21% (9/42) of the samples extracted from "cracking vines"
- 33% (15/46) of the samples taken away from "cracking and reddening vines"

Positive samples were detected at the three sampling dates but in an increasing proportion since May to October.

Nine of the PCR products obtained, chosen to be representative of all of the different samples (tissue, sampling period, degree of symptoms) were sequenced. They were then analysed by comparison with other sequences available on NCBI. Very strong similarities (more than 98%) were obtained with numerous 16SrRNA phytoplasma sequences, belonging to group I and group XII, identified in many different plants. These nine sequences show from 96 to 100% similarities to each other. Two sets can nevertheless be distinguished, the first one constituted of 4 sequences belongs to group I whereas the second one belongs to group XII. Thus, these analyses confirmed that the positives samples were infected with two distinct phytoplasmas: one belonging to 16SrI group (Aster yellow) and the other belonging to16SrXII-A group (Stolbur). No correlation could be established between the type of sample (tissue, sampling date and degree of symptoms) and the group belonging. It is worth noting that it is the first time the 16SrI group phytoplasma is reported on grapevine in France.

These experiments allowed us to identify phytoplasmas of two different groups in declining Syrah. However, there is still no obvious correlation between phytoplasma infection and Syrah decline; further investigations are in progress to be able to conclude.

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MONITORING THE SPREAD OF VIRUSES AFTER VINEYARD REPLANTING WITH HEAT-TREATED CLONES OF *VITIS VINIFERA* 'NEBBIOLO'

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Introduction

Planting the vineyard with virus-free plants (originally healthy or sanitated) is still the only available tool against the spread of viral infection. The cultivation of grapevine for decades or centuries in the same areas (due to the favorable climate and soil, and often to the lack of cultivation alternatives) lead to an established presence of grape parasites including viruses. When a vineyard is planted or re-planted with certified virus-free material but viruses are present – even sporadically – in the vineyards nearby, viral infection of the new plants can occur by means of the natural vectors. Mealybugs were shown to be able to transmit viruses associated to the grapevine leafroll disease (GLRaV-1 and 3) and rugose wood such as Grapevine Virus A (GVA); nematodes of the genus *Xiphinema* are vectors of *nepoviruses* such as the Grapevine FanLeaf Virus (GFLV) (Martelli, 2002). The possibility of an early reinfection can frustrate the efforts for producing healthy vines, reducing the advantages of their use. Therefore a better knowledge of the eventual natural spread of viruses in vineyards planted to healthy plants will be useful also from an economic point of view. The aim of this research is monitoring the field occurrence of natural viral reinfection in grapevine plants which were originally sanitated and certified as virus-free clonal material.

Materials and Methods

The study is being carried out in two vineyards located in the Langhe, an area of intensive viticulture in NorthWestern Italy. Both fields were previously planted to grape, and both were re-planted to clonal vines of the cultivar 'Nebbiolo' (*Vitis vinifera* L.). Vines are vertically trained and cane pruned (Guyot system); spacing is 2.7 m between rows and 1 m within rows.

The Neive vineyard was established in 1992 and is composed by 19 rows of 45 plants each. Five non-adjacent rows (5^{th}, 7^{th}, 11^{th}, 13^{th} and 17^{th}) were planted to infected vines (GVA + GLRaV-1 or 3), while all the other vines originated from heat-treated mother plants. The experimental field is surrounded by commercial vineyards having uncertain virological status. Viral analyses were performed in 2003 and 2004 on all the plants of the rows 4^{th}, 6^{th} and 12^{th}.

The Barbaresco vineyard was established in 1994. A nematocide fumigation was applied after the old vineyard pulling out and one year prior to planting because of the previous heavy presence of GFLV. Only clonal grapes, originated from heat-treated mother plants, were used for re-planting. Fanleaf disease symptoms are present in adjacent vineyards. Serological assays were performed since 1997; samples for viral tests were collected from 10 plants - scattered in the vineyard - out of 150. In this vineyard the nematode population was tracked from 1994 to 1999, in 2002 and 2005. Soil samples (500 cm^3) were collected during spring or autumn in 3 spots inside the fumigated vineyard and, as a control, in 3 spots in the bordering commercial vineyards.

Woody material, from mature canes collected during winter pruning, was tested by Double Antibody Sandwich-Enzyme Linked ImmunoSorbent Assays (DAS-ELISA). Commercial ELISA kits (Agritest, Valenzano, I) were used for detection of GFLV (Barbaresco) or GLRaV-1, GLRaV-3 and GVA (Neive). We conventionally assumed that a sample is positive in ELISA if its OD_{405} value is at least three times higher than the negative control value.

Results

Neive vineyard. ELISA assays were performed on 135 plants in winter 2003 and 134 in winter 2004. Ten vines resulted virus-infected in 2003 (7.4 %) and 15 in 2004 (11.2 %). The results of serological assays are reported in Table 1. Figure 1 shows the distribution of the infected plants in 2004: infected plants are usually gathered in groups along the row, sometimes close to the row ends.

Barbaresco vineyard. The ELISA performed in 1997, 1998, 2002 and 2003 always gave negative results for GFLV. The nematode analyses showed an increase of the nematode number in the soil samples over the years following soil fumigation (Table 2). The nematodes were classified as *Helicotylenchus* spp.,

Xiphinema pachtaicum, and saprophytes, the last being the most common ones. *X. index* was found only once.

Discussion

Grapevine leafroll and fanleaf diseases are widespread in main viticultural areas and this is mostly ascribed to the use of infected propagated material. Natural spread of these viral diseases in vineyards was reported (Kasdorf *et al*., 1990; Belli *et al*. 1993; Cabaleiro *et al*., 1997; Habili *et al*., 1997), but information on the frequency and characteristics of this spread is still scarce. We started a project aimed to monitoring the viral presence in two vineyards exposed to "standard" risks of reinfection.

After about ten years from vineyard planting, GVA, GLRaV-1 and 3 were detected in a site (Neive) in which rows of infected grapes were interplanted among rows of originally healthy plants. The low speed and the pattern of leafroll spread, which often involves a small group of plants close to the vineyard borders, can be indirect confirmation of the diffusion of the disease through slow-moving natural vectors. The size of the *foci* is probably related to the age of the vineyard and the mealybug population in the area (Cabaleiro *et al*., 2003).

No natural spread of the fanleaf disease was so far detected in a second vineyard (Barbaresco) which was subjected to soil fumigation before re-planting. A few nematodes were found in the soil even shortly after the treatment, and their number dramatically increased after some years. *X. index*, the main vector of GFLV, was detected only occasionally.

More detailed investigations are planned for next years, including monitoring the mealybug populations and viral molecular assays on the vines and on the possible vectors.

Year	Viral status of the vines				
	GLRaV-1 + GVA	GLRaV-3	GVA	GLRaV-3 + GVA	Healthy
2003	-	6	-	4	125
2004	1	8	1	5	119

Tab. 1 – Results of ELISA performed on plants growing in the Neive vineyard.

	1994	1995	1996	1997	1999	2002	2005
Inside	3.7	0.2	1	3.2	3.3	89	58.3
Outside	107	74	77.3	58.7	107.7	76.3	83.7

Tab. 2 – Total number of nematodes found in 500 cm^3 of soil collected in the Barbaresco fumigated vineyard or in the bordering commercial vineyards. Values are means of three samples collected during spring or autumn.

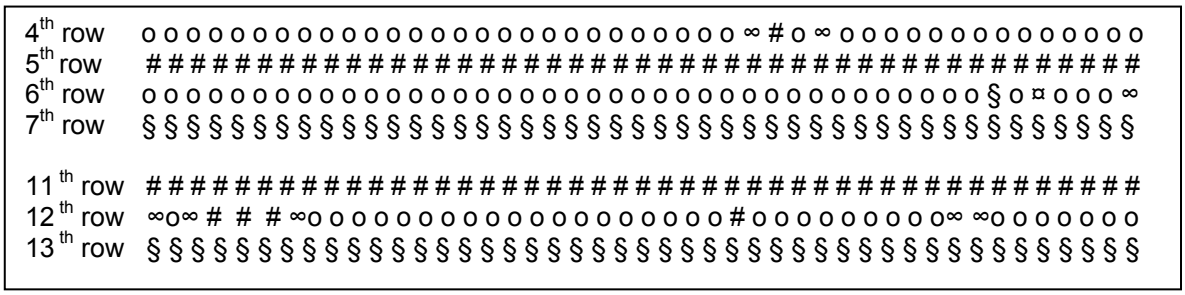

Fig. 1 – Results of serological assays performed on the plants at the Neive vineyard in 2004. Rows 5th, 7th, 11th and 13th were planted to infected vines; rows 4th, 6th, and 12th to healthy plants. #: GLRaV-3 + GVA; §: GLRaV-1 + GVA; ∞: GLRaV-3; ¤: GVA; o: healthy.

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PRODUCTION OF MONOCLONAL ANTIBODIES TO GRAPEVINE LEAFROLL ASSOCIATED VIRUS 7 (GLRaV-7)

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Grapevine leafroll associated virus 7 is a putative ampelovirus, a member of the Closteroviridae family (1). It was first found in Albanian grapevine accessions (2). Molecular and serological techniques have been used to detect the virus in grapevine. Polyclonal antibodies are commercially available from various sources. Here we describe the development of monoclonal antibodies and their evaluation in enzyme-linked immunosorbent assay (ELISA), immuno-precipitation electron microscopy (IPEM) and Western immunoblot analysis.

Material and methods

GLRaV-7 infected grapevine accession Y276, that was used for the immunization of mice, was kindly provided by Dr M. Fuchs from the collection at INRA at Colmar, France. Other *Vitis vinifera* were essentially from the collection of Agroscope RAC at Nyon. Viral nucleoprotein was purified from infected grapevine leaves as previously published (3). The production of antiserum to GLRaV-7 and hybridoma, purification and conjugation of immunoglobulins, double-antibody-sandwich ELISA (DAS-ELISA), triple-antibody-sandwich ELISA (TAS-ELISA), immuno-precipitation electron microscopy (IPEM) and electrophoresis and Western blot analysis were essentially done as described elsewhere (4; 5). Reference monoclonal antibodies were all from Agroscope RAC.

Results

Production and characterization of hybridoma cultures

Out of 960 micro-cultures, with an average of two growing colonies per well, 129 reacted dominantly to plant sap from V. vinifera Y276 infected by GLRaV-7. These virus-specific micro-cultures were expanded as 2 ml cultures, screened again by TAS-ELISA and frozen for later evaluation. Supernatants of 40 promising 2 ml cultures were analyzed by Western blots. Antibodies produced by culture 37 stained two dominant proteins of about 34 and 33 kDa specific to GLRaV-7 virions whereas those from culture 32 and 33 stained each a dominant protein of about 30 kDa (Table 1) corresponding to the coat protein of grapevine fleck virus (GFkV). The antibody activity of 8 hybridoma cultures was further assessed by IPEM (Table 1). Antibodes from hybridoma culture 6 and 37 heavily decorated the filamentous GLRaV-7 virions (Fig. 1).

Table 1. Activity of Mabs assayed by ELISA, Western immunoblot and IPEM

Hybridoma culture	ELISA	Western Immuno-blot	***IPEM of filamentous GLRaV-7 virions***	
			aggregated particles	decorated particles
3	+	-	-	-
5	+	-	+	-
6	+	-	+	+
7	+	-	+	-
26	+	-	-	-
32	+	+ (30 kDa)	-	-
33	+	+ (30 kDa)	-	-
37	+	+ (33/34 kDa)	-	+

(+) positive reaction; (-) no reaction

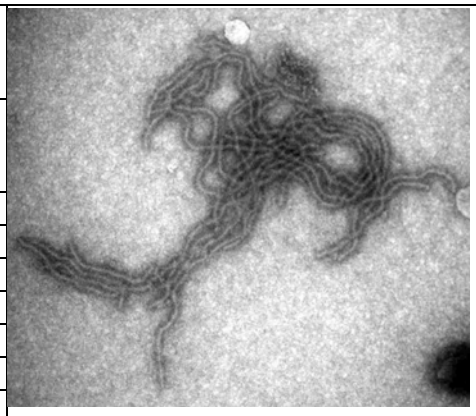


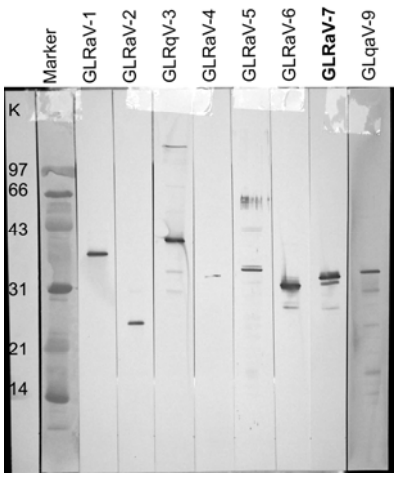
Figure 1. GLRaV-7 virions decorated and aggregated by Mab 6-5-1 (IPEM) (~34'000 x).

Subcloning by limiting dilution
Hybridoma cultures 3, 5, 6, 26, 32, 33 and 37 were at least once to eight times cloned under conditions of limiting dilution in order to assure their monoclonal status. Antibody activity was then confirmed by TAS-ELISA.

TAS- and DAS-ELISA
A sub-clone from hybridoma culture 6 (Mab 6-5-1-1-1-2-1-5-7 , shortly named Mab6A and sub-clones 37-15-4-3-3-3 (Mab37A) and 37-15-17-2-1-3 (Mab37B) were first evaluated as supernatants in TAS-ELISA against GLRaV-1 to GLRaV-9, except GLRaV-8 as well as unrelated grapevine viruses including GFLV, GFkV, GVA, GVB, TBRV, ArMV and RRSV). The new Mabs reacted only with *V. vinifera* Y276 infected by GLRaV-7 as well as with positive GLRaV-7 control sample purchased from Agritest. Mabs from sub-clones of culture 32 and 33 reacted however specifically to GFkV. The production of Mab6A, Mab37A and Mab37B was upscaled and immunoglobulines conjugated with alkaline phosphatase and further evaluated in either homologous or heterologous DAS-ELISA as primary and enzyme conjugated secondary antibody. Polyclonal primary rabbit antibody from Agroscope RAC or purchased from Agritest were also used in comparative tests. Primary antibody from Agritest and new Mabs as secondary allowed to detect specifically GLRaV-7. Mab6A as primary and Mab37A as conjugated secondary antibody yielded the best signal to noise ratio (Table 2).

Table 2. *Identification of GLRaV-7 in crude leaf extracts of* V. vinifera *by homologous DAS-ELISA using Mab6A, Mab37A and Mab37B (Mean values of* $A_{405/492}$ *ELISA readings after 1h substrate reaction, n=3).*

Coating antibody	Mab6A	Mab6A	Mab37A	Mab37B
Concentration: µg/ml	2	2	1	1
Conjugate	Mab6A	Mab37A	Mab37A	Mab37A
Concentration: µg/ml	1.5	0.75	0.75	1.5
GLRaV-7 Y276	1.25	3.02	2.90	3.34
GLRaV-7 Lyo 047375	1.05	2.53	2.23	2.60
GLRaV-7, Colmar	1.19	2.02	1.53	2.01
Healthy, Chasselas 9050	0.14	0.14	0.16	0.19
Healthy, St. George 4723	0.13	0.13	0.12	0.15
Healthy, St. George 4724	0.14	0.13	0.12	0.15
Extraction buffer grapevine	0.12	0.13	0.11	0.14
Performance	+	+++	++	+



Western blot analysis
In repeated Western blots, Mab37B reacted to a dominant structural protein of GLRaV-7 with an estimated molecular weight of about 34 k and a less prevailing protein of about 33 k. Comparatively, the next closest molecular weight was that of GLRaV-4 coat protein. No cross reaction was observed with the later, in whichever antibody combination. From an average of five Western blot repeats, the following estimated dominant coat protein molecular weights were determined for GLRaV-1: 38.9 k, GLRaV-2: 24.7 k, GLRaV-3: 41.6 k, GLRaV-4: 33.2 k, GLRaV-5: 35.0 k, GLRaV-6: 31.6 k, GLRaV-7: 34.4 k and GLRaV-9 (Australian isolate, origin Dr N. Habili): 34.4 k.

Figure 2. Mab37A in comparative Western blot analysis of GLRaV's 1, 2, 3, 4, 5, 6, 7 and 9 with respectively monoclonal antibodies GLRV-1: 2-4, GLRV-2: 29-2-6-4-1-2, GLRV-3: 2-8, GLRV-4: 3-1-2, GLRV-5: 43-1-1-1-3, GLRV-6: 36:117-14-1-2-1-1-1, GLRV-7: Mab37A and absorbed antiserum to GLRV-9.

Conclusions
The new monoclonal antibodies Mab6A, Mab37A and Mab37B complete the diagnostic reagents for fast and specific detection of grapevine leafroll associated viruses. The high signal to noise ratio obtained in DAS ELISA underline their potential. On-going large-scale comparative studies and field surveys should further characterize their specificity and performance. The new monoclonal antibodies also allow unambiguous comparison of GLRaV's by Western blot analysis, although strain specific variability in electrophoretic mobility corresponding to up to 1 k should be considered for GLRaV-1, GLRaV-2, GLRaV-3 GLRaV-4 and GLRaV-6 (Gugerli, unpublished).

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THE ASSOCIATION OF *GRAPEVINE VIRUS A* WITH AUSTRALIAN SHIRAZ DISEASE AND ITS APPARENT SPREAD IN A COMMERCIAL VINEYARD

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Introduction

Vitis Vinifera cv. Shiraz (syn. Syrah) is the most popular red wine variety in Australia. In Australia, a disease with the following symptoms has been observed in both own rooted and grafted Shiraz vines: delayed bud burst, stunted and zigzag growth of canes, poorly lignified wood. Also leaves, which hang on the vines during the winter, turn red and become brittle. *Grapevine virus A* (GVA) was detected in these vines using the reverse-transcription polymerase chain reaction (RT-PCR) assay (Symons & Habili, 2000; Habili & Randles, 2004). These symptoms resemble those described for Shiraz disease (SD) in South Africa (Corbett & Wiid, 1985), where GVA has also been detected in the infected vines (Goszczynski,& Jooste, 2003). Natural spread of the SD associated GVA by mealybugs has been reported from South Africa (Goszczynski & Jooste, 2003).

We have named the disease Australian Shiraz Disease (ASD) to differentiate it from its counterpart in South Africa. A similar disease has been found in Merlot and Ruby Cabernet in Sunraysia (Victoria). We have already reported the spread of GVA in our experimental vineyard (Habili *et al*., 2003). Here we report its apparent spread in a commercial vineyard in the Adelaide Hills (South Australia).

Materials and Methods

Dormant cane samples of the grapevine were sent to Waite Diagnostics from all the viticultural regions of Australia and were tested for GVA using RT-PCR as described (MacKenzie *et al*., 1997; Shi *et al*., 2003).

For the RT-PCR assay, a pair of GVA specific primers, GVA-H7038, 5'-AGG TCC ACG TTT GCT AAG-3' and GVA-C7273, 5'-CAT CGT CTG AGG TTT CTA CTA T-3' derived from the sequence of the ORF5 (putative RNA binding protein) of the Italian isolate of the virus (GenBank Acc. # X745433, Minafra *et al.*, 1994) was used.

The Shiraz vineyard was monitored for symptoms in the late autumn when the GVA associated symptoms were most conspicuous.

Results and Discussion

Table 1 shows the percentage of samples that tested positive for GVA has been on the rise since 2001. This is a cause for concern, and it maybe linked to our previous report that the virus was spreading

Table 1. The *Detection of Grapevine virus A* by RT-PCR in Australian grapevine samples sent to Waite Diagnostics for virus testing.

Year	*Total tested*	*+ve samples*	*%*
2001	728	25	3.4
2002	557	65	11.6
2003	1309	116	9.0
2004	1279	213	16.6

naturally, but at a slow rate, in our experimental vineyard (Habili & Randles, 2003).

Recently, another hot spot for the natural spread of ASD was detected in a Shiraz vineyard in the Adelaide Hills (South Australia). The Shiraz vines in that vineyard showed the typical symptoms of Australian Shiraz Disease. The RT-PCR assay detected GVA only in the symptomatic vines, while the apparently healthy neighboring vines of the same row tested negative for the virus. I also tested the samples for 11 other viruses and the results were negative (see also Habili & Randles, 2004). Up to date, no mealybugs were observed in the vineyard. The collection of data for the temporal and spatial analysis of GVA in Shiraz is in progress.

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AUSTRALIAN GRAPEVINE YELLOWS: FIRST DETECTION OF THE PHYTOPLASMA IN NON-VITIS HOSTS IN SOUTH AUSTRALIA

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Introduction

Australian Grapevine Yellows (AGY) is a widespread disease in Australian viticulture (Magarey, 1986). It is of most economic importance in the Riverland (South Australia), Riverina (New South Wales) and Sunraysia (Victoria). The varieties most severely affected are Chardonnay and Riesling which show symptoms of yellowed and downward curled leaves, dying bunches and stunted, unlignified shoots. Typically only sectors of vines are affected.

The presumed pathogen of AGY is *Candidatus* Phytoplasma australiense. Overseas, yellows diseases of grapevine are associated with similar but different phytoplasmas (Padovan *et al*., 1995). Some of the grapevine phytoplasmas have known vector-host relationships, which information is needed for AGY.

The lack of AGY outside Australasia and other evidence implies that AGY is native to the region and is transmitted by leafhopper or plant hopper insects. This paper reports investigations into native host plants of the phytoplasma and thus locating the source of disease.

Materials and Methods

Host-plant survey In search of the primary host(s) of AGY, during 2005 samples of mostly native plants were tested by the nested-PCR assay. The samples were collected from wastelands and swamplands adjacent to AGY 'hotspots' in the Riverland, South Australia. Samples were sent under ambient temperature to Waite Diagnostics for PCR analysis.

Nucleic acid extraction and PCR analysis RNeasy Mini columns (Qiagen, Germany) were used to extract total nucleic acids from young stems and leaf veins of native plants and phloem scrapings of the grapevine (MacKenzie *et al*, 1997). Nested polymerase chain reaction (PCR) was used to detect AGYp. First step PCR was carried out using fP1 (Deng & Hiruki, 1991) and rP7 (Schneider *et al.,* 1995) primers. The PCR products from this step were diluted 1:15 in water and subjected to a second PCR using the AGYp specific primers, AUSGYF1 and AUSGYR2, (Davis *et al*., 1997). These primers were derived from the16S ribosomal RNA and gave an amplicon size of 644 bp (Fig 1).

Results and Discussion

During 2001-2004, Waite Diagnostics received 907 grapevine samples for AGYp analysis using the PCR assay. Of these, 41 (4.5%) tested positive for the phytoplasma. We have been looking for AGYp in non-Vitis plants for some years. In early 2005, PCR-analyses of several Riverland native and non-native species reacted positively for AGY including some woody perennial native plants (Fig. 1).

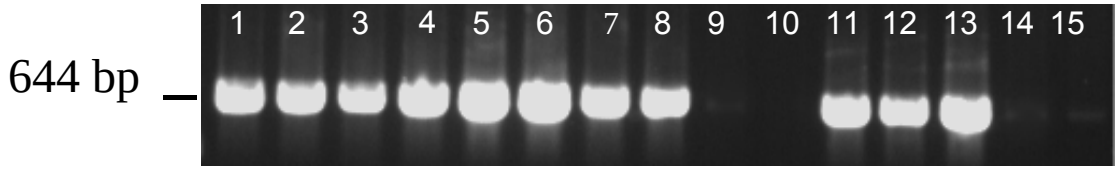

Fig 1. Nested PCR analysis of samples collected from Riverland using AGY specific primers. Samples 1-5: *Maireana brevifolia* (yanga bush); 6: *Enchylaena tomentosa* (ruby saltbush); 7 & 8: *Einardia nutans*; 9-10: Yanga and saltbush negatives; 11-12: *Vitis vinifera*; 13: AGY positive control; 14: Tomato big bud +ve; 15: buffer control. The amplicon size (644 bp) was calculated using a 100 bp DNA ladder (not shown).

A total of 8 samples from 88 native and non-native plants (9%) growing next to the AGY infested vineyards tested positive for the phytoplasma (Table 1). The major native plant in these areas was yanga bush or short-leaf bluebush (*Maireana brevifolia*). It is possible that yanga bush is the primary native host of AGYp. More samples from different plant species should be tested to obtain a better understanding of the primary sources of this indigenous phytoplasma.

Table 1. Incidence of AGY in non-Vitis and Vitis samples as detected by nested PCR using specific primers.

Host	Sample numbers	Numbers +ve for AGY	% +ve for AGY
Yanga Bush (*Maireana brevifolia*)	48	5	10.4
Ruby Saltbush (Enchylaena tomentosa)	28	1	3.6
Lucerne	2	0	0.0
Euphorbia terracina ?	1	1	100.0
Hopbush	1	0	0.0
Einardia nutans ?	5	1	20.0
Lignum	1	0	0.0
Sudax	1	0	0.0
Spiky leafed plant	1	0	0.0
V. vinifera cv. Shiraz ?	*7*	*2*	*28.6*
Total non-Vitis hosts	**88**	**8**	**9.0**

Our initial analysis on the pattern of disease spread within vineyard has indicated that AGY does not spread from vine to vine as no clustering of infected vines were observed. On the contrary, the presence of the disease hot spots in vines growing close to native vegetation in wastelands and/or swamplands prompted us to test randomly selected samples for AGY. The presence of PCR-positive native plants in these hotspots indicated that AGY phytoplasma might infect grapevines from these areas with the help of a leafhopper or a planthopper vector.

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THE FAILURE OF ELISA TO DETECT TWO OF THE THREE SEQUENCE VARIANTS OF *GRAPEVINE FLECK VIRUS* WHICH ARE ROUTINELY DETECTED BY RT-PCR

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Introduction

Grapevine fleck virus (GFkV) is a spherical, phloem-limited virus assigned to family *Tymoviridae* and genus *Maculavirus* (Martelli *et al.*2002). The virus occurs worldwide and has been detected in nearly 20% of Australian grapevine samples tested by Waite Diagnostics (Habili & Symons, 2000). Hewitt *et al.* (1962) detected the virus in vines showing a graft-incompatibility disorder. Long-term indexing for GFkV is done by grafting candidate buds onto *Vitis rupestris* St. George, the biological indicator for the virus. Short-term indexing is carried out using ELISA and RT-PCR. We reported that the RT-PCR assay could detect two sequence variants of GFkV, namely A and B of which GFkV-A comprised 75% of the GFkV positive vines (Habili and Symons, 2000; Shi *et al*., 2003). Here we provide evidence that a third variant, GFkV-C is detectable by RT-PCR in infected vine samples from Australia.

Materials and Methods

All grapevine sources were from South Eastern Australia as shown on Table 1. Sample 256-1 (Fig. 1) was from an unknown source introduced into Australia and was growing at a post-entry quarantine facility at Knoxfield (Victoria).

Total nucleic acid was extracted from the phloem layer of one-year-old dormant grapevine wood. The RT-PCR protocol was according to MacKenzie *et al*. (1997) as modified by Shi *et al*. (2003). The PCR products were analysed in 1.5% agarose gels containing 0.5 µg ml per ml ethidium bromide. The PCR bands were visualized with a UV light.

For the RT-PCR assay a pair of GFkV specific primers, GFkV-U279 (5'-TGG TCC TCG GCC CAG TGA AAA AGT A-3') and GFkV-L630 (5'-GGC CAG GTT GTA GTC GGT GTT GTC-3') derived from the sequence of the ORF1 (RNA polymerase) of the Italian isolate of the virus (GenBank Acc. # AJ309022, Sabanadzovic *et al*. 2001) was used.

Amplified products were cloned into the pGEMT Easy Vector (Promega, USA). The DNA sequencing was performed by deoxynucleotide chain termination and the sequences were analysed using the BLAST programs.

Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for GFkV was carried out using a Bioreba Ag kit (Basel, Switzerland) and following the protocol provided with the kit. The plates were read at an optical density of 405 nm using a Bio-Rad Model 3550 Micro plate Reader).

Results and Discussion

Fig. 1 shows the RT-PCR products from four different grapevine samples infected with three variants of GFkV (A, B and C). As shown, one sample contains both A and B variants. Lane H represents a healthy grapevine.

The occurrence of the A and B variants, as confirmed by sequence analysis, has already been reported (Habili and Symons, 2000; Shi *et al*., 2003). However, we observed that a number of samples from certain vine varieties consistently gave two RT-PCR bands using the same GFkV specific primers from the RNA polymerase gene, (Habili and Randles, 2002). Neither of these bands was present in healthy grapevines (Fig. 1). This indicated to us that a third variant might be present. To confirm this, we sequenced both the larger band with 353 bp and the smaller band with 175 bp (Fig. 1) from sample 256-1 sent by quarantine. The larger band had 90% similarity at amino acid level with a similar area in the ORF1 of *Grapevine red globe virus* (GRGV), which is another member of *Maculavirus*, in which GFkV is the type member (Sabanadzovic, per. Commun.; Ghanem-Sabanadzovic *et al.* 2003). We tentatively named this variant as GFkV-C. The sequence of the 175 kb band was apparently unrelated to any virus. The serological relationship between GRGV and GFkV is not known (Sabanadzovic, personal communication).

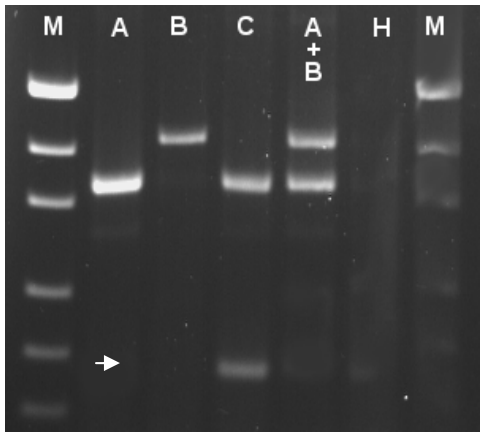

Fig. 1. Agarose gel analysis of the RT-PCR products of GFkV. Lanes A, B and C are products from samples 191-1263, 189-866 and 256-1, respectively. Lane A + B was from a Jade Seedless table grape. A, B and C represent the three sequence variants of GFkV. The smaller band present only in C (with a size of 175 bp) is shown by an arrow head. H, healthy *Vitis vinifera* cv. Shiraz. M, DNA size markers. Viral RNA from sample 256-1 (ex quarantine) was used for sequencing.

Further to our RT-PCR analysis, in order to confirm the presence of GFkV, a concurrent RT-PCR assay and ELISA were carried out on samples listed in Table 1. All the three variants of GFkV were present either singly or mixed as shown by RT-PCR (Fig. 1 & Table 1). However, ELISA was only able to detect GFkV-A. Interestingly, this is the variant that we find in 75% of our GFkV positive vine samples (Habili and Symons, 2000). It is possible that the use of monoclonal antibodies for the detection of GFkV may not be suitable when a vine is infected with a less prevalent strain or sequence variant of the virus.

Table 1. A comparison of the detection of GFkV by ELISA and RT-PCR

Sample code	Variety	GFkV strain	PCR result	ELISA (405 nm)
179-4	Cabernet Sauvignon	C	positive	0.13
187-1	Cabernet Sauvignon	C	positive	0.06
187-2	Cabernet Sauvignon	C	positive	0.07
187-3	Cabernet Sauvignon	C	positive	0.07
187-4	Cabernet Sauvignon	C	positive	0.12
190	Merlot	A	positive	0.16
191/1249	101-14 clone 100-3	**A**	positive	**2.80**
191/1256	Ramsey clone A11V2	B	positive	0.23
191/1257	Ramsey clone A11V2	B	positive	0.15
191/1258	Ramsey clone A11V2	B + C	positive	0.13
191/1263	Ruggeri clone Q45-3a	**A**	positive	**2.74**
191/1264	Ruggeri clone Q45-3a	**A**	positive	**2.80**
191/1265	Ruggeri clone Q45-3a	**A**	positive	**2.63**
191/1266	Ruggeri clone Q45-3a	**A**	positive	**2.87**
248-1, Jade seedless	Jade seedless	**A** + B	positive	**2.72**
248-2, Jade seedless	Jade seedless	**A** + B	positive	**2.90**
248-3, Jade seedless	Jade seedless	**A** + B	positive	**2.78**
248-4, Jade seedless	Jade seedless	**A** + B	positive	**2.65**
248-5, Jade seedless	Jade seedless	**A** + B	positive	**2.88**
248-6, Jade seedless	Jade seedless	**A** + B	positive	**2.93**
Negative tissue	Cabernet Sauvignon	no	negative	0.15

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GRAPEVINE VIRUS A IN RHINELAND-PALATINATE (GERMANY): OCCURRENCE AND IMPORTANCE FOR GERMAN VITICULTURE

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Introduction

Grapevine virus A (GVA) first described in 1980 (Conti et al.) is a member of the genus *Vitivirus* and transmittable by mealybugs (Rosciglione et al. 1983). It is limited to the phloem tissue of infected vines and associated with Kober stem grooving disease (Garau et al. 1994). The virus was frequently found in grapevine affected by leafroll (Milne et al. 1984) but is not strictly associated with this disease (Rosciglione & Gugerli, 1986). First the detection of Kober stem grooving was only possible by indexing with Kober 5BB as indicator. Now there are serological and molecular methods available for testing GVA. Although it is thought the virus has a worldwide distribution only few information is available about the occurrence of GVA in Rhineland-Palatinate, which has with more than 64 000 ha vineyards about 2/3 of the entire wine-growing area of Germany. One reason for this may be the fact that in Germany symptoms of Kober stem grooving or one of the other diseases of the rugose wood complex are not to be found on grapes.

Materials and Methods

GVA-positive grapevines coming from Italy were used for indexing trials with Kober 5BB as indicator variety. The experiments were carried out by wooden or green grafting, using the indicator as rootstock. The grafted grapevines were growing in the greenhouse or under semi-field conditions. For symptom analysis the plants were uprooted, autoclaved and peeled clean of cortex.

The detection of GVA exclusively was conducted with serology because in a routine-working lab it is not possible to additionally check some of the material by PCR. Commercial kits for the detection of GVA, GLRaV-1, -2 and -3, and *Nepoviruses* (Bioreba, Basel) were used according to manufacturer's instructions. To find out the most suitable tissue for GVA-detection petioles and blades from mature leaves of GVA-infected grapevines and/or cortical scrapings from dormant canes were used as antigen source. Some of the plants were repeatedly tested to confirm the results. To get a first survey on the occurrence of GVA in Rhineland-Palatinate grapevines coming from different leafroll-diseased vineyards were tested. Additionally each suitable grapevine in the lab tested for *Ampelo*- and *Closteroviruses* was checked for GVA too.

Results and Discussion

Indexing trials with donor grapevines definitely infected with GVA did not show any symptoms of Kober stem grooving four years after grafting no matter if the plants were growing in greenhouse, in a climate chamber with different temperature programs or under semi-field conditions. At the earliest five years after grafting the typical grooving symptoms could be seen.

Repeated tests showed that the most suitable tissue for detecting GVA was petioles from mature leaves or cortical scrapings from dormant canes. In all experiments the tests with petioles resulted in much higher extinction values compared to the blades. Even in some samples it was not possible to detect GVA in blades but in petioles. Generally the tests should not be conducted in Germany before June to make sure that mature leaves are available.

The results concerning the occurrence of GVA in Rhineland-Palatinate are summarized in Table 1 and 2. None of the tested plants showed any symptoms of rugose wood. By chance in the year 2004 grapevines of a vineyard more than 30 years old (variety Riesling, Piesport, Mosel) have to be checked for their virus status to find out some healthy plants for preserving the genetically diversity of the variety. The results of the virus tests using dormant canes are shown in Table 1. In summary a high percentage of GVA was detected, 57.1 % in mixed infections with GLRaV-1, 11.2 % of the plants showed additionally to GLRaV-1 an infection with one of the *Nepoviruses*. Only one of the tested plants had a single infection of GVA.

Grapevines collected from more than 17 different vineyards in Rhineland-Palatinate were checked on the presence of GVA (Table 2). It was shown that a high percentage of mixed infections of GVA and GLRaV-1 or –3 appeared. But not all grapevines having leafroll disease showed a simultaneous infection with

GVA. In Germany up to now no field spread of leafroll disease has been observed. Therefore it is assumed that the only way to infect grapevines with GLRaV's and GVA is by grafting. No different effects on symptoms or on must quality can be seen between vineyards infected with GLRaV's alone and such with mixed infections (GVA and GLRaV's). Therefore the importance of a GVA infection in Germany seems to be extremely low.

Table 1: Viruses detected by ELISA singly or in mixed infection, more than 30 years old vineyard, variety Riesling, Piesport, Mosel

Virus status	**Total**	**%**
ELISA negative (healthy)	6	9.5
Arm	2	3.2
RpRSV cherry strain	1	1.6
GLRaV-1	6	9.5
GVA	1	1.6
GLRaV-1, Arm	2	3.2
GLRaV-1, RpRSV cherry strain	2	3.2
GLRaV-1, GVA	36	57.1
GLRaV-1, GVA, ArMV	3	4.8
GLRaV-1, GVA, RpRSV cherry strain	3	4.8
GLRaV-1, GVA, GFLV	1	1.6
Total	**63**	**100**
Mixed infection of GVA, GLRaV-1 and Nepoviruses	**43**	**68.3**

Table 2: Results of serological tests of different vineyards in Rhineland-Palatinate

Varieties	**Locality of the tested Vineyards**	**Total**	**Elisa Neg[*1]**	**Elisa pos**	**GLRaV**		**GVA**	**GVA + GLRaV**	
					-1	**-3**		**-1**	**-3**
Riesling	Mosel	8	3	5	-	-	1	4	-
Cabernet Mitos	Monzernheim	2	1	1	-	-	-	1	-
Riesling	Schweigen	3	3	-	-	-	-	-	-
Primitivo	Essingen[*2]	9	-	9[*3]	-	-	1[*3]	2	5
Pinot Noir	Maikammer	4	4	-	-	-	-	-	-
Riesling	Maikammer	1	1	-	-	-	-	-	-
Dunkelfelder	NW-Hambach	4	-	4	4	-	-	-	-
Pinot Grigio	NW-Hambach	3	3	-	-	-	-	-	-
Riesling	NW-Haardt	6	5	1	-	-	-	1	-
Pinot Grigio	NW-Gimmeldingen	3	-	3	2	-	1[*3]	-	-
Pinot Noir	NW-Königsbach	1	-	1	1	-	-	-	-
Morio Muskat	NW-Mussbach	1	-	1	-	-	1	-	-
Pinot Blanc	NW-Mussbach	10	5	5	3	2	-	-	-
Pinot Madeleine	Ruppertsberg	22	8	14	-	-	-	14	-
	Total	77	33	44	10	2	4	22	5
	%	100	42.9	57.1	12.9	2.6	5.2	28.6	6.5

[*1] = tested negative for GVA and GLRaV-1, -2 and 3; [*2] = grapevines originating from South-Italy;
[*3] = additionally positive for GLRaV-2,

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DISTRIBUTION OF GRAPEVINE VIRUSES IN THE CZECH REPUBLIC

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Introduction:
A survey on a healthy status of grapevines in the Czech Republic continued on vineyards in a viticulture region of South Moravia. The presence of sixteen viruses was evaluated - seven nepoviruses, *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Raspberry ringspot virus* (RpRSV), Strawberry latent ringspot virus (SLRSV), *Tomato black ring virus* (TBRV), *Tomato ring spot virus* (TomRSV), *Tobacco ring spot virus* (TRSV), two vitiviruses, *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB), three closteroviruses, *Grapevine leafroll - associated virus 2* (GLRaV-2), *Grapevine leafroll - associated virus 6* (GLRaV-6), *Grapevine leafroll - associated virus 7* (GLRaV-7), three ampeloviruses, *Grapevine leafroll - associated virus 1* (GLRaV-1), *Grapevine leafroll - associated virus 3* (GLRaV-3), *Grapevine leafroll - associated virus 5* (GLRaV-5), and one maculavirus, *Grapevine fleck virus* (GFkV).

Material and methods
Dormant wood was sampled from grapevines (Burger and Thatcher, 1987) in the Czech Republic. Our survey was oriented mainly into a viticulture region of South Moravia. Ten vineyards from different parts of South Moravia were selected for the survey. 62 grapevines were sampled in 2004 year and 48 in 2005.

Scrapings from the wood were tested using DAS-ELISA for the presence of above mentioned viruses. Antisera were purchased from different producers: Agritest, Italy (antisera against GFLV, ArMV, GVA, GVB, GFkV, GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-7), Bioreba, Switzerland (GLRaV-6, TRSV), Bio-Rad, France (SLRSV, GLRaV-5 and TomRSV) and Loewe, Germany (TBRV, RpRSV) and they were used in DAS - ELISA method according to instructions of manufacturers.

Results and Discussion
Results of a two-year survey of occurrence of grapevine viruses in vineyards are given in a following table.

Virus	Number of positive grapevines / tested grapevines	Percent
GFLV	1 / 110	0,91
ArMV	1 / 110	0,91
GVA	17 / 110	15,45
GVB	2 / 110	1,82
GLRaV-1	31 / 110	28,18
GLRaV-2	1 / 110	0,91
GLRaV-3	3 / 110	2,73
GLRaV-5	2 / 110	1,82
GLRaV-6	0 / 110	0
GLRaV-7	0 / 110	0
GFkV	17 / 110	15,45
TBRV	2 / 110	1,82
SLRSV	2 / 110	1,82
RpRSV-g	0 / 110	0
TRSV	0 / 110	0
TomRSV	0 / 110	0

From 110 grapevines tested, 48 were found to be infected with at least one virus, mixed infections occurred frequently.

GLRaV-1, GVA and GFkV were found to be most widely spread viruses in grapevines in the Czech Republic. These viruses occurred in more then 15% of examined vines and are considered as

economically important for grapevine production in the Czech Republic. Other viruses were found in negligible number (GFLV, ArMV, GVB, GLRaV-2, GLRaV-3, GLRaV-5, TBRV, SLRSV).

Quarantine nepoviruses TRSV and TomRSV were never found in Czech grapevines. Similarly, GLRaV-6 and RpRSV were not found during our experiments.

We did not realize any difference in occurrence of viruses among individual vineyards. Similarly we did not realize any pattern of virus spread; two neighbouring grapevines gave different results of virus presence. Also we did not realize a connection of a certain cultivar with certain virus.

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SANITARY STATUS AND SANITATION OF LOCAL GERMPLASM IN SOUTHERN ITALY

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Introduction

The deteriorated sanitary status of autochthonous minor wine grape varieties is often the main cause of loss of interest, abandon, and consequent risk of genetic erosion. Restoring the heath of this germplasm is most desirable for evaluating its productive and oenological potential, and for producing sanitarily improved propagation material for the establishment of new vineyards. The results obtained in a three-year (2003-2005) investigation on the sanitary improvement of 22 cultivars locally grown in the southern Italian regions of Apulia, Basilicata, and Campania are reported.

Materials and methods

After field selection, attention was focused on 42 vines that were assayed serologically (ELISA or Western blot) for the presence of *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 2* (GLRaV-2), *Grapevine leafroll-associated virus 3* (GLRaV-3) and *Grapevine rupestris stem pitting associated virus* (GRSPaV). All selected plants, regardless of whether or not they were showing symptoms, proved to be infected by at least one of the above viruses (Table 1), confirming the alleged precarious sanitary conditions of southern Italian vineyards (Savino *et al*., 2002). All selections underwent sanitation using techniques tailored to their sanitary status, i.e.meristem tip culture for phloem-limited viruses and *in vitro* heat therapy for parenchymatous viruses (i.e., GFLV), as described by Bottalico *et al*. (2003). The 265 explants from treated selections were assayed twice by ELISA (Table 2) or Western blot (for GRSPaV).

Results and discussion

In accordance with what previously reported for major wine grape varieties (Bottalico *et al*., 2000), an overall average sanitation rate of 86% was obtained with selections affected by leafroll, rugose wood, and fanleaf, thus confirming the efficacy of the used protocols. For GLRaV-3 and GVA, sanitation efficency by meristem tip culture reached *c*. 97% and 89%, respectively. With GFLV, the sanitation rate of 84% obtained by *in vitro* heat therapy [(higher that 76% afforded by meristem tip culture (Bottalico *et al*., 2000)] and the shorter time required (6-8 months versus 10-12 months with *in vivo* heat therapy) confirms this to be the best technique actually available in our hands for knocking out this virus. Preliminary data (not shown) on the low sanitation level from GRSPaV, confirm the recalcitrant nature of this virus (Bouyahia *et al.*, 2005), which calls for the development of novel protocols for its efficient elimination. In conclusion, our results show that, despite a certain variability consequent to varietal behaviour in *in vitro* culture, and the need for additional *in vitro* multiplication steps, sanitation through meristem tip culture and *in vitro* heat therapy, regardless of the season, takes no more than 6-8 months from explant excision to acclimatation. A further shortening of the sanitation process seems difficult, while further improvements can be achieved in speeding up the successive steps , i.e. explant growth and diagnosis.

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Table 1. Results of the ELISA tests of selected grapevine accessions.

Variety	Selected vines (No.)	Infected vines (No.)							Explants obtained through sanitation (No.)
		GVA	GVB	GLRaV-1	GLRaV-2	GLRaV-3	GFLV	GFKV	
ANTINIELLO	2	1	-	-	-	-	1	1	**5**
BIANCO D'ALESSANO	1	1	-	-	-	-	1	1	**3**
BOMBINO BIANCO	1	-	-	-	-	1	-	1	**3**
BOMBINO NERO	1	1	-	-	-	1	-	-	**3**
CODA DI VOLPE	1	1	-	1	-	-	-	-	**1**
FALANGHINA	3	3	-	-	-	2	-	-	**14**
FIANO	1	-	-	-	-	1	-	1	**1**
FRANCAVIDDA	1	1	-	-	-	1	-	-	**28**
GRECO DI GERACE	1	1	-	-	-	-	-	-	**17**
GRECO DI TUFO	1	1	-	-	-	1	-	-	**12**
MALVASIA BIANCA	2	1	1	-	-	2	2	2	**15**
MALVASIA BASTARDA	1	1	-	-	-	1	1	1	**12**
MANTONICO	1	1	-	-	-	1	-	-	**9**
MARUGGIO	1	1	-	-	-	1	1	-	**6**
MONTONICO	1	-	-	-	-	1	-	-	**1**
MOSCARDELLA B.	1	-	-	1	-	-	-	-	**9**
NEGROAMARO	1	-	-	-	-	1	-	1	**1**
NOTARDOMENICO	2	2	-	-	-	2	1	-	**20**
PRIMITIVO	15	10	2	-	1	10	6	10	**62**
SANGIOVESE	2	2	-	-	-	1	2	-	**5**
SANTA TERESA	1	1	-	-	-	1	1	1	**25**
UVA ATTINA	1	1	-	-	-	1	-	-	**13**
total	**42**	**30**	**3**	**2**	**1**	**41**	**16**	**19**	**265**
infection rate %		**71,4**	**7,1**	**4,7**	**2,3**	**69,0**	**38,0**	**45,2**	

Table 2. Results of sanitation treatment (explants tested twice by ELISA).

	GVA	GVB	GLRaV-1	GLRaV-2	GLRaV-3	GFLV	GFkV
No. of explants from selected accessions infected by	216	20	10	2	203	94	96
ELISA-negative	193	20	10	2	196	79	95
ELISA-positive	23	0	0	0	7	15	1
Sanitation rate %	**89,4**	**100,0**	**100,0**	**100,0**	**96,6**	**84,0**	**99,0**

DETECTION AND CHARACTERIZATION OF GRAPEVINE PHYTOPLASMAS IN SICILY (ITALY)

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Introduction

Grapevine yellows (GY) are worldwide distributed and represent a serious limiting factor for grapevine production. The causal agents of GY are phytoplasmas, belonging to different taxonomic groups, which induce similar symptoms on the plants. In Sicily symptoms of grapevine yellows have been reported since 1980 on cv. Inzolia (Granata, 1982; Granata, 1985) and phytoplasmas have been observed by E.M. in the phloem of symptomatic plants (Granata and Grimaldi, 1991). Subsequently, molecular analyses confirmed the presence of phytoplasmas belonging to 16SrXII group (Albanese *et al.,* 1996) whereas investigations on *Homoptera Auchenorrhyncha* collected in sicilian infected vineyards on grapevine and wild plants showed a phytoplasma, genetically different from the phytoplasma found in infected grape, in *Psammotettix striatus* (L:) (Albanese *et al.*, 1997). In Calabria (South Italy) Stolbur (16Sr XII group) has been identified, by molecular analyses, in severely diseased tomato plants (Albanese *et al.*, 1998) but, until last year, there was a lack of information on phytoplasmas and their vectors on grape. *Flavescence doree* (FD) (Elm Yellow, 16Sr-V group), the other phytoplasma-associated grapevine yellows in Italy, has not been reported in Southern Italy, as well as its main vector, the leafhopper *Scaphoideus titanus* Ball. In order to verify the absence of FD (*sensu strictu*) and to evaluate the presence and distribution of GY phytoplasmas from 2004, in the framework of an Italian Project financed by the Ministry of Agriculture titled "Grapevine yellows: a limiting factor of grapevine productions - GIAVI", a survey on grapevine yellows diseases started in Sicily (R.U. 10 of the Project), as in other Italian regions. Results obtained from field surveys and phytoplasmas detection and characterization in Sicily during the first year of the project are here reported.

Materials and Methods

In late summer and autumn 2004 several samples in n. 18 (suspected as phytoplasma-affected) grapevine vineyards of Catania, Ragusa, Siracusa and Palermo provinces (Sicily) were randomly collected. Samples were taken from different cv. or selections, some of which are the most diffused in Sicily (as Moscato bianco, Nero D'Avola, Chardonnay). Some of the plants of white-berried cv showed symptoms as leaf margin downward with rolling yellowing coupled, in some cases, with necrosis along the veins or in intervenial sectors and/or desiccated fruit clusters. Nucleic acids were extracted from main leaf vein by the protocol of Barba *et al.* (1998). To identify and characterize phytoplasmas, direct and nested polymerase chain reaction, followed by restriction fragment length polymorfisms (RFLP), were used. Direct PCR was performed with primers R16F2/R16R2 (Schneider *et al.*, 1995) followed by nested-PCR with primers R16(I)F1/R1 (Lee *et al.,* 1994) and R16(V)F1/R1 (Lee *et al.,* 1994). Five symptomatic grapevine samples (cv Chardonnay) were further characterized by RFLP analysis of a partial sequence of the *tuf* gene, amplified in nested assays with primer pairs tufAY followed by tufu (Schneider *et al*., 1997), digested with the endonuclease *Hpa*II as detailed by Langer and Maixner (2004). In the same period, with the aim to identify the insect vectors, a survey has been also realised in most of the investigated Sicilian vineyards, both by the use of chromoattractive traps and netting the canopies (on grapevine plants and on close wild vegetation) at regular intervals of time.

Results and Discussion

In grape samples from Sicily out of total tested plants only 5, all belonging to cv. Chardonnay, were positive in nested-PCR for LN (16Sr XII group). Following RFLP characterization of the partial *tuf* gene, these samples showed an identical *HpaII* profile corresponding to that of the Stolbur isolate from field-bindweed as well as grapevine in Germany (Langer and Maixner, 2004) and known as VKII. As to the insects involved in the study, collections by net on grapevine plants from Sicily showed *Empoasca vitis* (Göthe) as the most frequent species in all the investigated areas; the termophilous *Jacobiasca lybica* (Berg. Zan.) has been largely detected as well, but only in the southern part of the island. Two additional polyphagous species have been also collected frequently, *Philaenus spumarius* (L.) and *Austroagallia*

sinuata (M.R.), both of them known as, or suspected to be, vectors of phytoplasmas. Species detected on wild vegetation largely varied with the floral composition of the agrosystem and with its general cultural management. Briefly, nine vector (or suspected vector) species have been totally detected on the row vegetation of the investigated orchards, of which one, *Psammotettix striatus* (L.), already showed in Sicily, during previous researches (Albanese *et al.*, 1997) to be positive for a phytoplasma of the subgroup 16SrI-B. Eleven vector (or suspected vector) species, including also the above mentioned *P. striatus*, have been detected on the edge vegetation. No collection has been made up to now of *Scaphoideus titanus* Ball.

Results obtained during the first year of the research in Sicily confirmed the presence of the LN phytoplasma (16SrXII group) in cv. Chardonnay corresponding, by RFLP characterization of the partial *tuf* gene, to the Stolbur isolate known as VKII. Eleven vector (or suspected vector) species have been detected, but not the main LN vector *Hyalesthes obsoletus* Signoret. Until now FD phytoplasma and *Scaphoideus titanus* Ball. are absent. As general remarks we underline that three isolates of Stolbur have been described in Germany (Langer and Maixner, 2004), each of them occurring in symptomatic grapevine and *H. obsoletus* vectors, but each associated to a specific weed: VKI to nettle, VKII to field-bindweed and VKII to *Calistegia sepium*. The existence of different populations of the vector, each living on nettle or field-bindweed and both occasionally feeding on grapevine, has been proposed (Langer and Maixner, 2004). On the basis of this hypothesis and from the obtained results, next step of the work (second and third years of the project) will be devoted, in addition to verify FD and its main vector absence, to improve our knowledge on LN epidemiology (diffusion, vectors, wild plants) in Sicily. The research will better address certification schemes of this economically important and traditional crop of South Italy.

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SPREAD AND TRANSMISSION OF BOIS NOIR PHYTOPLASMA IN TWO REGIONS OF SPAIN

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Bois Noir disease is caused by the stolbur phytoplasma, which belongs to the stolbur group, also referred to as the 16S rXII-A group (9). The disease is widespread in different vine growing regions in Spain, such as La Rioja, Navarra, Catalunya and Aragón (2). In Spain it has also been shown to affect different crops such as carrot, tomato, pepper, strawberry, avocado and asparagus (1, 5). The results of several epidemiological studies carried out over the last five years in grapevine plots in Spain have shown a high incidence of the Bois Noir disease in plots sampled in La Rioja, whereas in Catalunya the incidence was low (2). Several leafhopper and planthopper species are thought to be involved in the transmission of stolbur phytoplasma, but only *Hyalesthes obsoletus* has been found to be a transmitter of stolbur in grapevine (6). In this study we have compared the occurrence and population level of potential insect vectors of stolbur phytoplasma in the two regions, and the transmission to grapevines *in vitro*.

Two plots in Catalunya and three in La Rioja were selected for this study. In each plot the insects were captured at the beginning of July, which, in previous studies, corresponded to the highest population levels of species belonging to potential phytoplasma transmitters (10). The insects were captured with a D-vac aspirator, with five aspirations, of 2 minutes each, over weeds inside grapevine plots.

Transmission experiments to *in vitro* grapevine plants were carried out with field-collected insects of phytoplasma-carrying species (10). Some of these species had shown in previous studies, the ability to transmit stolbur phytoplasma in nutrient media. In these studies, stolbur transmission was obtained by *Euscelidius variegatus, Euscelis obsoletus, Hardya tenuis, H.obsoletus, Laodelphax striatellus, Macrosteles quadripunctulatus Peragallia sinuata, Psammotettix striatus* and *Zyginidia scutellaris* (10). For these assays, the insects were starved for one day and then individuals were placed in tubes with a single *in vitro* grapevine plant. After three days the insects were collected and analyzed by PCR, to determine the percentage of infection. Test plants were acclimatized and maintained in an insect-proof greenhouse for at least 6 months, in order to observe the appearance of symptoms and for periodical analysis by PCR to determine the presence of phytoplasma.

The PCR technique was used for phytoplasma detection in plants and insects. DNA was extracted following the methods used in previous studies (2,3). DNA from insects was extracted by grinding 1-10 insects, depending on the species. Nested PCR was used for specific detection of the phytoplasma. The universal primers for phytoplasma detection, fP1/rP7 located at the 16S rDNA and 23S rDNA gene respectively, were used in the first step. The second step was performed with the fstol/rstol specific primers for the Stolbur group (7). Negative samples with the specific primers were analyzed in the second step using the universal primer pair fU5/rU3 to determine the presence of phytoplasmas not from the Stolbur group. Ten µl of the mixture containing the amplified DNA in the second step were digested overnight at 37ºC, with 1 unit of T*ru* I enzyme. In order to classify the stolbur phytoplasma isolate in the samples, nested PCR with Tuf primers, and RFLP with H*pa*II enzyme were used (4,8).

Results of sampling showed that *H. obsoletus* was present at the highest levels in La Rioja region, where the mean of individuals per capture ranged from 1.6 to 5 individuals, whereas the population in the plots in Catalunya was lower (0 and 1.6 individuals per capture) (Table 1). This indicates a correlation between the presence of this species and the disease incidence. The disease incidence in the two plots in Catalunya were 15 and 25%, whereas in the three plots in La Rioja, it ranged from 40 to 80%. Other potential insect vectors captured within the plots in both Catalunya and La Rioja were among them *Aphrodes bicinctus, Agallia laevis, E. variegatus, E. obsoletus, Issus sp.,* and *P. striatus*. These species were caught mainly on *Convolvulus arvensis* and Graminea in the plots.

The transmission trials conducted in 2004 were carried out with insects captured from grapevines in Catalunya and showed that both *H.obsoletus* and *E. obsoletus* transmitted stolbur to *in vitro* grapevine plants, indicating that *Euscelis obsoletus* could be the transmitter of the disease in those areas where *H.obsoletus* is not common (Table 2).

The experiments carried out in 2005 involved species collected from La Rioja. The plants from these experiments are being maintained in a greenhouse to monitor the appearance of symptoms of the disease, and for periodical analysis. The first results showed that some plants exposed to *E.obsoletus* were infected by the phytoplasma corroborating the results previously obtained in 2004. *A. bicinctus, Issus* sp. and *E.variegatus* infected one plant each one (Table 2).

The transmission assays to *in vitro* grapevine plants are a reliable method for determining the ability of insects to infect and transmit the disease, but it is necessary to corroborate the results with transmission trials to adult grapevine plants.

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Table 1.-Leafhoppers and planthoppers species captured in July 2005 in two vineyards of Catalunya and three plots of La Rioja, affected by Bois Noir Phytoplasma

Insect Species		Catalunya		Stolbur detection[1]	La Rioja			Stolbur detection[1]
		Fransola	Poblet		Cardenas	Manjarrés	Aranzana	
		Population mean[2]			Population mean[2]			
Adarrus	*taurus*	2	1,2	1+/16(6,25%)	0,2		0,2	1+/2
Agallia	*laevis*	0,6	1,8	1+/12(8,33%)	0,2	2,6	2,2	2+/25(8%)
Aphrodes	*sp*		0,2	0+/1		0,2		0+/1
Cicadula	*divaricata*		0,2	0+/1				
Empoasca	*sp*	7,2	4,8	1+/60(1,66%)		1,4	1,4	0+/14
Euscelidius	*variegatus*		1,2	1+/6(16,6%)	0,6	1,2	1,2	4+/15(26%)
Euscelis	*obsoletus*		4,8	2+/24(8,33%)	0,8	0,8	1,4	6+/15(40%)
Graphocraerus	*ventralis*	1,2	0,2	0+/7				
Hyalesthes	*obsoletus*		1,6	2+/8 (25%)	1,8	1,6	5	15+/42(38%)
Issus	*sp*	0,8	1	1+/9 (11%)	0,8		0,8	2+/8 (25%)
Mocydia	*crocea*		0,2	0+/1				
Peragallia	*sinuata*		0,6	0+/3	1,6		0,8	1+/12 (25%)
Philaneus	*sp*	0,6	0,6	1+/6 (16%)	0,2		1,2	0+/7
Psammotettix	*striatus*	1,8	4,2	2+/30(6,6%)	31,2	1,2	4,2	(6,25-30%)
Tettigometra			0,4	0+/2				
Ulopa	*sp*				0,2	0,2		0+/2
Zyginidia	*scutellaris*		1,6	1+/8 (12,5%)				

[1].Stolbur detection was carry out by PCR with specific primers fstol/rstol (Maixner et al. 1995)
[2]-Data are means of five aspirations in the plots

Table 2.-Results of transmission trials. PCR detection of stolbur phytoplasma in grapevine plants incubated with different field collected leafhoppers and planthoppers species.

2004 Transmission tests		**2005 Transmission tests**
Insect Species	**PCR positive plants/ Total plants analyzed**	**PCR positive plants/ Total plants analyzed**
Adarrus taurus	0+/11	
Agallia laevis	0+/8	0+/17
Aphrodes bicinctus		1+/1
Eupelix cuspidata	0+/6	
Euscelidius variegatus		1+/7
Euscelis obsoletus	4+/37	3+/9
Hyalesthes obsoletus	2+/8	1+/3
Issus sp	0+/3	1+/3
Laodelphax striatellus	0+/10	
Philaneus sp	0+/5	0+/3
Psammotettix striatus		0+/14

GLRaV-3 SHOWS A LESSER AGGREGATED SPATIAL DISTRIBUTION THAN GFLV IN RIOJA ALAVESA VINEYARDS

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Introduction

Rioja Alavesa, in the Basque Country, Northern Spain, is renowned for its red wines, either young or oak matured. It has a mild mediterranean climate and Tempranillo variety is prevalent. Leafroll was targeted as a disease to be surveyed because of its effect on alcoholic content, acidity and colour intensity.

As the starting point of a long-term follow-up on grapevine leafroll epidemiology, commercial vineyards were surveyed in Rioja Alavesa in winter 2004-2005 for the most common grapevine viruses.

Material and Methods

Random samples of ca. 100 dormant canes were taken in two commercial vineyards, namely La Tejera, in Elciego and Durana, in Barriobusto. Both were trained in vase and older than 25 years. Two series of 50 plants, in two parallel rows, three rows apart from each other, were sampled in La Tejera. An X-shaped pattern was followed in Durana. ELISA tests were done for *Grapevine leafroll-associated virus 3* (GLRaV-3, genus *Vitivirus*), 2, and 1, *Grapevine fanleaf virus* (GFLV, genus *Nepovirus*), GFkV, and GVA.

The incidences of GLRaV-3 and GFLV were compared by confidence intervals of the binomial distribution. Their association was measured by Fisher's exact test. Their spatial randomness was assessed by the Wald-Wolfowitz's runs test. These tests were done on each of the two rows in La Tejera and each leg of the X in Durana.

Results

GLRaV-3 and GFLV were the most commonly detected viruses.

	GLRaV-3 incidence	GFLV incidence	Comparative incidence	Association between the two viruses	GLRaV-3 aggregation	GFLV aggregation
La Tejera	14%	10%	ns	ns	-2.45 *	-3.30 ***
	6%	28%	*	ns	0.49 ns	-5.40 ***
Durana	2.6%	44%	***	ns	0.23 ns	-4.02 ***
	11%	5.4%	ns	ns	-0.66 ns	-0.46 ns

Discussion

It is not possible to describe an epidemic from one single year's data; however, the initial distribution of leafroll is expected to be random, due to the infection of the propagating material. As the mealybug vectors transmitted the virus, foci would be formed around the initial sources of infection. Cabaleiro and Segura (1997) described GLRaV-3 epidemics in Galice, Northwestern Spain. One of them, planted healthy, reached 21 % incidence within 3 years, levelling off later. The other, of unknown initial health status, increased from 33 to 83% in 4 years. 11 to 25 % of the rows showed an aggregated spatial pattern. Habili and Nutter (1997) described a GLRaV-3 epidemic in Southern Australia, with an initial 23% infection, which increased from this level to 52% in years 8 to 10 after planting. This increase was linked to a transition from an unaggregated to an aggregated spatial pattern.

Our data show 6-14% GLRaV-3 incidence more than 25 years after planting. Moreover, aggregation is low in Rioja Alavesa. Compared to others researcher's, our data are suggestive of a very slow rate of field transmission. We used GFLV as a term of comparison. Its nematode vectors are slow-moving and show an aggregated pattern of distribution, the same as the mealybug vectors of leafroll. With an incidence only a little higher than GLRaV-3, GFLV showed a much higher spatial aggregation, which is suggestive of active vector transmission for this virus. As expected, the distributions of the two viruses were independent.

Climate, training system, grape variety or vector abundance might be responsible of this slow leafroll spread. The mentioned galician vineyards, though geographically close, have different varieties and are trained on a trellis under an atlantic climate.

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TEMPORAL BEHAVIOUR OF GRAPEVINES INFECTED BY TYPE II OF VERGILBUNGSKRANKHEIT (BOIS NOIR)

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The visible incidence of grapevine yellows (GY) in vineyards is a result of new and retained infection, but it is also influenced by phenomena such as remission and re-occurrence of symptoms as well as the complete recovery of infected vines. These parameters are likely to depend on grapevine variety and the type of GY. It was the objective of this study to quantify them and to estimate their contribution to the annual levels of disease incidence in two cultivars of different susceptibility. Such information helps to understand epidemiology but is also useful e.g. to estimate the risk of unintentional propagation of latently infected vines or to decide whether affected vineyards should be rogued or not (Osler *et al*., 2003).

Since grapevine infected by phytoplasmas of different groups or strains might behave differently, we restricted our study to type II of Vergilbungskrankheit (VK; =Bois noir) (Langer & Maixner, 2004). A vineyard was planted in 1995 with 609 vines of the highly susceptible cultivar Riesling (RI) and 822 vines of cv. Pinot noir (PN) that is only moderately susceptible. It is situated in an area of the Middle-Rhine valley where only VK type II occurred so far. Each vine was examined for visible symptoms of GY every year in September from 1996 to 2005. The planting of the vineyard fell in a period of an epidemic outbreak of VK in the whole region causing high incidence (RI: 50%; PN: 14%) within two years (table 1). The situation calmed down thereafter during an endemic phase with slowly decreasing incidence. Therefore, the data were analyzed for the entire period, but also separately for the epidemic (1996-1999) and the endemic phase (2000-2005).

A total of 82% of Riesling and 30% of Pinot noir vines showed symptoms of VK for at least one year, although the maximum yearly incidence was only 50% and 14%, respectively. The yearly incidence was 4 to 10 times higher in Riesling than in Pinot noir. On average, Riesling vines showed symptoms for 2.9 years compared to 1.6 years in Pinot noir. The annual rates of new infection in Pinot noir varied between a third and a sixth of those in Riesling. If we assume a constant inoculum pressure throughout the vineyard, the rates of new infection should represent the different inoculation efficiency for the two cultivars.

Remission of symptoms is a common feature of GY. In order to distinguish between temporary latent infection and permanent recovery, the frequency of symptom expression was calculated as a function of the number of consecutive asymptomatic years (Fig. 1). It exceeded the average incidence (100%) in the groups of vines that were symptomatic in the previous year or showed only one year of symptom remission. Longer gaps led to rates that were significantly less then average and similar to those of vines that were newly infected. Therefore, vines with a minimum asymptomatic period of three years were considered as completely recovered. This decision is supported by results of Osler *et al*. (2003) who found such vines to be PCR negative. Reinert & Maixner (1999) reported that most cuttings from VK infected shoots developed symptoms in the first year. A few developed symptoms in the second year but never later. It is therefore not likely that outbreaks of BN in young vineyards that often occur within two and five years after planting could be due to infected planting material. They are more likely the result of the favourable environmental conditions in young vineyards that attract the vectoring planthopper *Hyalesthes obsoletus*.

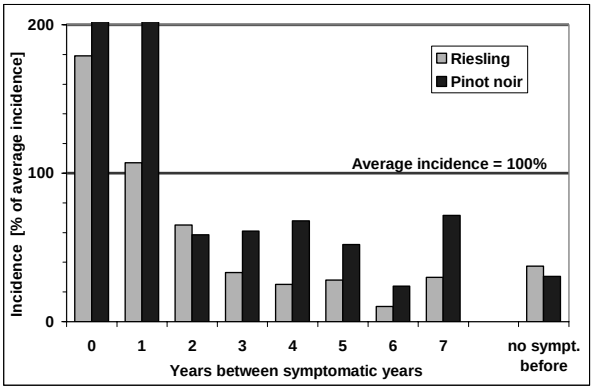

Fig. 1: Influence of consecutive asymptomatic years on the frequency of symptom expression

Recovery occurred in 18% of symptomatic Riesling and 53% of Pinot noir vines with no differences between the epidemic and the endemic period. Pruning is likely to support recovery. It could eliminate inoculum as long as it is limited to parts of the plant which is a common feature of BN. Recovery indeed

Table 1. Quantification of parameters that contribute to the visible incidence of VK in two different cultivars.

Parameter		Fig.1	Riesling						Pinot noir					
			1996 - 2005		1996 - 1999		2000 - 2005		1996 - 2005		1996 - 1999		2000 - 2005	
			Average %	Range %	Average %	Range %	Average %	Range %	Average %	Range %	Average %	Range %	Average %	Range %
Incidence		b	34	12 - 50	33	12 - 50	33	21 - 44	6	2 - 14	9	2 - 14	4	2 - 6
	yearly values		12 - 50 - 32 - 39 - 38 - 44 - 32 - 32 - 30 - 21						2 - 14 - 13 - 7 - 6 - 6 - 4 - 3 - 3 - 2					
New infection		2	14.8	4 - 47	24.3	12 - 47	8.4	4 - 16	3.5	2 - 14	6.8	2 - 14	1.3	1 - 2
Retained symptoms		3	61	45 - 76	60	45 - 76	62	57 - 70	40	22 - 67	38	31 - 48	42	22 - 67
Remission		4	39	24 - 54	40	24 - 54	38[a]	30 - 48	60	33 - 78	62	53 - 69	58[a]	33 - 78
Recovery	% of remission	5	50	33 - 64	44	33 - 59	58[b]	50 - 64	84	76 - 90	84	78 - 90	84[b]	76 - 90
	% of symtpomatic		18	13 - 23	17	13 - 23	20[b]	19-21	53	35 - 69	53	41 - 61	53[b]	35 - 69
Re-occurrence	% of remission	6	50	36 - 67	56	41 - 67	42[b]	36 - 50	17	11 - 32	18	12 - 32	16[b]	12 - 21
	% of symtpomatic		20	10 - 31	23	10 - 31	15[b]	11 - 21	11	4 - 20	11	7 - 20	10[b]	4 - 20
Re - infection		7	-[c]	-	-[c]	-	9.4	5 - 16	-[c]	-	-[c]	-	1.4	0 - 2

[a] Data from 2000-2004; Remission of 2005 unknown yet

[b] Data from 2000-2002; Recovery and re-occurrence cannot be distinguished later on

[d] No recovered vines for re-infection were available before 2000

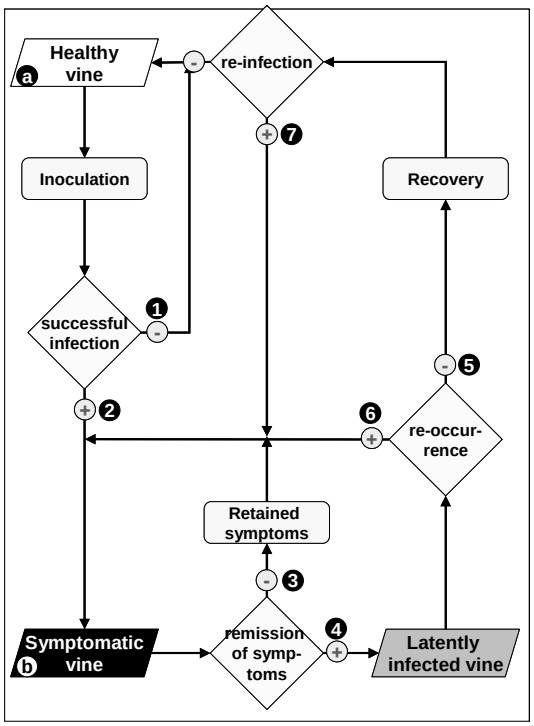

Fig. 2: Flowchart explaining visible incidence of VK as a dynamic equilibrium. Characters and numbers refer to table 1. (a: 609 vines RI; 822 vines PN)

decreases with increasing consecutive periods of symptom expression. For example, 21% of Riesling vines showing symptoms for up to two years recovered (PN: 52%) compared to only 7% (PN: 40%) of those with 5 or 6 continuous symptomatic years.

Proportions of 15% (RI) and 1.5% (PN) of all symptomatic vines exhibited symptoms for more than five years. They probably represent the 'baseline incidence' during endemic periods of BN. Replanting of such vines could be advisable to decrease disease incidence as long as infection pressure is low. If replanting of diseased vines is considered it should be taken into account that young vines have a significantly higher risk to develop chronic infection. 22% of up to three year old Riesling vines remained diseased for at least four years compared to only 13% of older vines. Young vines might develop a systemic infection more easily due to their smaller size.

The fluctuating incidence of VK is the result of a dynamic equilibrium (Fig. 2) which is influenced by antagonistic parameters such as new and retained infection, remission and reoccurrence of symptoms but also complete recovery. Only the level of infection pressure expressed as the frequency of new infections varied considerably during the observation, causing an epidemic outbreak of the disease. The years thereafter were characterized by only a low intensity of new infection and a constant rate of recovery resulting in decreasing incidence. The phenomenon of occasional sudden outbreaks followed by calm periods is typical for VK, but the reasons for the changing infection pressure are not yet clear. Moderate fluctuations during the endemic period are probably due to cultural and environmental influence on the phenomena of remission, re-occurrence and recovery. There is no evidence of any kind of "immunity" of previously infected vines since the proportions of new infection and re-infection did not differ.

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THE DECLINE OF "TEROLDEGO" GRAPEVINES IN TRENTINA (N.EAST ITALY)

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(Not received)

AGRONOMICAL AND ENOLOGICAL PERFORMANCES OF HEAT-TREATED VS. GFLV-INFECTED "GEWÜRZTRAMINER" CLONES IN TRENTINO (N.EAST ITALY)

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Introduction

Grapevine fanleaf virus (GFLV) is the most widespread nepovirus involved in the grapevine degeneration. The genetic variability within a cultivar is the basis of clonal distinction, but the virus infections may have great practical implication on phenotypical variability. The clonal selection carried out at IASMAA confirmed interesting differences, as for agronomical performances and analytical and sensorial profiles of wines, among "Gewürztraminer" clones (Malossini et al., 2002). Only a few clones (both healthy and affected by viruses) showed always varietal typicality. It was confirmed that the profile of bound forms of certain aroma compounds (e.g. geraniol and linalool) well discriminated the *aromatic* and *neutral* genotypes. The present study upgrades a previous communication (Malossini et al., 2003) and deals with the differences in agronomical and oenological performances between GFLV-infected (MP) and heat-treated healty (HT) progenies of two "Gewürztraminer" clones.

Materials and methods

In 1997, the originally GFLV-infected clones 920 and 921, respectively *aromatic* and *neutral*, were heat-treated according to *in vitro* thermo-therapy. Daughter vines of both the original (MP) and heat treated (HT) clones were tested by ELISA for GFLV and others viruses using commercial kits (Agritest, Valenzano-Bari, Italy). *Ex-vitro* MP and HT materials were green-grafted onto GLFV-free rootstocks (Kober 5BB, S.O.4). Three experimental vineyards were planted: one on the plain (year 2002, 210 m a.s.l., Guyot, San Michele a/A) and two on the hill (year 2002, 250 m a.s.l., simple pergola trained, Faedo), with at least of 10 plants for each MP and HT clone in each vineyard. ELISA tests were carried out on original (MP) and heat-treated (HT) vines in eight years (1998-2005). In 4 years (2002-2005), leaves from MP and HT plants were sampled at veraison and at harvest. Fresh weight and length of blades and petioles were measured. Bud fertility was measured in the three vineyards in 3 years (2003-2005). At harvest (2002-2005) grape yield and juice composition (Brix degree, total acidity and pH) after grape crushing, short skin-contact, pectolitic enzyme addition and overnight cool-settling of the juice were recorded for each plot. Free and bound (as aglycons) monoterpene aroma compounds have been quantified in the juice at June 2005, after frozen storage, by HRGC-MS according to bibliography (Versini et al., 1988).

Results and discussion

On the basis of 751 samples ELISA-tested, all the HT progeny of the two clones proved to be GLFV-free, while all (No. 39) MP samples confirmed to be infected.
HT vines had significantly longer petiols (mean value HT: 79 mm; MP: : 67 mm, p>0.000) and blade veins (mean value HT: 90 mm; MP: : 84 mm, p>0.000). **(Figg. 1-2).**
GLFV elimination induced a strong modification of vine production and bunch characteristics **(Table 1)**
In particular, significant increases were measured for bud fertility, both real and potential, and grape yield *per* vine due to the higher average weight of bunch, berry and stem. On the contrary, HT vines had a significantly lesser number of total buds *per* plant and *per* shoot, as a consequence of internode's lengthening. No significant differences were measured for the parameters related with plant vigour (Ravaz's Index, wood yield) and for the basic qualitative characteristics of the juices.
In the light of the available data, a certain and significant change in the aroma composition due to sanitation was not observed, while the difference among clones was confirmed (**Table 2)**
In conclusion, GFLV elimination from "Gewürztraminer" clones grown in a cool climate area resulted in lengthening of shoots and leaves, and increasing of fertility and yield. This happened without penalization in the quality characteristics of juices.

Acknowledgements:
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Fig. 1: lenght of leaf (at veraison)

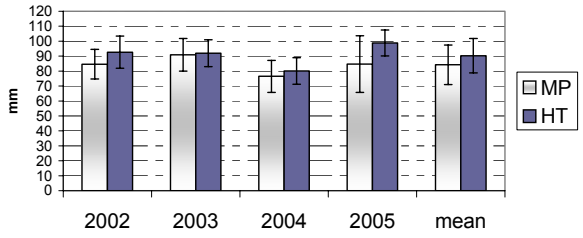
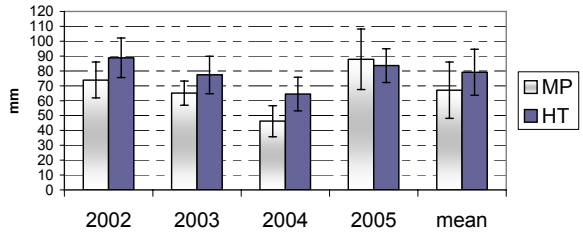


Fig. 2: lenght of petiol (at veraison)



Figg. 1 and 2: measures of leaves sampled on GLFV-infected vines (MP) in comparison with these on GLFV-free vines (HT) of two Gewürztraminer clones. (Vineyard on plain, Guyot, S.Michele a/A).

Table 1. Mean values ± std. dev. of some vine's and bunch's characteristics of two Gewuerztraminer clones (3 years:2003/2005 - 3 vineyards): MP (+GLFV) vs HT (GLFV-free)

Vine	**MP**	**HT**	statistic significance
No. of buds / shoot	13,6 ± 3,2	12,4 ± 2,8	* *
No. of flower cluster [A]	20,7 ± 12,9	21,0 ± 13,0	n.s.
No. of total buds [B]	20,8 ± 9,1	18,2 ± 9,1	*
No. of break buds [C]	15,7 ± 7,4	14,1 ± 6,9	n.s.
real fertility [A/B]	0,99 ± 0,37	1,16 ± 0,37	* *
potential fertility [A/C]	1,27 ± 0,38	1,44 ± 0,37	* *
bud burst (%)	75,9 ± 12,5	79,6 ± 12,9	*
grape yield (Kg) [1^]	1,50 ± 0,87	2,99 ± 1,68	* *
weight cluster (g)	73 ± 15	116 ± 25	* * *
wood yield (Kg) [2^]	0,43 ± 0,22	0,57 ± 0,29	n.s.
index of Ravaz [1^/2^]	6,1 ± 4,1	6,3 ± 2,4	n.s.
Bunch			
weight of bunch (g)	86,4 ± 14,1	134,2 ± 27,3	* * *
weight of berry (g)	1,21 ± 0,18	1,36 ± 0,19	*
weight of stem (g)	4,8 ± 1,2	6,3 ± 1,4	* *
% stem/ cluster (g / g)	5,6 ± 1,5	4,8 ± 1,1	n.s.
% must/ cluster (mL / g)	57,4 ± 9,6	63,0 ± 10,5	n.s.
Brix degrees	21,59 ± 1,71	21,88 ± 1,57	n.s.
total acidity (g/L)	4,40 ± 1,86	4,30 ± 1,90	n.s.
pH	3,57 ± 0,35	3,65 ± 0,34	n.s.

'Note: (n.s.) not significance (*) significance between 95 and 99% (**) significance of 99% or superior from ANOVA

Table 2 : Aroma compounds characterising Gewürztraminer juice (vineyard S.Michele, 3 years)

year	*2002*				*2003*				*2004*			
clone	*920*		*921*		*920*		*921*		*920*		*921*	
material	*MP*	*HT*	*MP*	*HT*	*MP*	*HT*	*MP*	*HT*	*MP*	*HT*	*MP*	*HT*
Compounds as free form (ug/L n-heptanol)												
linalool	0,9	1,1	<0,5	<0,5	1,5	2,2	< 0,5	< 0,5	< 0,5	< 0,5	< 0,5	< 0,5
citronellol	3,6	7,4	<0,5	<0,5	6,3	3,3	< 0,5	< 0,5	4,1	1,3	< 0,5	< 0,5
nerol	42,5	90	4,3	1,0	50	49	3,3	1,3	12	6,0	1,3	1,6
geraniol	137,5	260	16,5	3,3	136	174	10	6,9	77	44	3,2	3,1
Compounds as aglycons from bound forms (ug/L n-heptanol)												
linalool	5,2	13,5	1,2	<0,5	3,8	0,5	< 0,5	< 0,5	0,7	2,1	< 0,5	< 0,5
citronellol	18	3,5	2,1	0,6	19	5,3	0,7	0,6	14	11	< 0,5	< 0,5
nerol	130	297	19,5	3,9	159	48	6,6	5,5	76	66	2,5	2,3
geraniol	525	782,5	73,5	27	443	199	29	22	279	291	10	8,7

by HRGC-MS according to bibliography (Versini et al., 1988).

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PHYSIOLOGICAL AND AGRONOMIC MODIFICATIONS INDUCED BY PHLOEM-LIMITED VIRUS ERADICATION IN A CLONE OF ALBAROLA (*VITIS VINIFERA* L.)

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Introduction

Albarola is a white cultivar grown in the Eastern side of Mediterranean coastal Region of Liguria (North-West Italy). The climatic conditions of the area often induce summer water stress conditions in the vineyards. In addition to a difficult environment, another reason for the unsatisfying performances of the old vineyards of the area is the strong diffusion of viral diseases. Phloem-limited viruses such *Ampelovirus* (GLRaV-1, GLRaV-3) and *Vitivirus* (GVA, GVB), causal agent of leafroll and rugose wood diseases respectively, are particularly common in those vineyards. Recent studies have shown important negative modification on vine vegetative vigour and yield due to mixed infection of GLRaV-1, GLRaV-3 and GVA (Mannini, 2003), however little information under experimental conditions is still available on vine physiological response consequent to the presence of the pathogens. The present report deals with the effect induced by phloem-limited viruses on some important canopy parameters and physiological aptitudes of an Albarola clone grown in summer water stress climatic conditions and its consequences on crop quality and quantity.

Materials and Methods

A clone of the cultivar Albarola, formerly infected by a mixed infection of GLRaV-1, GLRaV-3 and GVA, was sanitated by means of meristem culture (Gribaudo *et al*., 2002). A trial was carried out over three years (2002-04) comparing the behaviour of infected vs. healthy plants of the clone grown in the warm environment of Liguria Region. The main agronomic performances (pruning weight, shoot fertility, yield, cluster number, cluster weight) and juice composition (s. solids, titratable acidity, pH, malic and tartaric acids) were assessed as well as a series of vine parameters to define canopy radiation microclimate (leaf contacts with point quadrat technique, total leaf area, leaf surface area, % light inside canopy). In addition leaf stomatal conductance, photosynthetic and transpiration rates were measured in open field by means of a ADC-LC3 gas analyser equipped by PLC in three different dates during growing season of 2003 and 2004.

Results and Discussion

The elimination of phloem-limited viruses resulted in a wider canopy (table 1, 2 and fig. 1). Total leaf area (LA) and exposed leaf area (SA) were in favour of healthy vines in both considered years (2003-04) allowing the canopy to performed higher leaf photosynthesis (P_n, fig. 2a) over the summer. The significant improvement of P_n,, however, was observed only in basal leaves, while in apical (i.e. younger) leaves the effect of virus eradication were not always detectable (data not shown). Stomatal conductance (g_s, fig. 2b) did not decrease in parallel to P_n, suggesting that P_n limitation could not be ascribed to stomatal limitation, but due to probable metabolic depletion progressing with leaf age.

The improved canopy efficiency of healthy vines resulted in a better degree of grape ripening as shown by juice higher soluble solids % and lower titratable acidity despite a similar amount of yield compared to infected plants (table 1). In terms of crop, in fact, the higher fertility of healthy vines was compensated by smaller clusters whereas the opposite happened in infected plants.

The results confirm the stress due to virus infection in grapevine and the positive response of the plants to virus eradication.

Table 1. Agronomic and qualitative characters of virus-infected and healthy plants of the same clone of Albarola (averages 2002-2004).

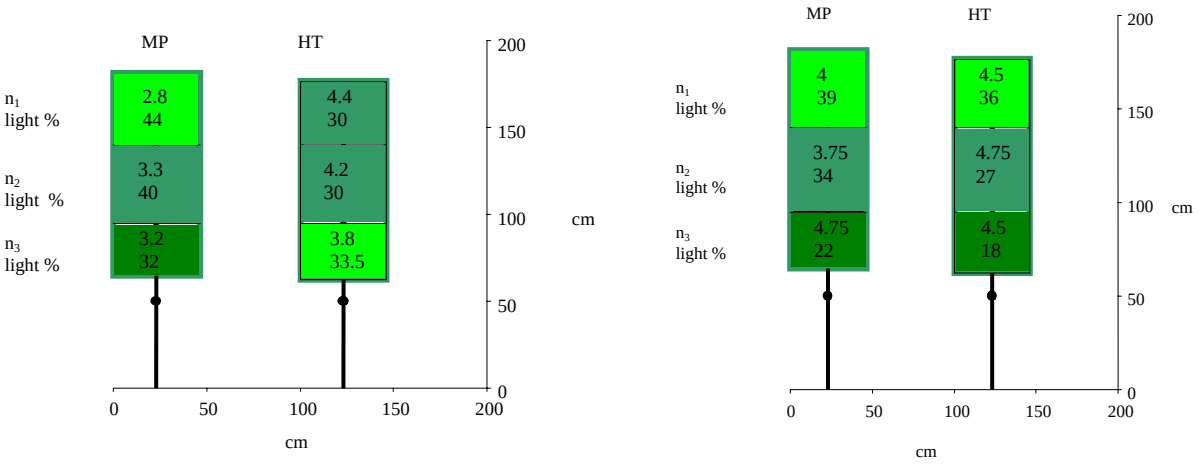
Data	Pruning wt (g/vine)	Fertility n.°inf./shoot	N.° of cluster/vine	Yield kg/vine	Cluster wt (g)	S. solids (%)	T. acidity (g/L)	Tartaric a. (g/L)	Malic a. (g/L)	pH
Infected	335	1.46	13.7	3.97	286	16.0	6.45	4.71	1.91	3.33
Healthy	401	1.64	14.0	3.80	243	17.0	5.76	4.12	1.65	3.39
Sign. F	*	*	n.s.	n.s.	n.s.	*	*	*	*	*

Table 2. Leaf area (LA), Surface area (SA) and shadow index (LA/SA) measured for 2003 and 2004 seasons on both healthy and infected plants.

2003	LA $m^2\ m^{-1}$	SA $m^2\ m^{-1}$	LA/SA
Healthy	2.7	1.7	1.6
Infected	2.4	1.2	2.0

2004	LA $m^2\ m^{-1}$	SA $m^2\ m^{-1}$	LA/SA
Healthy	4.0 (±0.03)	2.3 (±0.18)	1.7
Infected	3.3 (±0.03)	2.1 (±0.18)	1.5

Fig. 1. Graphs representing scale canopy dimensions, leaf contacts (n) and % of light inside the according to upper (n_1), intermediate (n_2) and lower (n_3) part of the canopy for 2003 (left) and 2004 (right) seasons on both healthy (HT) and infected plants (MP).



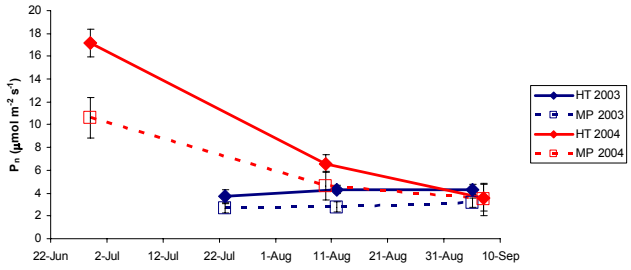


Fig. 2a. Net photosynthesis (Pn) measured for 2003 and 2004 in healthy (HT) and infected (MP) basal leaves.

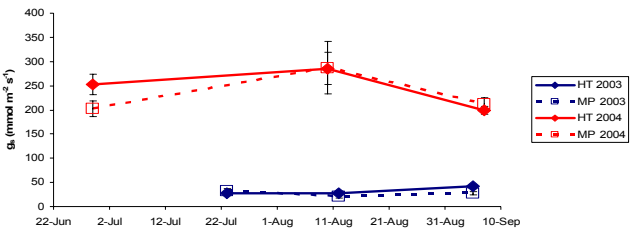


Fig. 2b. Stomatal conductance (gs) measured for 2003 and 2004 in healthy (HT) and infected (MP) basal leaves.

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STUDY OF THE RELATIONSHIP BETWEEN *GRAPEVINE RUPESTRIS STEM PITTING ASSOCIATED VIRUS* MOLECULAR VARIANTS AND VEIN NECROSIS

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Introduction

Grapevine rupestris stem pitting associated virus (GRSPaV), member of the genus *Foveavirus* (Martelli & Jelkmann., 1998) within the family *Flexiviridae* is the putative causal agent of rupestris stem pitting (RSP) (Meng *et al*., 1998; Zhang *et al*., 1998) a component of the rugose wood disease of grapevine. Genome analysis of GRSPaV suggests the occurrence of molecular variants, clustering in two or more groups according to the studied part of the genome (Rowhani *et al*., 2000; Nolasco *et al*., 2005). The study of the pathological role of those groups has never led to clear cut evidence of their association to RSP, due to their occurrence in mixed infections, to the complexity of biological indexing and to the presence of latent GRSPaV infections in the *V. rupestris* sources widely used for routine indexing trials. Recently, a strikingly high association has been detected between GRSPaV and vein necrosis (VN), suggesting that 110 Richter (110 R) is the best indicator of GRSPaV infection (Bouyahia *et al*., 2005). In the present study, we investigate the occurrence of molecular variants of GRSPaV in grapevine accessions previously indexed for vein necrosis on 110 R.

Materials and Methods

Object of the study were 26 Tuscany clonal accessions of 4 wine varieties of *V.vinifera* (Prugnolo gentile, Colorino, Sangiovese and Canaiolo), 3 mother plants of the biological indicator Kober 5BB (*V.berlandieri* x *V.riparia*) and 5 mother plants of the *V.rupestris* cv. St George indicator. All the above accessions previously indexed for major grapevine diseases and for vein necrosis on 110 R (Triolo & Materazzi., 2000) were tested for the presence of GRSPaV by RT-PCR. Total nucleic acid (TNAs) was extracted from cortical scrapings of mature canes collected in late autumn 2004 or from leaf petioles (3 basal leaves/sample/accession) collected twice, June and July 2005. After random primer cDNA synthesis, PCR was done using primers 13 and 14 designed in the helicase-like domain of ORF-1 and able to detect a wide range of variants (Meng *et al*., 1999). In order to study the genetic variability, PCR was done using three different sets of primers provided by Dr. Rowhani (University of California, Davis, USA) designed in the coat protein region and able to distinguish three different groups of virus sequence variants: group I, group II and group III.

Results and Discussion

Twenty two out of 26 (84,6%) *V.vinifera* accessions induced VN symptoms in 110 R. 4 *V.vinifera* accessions and 8 mother plants of the indicators Kober 5BB and *V.rupestris* St.George were negative to VN and to GRSPaV (Table 1). 100% of the VN infected accessions results positive to GRSPaV by RT-PCR using primers 13/14. When leaf petioles were used, amplification was obtained from 4 accessions. No changes in the virus titer was observed, repeating the test one month later (July 2005) did not improve the efficiency of RT-PCR and the same 4 accessions were the only ones which give positive reaction. Using group specific primers, group I was detected in all the 22 accessions infected with VN, 11 times in mix infection with group III, 3 times with group II, and twice as a single infection. Unexpected results were observed with group II, in fact this group is present in 6 accessions negative to VN and to GRSPaV with primers 13/14. This work confirms the association between GRSPaV and vein necrosis. Cortical scrapings from mature canes of *V.vinifera* seem to be the best tissues for the diagnosis of GRSPaV. The results obtained with the “strain” specific primers suggest that group I is likely to be the predominant variant among GRSPaV viral population and is closely related to VN. No single infection was, however, recorded for group III, the only available indication is its exclusive presence in VN infected accessions. The presence of group II in both symptomatic and symptomless plants is intriguing, serological and molecular characterizations are underway to obtain more information about the nature and pathological role of this group.

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Table 1. Results obtained by biological indexing and RT-PCR by universal and group specific primers

N°	Accessions	Bioindex Vein necrosis	RT-PCR				
			Leaf petioles	Cortical scrapings			
			13 and 14	13 and 14	Group I	Group II	Group III
1	*V.rupestris* 5V-20-1	-	-	-	-	-	-
2	*V.rupestris* 5V-20-2	-	-	-	-	-	-
3	*V.rupestris* 5V-20-3	-	-	-	-	-	-
4	*V.rupestris* 5V-20-4	-	-	-	-	-	-
5	*V.rupestris* 5V-20-5	-	-	-	-	-	-
6	Sangiovese-CC30	-	-	-	-	-	-
7	Prugnolo gentile-MP	-	-	-	-	+	-
8	Colorino-5/1	-	-	-	-	+	-
9	Sangiovese.grosso-SMH 22	-	-	-	-	+	-
10	Kober 5BB-BSK3-1	-	-	-	-	+	-
11	Kober 5BB-BSK3-2	-	-	-	-	+	-
12	Kober 5BB-BSK3-3	-	-	-	-	+	-
13	Prugnolo gentile-FA	+	-	+	+	+	-
14	Prugnolo gentile-KA	+	-	+	+	+	-
15	Sangiovese-CC26	+	-	+	+	+	-
16	Sangiovese-CC6	+	-	+	+	+	+
17	Sangiovese.grosso-SMF 13	+	-	+	+	+	+
18	Colorino-CC25	+	-	+	+	+	+
19	Sangiovese.grosso-SM 6/10	+	+	+	+	+	+
20	Sangiovese.grosso-SME 9	+	+	+	+	+	+
21	Prugnolo gentile-CA	+	+	+	+	+	+
22	Prugnolo gentile-WA	+	+	+	+	-	+
23	Prugnolo gentile-YA	+	-	+	+	-	+
24	Prugnolo gentile-RA	+	-	+	+	-	+
25	Sangiovese-CC19	+	-	+	+	-	+
26	Sangiovese-CC10	+	-	+	+	-	+
27	Colorino-CC24	+	-	+	+	-	+
28	Sangiovese-CC13	+	-	+	+	-	+
29	Canaiolo-CC4	+	-	+	+	-	+
30	Canaiolo-CC5	+	-	+	+	-	+
31	Canaiolo-CC29	+	-	+	+	-	+
32	Canaiolo-CC21	+	-	+	+	-	+
33	Sangiovese-CC8	+	-	+	+	-	-
34	Canaiolo-CC17	+	-	+	+	-	-

PRELIMINARY RESULTS SHOW IRREGULAR DISTRIBUTION OF *RASPBERRY BUSHY DWARF VIRUS* IN INFECTED GRAPEVINES

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Introduction

Grapevine is an important crop in Slovenian agriculture. Almost 58% of land under permanent crops is planted with grapevine, mostly for wine production (Statistical office of the Republik of Slovenia, 2004). In 2002 *Raspberry bushy dwarf virus* (RBDV) was identified on grapevine in Slovenia (Mavrič *et al*., 2003). This was the first report of natural infection of non-*Rubus* host with RBDV. Attempts were made to gather more information about RBDV infection of grapevine. Here we report about results that suggest irregular distribution of RBDV in infected grapevine plants of cv. Italian Riesling (Laški Rizling).

Material and Methods

A vineyard of cv. Italian Riesling located in north-eastern part of Slovenia was found to be infected with RBDV. 100 plants were tested for RBDV in autumn 2003 by DAS-ELISA using commercial reagents (Loewe Biochemica). 17 RBDV negative and 21 RBDV infected grapevines were selected for grafting on virus-free rootstock. 138 produced grafts (57 from negative and 81 from positive scions) were sampled in October 2004 and tested for the RBDV infection as before.

Results and Discussion

The results are presented in Table 1. The majority of tested grafts was found to be infected with RBDV or were RBDV-free as expected from the testing of mother plants. 31 grafts produced from 9 negative mother plants all tested negative and 14 grafts produced from 5 negative mother plants tested positive for RBDV. 3 negative mother plants gave 4 RBDV positive grafts and 8 RBDV negative grafts. Similar situation was observed on grafts produced from infected mother plants. 66 grafts from 17 mother plants tested positive, 3 grafts from 2 mother plants all tested negative. 12 grafts from 2 mother plants tested partly positive (4 grafts) and partly negative (8 grafts).

We believe that these results indicate an irregular distribution of RBDV either in mother plants used for grafting or in obtained grafts. Further analyses are still needed to clear the observed situation. 100 plants including all plants used as mother plants in this grafting are tested repeatedly every year at least once a year. The obtained grafts will be planted outside in next season and the testing for RBDV presence will be continued during their growth period.

Table 1: Testing of grapevine grafts for RBDV

Number of mother plants	Number of grafts
9 negative	31 negative
5 negative	14 positive
3 negative	4 positive / 8 negative
17 positive	66 positive
2 positive	3 negative
2 positive	4 positive / 8 negative

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CUSCUTA SPECIES AS VECTOR FOR TRANSMITTING *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS* 7 TO HERBACEOUS PLANT HOSTS

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Sequence analysis of plant viruses requires high amounts of purified double-stranded RNA for efficient modification and cloning of the genetic material. Thus constant virus supply is indispensable. For dsRNA extractions of grapevine leafroll-associated closterovirues phloem scraping is time-consuming and did not always lead to satisfactory results. Therefore, a virus source from which the nucleic acid material is easy to extract and available in high quantity is desired.

We chose *grapevine leafroll-associated virus* 7 (GLRaV-7; Y15987) for transmission experiments as availability of high amounts of dsRNA was required. GLRaV-7 could not yet be allocated to any genera of the family *Closteroviridae* [1]. Within the three genera of this family, only the genus *Closterovirus* contains few species that are transmissible by mechanical inoculation, though with difficulty, whereas none of the species of the genera *Ampelovirus* and *Crinivirus* can be [1].

There is no indication for the transmissibility of the unassigned GLRaV-7 and the nature of a vector. Successful transmission of grapevine leafroll associated agent(s) to healthy herbaceous plants like Nicotiana species are available [2]. Due to this information we carried out mechanical inoculation of GLRaV-7 infected sap from grapevines to Nicotiana species. Since these experiments did not lead to infection of the herbaceous plants alternative transmission pathways were necessary.

Publications report on investigations of the parasitic dodder *Cuscuta spp*. as vector to transmit plant viruses with partly positive results [3-5]. Cuscuta has little to none photosynthetic activity and draws most of its nutrients from the host. No general relationship between the virus and the vector could be found concerning successful virus transmission. The vector efficiency depended strongly on the season when the transmission took place. The degree of inhibitory dodder compounds play an important role by influencing the virus-vector interaction [6].

Earlier experiments of grapevine leafroll transmission by dodder was successful from grapevine to grapevine, whereas herbaceous receptor plants remained healthy [7]. These previous investigations did not differentiate between grapevine leafroll associated virus species, thus no conclusion about the transmitted virus species was possible.

In our experimental design we chose three different dodder species, *Cuscuta campestris* (CC), *Cuscuta europea* (CE) and C*uscuta reflexa* (CR), as vector and *Nicotiana occidentalis* as receptor for the GLRaV-7 transmission experiments. GLRaV-7 infected grapevines were scion propagated. Positively tested material (RT-PCR) was used for virus donation. Each Cuscuta species acted as “phloem bridge” between the GLRaV-7 infected grapevine and the tobacco plant for at least two month in the greenhouse. We carried out RT-PCR on total nucleic acid (TNA) extracts and on double-stranded RNA (dsRNA) extracts from Nicotiana plants as well as from Cuscuta. The virus was detectable in CC and CR but not in CE and the whole group of Nicotiana receptor plants.

DsRNA extractions from CC and CR show strong visible bands in agarose gel electrophoresis, corresponding to high molecular weight virus genomic and to subgenomic RNAs. These results suggest replication of the virus in dodder.

So far, we were able to establish GLRaV-7 in CR and CC cultivated on *Urtica dioica* for almost one year with constant yield of dsRNA. CR turned out to be the most suitable vector due to its nonbudding appearance under greenhouse conditions. CE and CC senesce naturally following flowering and seed production and are not suitable for further use. The two species could be maintained by constant vegetative propagation, however, with difficulty.

Extraction of dsRNA is less difficult from Cuscuta compared to grapes, however, dodder has disadvantages. DsRNA extracts from dodder contained compounds inhibiting RNA and DNA modifying

enzymes. Therefore, host range transmission studies were conducted and resulted in infection of *Tetragonia expansa* (New Zealand spinach, *Aizoaceae*) with GLRaV-7.
The receptor plants remained symptomless.

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ETIOLOGY OF RUGOSE WOOD DISEASE IN JAPANESE GRAPEVINES

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Introduction

Rugose wood (RW) of grapevine is a complex of graft-transmissible diseases characterized by corky bark, stem grooving, and stem pitting. These diseases are responsible for graft incompatibility, delayed budburst, severe decline, and even death of vines. Although RW is one of the most widespread graft-transmissible diseases of grapevines, the etiology of RW in Japanese grapevines has not been determined. Recently, reverse transcription-polymerase chain reaction (RT-PCR) diagnosis revealed various viruses in Japanese table grape cultivars (Nakaune et al., 2003). In this study, we survey viruses associated with RW in Japanese table grape cultivars.

Materials and methods

Vitis labruscana 'Pione' and 'Kyoho,' important table grape cultivars in Japan, were obtained from vineyards in Hiroshima and Okayama prefectures. Fifty-seven grapevines with rugose wood (RW) symptoms, stem pitting and corky bark, and 31 grapevines without symptoms were tested by RT-PCR. Plant extracts and RT-PCR were prepared according to the previous study (Dovas et al., 2003; Nakaune et al., 2003). All PCR products were sequenced. The sequences were compared for similarity against the sequence database of the DNA Data Bank of Japan (DDBJ) (National Institute of Genetics, Shizuoka, Japan) using the BLAST program.

Results and discussion

Figure 1 shows the typical symptom of stem pitting on a trunk of Japanese table grape cultivar Pione. RSPaV was detected from all 57 grapevines with RW (Table 1). Forty-one grapevines of the 57 grapevines were infected with RSPaV alone. On the contrary, RSPaV was not detected from 22 of 31 grapevines without symptoms (Table 1). These results suggest that RSPaV is closely associated with RW diseases in Japanese cultivars Pione and Kyoho. Some of the grapevines were infected by *Grapevine leafroll-associated virus* (GLRaV)-1, GLRaV-2, GLRaV-3, *Grapevine virus B* (GVB), *Grapevine fleck virus* (GFkV), or *Grapevine asteroid mosaic-associated virus* (GAMaV) (Table 1). However, these viruses are not necessarily associated with stem pitting.

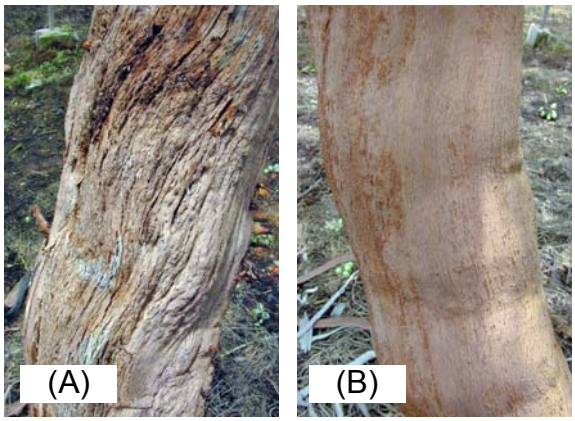

Figure 1. RW in Japanese cultivar Pione. (A) Stem pitting symptoms on Pione infected with RSPaV. (B) Pione with no virus.

Recently, sequencing analysis of RSPaV isolates revealed that RSPaV was comprised of many sequence variants. RSPaV isolates grouped into three or four different clusters based on the phylogenetic analysis of nucleotide sequences of RT-PCR fragments (Casati et al., 2003; Meng et al., 1999; Rowhani

et al., 2000; Terlizzi et al., 2003; Santos et al., 2003). Sequences, amplified with primers RSP-up1 (5'-TGAGATGGTYGCTAATATCG-3') and RSP-do2 (5'-CTATTAGTACGGTATTCCAG-3'), that encode the 3′ region including part of the CP gene, were separated into at least four groups. Nucleotide sequences of tentative group 1, group 2, group 3, and group 4 showed approximately 83%, 84%, 92%, and 93% identity with the sequence from the database (accession number: AF057136), respectively.

Table 1. Results of RT-PCR detection of grapevine viruses on 88 grapevines with or without (w/o) RW

No. of plants tested		No. of plants positive for virus						
		RSPaV	GLRaV-1	GLRaV-2	GLRaV-3	GVB	GFkK	GAMaV
Grapevines with RW	57	57	1	9	6	3	11	0
Grapevines w/o RW	31	9	0	0	4	1	0	1

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GENERATION OF A SCFV ANTIBODY WITH HIGH SPECIFICITY TO VIRUSES OF THE FAMILY *CLOSTEROVIRIDAE*

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Pathogen-specific recombinant antibodies (rAbs) expressed ectopically in plant cells are an alternative approach to affect pathogen infectivity and to engineer resistance in crops (Nölke *et al*. 2004). However, successful use of antibodies to generate plant pathogen resistance relies on appropriate target selection, careful antibody design, efficient antibody expression, stability and targeting to appropriate cellular compartments.

We have chosen the coat protein (CP) of *Grapevine leafroll-associated virus 3* (GLRaV-3) as a target for generation of specific rAbs that could interfere with the virus life cycle and confer resistance to the host. The single chain antibody fragment (scFvLR3cp-1) specific to the CP of GLRaV-3 was selected by phage display technology. The specific binding of the scFvLR3cp-1 to the surface of the GLRaV-3 particles was shown by electron microscopy.

Surface plasmon resonance analysis indicated that the scFvLR3cp-1 is a high-affinity antibody with a dissociation constant 10^{-8} M. Furthermore, pepscan analysis demonstrated that the scFvLR3cp-1 binds to a linear epitope located between the amino acid 69 and 75 of the GLRaV-3 CP. Transient transformation experiments were performed to determine scFv stability and accumulation levels in the cytosol of tobacco leaves. The functionality of scFvLR3cp-1 in the plant cell cytosol was confirmed by ELISA.

Finally, binding analysis of scFvLR3cp-1 to different viruses of the family *Closteroviridae* revealed that the recombinant antibody also recognizes Grapevine leafroll-associated virus 1 (GLRaV-1), -6 (GLRaV-6), and -7 (GLRaV-7). Therefore, scFvLR3cp-1 can be regarded as a good candidate for mediating broad range virus resistance when produced in transgenic grapevine plants.

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SOME ASPECTS OF THE PRESENCE OF *GRAPEVINE B VITIVIRUS* IN SPAIN: DETECTION OF GVB IN *PLANOCOCCUS CITRI* RISSO

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Introduction

Grapevine B vitivirus is a causal agent of corky bark disease in grapevine belonging to the rugose wood complex (Boscia *et al*., 1993). A survey carried out in Murcia (Spain) revealed the presence of plants showing the characteristics symptoms of corky bark and GVB was found associated to it (Velasco *et al*., 2004). Additional vineyards have been located with symptomatic plants, belonging to different table grape varieties. In addition we have observed an abundant presence of mealybugs, described in the literature as corky bark vectors (Engelbrecht & Kasdorf, 1990; Tane *et al*., 1989; Minafra & Hadidi, 1994; Golino *et al*., 1995; La Notte *et al*., 1997). In this report RT-PCR was used as a diagnostic tool which allowed establishing a causal relation between GVB presence and disease symptoms. Similar analysis was carried out to detect GVB in mealybugs.

Material and Methods

Mealybug individuals of adult females, instar larvae or crawling nymphs were sampled from corky bark symptomatic vines. RNA was isolated from corky bark diseased vines or mealybugs with a modified *Concert Plant RNA Reagent* protocol (Invitrogen). For GVB detection in mealybugs, 1 to 5 individuals were grinded in 100 uL extraction buffer using a hand-operated homogenizer (SIGMA Z35.997-1). For GVB detection in grapevine, plant petioles or bark scrapings were grinded with liquid nitrogen and extraction buffer. RT-PCR was performed with GVB specific primers (H6980; C7439; MacKenzie *et al*., 1997) following standard procedures.

Results and Discussion

Mealybugs tested positive to GVB were taxonomically identified as *Planococcus citri* Risso (*Hemiptera: Pseudococcidae*). RT-PCR test proved to be sensitive enough to detect GVB in single individuals. Every symptomatic plant so tested gave positive to GVB. Moreover, in one of the surveyed vineyards, less than one year old scions grafted over diseased vines (previously tested the year before) resulted positive to GVB. Although mealybugs are inefficient virus vectors due to its reduced mobility and require plant to plant contact to disperse, traditional cultivation of table grape and environmental circumstances in this area are adequate for vector movement because vines are in physical contact. On the other hand, growers are not prevented from this disease and make use of diseased vines for grafting different varieties when a new one is demanded by the market or when the vine starts to decline because of the disease, which results in a high rate of graft abortion. Additionally, a much extended custom is the gift of vines among growers, thereby contributing to disease spread.

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FURTHER EVIDENCE OF THE INVOLVEMENT OF *GRAPEVINE LEAFROLL ASSOCIATED VIRUS 2* IN GRAFT INCOMPATIBILITY

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Introduction

Grapevine leafroll-associated virus 2 type strain (GLRaV-2-T) and its variant GLRaV-2-RG (genus *Closterovirus*, family *Closteroviridae*), have frequently been reported in association with graft-incompatibility (GI) conditions in grapevines. Ultimate evidence of the aetiological involvement of this virus in GI is, however, lacking. Thus, taking advantage of the numerous cases of GI registered the last few years in Apulia (south-east Italy) in vines of different varieties that invariably hosted GLRaV-2-T and/or GLRaV-2-RG a specific study was carried out, as reported hereafter.

Materials and Methods

Diseased plant material: mature canes were collected from GI-affected vines in three different young vineyards of cv. Red Globe (RG-I, RG-II and RG-III), one of cv. Primitivo (PRM), and one of cv. Vermentino (VRM). Samples were taken also from a self-rooted vine of cv Cardinal (CRD) from the varietal collection of the University of Bari. All accessions reacted positively in ELISA when tested with the GLRaV-2-specific monoclonal antibody R19 (Zhou *et al*., 2000). Concentrated extracts from all accessions, except PRM, were mechanically inoculated to *Nicotiana benthamiana*.

RT-PCR using the specific primers RGHSP227V/777C (Rowhani *et al*., 2000) confirmed that RG-I and RG-III were infected by GLRaV-2-RG, while VRM and PRM were amplified by the GLRaV-2-T specific primers LR2-U2/L2 (Abou-Ghanem *et al.*, 1998). RG-II and CRD were amplified by both sets of primers.

Sanitation: meristem tips from infected accessions of cvs Red Globe (RG-III) and Cardinal were grown *in vitro* as described by Bottalico *et al*., (2000, 2003). Acclimatized rooted explants were analysed by ELISA and RT-PCR to assess their sanitary status and two of them (RG-2B and CRD-111) that were negative with both detection techniques, were selected for indexing trials.

GLRaV-2-free plant material: in addition to the above GLRaV-2-free accessions of Red Globe and Cardinal, a virus-free clone of cv. Primitivo (UBA 46H) previously sanitized by heath therapy was chosen. A virus-free clone of cv. Vermentino (CTV 84), kindly provided by Dr. F. Mannini (CNR, Grugliasco) was also included in the trials.

Indexing: a total of nearly 200 plants of virus-free rootstocks belonging to nine different hybrids of *V. berlandieri*, *V. riparia,* and *V. rupestris* which are by far the most popular grape rootstocks in Apulia, were top-grafted in February 2005, in different combinations, with each GI-source and sanitized accessions (Tab. 1). Monthly observations were made for the appearance of GI symptoms. Late in August, the soil around the graft union was removed and roots emerging from scions were excised.

Results and Discussion

Mechanical transmission: successful transmission to *N. benthamiana* was obtained from RG-I, II and III, VRM and CRD extracts. Symptoms were comparable to those described by Abou-Ghanem *et al.* (2000) and, in all cases, infected plants reacted positively in ELISA and RT-PCR to GLRaV-2. No differences in the type and severity of symptoms were observed between the five isolates.

Indexing: no GI symptoms were observed in the grafted vines until the of soil around the graft union was removed (end of August). Soon afterwards, several plants in the group of GI-affected sources (32 of 101) declined quickly and died, while no reaction was apparent in any of the sanitized sources (Tab. 1). No significant differences in behaviour were observed among the three different sources of GI-affected Red Globe (data not shown).

The incidence of GI differed in the different rootstocks, reactions ranging from latency in 157.11 and 420A (both hybrids *V. berlandieri* x *V. riparia*), to severe (7 dead out of 11), or extremely severe (11 dead out of 13) in 1103P (*V. berlandieri* x *V. rupestris*) and Kober 5BB (*V. berlandieri* x *V. riparia*), respectively (Tab.

1). These data are in agreement with the already reported sensitivity of 1103P and Kober 5BB to GI (Uyemoto *et al*., 2001).

No apparent difference was found in the biological behaviour of the two GLRaV-2 variants as both were transmitted mechanically, eliciting the same symptoms in *N. benthamiana,* and induced GI reactions. Lack of GI development in grafting trials with sanitized sources, seems to exclude the genetic origin of GI, thus strongly supporting the notion that GLRaV-2 is the causal agent of this disorder.

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Table 1: Results of graft transmission trials with GLRaV-2-infected accessions and sanitized sources on nine different rootstocks. Numbers in brackets correspond to dead vines.

GLRaV-2 infected accessions

Scion	5BB	140Ru	225Ru	775P	1103P	779P	157/11	420A	34EM	Total
RG	8(6)	7(1)	6(3)	6(4)	8(6)	8(1)	9	4	6(2)	62(23)
PRM	2(2)	1	1	2	2	-	2	-	2	12(2)
VRM	1(1)	1	3(1)	3	1(1)	1	2	2	2(2)	16(5)
CRD	2(2)	-	-	6	-	-	2	1	-	11(2)
Total	13(11)	9(1)	10(4)	17(4)	11(7)	9(1)	15	7	10(4)	101(32)

GLRaV-2 free accessions

Scion	5BB	140Ru	225Ru	775P	1103P	779P	157/11	420A	34EM	Total
RG	7	6	6	2	5	9	5	9	6	65
PRM	2	1	-	-	1	1	-	1	1	7
VRM	2	1	2	2	-	-	1	2	3	13
CRD	2	-	-	4	-	2	1	2	2	13
Total	13	8	8	8	6	12	7	14	12	98

DETECTION OF RUPESTRIS STEM PITTING ASSOCIATED VIRUS IN SEEDLINGS OF VIRUS-INFECTED MATERNAL GRAPEVINE PLANTS

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Introduction

Rupestris stem pitting associated virus (RSPaV) is a species in the genus *Foveavirus* (Martelli and Jelkman, 1998) and the family Flexiviridae. The virion has a positive sense, single stranded, polyadenylated RNA genome of 8.7kb in size and a coat protein of 28kD (Martelli and Jelkman, 1998). The virus has been reported to be present in pollen (Rowhani et al., 2000) and seeds (Stewart and Nassuth, 2001), however, it has not been proved to be seed-transmitted. In our investigation reported here we have proven that RSPaV transmits by seed from RSPaV-infected mother plants to their siblings.

Methods

Seeds were collected from RSPaV-infected grapevine cultivars Pinot Noir, Muscadelle and Cabernet Sauvignon. Before stratification, some seeds from each grape cultivar were tested by RT-PCR for the presence of RSPaV. For the test, the seed coat surface was cleaned and disinfected in a solution of 3.5% sodium hypochlorite for 5 min, then each individual seed was ground and total RNA was extracted using RNeasy column (Qiagene Inc) followed the manufacturer's instruction and used as a template for RT-PCR tests.

For germination, seeds were treated with 1.5% hydrogen peroxide and 350 ppm gibberelic acid solutions for 24 hr and then stored at about 4-6 C for stratification. After stratification, they were germinated on wet sterile filter paper in sterile petri dishes in the growth chamber. Afterwards, part of the germinated seeds were selected, their seed coat were removed and stored at -80 C for further analyses. The remaining germinated seeds were transplanted into trays with individual cells containing sterile soil and maintained in the growth chamber for about two weeks to grow. The seedlings were then transferred into 5x5 cm pots and transferred to a greenhouse. To test the germinated seeds kept in the freezer, total RNA was extracted from individual small plantlets using RNeasy extraction kit (Qiagene) and tested by RT-PCR. For the seedlings grown in the greenhouse, petiole samples from each 5 plants were combined and total RNA was extracted as described above and tested. The one step RT-PCR as described by Rowhani et al (2000) using RSPaV 48/49 primers (Zhang et al, 1998) was used to test the samples. For confirmation, purified RT-PCR products from positive seedlings were cloned, sequenced and the sequences were compared with those present in the GenBank.

Results and Discussion

RSPaV was detected in total RNA obtained from seeds originated from infected plants (Table 1) indicating the presence of virus in the seed. Some of the seedlings grown from seeds of infected plants were also tested positive for the virus (Table 1). The percentage of infection varied from 0.4% (seedlings grown in the greenhouse) and 6.2-14.3% (small plantlets stored in the freezer). Clones obtained from the RSPaV-positive seedlings were compared to sequences reported in the database. Comparative analysis of amino acid sequences of 22 clones revealed percent identity varying from 94% to 100% to RSPaV. As a conclusion, the data indicated that RSPaV is present in the seed and could tramsmit from infected mother plants to their siblings.

Cultivar	Seed	Small Plantlets	Seedlings
Cab. Sauv.	100%	14.3%	0.4%
Muscadelle	100%	6.2%	0%
Pinot Noir	80%	0%	0%
Pinot Noir (healthy control)	0%	0%	0%

Table 1. Percent of RSPaV-infection in seeds, and its transmission to plantlets and seedlings.

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PARTIAL CHARACTERIZATION OF TWO DIVERGENT VARIANTS OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 4*

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Leafroll is a widely known major disease of the grapevine causing significant quantitative and qualitative crop losses. Nine distinct viruses belonging to the genera *Closterovirus* (GLRaV-2 only) and *Ampelovirus* are associated with this disease (Gugerli, 2003).

The existence of a possibile new species (putative GLRaV-10) in a Turkish vine of cv. Koussan (accession Y253) was hypothesized and reported at the 14th Meeting of ICVG (Cornuet *et al*., 2003) based primarily on the folllowing serological evidence: (i) lack of ELISA reaction of Y253 with antisera to Grapevine leafroll-associated viruses 1 to 7 (GLRaVs 1-7), and, viceversa, of GLRaVs 1 to 7 with an antiserum to Y253; (ii) no decoration by immunoelectron microscopy (IEM) of GLRaV-1 and GLRaV-3 particles with the Y253 antiserum, and no decoration of Y253 virions with antisera to GLRaV-1, -2, -3 and -4. However, a survey of 80 grapevine accessions of the INRA collection at Colmar, disclosed that a vine of cv. Koudsi from Israel reacted with the Y253 antiserum.

More recent studies encompassing partial sequencing of the Y253 genome showed that a 549 bp fragment of its HSP70 gene had a high level of identity at the amino acid level with the comparable gene of GLRaV-4 (95%), GLRaV-5 (91%), GLRaV-6 (84%), and GLRaV-9 (90%), but a much lower identity (27-35%) with the HSP70 gene of other GLRaVs.

An independent study was in the meantime in progress at Bari for raising a polyclonal antiserum to GLRaV-4 since the antiserum CA-4 to GLRaV-4 produced in the United States (Hu *et al*., 1990), which is the only serological reagent available for this virus so far, gave inconsistent results in ELISA. An IEM survey using the CA-4 antiserum with a group of grapevine accessions from a varietal collection of the University of Bari, that were ELISA-negative for GLRaV-1 to -7, showed that the locally grown accession Koudsi Y252 (Y252), originally obtained from Colmar, was in fact a GLRaV-4 source that could be used for immunization.

An antiserum to Y252 was therefore obtained, which reacted as follows: (i) in IEM, it decorated the homologous antigen at a dilution 1:100, but the GLRaV-4 type (source LR106, Routh *et al*., 1998) only to a dilution 1:10; (ii) in ELISA, it recognized the homologous isolate Y252 but not LR106; (iii) in a ELISA survey of 320 grapevine accessions, it reacted with nine additional grapevine sources, including the Turkish accession Koussan from Colmar (Y253).

A set of low-degeneracy GLRaV-4/GLRaV-5 primers designed on the HSP70 gene (Routh *et al*. 1998) amplified all three isolates (Y253, Y252, and LR106). When amplicons from Y252 and LR106 were cloned and sequenced, they showed 88% and 95% homology to one another at the nucleotide and amino acid level, respectively. A sequence extension of 486 bp of the HSP70 gene of Y253, showed 99% and 95% homology at the amino acid level, respectively, with Y252 and LR106.

These results led to the conclusion that virus isolates Y252 and Y253 under investigation at Colmar and Bari were very close but, because of the inconsistent serological results, further studies for a better molecular characterization were desirable.

Initial attempts to amplify the complete CP genes from Y252 and Y253 using primers designed on the LR106 CP sequence (Dr. A. Rowhani, University of California, Davis) were unsuccessful, By contrast, a

partial amplification of the Y252, Y253, and LR106 CP gene, comprising about two thirds of the sequence that included the 3' termini, was sucessfully obtained. These sequenced amplicons had a homology higher than 99% at the amino acid level among each other.

Further amplification attempts for completing the CP sequence, so as to include the 5' terminus, were made with a set of low degeneracy primers kindly suggested by Dr. S. Sabanadzovic (Mississippi State University, Starkville) that were designed on the HSP90 (forward) and CP (reverse) gene sequences, respectively. A fragment from the Y253 genome was successfully amplified and sequenced. The assembled complete CP gene sequence of Y253 differed only in a few amino acid residues at the extreme 5' end of the CP from the LR106 sequence determined by A. Rowhani.

Predictive analysis of these amino acid differences using the Peptidestructure software (UW-GCG Package) seem to explain the serological inconsistency observed in the trials made at Colmar and Bari, because antigenic sites existing in LR106 CP were absent in the 5' terminus of Y253 CP.

Thus, the conclusion can be drawn that Y253, Y252 are variants of GLRaV-4 type (LR 106) and that the source of variation lies in their extreme 5' CP terminus. Since pylogenetic analysis of HSP70 genes indicates that a close relationship exisits between GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-9, additional studies for the obtention of all CP sequences are desirable. Based on our study, the availability of the HSP70 and CP sequences seems to constitute the minimal molecular information required for the establishment of a new closterovirid species.

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MORPHOLOGICAL CHANGES OF GRAPEVINE LEAVES (CV. ARINTO) IN CONNECTION/IN RELATION TO/ WITH INFECTIONS BY VIRUSES AND WEATHER CONDITIONS

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Leaf morphology changes induced by viruses are often reported but seldom quantified. Following a previous study of the effects of *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV), and *Grapevine leafroll-associated virus 3* (GLRaV3) on leaf morphology using a multivariate discriminant analysis on leaf measurements (1), the same population of the Portuguese cv. Arinto was used to investigate the influence of weather conditions on symptom expression due to infection by the same viruses plus *Grapevive leafroll-associated virus 2* (GLRaV2). Morphometric variables used in this study provide an overall description of the shape of the leaves based on a limited number of measurements.

These variables are Total Leaf Area ("Area"), "Asymmetry" (as the percentage area on the smaller half blade), Leaf Outline ("Outline", a measure of the depth of the sinuses, calculated as the ratio of leaf perimeter to leaf total area), and "Elongation" (the ratio of the maximum length to the maximum width of the leaf blade). Details on the composition of the studied collection, the virus-testing techniques, and the measurement of the leaves were reported by Santos et al. (2003). Observations were made in three years (1997, 1999 and 2000), thus providing different weather conditions (see lower part of Fig. 1). Only clones free of viruses or with one single virus infection were used in the present work. All calculations were carried out using NCSS, a program developed by Hintze (2004).

Two-way analyses of variance (ANOVA) using "Year" and "Virus" as sources of variation and each of the four morphometric variables as response were performed. Significant differences (P<=0.05) were found between the effect of "Years", but not as a result of virus infection except for "Elongation", where differences between the effect of virus were significant, and those between "Years" were not. As a consequence, further analyses were done in each year independently (Table 1).

Table 1 – Summary of the results of one-way ANOVA comparing virus-free clones with those showing single-virus infection using the four morphometric variables as response, in the three years of study

	1997				1999				2000			
	Area	Asymmetry	Outline	Elongation	Area	Asymmetry	Outline	Elongation	Area	Asymmetry	Outline	Elongation
GFLV				●				●				●
GFkV												
GLRaV2				●		●						
GLRaV3				●								

●: significant; empty cells: non-significant (P<=0.05).

Only GFLV showed a systematic, significant effect on "Elongation" in the three years of observation. "Elongation" was also sensitive to the presence of GLRaV2 and GLRaV3 in 1997, which had the driest spring. In each of the three years of study, no significant differences due to GFkV were observed on the four morphemetric variables. Plants with a mixed infection by GFkV and another virus, were similar to those having a single infection by the second virus in all performed ANOVA (data not shown). These results substantiate the generalised reference to the absence of observable effects of GFkV on *Vitis vinifera*.

Response of infected and non-infected plants to weather conditions in spring can be deduced from data presented in Fig.1. As could be expected, "Area" has a close relation with the amount of rainfall during April, May and June. "Asymmetry", "Outline" and "Elongation" seem to be more connected with the average temperature of the same period, their values as a rule decreasing with temperature. Although leaves used in this study (about 12 per clone per year) were selected among those in the same position

in the plant that did not show any apparent malformation, seeming normal specimens in the scope of the natural shape variation of the cultivar, some differences can be revealed through a finer analysis and measurement: the two leafrolls (GLRaV2 and 3) are correlated with longer leaves in a hot and dry spring (1997); GLRaV2 reduces differences of area between leaf half blades in an "average" year (1999); and GFLV seems to induce systematically shorter leaves (lower "Elongation" values), irrespective of the prevailing weather conditions, a difference that was not apprehended by the naked eye when collecting leaves in the field.

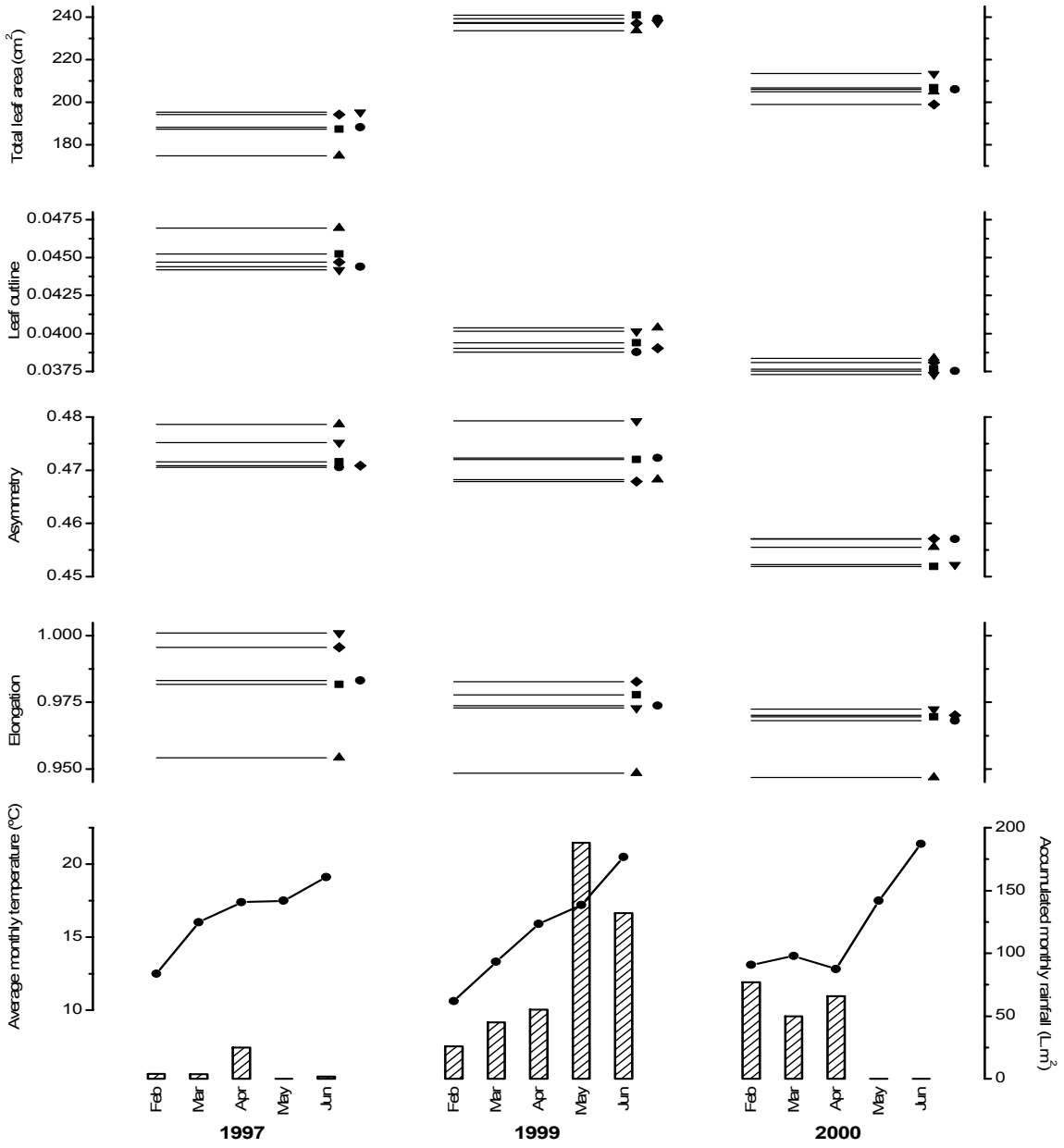

Figure 1 – Morphology of grapevine leaves infected with viruses under different weather conditions. Virus-free (■), GFkV (●), GFLV (▲), GLRaV2 (▼), GLRaV3 (♦).

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INFECTION OF CP-TRANSGENIC GRAPEVINES WITH VITIVIRUSES BY GRAFTING, MEALYBUG TRANSMISSION AND *NICOTIANA* HETEROGRAFTING

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Introduction

Grapevine plants transformed with virus genes for resistance induction need to be assessed for detection of recombination and heteroencapsdidation events between infecting viruses and transgenic products (Fuchs, 2003; Buzkan *et al.,* 2001). Accurate evaluation of the replication, accumulation, and translocation of a challenging virus in transgenic plants is therefore needed for gathering information on possible interactions between transgene and infecting viral RNAs.

Vitis vinifera transgenic lines transformed with CP genes of *Grapevine viruses A* (GVA) and *B* (GVB) (Goelles *et al*., 2000), were investigated for verifying successful gene insertion and expression. Moreover, the level of potential resistance was evaluated by challenge infection with homologous viruses using different inoculation techniques. Selection of transgenic plants that correctly process the transgene and do not affect replication of challenging virus(es) will help in defining parameters for risk assessment.

Materials and Methods

Molecular analysis of available lines of *Vitis vinifera* cv. Russalka 3, transformed with the CP gene of GVA or GVB, was done by Southern blot hybridization. About 10 □g of total DNA extracted form six lines of GVA and four lines of GVB transgenic grapevines, were digested with *EcoRI, DraI, AccI, Bcl I and Bgl II,* blotted and hybridized with digoxigenin-labelled DNA CP probes. Western and Northern blot were also done with antisera and riboprobes to both viruses.

In Spring 2005, budwood from four GVB and six GVA transgenic lines was grafted on rooted cuttings of GVA- or GVB-infected sources. Graft take was low and the few successfully grafted plants grew slowly. A mealybug transmission trial was done in Autumn 2004. Young sprawlers of *Pseudococcus longispinus*, were allowed to feed on GVA- or GVB-infected grapevine sources for a month for virus acquisition, prior to tranferring to self-rooted transgenic grapevine cuttings (6-8 plants per line), on which they were allowed to feed for an additional month. Plants were tested for infection either by Western blot with antisera to the recombinant movement protein (MP) of both viruses (Saldarelli *et al*., 2000) or by RT-PCR using virus-specific primers designed on the sequence of MP genes.

Heterografting was also attempted, whereby two-node green shoots of transgenic grapevines or healthy indicators (LN33 and Kober 5BB) were top grafted on stems of GVA- or GVB-infected *Nicotiana* plants. Heterografted plants were grown under controlled temperature (25°C) and light (16h photoperiod) for at least 20 days. Grape cuttings were then removed, planted in Jiffy pots, rooted, and grown in a climatized greenhouse at about 24 °C.

Results and Discussion

Molecular analysis of transgenic grapevine lines showed that CP genes of both viruses were successfully inserted. Specific CP bands (22 kDa) were identified by Western blot, whereas Northern blots hybridized the transgenic RNA transcripts (*c.* 600 nt) in all lines transformed with either viruses, indicating that the transgene was correctly processed in engineered plants.

Southern blots of GVA lines were more controversial. GVA transgenic lines cut with *EcoRI* (with a single cut inside the cassette, outside the CP gene) did not differentiate these lines from one another. Likewise, when *DraI* was used (a single cut inside both viral sequences) together with DNA hybridization probes designed on the left and right sides of the *DraI* site, the same hybridization pattern was shown by the 6 GVA lines. However, using *AccI,* two GVA lines (77.16 and 74.13) showed an additional band in the electrophoretic profile that differentiated them from the other four (70.14, 85. 3/4 , 50.13/4, 25.16/7).

As to GVB lines, one (43.15) could be differentiated from the others (3.17, 11.49 and 14.29) when the 5' CP gene probe was used (DNA cut by *DraI*).

Challenge inoculation of transgenic grapevine lines by grafting gave inconclusive results, mostly due to the low graft-take. More encouraging were the results of the preliminary analysis of transformed vines that had been inoculated by mealybugs. In two GVA transgenic lines (50.13/14 and 25.16/17) the number of uninfected plants was high (5/6 and 8/10, respectively), whilst other lines transformed with either GVA or GVB had an infection rate of about 50%. All healthy controls exposed to viruliferous mealybugs became infected.

Heterografted plants had a very low survival rate due to technical problems (poor selection of green grapevine scions with the right degree of maturity, rotting). Thus, the evaluation of the sanitary status of the few surviving grape internodes that were successfully rooted, had to be postponed to the next vegetative season.

Acknowledgements
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PLANOCOCCUS CITRI (RISSO) (HOMOPTERA: PSEUDOCOCCIDAE) AS GLRAV-3 VECTOR

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Introduction

The citrus mealybug appears occasionally in the vineyards of Galicia (NW of Spain) but as a pest it is not a great concern for the wine-grape growers because it does not reach very high infestation levels.

The importance of this insect is determined by the aptitude of *Planococcus citri* to transmit the GLRaV-3, which has a high incidence in the vineyards of Galicia (Cabaleiro and Segura, 1997a). In 1997 we described the acquisition and transmission capacity of this mealybug (Cabaleiro and Segura, 1997b), what allowed to explain the field spread of GLRaV-3 in a full production vineyard (Cabaleiro and Segura, 1997a). Since the field spread of the virus was quite fast in relation with the apparent low mealybug population levels (Cabaleiro and Segura, 2003) in 2003 and 2004 the populations of mealybugs were monitored and the acquisition and retention of the virus by the vectors were evaluated in order to obtain as much information as possible about the factors affecting the field transmission of the virus and therefore help to control the vector or limit the virus transmission.

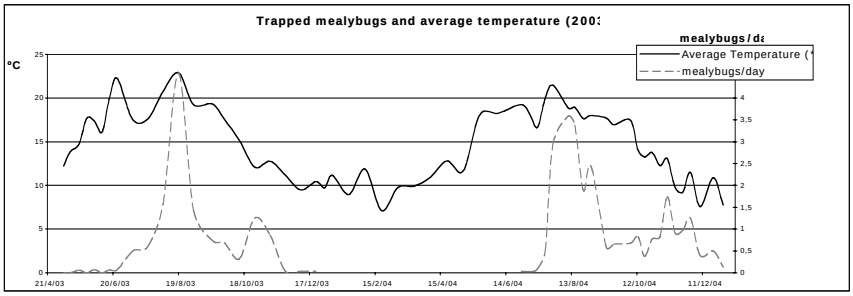
Materials and Methods

The mealybugs population monitoring was carried out by means of adhesive tape traps placed around the trunk (Lucas, 2002) and one vine shoot by plant. The tapes were removed and renewed at variable intervals: (from weekly even monthly) depending on the climatic conditions and on the activity of the mealybugs populations. In 2003 the study was carried out with two plants, and in 2004, with 18 plants. The number of mealybugs was recorded in each of the collected tapes. The number of mealybugs trapped by day was obtained as the quotient of the number of mealybugs of each tape and the days that the tape remained on the plant. Three mealybugs from five plants of the local cv. Tinta femia infected with GLRaV-3 where collected during the growing period and were analyzed by IC-RT-PCR (Cid *et al*., 2003) to calculate the percentage of potentially infective mealybugs.

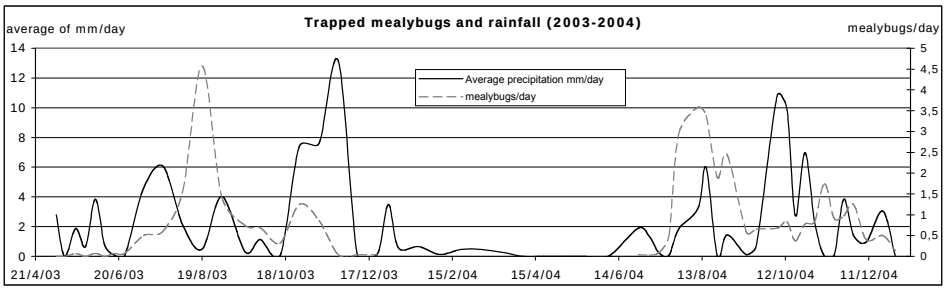
Analysis of acquisition and lost of potential infectivity of the vectors using IC-RT-PCR. Six mealybugs belonging to all stages of development (crawlers, 2nd and 3rd instar nymphal stages, female adults and female adults with laying) reared in potato sprouts were allowed to feed on leaves from a GLRaV-3 infected plant (cv. Albariño C1) for more than three days and then removed and analysed. As negative control we used mealybugs feeding in healthy leaves. To evaluate the lost of potential infectivity, mealybugs reared in potato sprouts were transferred to GLRaV-3 infected leaves and allowed to feed until they reached the third nynphal stage or the adult stage; then, they were transferred to non-infected leaves and five individuals were removed and analysed for the presence of GLRaV-3 after feeding periods of 3, 6, 12, 24, 48, 72 or 96 hours. Small pieces from the leaves where the insects fed for the longest period (96 h) were also tested for the presence of GLRaV-3.

Results and discussion

The mealybugs showed a polymodal growth pattern (Graph 1). There were no discrete generations but an overlap of succeeding generations. The environmental factor that had more influence on the population dynamics was the rainfall. The rain caused important and sudden descends in the number of mealybugs trapped (Graph 2). This is due both to the direct effect of the water on the mealybugs but also to parasite fungi proliferation. The average temperature had also influence in the population fluctuation or movement with higher captures at higher temperatures. Moreover we see the predator effect of some mites on the laying with raises in the average temperature.

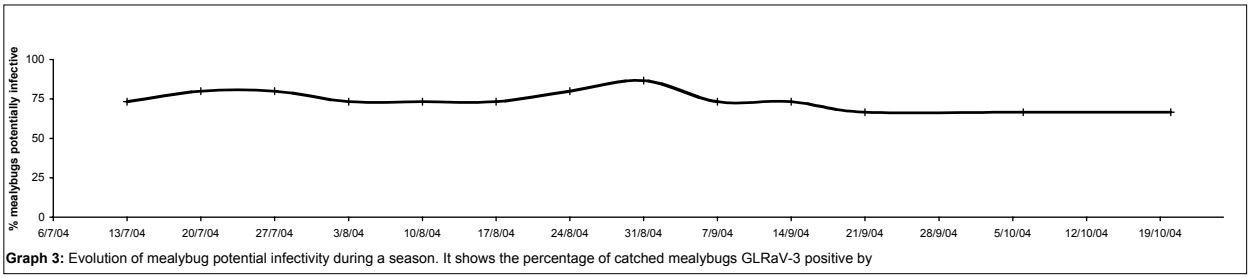


Graph 1: Temperatures evolution and trapped mealybugs by day during 2003 and 2004. The showed temperature is the temperature average during the period in which the adhesive tape is placed in the plant. The peaks of mealybugs growth corresponds with raises in the average temperature.



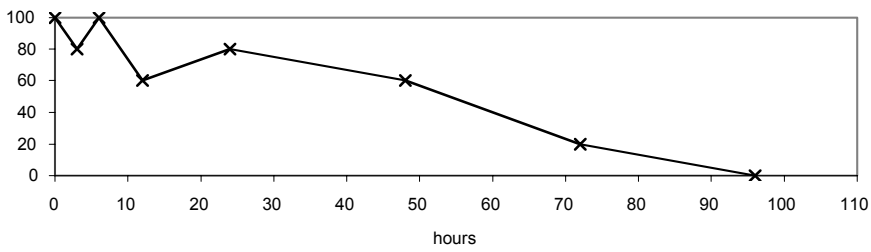
Graph 2: Rainfall evolution and trapped mealybugs by day during 2003 and 2004. The represented rainfall is the average of daily rainfall during the period in which the adhesive tape is placed in the plant. The high rainfall gave rise a loss in the population curve.

The percentage of potentially infective mealybugs was fairly constant through the season (Graph 3). About 70% of the mealybugs feeding in a GLRaV-3 positive plant were potentially infective in a certain moment.



Graph 3: Evolution of mealybug potential infectivity during a season. It shows the percentage of catched mealybugs GLRaV-3 positive by

In the assays in controlled conditions, all the individuals tested belonging to any of the stages of development were positive for GLRaV-3 after three days acquisition period what means that all are at least potential GLRaV-3 vectors. The lost of potential infectivity (Graph 4) was gradual; after 96 hours of feeding in a healthy leaf, none of the insects retained the virus but the virus could not be detected in any of the samples taken from the leaves where those mealybugs had been feeding.



Graph 4: Lost of potential infectivity. It shows the percentage of mealybugs GLRaV-3 positive according to hours of feeding on health leaf

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PHYTOPLASMAS AND THEIR POTENTIAL VECTORS IN VINEYARDS OF INDIGENOUS CROATIAN VARIETIES

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Introduction

Grapevine yellows (GY) symptoms have been observed in most viticultural regions of Croatia. First molecular evidence of aster-yellows (16SrI-B) and stolbur (16SrXII-A) phytoplasmas infecting Croatian grapevines were presented by Šarić *et al*. (1997) and Škorić *et al*. (1998), respectively. Visual and molecular surveys of indigenous grapevine cultivars from Croatian coastal vine-growing regions in the period from 1998 to 2002 demonstrated only the presence of phytoplasmas belonging to the ribosomal subgroup 16SrXII-A (Šeruga *et al*., 2002). In the years 2003 and 2004, the survey continued on a larger scale encompassing indigenous cultivars from northern coastal, southern coastal and continental viticultural areas of Croatia. In 2004, investigations of potential phytoplasma natural vectors were started in three vineyards, one in each region.

Materials and methods

Leaves were collected from 20 symptomatic vines of 10 indigenous Croatian grapevine varieties from different regions in September 2003 and 2004. In 2004, potential phytoplasma vectors were collected by using yellow sticky traps and entomological net from three vineyards: Voloder (continental Croatia), Novigrad and Pelješac (northern and southern coastal Croatia, respectively).

Total nucleic acids (TNA) were extracted following the procedure described by Šeruga *et al*. (2003). Approximately 0.2 g of $CaCl_2$-dried leaf midribs was used instead of the frozen/fresh material. TNA from insects were extracted as described by Zhang *et al*. (1995).

Amplification of phytoplasma 16S rRNA gene was performed in a direct PCR using R16F1/R0 phytoplasma universal primer pair (Lee et al. 1995). First nested PCRs were performed by using universal primer pairs R16F2n/R2 (Lee et al. 1993) and second nested PCRs were primed by group-specific R16(I)F1/R1 and, in some cases, by R16(V)F1/R1 primers (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 (=*Mse*I) for 16 h at 65°C. Obtained fragments were separated by electrophoresis through 5% polyacrylamide gel, stained with ethidium-bromide and compared with RFLP patterns of standard phytoplasma strains: SA-1, Stol, PTV (16SrXII-A), Hyd-B (16SrI-B), PPT (16SrI-C), EY-C (16SrV-A) and KVI (16SrIII-B) (Bertaccini, 2003).

Results and Discussion

GY symptoms were observed in all examined vineyards of indigenous Croatian varieties from different viticultural areas (Table 1). In Plavac Mali and Babić milder GY symptoms (only leaf reddening) were observed and phytoplasmas belonging to subgroups 16SrI-B and 16SrXII-A were detected, respectively.

In direct PCR assays, no visible products were obtained. In nested PCR assays using generic primers, only three samples (Malvasija and Škrlet) produced visible 1.2 kb products. Further PCR amplifications using group–specific primer pair R16(I)F1/R1 yielded additional fifteen positive samples. Plavec Žuti and one of the Plavac Mali samples tested negative. For samples showing R16(I)F1/R1/ *Tru*9I profiles characteristic of 16SrI-B subgroup, additional second nested PCR using R16(V)F1/R1 primers on R16F2n/R2 templates was done to exclude the possibility of mixed infections. The absence of visible products primed by R16(V)F1/R1 indicates the presence of only 16SrI-B subgroup phytoplasmas in these samples.

Six species of potential phytoplasma insect vectors were analyzed (Table 2). Insects collected in vineyards not indicated in the literature as potential phytoplasma vectors, were not subjected to laboratory analyses. In all vectors that resulted positive for phytoplasma presence, the agents belonging to the

subgroup 16SrXII-A were detected. The number of analyzed vectors was too small to draw definite conclusions from these results. Nevertheless, *Reptalus cuspidatus* is abundantly present and consistently harbors phytoplasmas from the subgroup 16SrXII-A suggesting its important role in some of the pathosystems indigenous vines – vector – herbaceous hosts. Although our previous research (not shown) preformed on a limited number of vineyard weeds indicates the involvement of *Taraxacum officinale*, *Stellaria media* and *Convovulus arvensis*, the definite role of these, and possibly other weeds for the molecular epidemiology of phytoplasmas in Croatian indigenous vines remains to be determined.

Acknowledgements
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Table 1. Phytoplasma identification in grapevine samples

Year	Region	Vineyard	Cultivar	Number of samples	R16F2nR2/Tru9I	R16(I)F1/R1/Tru9I
2004	Northern coastal	Rovinj	Malvasija	2	16SrXII-A	not tested
		Vrbnik	Žlahtina	2	-	16SrXII-A
	Southern coastal	Pelješac	Plavac Mali	1	-	16SrI-B
		Čara	Pošip	1	-	16SrXII-A
		Lumbarda	Grk	1	-	16SrXII-A
		Primošten	Babić	2	-	16SrXII-A
	Continental	Zelina	Kraljevina	2	-	16SrXII-A
		Nespeš	Kraljevina	1	-	16SrXII-A
		Voloder	Moslavac	1	-	16SrI-B
		Križevci	Kraljevina	1	-	16SrXII-A
			Plavec Žuti	1	-	-
2003	Southern coastal	Pelješac	Plavac Mali	2	-	16SrI-B
			Plavac Mali	1	-	-
		Čara	Pošip	1	-	16SrI-B
	Continental	Voloder	Škrlet	1	16SrXII-A	not tested

Table 2. Phytoplasma identification in insect samples

Region	Vineyard	Species	R16F2nR2/Tru9I	R16(I)F1/R1/Tru9I
Northern coastal	Novigrad	Reptalus cuspidatus	-	16SrXII-A
		Scaphoideus titanus	-	-
		Metcalfa pruinosa	-	-
Southern coastal	Pelješac	Empoasca vitis	not tested	
		Arboridia dalmatina	not tested	
Continental	Voloder	Reptalus cuspidatus	-	16SrXII-A
		Reptalus cuspidatus	-	-
		Cicadella viridis	-	16SrXII-A
		Reptalus panzeri	-	-
		Hyalestes obsoletus	-	-

GENETIC DIVERSITY OF RDRP ORF AMONG RUPESTRIS STEM PITTING ASSOCIATED VIRUS ISOLATES FROM ARGENTINA

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Rupestris stem pitting is one of the four individual diseases involved in the rugose wood complex as described by Walter (2000). Two nearly identical viruses called Rupestris Stem Pitting associated Virus 1 (RSPaV-1)(Meng *et al.* 1998) and Grapevine Rupestris Stem Pitting Virus (Zhang *et al.* 1998) have been consistently associated with the disease, as determined by molecular, serological and electron microscopic techniques. RSPaV-1 was also found in RSP diseased vines in Argentina (Tarnowski *et al.* 2002). A paper recently published (Meng *et al.* 2005) reports the genetic variability along the entire genome of the virus between two newly described strains (RG1 and BS) and a reference sequence (RSPaV-1). The amino acid similitude of ORF1 ranges between 91.6 and 92.7%. The similitude in the region between polymerase domains II and V of these strains ranges between 98 and 99%, showing a conserved region among the virus isolates.

Partial degenerated PCR primers have been developed targeted to amplify this region of the Flexiviridae. An alignment including all available sequences was constructed using the BLOCKS algorithm (Henikoff *et al*. 1995), and the output directly used to design the primers "K2" TCATGAAGCAACAACTTTGCACNAARWTBG and "H" ACATRTCATCMCCNGCAAA with the CODEHOP package (Rose *et al*. 1998).

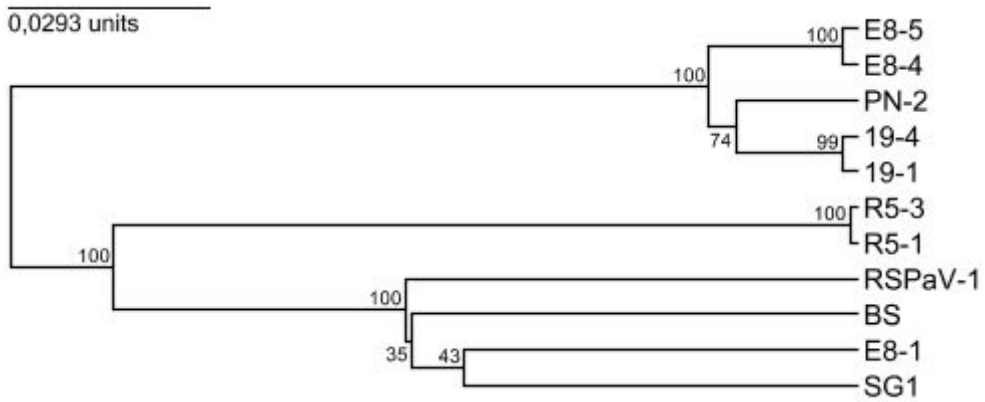
The PCR product was restricted individually with EcoRI and HinfI, and the pattern obtained was compared with the theoretical pattern of the sequences in the databases using the gel simulation tool of the Vector NTI software. In several cases, the pattern was not similar to any individual accession. Also several samples showed an electrophoretic profile suggesting a multiple infection.
PCR products from five plants, comprising 12 unallocated patterns were cloned using the pGEM T-Easy Vector System (Promega) according to the manufacturer instructions. Colonies were screened by restriction analysis using EcoRI and HinfI. The selected clones were sequenced with an ABI 3130XL genetic analyzer.

Eight of the resultant sequences showed homologies with RSPaV-1 and the other four with GVA. The identities at amino acid and nucleotide level between the isolates under study and three reference sequences (SG1, BS, and RSPa-V-1) are shown in Table 1. In three cases, a single nucleotide substitution between two sequence variants found in one plant (19-1 & 19-4, E8-4 & E8-5 and R5-1 & R5-3), yielded one amino acid change. Otherwise, two sequence variants from the same plant (E8-1 vs. E8-4 and E8-5) have shown one of the highest divergences between the analyzed sequences. Previously, the genetic variability of the coat protein ORF was reported (Santos *et al*. 2003). We report here a higher than expected diversity in Argentine isolates of RSPaV-1 in a putative conserved domain of the polymerase ORF.

Table 1
Identity percentages of nucleotide (below diagonal) and deduced amino acid (above diagonal) between analyzed clones and reference accessions.

	Percentage of identity										
Clone - Accesion	19-1	19-4	E8-4	E8-5	PN-2	R5-1	R5-3	E8-1	SG1	BS	RSPaV-1
19-1		**99**	99	98	98	95	95	95	95	95	95
19-4	**100**		99	99	99	95	96	95	96	96	95
E8-4	96	96		**99**	99	96	97	**95**	97	97	96
E8-5	96	96	**100**		99	95	96	**95**	96	96	95
PN-2	96	97	95	81		95	96	95	96	96	95
R5-1	81	81	81	80	77		**99**	95	97	97	96
R5-3	81	81	81	81	77	**100**		96	97	97	97
E8-1	77	78	**78**	**78**	79	80	81		**98**	**99**	**98**
SG-1 (AY881626)	79	79	79	79	79	81	81	**89**		**99**	**99**
BS (AY881627)	77	78	78	78	78	82	82	**88**	**88**		**99**
RSPaV-1 (NC_001948)	77	78	79	79	78	81	82	**87**	**88**	**88**	

Figure 1
Dendrogram obtained by UPGMA including the analyzed clones and the three reference sequences.



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HIGH INCIDENCE OF GLRAV-2 IN A CABERNET SAUVIGNON VINEYARD OF ARGENTINA

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Cabernet Sauvignon is one of the most worldwide appreciated wine cultivars. Grapevine leafroll associated virus 2 (GLRaV-2) was recently reported infecting Cabernet Sauvignon vineyards of Mendoza, Argentina (Gómez Talquenca et al 2003).

The aim of this study was to know the incidence of this virus in three Cabernet Sauvignon clones lately imported from France, to validate the ELISA technique for the detection of diseased plants and finally to compare the water potential of diseased and healthy vines.

Three parcels with 30 to 32 plants each, implanted in 2001 with the Cabernet Sauvignon clones 337 grafted over Gravesac, 191 over RGM and 341 over 101-14, were delimited inside a commercial vineyard. Samples were taken in December 2004 and February 2005 from each plant. ELISA test were performed from internodal cambial scrapings and petioles with reagents and directions from Bioreba AG. The water potential was measured by a Scholander chamber at 4:30 – 5:30 h and 12:00 – 13:00 h each four days from January 6 to February 28, 2005. Ten plants were measured per parcel being five infected and five ELISA negative ones. Plants were previously stressed by the suppression of regular irrigation

ELISA test performed suitably, without significative differences from samples taken in December or February, using stem as well as petiole tissue. Results indicate 83, 75 and 50% of GLRaV-2 infected plants of 337, 191 and 341 clones respectively in the surveyed parcels. The water potential measured did not evidence statistical differences between diseased an healthy plants.

The high percentage of GLRaV-2 infection in the Cabernet Sauvignon clones studied may be related with the importation of nursery plants from France. The infection of several certified cultivars and clones with GLRaV-2 in France was reported (ONIVINS, 2002) including the clones here surveyed. The disease does not show particular severity in France. Probably under our ecological conditions severe detrimental effects would appear. We postulate that the hydric condition of infected plants could be reduced inducing viral symptoms and vine decay. The water potential as measured in our trial did not show such differences. We continue with the evaluation of detrimental effects of the infection over plant yield and grape quality.

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DEVELOPMENT AND EVALUATION OF A BULGARIAN GRAPEVINE GENEBANK

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Introduction

Grapevines have been one of the most important crops of Bulgaria both from the economic and social point of view. The total area under vines is about 131 069 ha of which 110 505 ha yield wine grapes and only 20 564 ha table grapes. The range of **wine grapes** cultivated is presented by local and introduced varieties. Red wine varieties cover 52% of the total wine grape area. The most distributed varieties are "Pamid" (35%), "Merlot" (18%) and "Cabernet Sauvignon" (16%). White wine areas is occupied by "Rkatzitelli"- 25% from the total white wine areas, "Dimijat" (21%), "Chardonnay" (5%), "Muscat Otonel" (5 %) (1).

Regarding the 20 564 ha planted with **table grapes,** "Bolgar" leads the way representing 75% of them, followed by "Muscat Hamburg" (7%), "Cardinal" (5%), "Sultanina (1%)". Last, but not least, the new local table varieties such as "Velika", "Nadezda", "Hybrid VI-IV", "Muscat Roussenski", "Rusalka 1" and others are much appreciated in the local and foreign market. At the period lots of viticulture problems are still waiting for decision. Large part of the vineyards is too old and emergency needed to renew (65%). Development and maintenance of Bulgarian pre-basic grape genebank and a certification scheme related to the European Legislation for the marketing of material for the vegetative propagation of grapevines [Directives 68/193, 71/140, 77/129 and 02/11] appeared to be a promising step for solving the key problem for revitalisation of Bulgarian viticulture. The objective of the present study was the development and evaluation of a Bulgarian grapevine genebank trough preliminary field selection, ELISA tests for 16 viruses, bacterial analyses, DNA identification, *in vitro* micropropagation and establishment of nuclear pre-basic plants. This investigation was carried out within the framework of a previously reported grapevine selection programme of the AgroBioInstitute (ABI) (2).

Materials and Methods

The mature cuttings from preliminary field selected plants placed on all parts of Bulgaria were used as a basic source for plant material development. A grapevine certification scheme based on Martelli *et al*. (1999) and on EEC Directives 68/193 and 02/11 was employed. ELISA analyses for 16 viruses were carried out as described by Voller *et al*. (1976) using DAS ELISA and RTA ELISA kits obtained from BIOREBA®. Bacterial analyses and DNA identification were based on modified protocols, reported by Genov *et al.* (2005) and Hvarleva *et al.* (2004). For grapevine clonal micropropagation a modification protocol based on Samson & Gasteran (1971) was used. The acclimatisation of grapevine *in vitro* plants was based on a modification of the protocol described by Babrikov *et al*. (2000).

Results and Discussion

Results from ELISA tests indicated the high presence of viral diseases – totally 75.50% of the cuttings were infected. In opposite, only 7 % carrying crown gall disease. Thanks to the previous ABI selection programme, there are not find non- authentic ones after SSR molecular analyses. Healthy plants for the establishment of nuclear pre-basic stock were selected. Genetic and health status of the established ABI grapevine genebank up to 25.07.2005 were showed on Table 1. The genebank has been maintained on the three level (*in vitro*, greenhouse and field). Since 2004, there are two field replication of the genebank

placed both on ABI grapevine bases Bijala (North part of Bulgaria- Rousse region- Tsenko'95 Ltd.) and on village Nayden Gerovo (South part of Bulgaria- Plovdiv region- Pepinier- Consult Agorbiotech Ltd.).

Table 1. Development of ABI grapevine genebank /up to 25.07.2005/

Virus Diseases	Authentity	Bacterial diseases
1. Grapevine Fanleaf Virus (GFLV) **2.** Arabis Mosaic Virus (ArMV) **3.** Grapevine Leafroll assoc. Virus 1 (GLRaV-1) **4.** Grapevine Leafroll assoc. Virus 2 (GLRaV-2) **5.** Grapevine Leafroll assoc. Virus 3 (GLRaV-3) **6.** Grapevine Leafroll assoc. Virus 6 (GLRaV-6) **7.** Grapevine Fleck Virus (GFkV) **8.** Grapevine Virus A (GVA) **9.** Grapevine Virus B (GVB)* **10.** Raspberry ringspot virus-ch strain (RpRSV-ch) **11.** Raspberry ringspot virus- g strain (RpRSV-g) **12.** Strawberry latent ringspot virus (SLRSV) **13.** Tobacco ringspot virus (TRSV) **14.** Tomato ringspot virus- Chikadee strain (ToRSV-Ch) **15.** Tomato ringspot virus-peach yellow bud mosaic strain (ToRSV-PYBM) **16.** Grapevine Bulgarian Latent Virus (GBulLV)**	3 years preliminary selection on the base of ampelographic and economic characteristics	**Crown gall** - Agrobacterium vitis - Agrobacterium tumefaciens
Methods		
1. ELISA analyses - ELISA kits BIOREBA® - Switzerland * AGRITEST®- Italy **AGROBIOINSTITUTE- Bulgaria **2. Molecular diagnostics** under the way	**1. Visual observation** - Prof. V. Vulchev - Prof. I. Todorov - Prof. L. Radululov - Prof. K. Katerov - Prof. S. Pandeliev **2. DNA identification** - microsatellite /SSR/ markers: VvUCH11; VvUCH29; VrZAG21; VrZAG47; VrZAG62; VrZAG64; VrZAG79; VrZAG83; VVS2	**1. Isolation on semi-selective medium** - Roy & Sasser **2. Molecular diagnostic** -PCR detection **polygalacturonase gene* **virD 2 gene* **vir E 2gene* **vitopine synthase gene*

Except mentioned above companies, working under strict control, ABI has a long term contracts also with other leading Bulgarian nurseries such as AMV- Agro Ltd., PPK Korten, Zaychev & son Ltd. Genetic and health status of the genebank is presented on Tab.1. It was showed also methods and the names of breeders included in preliminary field selection of the mother plants. The genebank is already used for production of pre-basic and basic material in contribution with ABI first pre-propagation bases.

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HAIRPIN DSRNA AS A TOOL FOR CONFERRING GFLV RESISTANCE TO A GRAPEVINE ROOTSTOCK (1103 PAULSEN), A TABLE GRAPE CULTIVAR (CRIMSON SEEDLESS) AND *NICOTIANA BENTHAMIANA*

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Introduction

Grapevine fanleaf nepovirus, Comoviridae causes the most severe virus disease to the grapevine industry (reviewed in Andret-Link et al., 2004). In spite of intensive control programs in order to prevent virus spreading it is still a serious problem in many grapevine growing areas. In the Spanish clonal selection program an 8% of clones resulted GFLV infected, always keeping in mind that these clones were subjected to visual inspections and many positive ones were rejected prior to sanitary analysis so this result is undervalued (Padilla et al., 2003). On the other hand *Xiphinema* vector presence is rising due to fertirrigation increase in many Spanish grapevine vineyards (Arias et al., 1997).

In the practice there are not good sources of GFLV resistance both for rootstocks and cultivars. In order to control GFLV dispersion grapevine transformation strategies have been proposed based on pathogen derived resistance (PDR). Transgenic expression of *Grapevine fanleaf nepovirus* genes, mainly derived from the translatable or untranslatable expression of coat, RNA polymerase or movement protein genes, are on study and even on field trials (Krastanova *et al*., 1995; Xue *et al*., 1999; Vigne *et al*., 2004). Protection of non transgenic scions over transgenic rootstocks is a promising strategy which is currently on grapevine field trials (Vigne *et al.*, 2004). This task has been recently accomplished on cucumber (Gal-On *et al*., 2005), watermelon (Park et al., 2005) and is presently in our planning.

We have adopted the PDR strategy with the intention of conferring GFLV resistance to a widely distributed and appreciated rootstock in Spain, 1103 Paulsen, and for a table grape variety (Crimson Seedless). A pHellsgate8 derived construction, pHEgflv1103, has been obtained containing an inverted-repeat of the partial coat protein gene from a Spanish GFLV isolate. In order to test resistance in an herbaceous host we have transformed and regenerated *Nicotiana benthamiana* plants with pHEgflv1103. Work on these plants is on progress. Besides we have transformed embryogenic calli of 1103 Paulsen and Crimson Seedless with pHEgflv1103.

Material and methods

RT-PCR and pHEgflv1103 vector construction

RNA was isolated from GFLV infected grapevine tissues following the Concert Plant RNA Reagent protocol (Invitrogen). RT-PCR with specific primers resulted in a cDNA of 367 pb length corresponding to the CP gene fragment (positions 2048-2415 of GFLV RNA2 according to sequence NC_003623 (Serghini *et al*., 1990) as confirmed by sequencing after pGEMT-Easy cloning. This construction allowed the amplification of a new PCR fragment with similar equivalent primers designed with additional attB adaptors (Gateway, Invitrogen). This PCR product was used for attBP recombination with pDONR221 (Invitrogen) resulting in pEgflv0202. After confirming by restriction analysis and sequencing the goodness of this construction, the same was used for attLR recombination for its cloning into pHellsgate8 (Helliwell and Waterhouse, 2003). After *E. coli* DH5α transformation, restriction analysis of several clones with *Xho*I and *Xba*I followed by sequencing resulted in plasmid pHEgflv1103 which was used for all subsequent steps. Strains C58(pMP90) and EHA105 of *Agrobacterium tumefaciens* were transformed by electroporation with pHEgflv1103.

N. benthamiana transformation

Leaves of in vitro *N. benthamiana* plants were cut into pieces (0.5 to 1.5 mm^2) in sterile conditions with a razor blade and inoculated with the transformed *Agrobacterium tumefaciens* for 10 min. Leaves explants were co-cultivated on agar/MS medium plates (Murashige and Skoog, 1962) supplemented with 0.1 mg/l NAA for 2 days. The gene for neomycin phosphotransferase II (*nptII*) was used as the selectable marker. Explants were transferred to selection medium with 1mg/L IAA, kanamycin (100 mg/L), cefotaxime (300 mg/L) and vancomycine (200 mg/l). Renewal transfers were made every 25 days. Resistant shoots were transferred to rooting medium. Regenerated plants, which did not showed observable phenotypic

response to the insertion of the genes, were grown in test tubes and finally transferred to pots in a growth chamber.

Grapevine transformation
Embryogenic calli of 1103 Paulsen and Crimson Seedless were co-cultivated for two days with *Agrobacterium tumefaciens* strains EHA105 or C58(pMP90) carrying the pHEgflv1103 plasmid in ½ MSAC medium (López-Pérez et al., 2005). Calli were transferred to the same medium supplemented with kanamycin (20 mg/L) and cefotaxime (300 mg/L). Embryos were transferred to germination medium added with cefotaxime (300 mg/L) and plantlets were developed in ½MS medium.

Results
Nicotiana transformation with Agrobacterium strain EHA105 resulted in early explant dead. However, transformation with strain C58(pMP90) succeeded and regenerated plants could be obtained. These plants are currently under analysis to test the presence of the transgenes. Grapevine transformed somatic embryos are growing and will be subjected to further analyses.

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THE OCCURENCE OF *RASPBERRY BUSHY DWARF VIRUS* IN DIFFERENT GRAPEVINE VARIETIES IN SLOVENIA

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Introduction

In 2002 *Raspberry bushy dwarf virus* (RBDV) was confirmed in Slovenia in grapevine grafts showing typical virus symptoms. This was the first finding of natural infection with RBDV outside the genus *Rubus* (Mavric *et al*., 2003), therefore the information about RBDV in grapevine is still highly limited.

In Slovenia grapevine is grown on approximately 16.600 ha (Statistical office of the Republik of Slovenia, 2004), mostly in the three winegrowing regions: Podravje, Posavje and Primorje. Due to the various microclimatic and soil conditions as well as different viticultural traditions a lot of different grapevine varieties are grown in Slovenia, including local varieties grown nowhere else.

Here we present the study of the distribution of RBDV in different winegrowing regions of Slovenia and its occurrence in different grapevine varieties.

Material and Methods

996 leaf samples were taken in numerous vineyards in the years 2003, 2004 and 2005 from plants with and without visible symptoms. Samples were tested for the presence of RBDV by DAS-ELISA using polyclonal antiserum (Loewe Biochemica).

Results and Discussion

The results are presented in Table 1. RBDV was confirmed in approximately one quarter of collected samples. None of the 314 samples of red varieties showed the presence of RBDV. Out of the 17 white varieties following seven varieties were found to be infected for now: Beli Pinot (= White Pinot; 14,3%) Chardonnay (65,7%), Laski Rizling (= Italian Riesling; 53,7%), Malvazija (= Istrian Malvasia; 5%), Renski Rizling (= White Riesling; 51,1%), Sauvignon (46,4%) and Sipon (= Furmit; 50%). The presence of RBDV was also confirmed in one out of 139 samples for which the information about variety was not available.

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Table 1: Results of testing different grapevine varieties for the presence of RBDV.

Winegrowing region	**PRIMORSKA**		**PODRAVJE**		**POSAVJE**	
Variety	**No. of positive samples**	**Number of tested samples**	**No. of positive samples**	**Number of tested samples**	**No. of positive samples**	**Number of tested samples**
Red varieties:						
Barbera	0	19				
Cabernet Sauvignon	0	18				
Gamay					0	20
Merlot	0	35				
Modra Frankinja					0	42
Modri Pinot	0	4				
Refosk	0	92				
Sentlovrenka					0	22
Zametna Crnina					0	62
White varieties:						
Beli Pinot			**1**	7		
Chardonnay	0	6	**23**	29		
Kraljevina					0	20
Laski Rizling	**45**	68	**76**	127	**16**	60
Malvazija	**1**	20				
Pinela	0	2				
Ranfol					0	20
Rebula	0	25				
Renski Rizling			**11**	25	**13**	22
Rumeni Muskat	0	18				
Sauvignon	**11**	24	**2**	4		
Sivi Pinot	0	15				
Sipon			**4**	8		
Tokaj	0	26				
Traminec			0	10		
Vitovska Grganja	0	1				
Zlahtnina					0	6
Unidentified	**1**	80	31	56	0	3
Total	**58**	**453**	**148**	**266**	**29**	**277**

AUTHOR INDEX

LIST OF SPONSORS

Bioreba

Bellevue Wine Estate

Labretoria

KWV

Laborie

Mischa Wines CC

Nederburg

Lourensford

Spier

Vergelegen

Voorgroenberg Nurseries

Winecorp