SEROLOGICAL RELATIONSHIP AND DIFFERENCES BETWEEN PHYTOPLASMAS OF THE ELM YELLOWS GROUP OBSERVED WITH POLYCLONAL AND MONOCLONAL ANTIBODIES TO FLAVESCENCE DORÉE PHYTOPLASMA.

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Flavescence dorée (FD) phytoplasma is specifically transmitted to grapevine in natural conditions by the ampelophagous leafhopper *Scaphoideus titanus* Ball. The broad-bean *Vicia faba* L. and the leafhopper *Euscelidius variegatus* Kbm, used as experimental hosts, contain high titres of FD phytoplasma which have been used to obtain a number of FD-specific polyclonal and monoclonal antibodies (1, 2) and FD-specific DNA probes and primers (3, 4) which allowed the diagnosis of FD phytoplasma in naturally-infected leafhoppers and grapevines (1, 4, 5, 6) as well as the characterisation of FD phytoplasma (4, 6, 7, 8, 9, 10).

Southern blot analysis of total DNA (3), as well as RFLP analysis of ribosomal and non-ribosomal DNA fragments have shown that FD is related to the Elm Yellows (EY) phytoplasma (4, 9). Preliminary attempts in ELISA using polyclonal antibodies to EY had nevertheless failed to demonstrate a serological relationship between FD and EY. More recently, EY-group phytoplasma have been identified in grapevines in Germany in the Palatinate region, where the leafhopper species *S. titanus* does not live. These isolates were found to react positively in ELISA (11). However, DNA analyses showed that they were different from FD *sensu stricto* isolates (i.e. transmitted by *S. titanus*).

The present work aimed at a closer study of serological relationships between FD and other grapevine and non-grapevine isolates.

Various rabbit antisera (Pab) (1) and a first series of mouse monoclonal antibodies (Mabs) (2) to FD phytoplasma have been raised in the animals with partially purified phytoplasma. A second series of Mabs were generated using immuno-affinity-purified FD phytoplasma cells (7) which had retained their physical and biological integrity (8). Western blots with rabbit Pab of proteins of the homologous FD strain detected two major components of 55 and 19 Kda (PP1 and PP2 respectively) and several minor components (7, 8). FD-Mabs from the first series would label PP1 or PP2 but no minor components, while Mabs of the second series would label PP1 or PP2 or else one of two minor components (10). Immuno electron microscopy showed that PP1 and PP2 were located on the phytoplasma membrane (8).

The different antibodies have been used in ELISA and Western-blotting assays on phytoplasmas in the EY group : two FD *sensu stricto* isolates originating from grapevine, two grapevine isolates from Palatinate and three non-grapevine isolates maintained in periwinkle, namely AEY (American elm yellows), ULW (French Elm witches-broom) and HD1 (12) (kindly provided by W. Sinclair).

The assays demonstrated that the strains are differently related. Some of the antibodies failed to give a positive response on heterologous isolates. Reaction with ULW was weak or negative. Western blot patterns were different, and the same Mab labelled peptides of different MW in the different strains. The biological significance of these differences in membrane proteins will be investigated, especially with regards to transmission specificity by vector leafhoppers.

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THERMOTHERAPY TRIALS TO ELIMINATE PHYTOPLAMAS FROM PROSECCO, CHARDONNAY AND INCROCIO MANZONI 6.0.13 GRAPEVINE CULTIVARS: PRELIMINARY RESULTS

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In the last four years severe epidemic of grapevine yellows disease associated with genetically distinct phytoplasmas have been reported in North Italy (1, 3, 7, 11). The situation is very severe in Veneto region where grapevine cultivars very susceptible to phytoplasma infection are grown and the local production of cuttings is very important: grapevine yellows phytoplasmas (aster yellows: 16SrI-B and 16SrI-G) are often associated with Flavescence dorée (FD) type phytoplasmas (elm yellows group: 16SrV) and were detected in single and mixed infection (2, 4). The viticulture is very seriously affected by the presence of this disease that causes high percentage of death in plants of cultivars Chardonnay and Incrocio Manzoni (IM) 6.0.13 and also causes severe and widespread losses of production in cultivars Garganega and Prosecco.

The possibility to produce grapevine materials without symptoms of yellows through thermotherapy has been demonstrated (6) on cultivar Baco 22A, therefore thermotherapy trials on some of the most important and affected cultivars such as Prosecco and I. M. 6.0.13 together with Chardonnay were carried out. Controls on samples before and after the thermotherapy were performed by PCR assays that has been demonstrated sensitive and reliable to detect phytoplasma presence in grapevine all over the year (10). The preliminary results after two growing seasons are reported.

During winter 1996 grapevine ripened canes from 3 Chardonnay, 6 Prosecco and 2 I.M.6.0.13 symptomatic and asymptomatic plants were collected in FD infected vineyards in Treviso province (5) and after storage at 4°C for 50 days were employed for thermotherapy. Three batches of cuttings (20-30 cm long) were obtained from each original plant and were treated with hot water at the temperatures of 45°C for 3 hours (B) or 50°C for 40 minutes (C) or maintained untreated as control (A). The cuttings were then kept in forcing boxes in hot house for 40 days and after rooting were planted under an insect-proof screenhouse in a soil free from pathogens and treated against pathogens and pests with regular agricultural practices for grapevine.

Before hot water treatment the nucleic acid extracted from woody canes from each source plant was tested to verify phytoplasma presence using nested-PCR with general R16F1/R0 and/or R16F2/R2 (9) and specific primer pairs R16(I)F1/R1 and R16(V)F1/R2 (8) to identify major phytoplasma groups already detected in grapevines in this area (16SrI-G, 16SrI-B and 16SrV). The experiments were carried out following procedures already described for nucleic acid extraction and for PCR amplification from grapevine (12). In each experiment negative controls devoid of DNA template and positive phytoplasma controls (AY, IPVR and EY) for the phytoplasma groups mentioned above were also employed. After re-establishment in soil the molecular tests were carried out 3 times in different periods of the year using samples collected from batches of 1 to 3 rooted cuttings.

Immediately after hot water treatment the plants in thesis A and B were in good conditions, while those from thesis C show reduction in viability indicating a dangerous effect of high temperature (table 1). The second control, performed in October 1996, show that high mortality percentage was present in A and C plants, especially in cultivars Prosecco and Chardonnay confirming that the lower temperature, even if for longer periods, gave better agronomical results; this data were confirmed in Spring 1997 when almost all the survived plants sprouted.

Table 1: effects of thermotherapy treatment on survival of ungrafted grapevine cuttings in May and October 1996.

Grapevine cultivars	A (untreated) % of plant surviving			B (45°C for 3 hours) % of plant surviving			C (50°C for 40 minutes) % of plant surviving		
	n. of plants	5 1996	10 1996	n. of plants	5 1996	10 1996	n. of plants	5 1996	10 1996
Prosecco symptomatic	19	100.0	26.3	26	84.6	53.8	21	66.6	42.8
Prosecco asymptomatic	32	84.4	31.2	32	82.3	44.1	37	54.0	16.2
Chardonnay symptomatic	13	92.0	38.5	11	100.0	45.5	16	50.0	31.2
Chardonnay asymptomatic	17	100.0	41.2	20	90.0	45.0	17	64.7	29.4
I.M.6.0.13 symptomatic	12	92.0	50.0	12	100.0	67.0	10	90.0	80.0
I.M.6.0.13 asymptomatic	10	100.0	80.0	10	100.0	33.3	10	100.0	0

Molecular analyses before the treatment to verify phytoplasma presence revealed that all the plants employed were singly or double infected with phytoplasmas, mainly belonging to the 16SrI or AY group, also those that were asymptomatic during the growing season (table 2). Nevertheless PCR tests carried out after re-establishment of plants revealed that only after one year (May 1997) it was possible to detect phytoplasma presence in some of the untreated control (A) since the tests carried out in August 1996 and February 1997, respectively on leaves and on dormant wood after pruning, were negative for all the materials. This results indicates that the phytoplasma concentration was remarkably reduced after the cutting procedures.

The results of May, indicating that no phytoplasma could be detected in the treated material, are encouraging but it is necessary to perform a further molecular tests later in the growing season 1997, to verify if all the untreated plant batches will give positive results for phytoplasma presence, and to further confirm that phytoplasmas have been eliminated with the heat treatments.

Table 2: preliminary results of thermotherapy to eliminate phytoplasmas from three grapevine cultivars

Grapevine samples		Symptoms presence	Phytoplasmas detected before treatment	A (untreated)	B (45°C for 3 hours)	C (50°C for 40 minutes)
Prosecco	27	symptomatic	16SrI + 16SrV (AY + EY)	+	-	-
Prosecco	29	asymptomatic	16SrI (AY)	+	-	-
Prosecco	30	symptomatic	16SrI (AY)	-	-	-
Prosecco	40	symptomatic	16SrI (AY)	-	-	-
Prosecco	41	asymptomatic	16SrI (AY)	-	-	-
Prosecco	42	symptomatic	16SrI (AY)	-	-	-
Chardonnay	33	symptomatic	16SrI + 16SrV (AY + FD)	-	-	-
Chardonnay	36	asymptomatic	16SrI (AY)	+	-	-
Chardonnay	37	symptomatic	16SrI + 16SrV (AY + FD)	+	-	-
I.M.6.0.13	46	asymptomatic	16SrI (AY)	-	-	-
I.M.6.0.13	47	symptomatic	16SrI (AY)	-	-	-

+ , amplification of phytoplasma DNA; -, no amplification.

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GRAPEVINE LEAFROLL ASSOCIATED VIRUSES IN AUSTRALIA: DETECTION TOOLS DEVELOPED AND VIRUS TYPES IDENTIFIED

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Grapevine leafroll disease was first identified in Australia during the 1960s and reported subsequently (Woodham et al., 1973). Later, biological indexing data revealed distinct disease types (Krake, 1993) and more recently the presence of five leafroll associated viruses (GLRaV-1, 2, 3, 4 and 5) have been determined (Habili et al., 1996).

Our approach to characterising leafroll associated viruses at the molecular level has been via isolation of viral dsRNA (Rezaian et al., 1991), cloning and partial sequencing of the viral genomes. A random-PCR cloning method has been adopted to the dsRNA template, selectively yielding larger PCR products (Fig. 1). This procedure requires minimal quantity of tissue extract and is being used systematically to produce clones of leafroll virus types.

A cDNA clone of GLRaV-1 was found to correlate with the incidence of the disease and allowed the application of DNA based detection for this virus (Habili et al. 1997). The clone, LR1, hybridised in northern blots to RNA species of approximately 19.5 and 8 kbp. The latter RNA is polycistronic (Fig. 2) containing 7 open reading frames including one encoding a putative protein that resembles the coat protein of lettuce infectious yellows closterovirus. The ORFs for the coat protein and its putative duplicate, as occur in closteroviruses, have been expressed in a bacterial system and are being characterised.

GLRaV-1 has been detected in major grapevine varieties grown commercially in Australia (Habili et al., 1996). Some vines of previously symptomless grapevine clones, have been recently found to show leafroll symptoms and to be infected with GLRaV-1. This suggests the virus may be spreading naturally and, if proven, would be the first incidence of GLRaV-1 spread by a vector.

GLRaV-2 was detected by ELISA in the variety Black July, from Victoria, showing typical leafroll symptoms. No further surveys have been carried out because of the lack of reliable detection method.

GLRaV-3 has generated a considerable interest because of its natural transmission. A probe made to the type 3 virus has been used, in an epidemiological survey demonstrating the spread of leafroll 3 in a clonal trial of Pinot Noir. The occurrence of virus spread since early 1990s in Australia has now been well established (Habili et al., 1995). GLRaV-3 has mostly been found in germplasm collections and is spreading in these vineyards . In the current season the virus spread has been observed in commercial vineyards in Victoria, in South Australia and in Western Australia. The identity of the virus vector is not known and several virus transmission attempts using mealybugs have failed to transmit the virus to healthy grapevines (Ewart, Bailey and Habili, unpublished).

GLRaV-4 has been detected in most Sultana clones known to have a mild leafroll (Krake, 1993; Habili et al., 1996). GLRaV-4 is present in most selected high yielding Sultana clones (Habili et al., 1996). In fact, no Sultana clone free of one or more leafroll types has been found in commercial plantings in Australia (Woodham et al., 1984).

A DNA clone (LR4) constructed to the type 4 virus has significantly improved the reliability and sensitivity of detection. Northern blot analysis showed hybridisation of LR4 to the dsRNA extract of a Thompson Seedless grapevine clone from which GLRaV-4 was originally isolated. The cDNA clone was sequenced and shown to be specific to GLRaV-4 by reverse-transcription-PCR using GLRaV-4 particles enriched by the virus antibody coupled to magnetic beads. RT-PCR was used successfully to screen different varieties of grapevines for the virus thus overcoming detection ambiguities for this virus by serological means. Western blot analysis of GLRaV-4 extracts from different sources of infected grapevines revealed two distinct species of capsid protein (Fig. 3) with estimated Mr of either 35,400 or 38,000 depending on the variety used. Both proteins reacted with polyclonal as well as monoclonal antibodies.

GLRaV-5 occurs in the table grape variety Emperor in Western Australia where it is considered to enhance the quality this grape. The detection of these two virus types was restricted by the lack of reproducibility using ELISA tests. The DNA cloning procedure described here will be applied to develop alternative detection tests.

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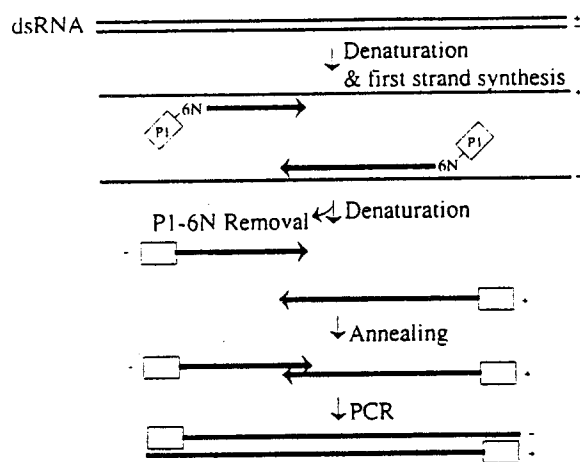
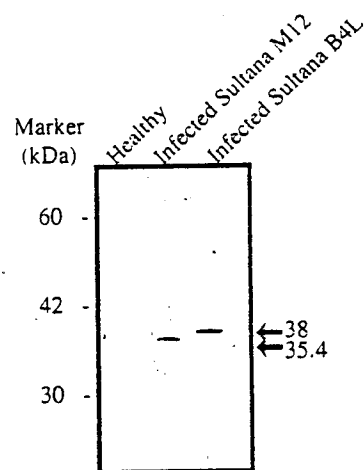Figure 1 The scheme for synthesis of dsDNA from dsRNA template using PCR

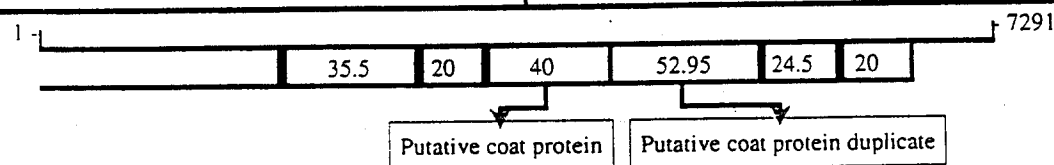Figure 3 Western blot of partially purified GLRaV-4 particles showing two types of coat protein

Figure 2 Partial nucleotide sequence of an 8 kb subgenomic RNA isolated from GLRaV-1 infected grapevine

APPLICATION OF ELISA FOR VIRUS DETECTION USING A POLYCLONAL ANTIBODY PRODUCED FROM A RECOMBINANT COAT PROTEIN OF GRAPEVINE LEAFROLL VIRUS 3 EXPRESSED IN *ESCHERICHIA COLI*

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Diagnosis of grapevine leafroll is complicated by the fact that several serologically distinct types of closterovirus-like particles (GLRaV 1 to 6) are associated with the disease. Recently, we sequenced the GLRaV-3 genome and identified its coat protein gene. During the identification of the coat protein gene, several immunopositive clones in pBluescript SK- were selected. Clone pCP10-1 produced a protein that reacted to GLRaV 3 antibody. Nucleotide sequencing of this clone revealed an open reading frame (ORF). A fusion protein encoded by this ORF consisted of the N-terminal portion of the GLRaV 3 coat protein (35.3 kDa) and the C-terminal portion of the β-galactosidase (14.7 kDa). The 50 kDa fusion protein was over-expressed in *E. coli* and purified through ammonium sulphate precipitation and SDS-polyacrylamide gel electrophoresis. The purified fusion protein was injected into a rabbit to produce a polyclonal antibody (As163). This antiserum decorated GLRaV 3 virus particles on immunosorbent electron microscopy. The sensitivity and specificity of As163 were compared with a polyclonal antibody produced to purified virus on ELISA and Western blot. As163 could be used for coating and/or conjugate in ELISA tests. However, the antibody was most effective as a coating antibody combined with a monoclonal antibody conjugate. This newly produced antibody was highly effective in validation tests of a diverse collection of grapevine germplasms. Results indicated that unlimited supply of recombinant coat protein for antigen can be produced from its expression in *E. coli* and the antibody so developed can be used for routine virus detection.

COMPARATIVE EFFECTIVENESS OF ELISA AND PCR FOR DETECTION OF GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUS 3

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Several serologically distinct types of closterovirus are thought to be the causal agent of grapevine leafroll, this makes diagnosis of the disease more difficult. Methods such as enzyme linked immunosobent assay (ELISA) and polymerase chain reaction (PCR) have the potential to streamline the virus detection. However because of the difficulty in purifying viruses from a grapevine, most of antibodies developed are either of poor quality or the supply of an antiserum is limited. Strategies aimed at control of the disease including regulatory certification programs are heavily dependent on appropriate disease diagnosis. In our earlier work, we sequenced most of the GLRaV 3 genome. Also we have observed that several sets of primers derived along the GLRaV 3 genome were suitable candidates for PCR. Two simple methods of sample preparation, immunocapture and proteinase K-treatment were developed for application of PCR. To further increase the sensitivity and accuracy of PCR for detection of large scale field collected samples, Nested-PCR was developed. Sensitivity of Nested PCR was high, which could provide 10^3-10^6 times more efficiency over ELISA. However, inconsistency and sometimes contamination of PCR reactions made data interpretation more difficult. Therefore a rigorous validation test was done to compare PCR and ELISA on samples that had been well defined for their virus status through woody indicator indexing. In a validation test, three symptom showing vines and three symptomless vines were selected from field. Over one thousand samples were simultaneously tested using ELISA and PCR for the presence of GLRaV 3 throughout the year on different tissues. PCR and ELISA were both effective in testing symptom showing or symptomless vines. However PCR could detect GLRaV 3 in an early plant developmental stage when virus titer was low and not detectable by ELISA. Compared to ELISA, PCR was more costly and sometimes PCR results were not consistent, especially with leaf samples. In another screening with over 50 germplasms collected around the world, results from Immunocapture PCR and ELISA were in a good agreement. All these evidences suggest that the PCR can be an alternative, complementary detection method over ELISA, but it has drawbacks in large scale application.

IC/RT-PCR COUPLED TO EXONUCLEASE FLUORESCENT ASSAY. EARLY-SPRING DETECTION OF GLRAV 3 IN LEAF PETIOLES.

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INTRODUCTION

Genomic amplification by PCR methodology has proven to be a very sensitive tool for the detection of plant viruses. However, although the first reports of its use began to appear some five years ago, it has not yet been adopted in large scale screenings. It is generally admitted that PCR based methods require a special technical skill and are much more time consuming and laborious than the widely used ELISA assay. Two main reasons stand for that: Laborious nucleic acids extractions and the analysis of PCR products by electrophoresis, which is very difficult to automate for large number of samples. In this work we present a format of PCR based assay that completely overcomes the above stated drawbacks: the nucleic acids extraction is completely avoided by an immunocapture step (Immunocapture Reverse Transcriptional Polymerase Chain Reaction - IC/RT-PCR) performed by antibodies immobilised on the surface of PCR tubes (Nolasco et al., 1992 and 1993 and the analysis of PCR products is performed by an homogeneous fluorogenic 5' nuclease assay (Holland et al., 1991) instead of electrophoresis. This assay takes advantage of the 5' nuclease activity of Taq DNA Polymerase to cleave a probe doubly labelled with a fluorescent dye and a quencher on its extremities (Taqman [TM] probe). During amplification the probe specifically anneals to the PCR target and is cleaved by Taq Polymerase during primer extension. The physical separation of the fluorescent group from the quencher results in a net increase of the fluorescence of the sample; in healthy samples the probe is not cleaved and fluorescence remains at a low level. Compared with previous approaches based on PCR detection, this procedure has the advantage of avoiding any post-PCR steps.

The assay was developed taking as a model the Grapevine Leafroll Associated Virus 3 (GLRaV 3).

Diagnosis of GLRaV 3 is usually done by ELISA in wood shavings obtained from dormant canes in fall, when the virus titer is higher. In order to assess the usefulness of PCR based diagnostics we compared against ELISA its ability to detect the virus in leaf petioles in early spring.

METHODS AND MATERIALS

Infected plant material with GLRaV 3 was obtained in most of the cases from naturally infected field plants. A few isolates kept in a collection representative of different parts of the country were selected for sequencing purposes. Leaf petioles were always used as source of infected tissue.

Sequencing part of the GLRaV 3 genome..

An initial set of primers was designed taking as basis a small sequence available in the GenBank Data Library under accession number U22158. This set of primers was used in IC/RT-PCR assays to amplify a part of the viral genome of several isolates. The cDNA so obtained was ligated via A-overhangs into the pCRII vector (Invitrogen) previously linearized and displaying T-overhangs and used to transform Invα F' competent E.coli cells. Sequencing of the insert was achieved through conventional methods.

One tube - Single step IC/RT-PCR assay.

The assay developed through this work was based in (Nolasco et al., 1992; 1993). In its optimised version the thermoresistant polypropylene PCR tubes or plates (QSP, USA Scientific or Perkin Elmer) are coated with 50 µl IgG (Bioreba). Sample extraction is done the same way as for ELISA and antigen trapping at the inner surface of the PCR tubes occurs for 3h at room temperature. At the end of this period the tubes are washed twice with PBS-T and once with MiliQ quality water; 50 µl of the RT-PCR mix (10 mM Tris HCl pH 9.0 at room temperature, 50 mM KCl, 4 mM Mg Cl_2, 200 mM each dNTP, 200 nM each primer, 40 nM of fluorescent probe containing 7.5 units MMLV RTase. (Perkin Elmer), 3.5 units ribonuclease inhibitor (Pharmacia) and 1.25 units of Taq DNA Polymerase, from Perkin Elmer) were added and the tubes incubated in the thermocycler at 38 °C for 45 min, 94 °C for 2 min, 40 cycles of 92°C for 30 S and 58 °C for 1 min, with a final step of 58 °C for 10 min.

Detection of PCR products by fluorescence.

The probe used in this assay (obtained from Perkin Elmer) has 27 nt and is labelled at its 5' end with 6-carboxyfluorescein (FAM, reporter dye) and at its 3' end with 6-carboxytetramethylrhodamine (TAMRA, quencher). Fluorescence was measured with a luminescence spectrometer equipped with a microwell plate reader. Excitation was performed at 488 nm and the emission recorded at 525 nm (reporter dye) and 582 nm (quencher). A low-cut filter of 515 nm was used during the measurements. The increase in fluorescence for each sample (Δ RQ) is calculated by dividing the value of the fluorescence of the reporter by that of the quencher (R/Q) followed by subtraction of the R/Q value corresponding healthy sample. The

positive / negative threshold was set at 6.965 times the standard deviation of the healthy samples (3 repetitions).

RESULTS AND DISCUSSION

It is well known that some degree of mismatching of the primers near their 5' end does not interfere significantly with the PCR amplification. However there is no much data that enables to predict the behaviour of the 5' fluorescent nuclease assay in a non conserved region. It was suggested that the occurrence of mismatching would result in probe displacement rather than its cleavage (Livak et al., 1995). Our experience (unpublished results) supports the same conclusion; with certain probes the occurrence of only one mismatch, irrespective of its position, is enough to impede the cleavage of the probe. To design this kind of assay it is then necessary to find a very conserved genomic region. This can become a problem with RNA viruses, which usually display a high number of nucleotide substitutions along the genome.

Until now only one a small part (1Kb) of the GLRaV 3 genome from an Australian isolate is available at the gene bank database. It was reported that this region had 99.5% nucleotide homology with an Italian isolate (Habili et al., 1995). This could be an adequate region to design a fluorescent assay. To further confirm the high degree of homology displayed in this genomic region, four additional isolates from different parts of Portugal were sequenced. A segment of about 500 nucleotides was found to be very conserved among the six isolates (homology higher than 99%) and it was used to design a set of primers and fluorescent probe.

To compare the ability of the PCR assay and ELISA to diagnose GLRaV 3, a set of twenty field grown grapevines known by ELISA to be infected in the fall of 1996 was weekly monitored during the spring of 1997 by both techniques. At the onset of spring (24 Mars) the virus was already detected by PCR in one plant. In general detection by PCR preceded the detection by ELISA for 3 weeks (1 plant) , 2 weeks (4 plants) or 1 week (3 plants). The first positive values obtained by ELISA occurred only at the 17th April. At the beginning of May the virus was detected by ELISA in all the samples. In the following weeks ELISA's detecting ability decreased progressively. At June 12th, ELISA could detected the virus only in 12 plants. At this date some of the ELISA negative samples could still detected by PCR .

On the other hand, the PCR-fluorescent assay could not detect always all the samples positive by ELISA. Two cases could be distinguished. One situation corresponded to a set of 3 samples that could be detected by PCR followed by electrophoresis but whose fluorescence was very weak to be considered positive in most of the times these samples were assayed. These samples have probably an unreported mismatch in the zone of probe hybridisation. The other case corresponds to samples that could not be amplified by PCR even using five different combinations of primers. This situation is intriguing because it occurs in a conserved region for most of the isolates.

In conclusion, IC/RT-PCR coupled to 5' nuclease fluorescent assay has the advantage of being able to extend the period for diagnosis and to simplify the preparation of the samples in comparison with ELISA, but some molecular aspects of GLRaV 3 variability need still further elucidation.

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DETECTION OF GRAPEVINE VIRUS A USING ANTISERUM TO THE PUTATIVE MOVEMENT PROTEIN

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Detection of rugose wood in grapevines is difficult and expensive: budwood from tested plants is grafted onto sensitive *Vitis* indicators and the appearance of symptoms is monitored for 3 years (8). Much effort has been invested in developing serological and nucleic acid-based methods for detection of viruses implicated in the rugose wood complex (1,2,3,9,10,11). Grapevine virus A (GVA), is closely associated with Kober stem grooving, a component of the rugose wood complex (3,7). Initially, serological detection of GVA was found difficult because of the low immunogenecity of the virus and low concentration of particles in affected plants, but recently the method has been improved by the introduction of monoclonal antibodies (2). PCR-based detection protocols have been developed which resulted in the more sensitive detection of GVA (3,9,10).

GVA coat protein (CP) and movement protein (MP) genes were cloned by RT-PCR (15) using oligonucleotide primers based on the sequence information published by Minafra et al. (12) in the T7 RNA polymerase expression vector pET3a (14). The expression of the CP and the MP genes in *E. coli* resulted in production of proteins of apparent molecular weights of approximately 26 and 35 kDa., respectively. Antisera induced in response to the two *E. coli*-produced proteins reacted specifically with GVA CP and MP in total protein extracts (6) from GVA infected *N. benthamiana* plants.

In order to examine the subcellular distribution of the GVA-MP, inoculated leaves were fractionated using a method, previously employed by Sato *et al*. (13) to study the subcellular distribution of apple chlorotic leaf spot trichovirus. Protein extracts were fractionated to cell membrane, soluble and cell wall fractions. The GVA-MP protein was detected in all fractions but was consistently most abundant in the soluble protein fraction. Alternatively, the procedure used by Deom, *et al*. to study the subcellular distribution of TMV MP was used (5). Again, most of the GVA MP was detected in the soluble protein fraction. Cell fractionation experiments, with several plant viruses, detected a large proportion of MPs in the cell-wall fraction (4,5,13). Recently it was demonstrated, by means of the same extraction methods used in the present work, that the distribution of CMV and TMV MP in infected plants is complex: the distribution was found to differ among viruses, among leaf positions and also between transgenic plants and virus infected-plants (4). More detailed study of GVA-MP localisation during different stages of the infection is needed to determine whether GVA-MP has a distinct subcellular distribution compared with other viral MPs

The GVA-MP and the CP were detected by western blot analysis of GVA infected grapevines. However, in each case where GVA could be detected, the immuno-reaction with the MP antiserum appeared as a stronger band relative to the detection signal obtained by the CP antiserum. suggested that the MP may be a preferred antigen for the detection of GVA in grapevine. Samples from rugose wood-affected vines (cv. Thompson seedless) collected in the Lachis region were analysed by western immunoblotting, using MP and CP antisera. The MP could be detected in all five vines tested, while the CP could not be detected in the same extracts. The same plants were also tested with a commercial ELISA kit for the detection of GVA (based on anti-GVA antisera) and all five plants tested negative. Phloem rich tissues - phloem shavings, petioles and older leaf veins - were the best source of material for testing for the presence of GVA-MP in grapevines. The MP was not detected in young leaves or in developing fruits. The MP antiserum was virus specific and did not cross reacted with the GVB-MP in protein extracts from GVB infected vine.

Immunoblot analysis showed that the MP may provide a sensitive mean of detection of GVA in infected grapevines. The MP antiserum proved to be superior both to the CP antiserum and to a commercially available ELISA kit based on anti-GVA antiserum. To our knowledge this is the first demonstration that a non-structural protein may be the preferred target for serological detection of a plant virus.

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IMPROVEMENTS IN THE MOLECULAR DIAGNOSIS OF GRAPEVINE CLOSTERO- AND TRICHOVIRUSES

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Efficient detection and identification of phloem-limited clostero- and trichoviruses of *Vitis* with molecular techniques is hampered by the lengthy sample preparation and processing, and the number of tests to be made if the whole series of these viruses (7 closteroviruses and 4 trichoviruses) is to be looked for (1, 2). On the other hand, the increasing emphasis on implementation of grapevine certification schemes and the need for large-scale testing with rapid, sensitive, and reliable methods, call for the adoption protocols as simple and wide range as possible. To this aim two methods were developed which greatly simplify sample preparation, and multiple virus detection.

SPOT-POLYMERASE CHAIN REACTION (spot-PCR)

Specific amplification of genomic fragments of grapevine trichovirus A (GVA), B (GVB) and grapevine leafroll associated virus 3 (GLRaV-3) was obtained by RT-PCR of total nucleic acid extracts solubilized from a charged nylon membrane pre-wetted with NaOH, on which a drop of unbuffered sap expressed from grapevine leaf petioles had been deposited. Small pieces of the spotted membrane were incubated in glycine buffer for releasing adsorbed nucleic acids that served as template for standard RT-PCR. Consistent amplification of the expected GVA, GVB, and GLRaV-3 genome fragments was obtained up to one month after spotting, with a detection threshold comparable to that of regular PCR. Dilution of the sample produced a more efficient amplification, probably because of the removal of inhibiting factors contained in grapevine extracts. Duplex PCR (i.e. amplification of different viruses from a grapevine source with mixed infection) was also found to be effective, since GVA and GLRaV3 were amplified by a mixture of specific primers in the same reaction. Spot-PCR speeds up sample preparation allowing to spot membranes directly in the field. Screening of several hundred samples can be done in a day, thus expediting certification schemes and quarantine controls. Spotted membrane can be shipped in places far away from the site of sample collection and processed after extended periods of time without apparent loss of sensitivity.

DEGENERATE PCR

Degenerate oligonucleotides targeted to conserved sequences of certain viral genes, successfully amplified specific genomic sequences of grapevine closteroviruses and trichoviruses (vitiviruses) as well as trichoviruses from other hosts. Degenerate primers for closterovirus detection were those chosen on the phosphate motifs 1 and 2 of the HSP70 homologue gene (2), slightly modified. Highly degenerate primers were also selected on conserved motifs II and V (3) of the RNA-dependent RNA polymerase genes of vitiviruses and trichoviruses (Fig. 1).

```
GVA    1411 AKAGQTIACFAHSVLCRFGPILRQTEKALRELLPEKLMIYSQKKYMDLDK
GVB    1575 AKAGQTIACFCHAVLCRFGPLLRQTEKALRDQLGPNVMIYSQKNYTDLDK
AC1SV  1406 AKAGQTLACFPHKILVEFSPWCRYTEKVLTANLPDNYYIHQRKNFSELED
            ****** ***  * .*  *.*  * *** *    *  .  *  .*.. .*.

GVA         WAKTWVESMMGTDSDYEAFDRSQDEKVLDLEVEVLRFFLWPEDLIREYEE
GVB         WCKGFVHTLDGTDSDYEAFDRSQDEKVLDFEVNVLKFFLWPEEMIEEYVT
ACLSV       FARRFSNGSICVESDYTAFDVSQDHTILAFEVELLRHFGWDDRVLQSYIK
              .     .   .*** *** ***. .*  .**..*. * *  . ..  *

GVA         LKLMMGCALGDLAVMRFSGEF 1531
GVB         LKLMMGCSMGSLAVMRFSGEF 1526
ACLSV       MKCTLGCRLGGFAIMRFTGEF 1695
            .*  .** .*  .*.***.***
```

Fig. 1. Motifs in the RdRp protein of trichoviruses selected to design primers (in bold).

cDNAs sequences of the expected size were successfully amplified by RT-PCR on total nucleic acid extracts or dsRNAs of the following: GLRaV-1, GLRaV-2, GLRaV-3, and GLRaV-7 closteroviruses; different strains of GVA, GVB, and GVD vitiviruses; ACISV, PVT, and HLV trichoviruses. These sequences were

cloned and used to generate digoxigenin-labelled riboprobes that reacted specifically with the homologous viruses. Cloned cDNAs were sequenced and found to contain the original conserved motifs of the HSP70 and RdRp genes.

In addition to diagnosis, this wide range PCR system can be used also for sequencing work. For instance, is now being utilised for amplifying trichovirus-related and unknown sequences of the GVD and HLV genomes and closterovirus-related sequences in the genomes of GLRaV-2 and GLRaV-7.

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PRODUCTION AND APPLICATION OF AN ANTIBODY TO THE GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUS 2 COAT PROTEIN EXPRESSED IN *ESCHERICHIA COLI*

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Grapevine leafroll associated closterovirus 2 (GLRaV 2) is one of the several serologically distinct closteroviruses that are associated with leafroll disease. GLRaV 2 contains a single species of 22 kDa protein and is phloem limited. Because the virus purification is difficult and yields of purified are usually low, it makes the antibody production difficult and unsatisfactory for accurate detection of GLRaV 2. Clearly, there is a need to develop an alternative method for production of antibody. In our previous work, the genome of GLRaV 2 was sequenced and the coat protein gene was identified. The coat protein gene of GLRaV 2 (local isolate) was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) from dsRNA extracted from GLRaV 2 infected grapevines. The PCR product was cloned into the fusion protein expression vector pMAL-c2 at EcoR I and BamH I sites. The coat protein genome sequence of GLRaV 2 was confirmed by sequencing. Fusion protein which contained a maltose binding protein and GLRaV 2 coat protein was expressed in *E. coli* by IPTG induction, and reacted specifically with GLRaV 2 antiserum in Western blot. The fusion protein was purified by a one step affinity column chromatography and concentrated with Ultrafree centrifugal filter. The purified fusion protein was subjected to immunisation of a rabbit and an antiserum was produced. This antibody was shown to give specific reaction to GLRaV 2 in Western blot. With this antibody we were able to use ELISA to detect GLRaV 2 in field collected grapevine samples. This antibody also reacted with other GLRaV 2 isolates from South Africa and France. This method demonstrates the advantage of molecular biology as an alternative approach to obtain pure antigen for production of high quality antisera, especially in the case of low titer viruses.

DETECTION OF GRAPEVINE VIRUSES USING COLORIMETRIC PCR.

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Polymerase chain reaction (PCR) provides a very sensitive methodology for the detection of plant virus RNA. The development of immunocapture-PCR (IC-PCR) has greatly simplified and improved our capability to process large numbers of samples. Although IC-PCR offers vast improvement in sensitivity over ELISA, it is substantially more time consuming in requiring gel electrophoresis to analyse the test results. To overcome this limitation, we have investigated and developed a colorimetric PCR assay. In this assay, digoxygenine (dig) is incorporated in amplified DNA. For detection of an amplified segment, a previously prepared oligonucleotide probe (about 20-25 base long) which is biotinilated and complement to an internal sequence of amplified segment is added to a previously streptavidin-coated ELISA plate. Denatured PCR products are added to the plate and the presence of specific amplified DNA is detected by adding anti-dig antibody conjugated to an enzyme followed by an incubation, wash and addition of specific substrate for the enzyme. Finally, the colorimetric reaction is analysed by an ELISA reader. We have tried this method for the detection of grapevine fanleaf virus, grapevine leafroll associated virus-3, and grapevine viruses A and B. We found that colorimetric PCR usually gave greater sensitivity than gel analysis of PCR products for the detection of these viruses.

SEROLOGICAL DETECTION OF DOUBLE-STRANDED RNA FROM GRAPEVINE VIRUSES

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Most plant viruses have RNA in their genomes and when they replicate double-stranded RNA (dsRNA) occurs. Considering that healthy plants do not usually have detectable amounts of dsRNA, its analysis is a reliable procedure for screening plants, once it is not specific, and so, allows the detection of any viral RNA, including viroids (1). However, dsRNA detection requires laborious extraction procedures and electrophoresis. Thus, serological detection of dsRNA seems to be a reliable alternative for testing plants, when the identity of the pathogen is of little consequence (2, 3, 4).

In this report, we present the results of different procedures for serological detection of dsRNA of three grapevine viruses - grapevine leafroll associated virus 3 (GLRaV-3), grapevine fleck virus (GFkV) and arabis mosaic virus (ArMV), using polyclonal and monoclonal antibodies.

Antiserum to polyinosinic-polycytidylic acid (poly[I].poly[C]) was prepared by weekly injections of a 10 weeks old female Californian rabbit with 1 ml of 2 mg/ml solution of poly[I].poly[C]. In the first immunisation (intramuscular), an equal volume of Freund's incomplete adjuvant was used. The last four injections were intravenous and weekly bleedings started 7 days later. IgG was purified by standard ammonium sulphate precipitation/DEAE-celulose chromatography and biotinylated. Ascite fluid with IgM antibodies was kindly provided by Dr. Noemi Lukacs (3). DsRNA extraction and purification was done from 5 g of mature canes tissue as reported (5). Serological detection was performed in 96 well polystyrene plates precoated with 2 μg/ml of poly-L-lisine, as described (2), with minor modifications. Wells were incubated with samples for 3 h at 37°C and a blocking step was performed for 1 h at 37°C with 4% non fat milk (NFM) in PBS-T (phosphate-buffered saline, pH 7.4 with 0.05% Tween 20). IgG-biotin or ascite fluid solutions were then added in PBS-T + 1% NFM in an appropriate dilution. Streptavidin or rabbit anti-mouse alkaline phosphatase conjugates in PBS-T + 1% NFM were incubated for 2 h at 37 °C and finally, substrate solution was added (1mg/ml of p-nitrophenil phosphate in diethanolamine buffer, pH 9.8). Between each step, plates were washed 3 times with PBS-T. A volume of 100 μl was used to fill each well. Virus infected and healthy samples were tested in three conditions: aqueous extracts (Tris-HCl 0.2 M, pH 8,2 with 2% PVP, 1% PEG and 0,05% Tween 20), phenol-chloroform extracts and purified nucleic acids.

In tested conditions, it was impossible to make a clear and consistent differentiation between infected and healthy samples either in aqueous or phenol-chloroform extracts with the two antibody systems. Backgrounds from healthy samples (determined by polyacrylamide gel electrophoresis) could not be eliminated (data not shown). However, this problem was overcomed after further purification of samples by CF-11 cellulose chromatography and ethanol/sodium acetate precipitation (Figure 1). Despite the lower sensitivity, as compared to the monoclonal antibodies, our biotinylated polyclonal antibodies could effectively detect dsRNA.

Two main limitations could be associated with aqueous or organic extraction media- the low concentration of these nucleic acids, that cannot be consistently detected by ELISA, and the strong influence of polyphenolic and polysaccharides compounds. These two compounds might difficult the isolation of dsRNA. Also, the origin of backgrounds is not clear. The antibodies could be reacting with low molecular weight nucleic acids that could not be detected by electrophoresis. Although the main goal of this work was not achieved (rapid non-specific detection of viral dsRNA in aqueous extracts), this methodology can be of some interest in sanitary selection programs, when the identity of the pathogen is irrelevant. Considering that actually more than 40 viral agents are associated with grapevines (Walter and Martelli, 1996, pers. com.) and that probably all of them have RNA in their genomes, they could hypothetically be simultaneously detected. So, we believe that further studies in other grapevine viruses and in the enhancement of this ELISA procedure should be carried.

A405 (2h)

1.60
1.40
1.20
1.00
0.80
0.60

GLRaV-3   GFkV   ArMV   Healthy
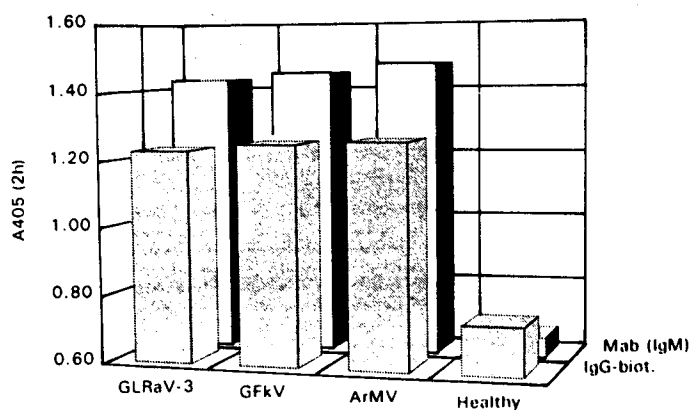
Mab (IgM)
IgG-biot.

Figure 1. Serological detection of purified dsRNA from grapevine woody tissues infected with GLRaV-3, GFkV and ArMV with biotinylated polyclonal IgG and ascite fluid with IgM (mean of four repetitions; standard deviations ranged from 0.199 to 0.008)

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ELISA FOR THE DETECTION OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUSES 1, 2, 3 AND GRAPEVINE VIRUS B BASED ON POLYCLONAL ANTIBODIES.

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Grapevine leafroll-associated viruses 1, 2 and 3 (GLRaV-1, -2 and -3) and grapevine virus B (GVB) are of concern to the grapevine industry. The consistent connection of GLRaV-1 and -3 with grapevine leafroll observed in laboratories world-wide has led to the conclusion that these viruses can be regarded as genuine agents of this disease (2). Studies in laboratories in France and Italy revealed that GLRaV-2 and GVB are associated with graft incompatibility of Kober 5BB rootstock (6) and grapevine corky bark disease (1) respectively.

Easily available, specific, sensitive and reliable method for the detection of GLRaV-1, -2, -3 and GVB are needed for the further study and effective control of these viruses in vineyards. In spite of the rapid development of molecular techniques, ELISA is still the most commonly accepted method for the detection of grapevine viruses.

In this paper we describe the indirect antibody-trapped antigen (ATA)- ELISAs (8) for the detection of GLRaV-1, -2, -3 and GVB which can be prepared relatively quickly, at low cost in standardly equipped laboratories.

Purification of viruses, immunoelectronmicroscopy (IEM), SDS-PAGE and Western blot were as described previously (3,4,5).

ATA-ELISA FOR THE DETECTION OF GLRaV-1, -2, AND -3

Multiple virus-infected grapevine cv. Black Spanish (3) was the source of GLRaV-1 and -3 used for the production of goat and rabbit antisera to these viruses. Coating antibodies for ELISA comprised a mixture of immunoglobulins from goat antiserum to viruses of grapevine cv. Black Spanish and from goat antiserum to GLRaV-2 that had been mechanically transmitted from grapevine to *N. benthamiana* (4). Crude rabbit antisera to electrophoretically separated capsid proteins of GLRaV-1 and -3 (3) and rabbit antiserum to GLRaV-2 (4) were used as second antibodies. Unlike to GLRaV-1 and -3, the composition of extraction buffer had a pronounced effect on the sensitivity of detection of GLRaV-2. 0.1 M Tris-HCl (pH7.6) buffer containing 0.01M $MgSO_4$, 0.2% 2-mercaptoethanol, 2% Triton X-100, 4% PVP-P was determined to be the best common buffer. Table 1 illustrates the application of our ELISAs for the detection of GLRaV-1, -2 and -3 in selected grapevines containing known combinations of these viruses.

Table 1. Specific or simultaneous detection of GLRaV-1, -2 and -3 in grapevines by ATA-ELISA.

| Grapevine | Virus status[1] | ATA-ELISA | | | |
		GLRaV-1	GLRaV-2	GLRaV-3	GLRaV-1,-2,-3
Neo Muscat	GLRaV-1,-2	1.585[2]	3.647	0.077	3.072
Tinta Barocca	GLRaV-2	0.078	2.949	0.067	2.169
Colombard	GLRaV-2,-3	0.076	0.857	2.202	1.307
Cabernet Sauvignon	GLRaV-3, GVA	0.082	0.067	2.846	1.840
Golden Hill	GLRaV-1,-2,-3	1.529	3.301	3.222	3.166
Cabernet Franc	healthy	0.073	0.067	0.066	0.068
Cabernet Sauvignon	healthy	0.076	0.076	0.067	0.073

[1]As determined by IEM

[2]Absorbance values (A_{405}) obtained after 45 min of substrate incubation

The survey of 117 grapevines from a collection block of table grape cultivars (VOPI, Pretoria), revealed unexpectedly high incidence of GLRaV-2 (72.6%), followed by GLRaV-3 (70.0%) and GLRaV-1 (10.1%).

ATA-ELISA FOR THE DETECTION OF GVB

Preparations of partially purified GVB from *N. benthamiana* were used for the production of goat and rabbit antisera to this virus. Immunoglobulins from goat antiserum to glutaraldehyde-fixed GVB were used in ELISA as coating antibodies. Crude rabbit antiserum to capsid proteins of GVB was used as second antibodies. GVB capsid proteins were purified by electrophoresis using SDS-PAGE gels as described by Harlow and Lane (7).

An effective and simple method of elimination of antibodies cross-reactive with grapevine virus A (GVA) from antiserum to capsid proteins of GVB, reported also for other antisera to this virus (5), was used.

A preparation of partially purified GVA was incubated in a boiling water bath until precipitation of proteins occurred and then mixed with the antiserum. After overnight incubation, the mixture was subjected to low speed centrifugation. The supernatant was used in ELISA.

Ten grapevines, all indexed corky bark positive were tested by our GVB ELISA and by an ELISA using monoclonal antibody (MAb1) (1) to this virus as second antibodies (Table 2).

Table 2. Detection of GVB in various grapevines by ATA-ELISA based only on polyclonal (Pab) or polyclonal and monoclonal (Mab) antibodies.

Grapevines	ATA-ELISA	
	PAb	MAb
Corky bark-positive:		
Shiraz	1.396[1]	1.528
Cabernet Sauvignon	0.626	1.563
Riparia Gloire de Montpellier	0.122	0.267
Colombard	0.209	0.838
Cape Riesling	0.367	0.912
Pais	0.156	0.459
Queen of the Vineyards	0.406	0.930
Alphonse Lavallee	0.311	0.815
Jubily	0.256	0.562
LN33	0.097	0.263
Healthy:		
Cabernet Sauvignon	0.052	0.069
Cabernet Franc	0.058	0.070
LN33	0.058	0.073

[1] Absorbance (A_{405}) values obtained after 50 min of substrate incubation

Despite the fact that ELISA based on polyclonal antibodies showed lower absorbance values to GVB than ELISA using monoclonal antibody, the results proved that antiserum to electrophoretically separated capsid proteins of GVB has diagnostic value and is useful for ELISA. It is necessary to point out that in the present study only one animal was immunised with capsid protein of GVB. As immunological response to injected antigen can be very variable between outbred animals such as rabbits, it is possible that better antiserum than described here may be obtained.

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PRODUCTION OF POLYCLONAL ANTIBODIES TO GRAPEVINE LEAFROLL ASSOCIATED VIRUS ISOLATED IN HUNGARY AND DEVELOPMENT OF HRPO-BASED ELISA SYSTEM

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Grapevine leafroll disease occurs world-wide and is of great economic importance (Goheen 1970). The causal viruses currently referred to as "leafroll associated" belong to two distinct genera: Closterovirus (particles 1400-2200nm) and Trichovirus (particles less than 800 nm) (Martelli, 1993).

In Hungary grapevine leafroll disease (GLD) is one of the most widespread disease on grapevine. Nowadays sanitary selection is the only preventive strategy useful against this grapevine disease. Selection is usually based on indexing method by grafting canes on indicator varieties. An indexing is time consuming because the expression of leafroll symptoms needs 2 or 3 years.

In this paper the production of polyclonal antibodies specific for closterovirus particles isolated from diseased Zweigelt (Hungary) showing leafroll symptoms and contained GLRaV-III antigens (Lehoczky et al., 1993) were describe.

MATERIALS AND METHODS

Virus source. Leaves of GLRaV-III infected Zweigelt plants were collected (2200 g) near Kecskemét at the beginning of September and kept frozen at -70 °C.

Virus purification and antiserum production. Virus purification was done according to Gugerli's procedure (Gugerli et al., 1984) for grapevine closteroviruses. Antiserum was prepared in six months old New Zealand rabbit.

Serology. The specificity of antiserum was determined with protein-A sandwich ELISA as it was described by Edwards and Cooper (1985). Extinction values twice higher than the average extinction of the healthy control plants were considered as positives (Cut off = $2 \times X_{healthy}$).

Plant material. Hungarian GLRaV isolates, identified previously on the base of woody indexing and of ELISA as type III as well as type isolate of GLRaV III, kindly provided by Dr. Gugerli and healthy plants of different grape varieties were applied for standardisation of the experiments. In order to check the reliability of the HRPO-ELISA system, simultaneous testing of symptom-showing Pinot Noir plants, inoculated with GLRaV III at PHSCS and about three hundred plants of different grape varieties, originating from various vine regions was carried out with BIOREBA GLRaV kit. Autumn leaf veins, petioles and shavings from mature canes (phloem) were homogenised in ratio 1:5 with "TRIS extraction buffer" (pH 8.2) suggested by BIOREBA and than diluted further for 1:20 and 1:50.

RESULTS

Virus purification. ELISA and electron microscopy were used to monitor virus particles in the purification procedures. Particles of semi-purified virus preparations were about 2000 nm (Fig.1) and remained intact in all steps of purification procedure.

Antiserum production. Specificity of obtained antisera in different bleedings were tested with leaf samples and bark scrapings from cane of grapevine leafroll diseased plants and virus free grapes. The average extinctions of healthy leaves (0.047 - 0.168) were relatively low and diseased leaves ranged between 0.221- 0-704. Extinction values of infected samples reached 1.48-2.3x higher than the cut off (2x $X_{healthy}$).

The ELISA tests. Grapevine leafroll diseased plants kept in collection in Kecskemét were tested by GLRaV-III antisera produced in our experiments and compared with Bioreba kit. Results are shown in Table 1.

In the Hungarian polyclonal HRPO ELISA system the average extinctions of the blanks (0.046) and the healthy controls (0.049-0.121) were acceptable low. Extinctions of the infected samples ranged between 0.437 and 0.797 it means that they reacted 2.7 - 4.8x higher value than the cut off (0.164). Standard deviation at the blank and healthy controls did not exceed 0.010 and in case of the infected samples this was between 0.001 and 0.054. In the alkaline phosphatase-based polyclonal/monoclonal BIOREBA ELISA system blanks and healthy controls had low extinctions (0.055 and 0.078-0.095). In the infected plant saps much higher extinction values (1.111 - 2.618) were measured than at the HRPO system. The infected plants showed 6.2 - 14.7x higher extinctions than the cut off value (0.178). Low standard deviation values were obtained in case of blank (0.004) and healthy controls (0.001-0.007) as well as at infected samples (0.016-0.272).

Although with the monoclonal/polyclonal BIOREBA reagents the extinction values of infected plants were much higher than compared to the extinctions got in the HRPO-based polyclonal ELISA system, it was

also able to detect all of the infections. The higher extinction values of the BIOREBA system most probably can be partly explained with combination of polyclonal and much more sensitive monoclonal antibodies.

Reliability of the HRPO system was further studied by comparative testing of about 300 grape samples using BIOREBA kit. Results of both system was the same in case of 287 samples out of 309 wich is 92.9% of agreement. At two samples BIOREBA gave positive reaction as those in HRPO system were negative, while at 20 samples GLRaV could not be detected with BIOREBA reagents but Hungarian HRPO ELISA system reacted positively. In spite of the generally lower extinction values of the HRPO system than measured with BIOREBA kit more virus infected plants were recognised by the Hungarian antiserum. Therefore we may assume that the serological differences between Swiss and Hungarian GLRaV isolates resulted in less positive reaction with the BIOREBA kit. To final clear up of this question further serological experiments and woody indexing of the above mentioned grapes are planned.

Table 1 Extinction values in DAS-ELISA obtained when comparing different grapevine plants showing severe leaf symptoms. Healthy control was virus free grapevine.

Samples	GLRaV-III / Hung	GLRaV-III / Bioreba	Symptoms
Healthy control 1	0.121 +/-0.005	0.092 +/-0.006	no
Healthy control 2	0.095 +/- 0.003	0.091 +/-0.001	no
Healthy control 3	0.061 +/-0.010	0.095 +/-0.003	no
Healthy control 4	0.049 +/-0.006	0.078 +/-0.007	no
Pn.25/1	0.560 +/-0.021	2.203 +/-0.272	severe
Pn.25/2	0.609 +/-0.015	2.618 +/-0.074	severe
Pn.25/3	0.530 +/-0.011	2.016 +/-0.269	severe
Pn.25/4	0.580 +/-0.010	2.357 +/-0.069	severe
Pn.25/5	0.437 +/-0.001	1.261 +/-0.073	severe
Pn.25/7	0.498 +/-0.034	1.688 +/-0.030	severe
Pn.25/8	0.477 +/-0.001	1.596 +/-0.130	severe
Pn.25/12	0.498 +/-0.037	1.783 +/-0.060	severe
Pn.25/14	0.488 +/-0.031	1.826 +/-0.177	severe
Pn.25/18	0.631 +/-0.026	2.595 +/-0.023	severe
Pn.25/19	0.489 +/-0.004	2.142 +/-0.016	severe
KelR-1	0.935 +/-0.023	1.246 +/-0.081	severe
ZolR-1	0.727 +/-0.014	0.788 +/-0.049	severe
1/37	0.714 +/-0.027	1.246 +/-0.081	severe
1/659	0.797 +/-0.054	1.111 +/-0.136	severe
Buffer control (blank)	0.046 +/-0.004	0.055 +/-0.006	-

GLRaV-III / Hung is horseradish peroxidase conjugate (1 µg/ ml IgG for coating, 5000x dilution of conjugate) Substrate was incubated 10 min.
GLRaV-III / Bioreba alkalic phosphatase conjugate (IgG 1:1000; conjugate 1:1000)
Data representing of average of two wells in one experiment.
Cut off = 2 X $_{healthy\ controls}$ (Hungarian HRPO: 0.164; BIOREBA:0.178)

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PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC TO GRAPEVINE ASSOCIATED CLOSTEROVIRUSES.

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Western blot immunoassay and ELISA using different monoclonal antibodies (mAb) and polyclonal antisera (PA) revealed mixed infections of serologically related and unrelated grapevine leafroll associated viruses (GLRaVs) and grapevine corky bark associated virus (GCBaV) in symptomatic grapevines (4, 5).

Discrepancies were found between work performed in Europe and the USA. Boscia *et al.* (1) concluded that the GLRaV-2 antiserum produced in the United States (GLRaV-2 US) (2) reacted mainly with GLRaV-1 and that the French isolate was the authentic GLRaV- 2. Western blot studies performed in our laboratory showed that the GLRaV-2 US PA had different reactivity (4, 5). For example the GLRaV-2 US PA detected a previously uncharacterised 37 kD polypetide when the LR 102 isolate was used. In addition, the 38 kD associated with GLRaV-1 and the 24 kD associated with GLRaV-2 and GCBaV were detected. The polyclonal antiserum also reacted to the 36 kD polypeptide associated with GLRaV-4 and the 36 kD polypetide associated with GLRaV-5 (4).

The uncharacterised 37 kD polypetide (p37) was separated from the 38 kD polypeptide associated with GLRaV-1 using a novel polyacrylamide gel electrophoresis system (SDS-PAGE). Preparative SDS-PAGE was used to isolate high concentrations of the purified 37 kD protein. The p37 preparations were used to immunise mice for the production of monoclonal antibodies. An immunoassay was developed for the screening of p37 antibody reactivity in mice serum and hybridoma cultures. Serum collected from the immunised mice showed reactivity with the 37 kD polypetide but no reactivity with the 38 kD polypetide associated with GLRaV-1 using the Western blot assay. Monoclonal antibodies were cloned using the limiting dilution method.

Monoclonal antibodies with single reactivity to the p37 were obtained but other mAb cell lines cloned had broad spectrum reactivity to GLRaV -4, -5, and p37. These results suggest that the 36 kD polypetides associated with GLRaV-4 and GLRaV-5, and the 37 kD polypetide share at least one epitope. The mAbs did not react to GLRaV-1, GLRaV-2, GLRaV-3, or the virus(es) found in the Swiss Chassellas cultivar (1). Because the 37 kD polypetide did not react with the Italian GLRaV-7 polyclonal antibodies (3) we named the putative grapevine associated virus with a p37 coat protein, GLRaV-8.

We have developed an ELISA and Western blot assay using the new mAbs which are used to screen nursery plants for the presence of grapevine associated closteroviruses. The WB assay has allowed our laboratory to detect viruses that generally remain undetectable using the traditional indexing and ELISA methods.

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EFFICIENCY OF DIAGNOSIS OF GRAPEVINE LEAFROLL VIRUS (GLRaV3)

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Grapevine Leafroll is a widespread disease in Portuguese vineyards. Among the viruses involved in this disease, GLRaV1 and GLRaV3 have already been identified in Portugal, the latter being the most frequently found.

Having in mind the importance of an early diagnosis of the disease, as far as control and certification of grapevine vegetative propagation material are concerned, the aim of this work is to study possible conditions to improve detection of GLRaV3. The use of appropriate samples for testing is critical so, we tried to find out the most convenient organ, tissue and season, for reliable utilisation of ELISA in diagnosis. At different periods during 1995,1996 and 1997, several kinds of organs were collected from a red wine variety growing in the field, in Lisbon's area. Various types of samples for DAS-ELISA were used: scrapings from the active cambium area of one year old bark; upper leaves with petioles, basal leaves with or without petioles, leaf blade collected near the point of attachment of petioles and grapes and rachis.

One year old bark was found to be a good source of virus for ELISA during winter, with minimal unspecific reactions. Upper leaves gave very erratic results in ELISA, especially during spring. From April to November, basal leaves with or without symptons, were the best source for virus detection. Basal leaf petioles were the most reliable plant material for ELISA, when compared with leaf blades that gave lower readings during spring. As summer progresses these differences became minimal. In spring and early summer, green upper stems gave higher readings comparatively to leaves from the same area of the branch. We have verified remarkable differences in a single branch between the values from symptomless leaves picked up at random and basal leaves showing symptoms.

It was only possible to detect GLRaV3 in bunches when the grapes were mixed together with rachis. The rachis by itself, was a good virus source. The virus has never been detected in the grapes.

It was possible to detect GLRaV3 by DAS-ELISA all over the year in petioles of basal leaves collected in fields in Lisbon area. The ELISA test was more reliable whenever applied to leaves with symptoms which, in our climatic conditions, usually occur from July to August. The appearance of the first symptoms vary strongly according to the wheather conditions.

It was found that the efficiency of diagnosis of GLRaV3, based on ELISA, largely depends on the time of the year in which the sampling is performed and the tissue used as source of virus (cuttings, upper leaves *versus* basal leaves, leafblades *versus* petioles, grapes *versus* rachis, leaves with and without symptoms). The appearance of symptoms in the field depends also largely upon the early climatic conditions, and so the periods of field inspections should be established accordingdly.

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STUDY OF BOIS NOIR EPIDEMIOLOGY IN FRANCE: SEARCH AND BIOLOGY OF A VECTOR SPECIES

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Bois noir (BN) disease was known until the 1980's as a yellows occurring on grapevine (*Vitis vinifera* L.) in France in northern areas. Symptoms were similar to those observed on flavescence dorée (FD), but epidemiology was different and experimental transmission by the leafhopper *Scaphoideus titanus* Ball, vector of FD, was not achieved. Thus, the etiology of the disease and the vection of the pathogen were unknown for several years. Detection and characterization of the pathogenic agent by PCR procedures revealed that a phytoplasma closely related to the stolbur agent was present in grapevines affected with BN disease in France (1) and with a yellows called vergilbungskrankheit (VK) in Germany (6). The stolbur agent, first studied and referenced as a virus by Suchov and Vovk in 1946, was considered to cause diseases in East Europe on crops of economic importance such as tomato, tobacco, red pepper (10). Many phloem-feeding insects were suspected as vectors of the stolbur agent. A Fulgoromorpha species belonging to Cixiidae, *Hyalesthes obsoletus* Sign., was shown to be an efficient vector to the latter crops in East Europe. Later, its role as a vector of a stolbur phytoplasma (SP) in West Europe was at first showed for solanaceous crops in France (3), and thereafter for grapevine in Germany in the case of VK (5).

Considering these previous data, the aim of our study was to identify one or several vectors of BN in France, to determine the period of contamination of grapevine and to suggest methods of control of the disease.

During 1995 and 1996, we investigated the presence of potential vectors in experimental blocks located in the Rhone valley. Blocks were 3 vineyards of Chardonnay and 4 fallow lands nearby the vineyards. Seasonal captures from April to October were performed with both yellow sticky traps and D-vac suction. Sticky traps were replaced weekly and D-vac suction was used every 15 days during the experimental period. All the insects belonging to Euhemiptera were identified and some of them were used for experimental transmission and/or monitored for phytoplasma DNA by PCR. Bait plant experiments were performed each year; bait plants of different species sensitive to stolbur agent were exposed every 2 weeks in vineyard blocks and then transferred to a greenhouse for a possible expression of yellows symptoms. Moreover, we collected samples of grapevine stocks, wild shrubs and trees which were checked for natural infection. Detection of stolbur phytoplasma in insects and plants was carried out using primers STOL11 f2/r1 (2). In addition, a nested PCR was used with primers P1/P7 and fU5/rU3 for detection in stolbur-infected plants with low titre (8).

Over 2 years, we captured about 40,000 specimens of Euhemiptera, identified at genus (7 genera) or species level (103 species). PCR assays for SP detection on specimens of forty wild species showed that the cixiid *H. obsoletus* was by far the species with the higher ratio of natural infection. SP infection could also be detected on nymphs of the 5th instar captured on roots of symptomatic weeds. Table 1 gives the comparison between percentages of stolbur infected populations of adults of *H. obsoletus* in 6 sites in France. Apart from *H. obsoletus*, two leafhopper species namely *Mocydia crocea* (H. S.) (1/10; 10%) and *E. lineolatus* Brullé (4/309; 1.5%) were found positive in 1995. Successful experimental transmission of SP with *H. obsoletus* to grapevine (27/96), periwinkle (*Catharanthus roseus* L.) (15/35) and thorn apple (*Datura stramonium* L.) (3/4) were obtained with specimens captured all along the activity period of adults. All transmission trials with all other wild species failed. Inoculation period was determined by bait plant experiment. In 1995 and 1996, symptomatic bait plants were obtained only from mid June to the beginning of August. The SP infection of the inoculated plants and of bait plants was confirmed by PCR. Checking of wild naturally-infected plants showed that weeds such as bindweed (*Convolvulus arvensis* L.) and hoary cress (*Lepidium draba* L.), trees such as cherry, elm, lilac, fig tree were infected by a SP. Seasonal captures showed that adults of *H. obsoletus* were present in vineyards and fallow lands from mid June to mid August. Some fallow lands with high numbers of infective *H. obsoletus* and infected weeds were found.

Our results show for the first time the role of *H. obsoletus* in BN disease occurrence in France. It is interesting to note that specimens of *H. obsoletus* were readily infective after emergence. These data were supported by detection of SP in nymphs of 5th instar. Thus, it is more probable that the main acquisition process takes place at nymphal stage on roots of infected host plants. We confirmed that bindweed was an important host plant as already reported (9; 3). Hoary cress described as a host plant in Eastern Europe (4), was to our knowledge for the first time shown to be a significant host for adults and nymphs of *H. obsoletus* in France. In vineyard areas, where *H. obsoletus* was present, the inoculation period lasted for about 2 months and was correlated with the adult activity of this monovoltine species (8). Moreover, the potential threat of *H. obsoletus* was also effective in non-wine-growing areas. We showed that some fallow lands nearby the vineyards can be considered as a representative source of inoculum for SP. From such a site, where high numbers of specimens were found, infected adults would fly to and from the vineyards and

occasionally transmit the phytoplasma. In this case, control of the BN disease by traditional methods would not be efficient; we suggest as a prophylactic measure a ploughing of fallow lands considered as a seat of infection during winter. This measure would reduce the population of radicicolous nymphs of *H. obsoletus* and the presence of main host plants.

Table 1: Comparison between percentages of stolbur infected adults of *Hyalesthes obsoletus* in 6 sites in France in 1995 and 1996. Insects were captured by D-vac suction and yellow traps and tested by PCR with primer STOL11 f2/r1.

	Wine-growing areas				Non wine-growing areas	
	Rhone valley	Languedoc	North Burgundy	South Burgundy	Rhone valley	North Provence
1995	102/381 [a] (26.7%)	-	-	1/4 (25%)	7/23 (30.4%)	-
1996	152/393 (38.7%)	3/30 (10%)	3/9 (33.3%)	5/41 (12.2%)	nc	17/187 (9%)

[a] number of stolbur infected insects/total number of insects tested
nc: no adult captured

We can advance regard to similarities of pathogenic agent and of insect vector that BN and VK are probably the same grapevine yellows occurring in different geographical areas on different cultivars. This suggestion would be thereafter confirmed by experimental transmission by healthy specimens of *H. obsoletus* obtained from a rearing now under development (7).

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PRELIMINARY STUDIES ON THE APPEARANCE AND SPREAD OF SYMPTOMS OF GRAPEVINE YELLOWS IN AN AUSTRALIAN VINEYARD

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Grapevine yellows (GY) is the common term used to describe a group of diseases of grapevines associated with the presence of phytoplasmas in the phloem cells. While these diseases are each associated with discrete and partially characterised different phytoplasmas, the presenting symptoms of all these diseases are very similar and are well described.

We have been observing GY symptoms in a young plantation of Chardonnay vines in the Sunraysia region of Australia for the last two years and we have noticed that in addition to the usual symptoms of GY diseases, a number of other symptoms that occur at different times of the year can also be observed. These additional symptoms include a condition described as late season leaf curl (LSLC) that is seen in autumn, unusual out of season growth seen in winter, abnormal bud burst in spring and a pale discoloration of whole vines seen in summer. We have tested sub-sets of vines showing usual GY symptoms and subsets of vines showing the additional symptoms by polymerase chain reaction (PCR). We have extensively mapped and recorded all the symptoms on each individual vine on the plantation and we have used a variety of computer programs to analyse the spatial and temporal distribution of both the usual and the additional symptoms. We have used evolutionary algorithms and linkage analysis to explore for any clustering among the symptoms and we have used correlation methods to look for associations between the usual GY symptoms and the additional symptoms.

Our mapping studies showed that there was an approximately 10 fold increase in the incidence of the usual GY symptoms from the first year of observation to the second. A similar increase in incidence was also seen for the additional symptoms.

PCR analysis confirmed that phytoplasma was present in many of the vines showing the usual GY symptoms as well as in some of the vines showing the additional symptoms. The efficiency with which we were able to detect phytoplasmas in vines which were showing visual symptoms of disease by PCR was, however, not very high, with less than 50% of the vines showing the usual GY symptoms being recorded as positive for phytoplasma by PCR. The computer analysis of the mapping data was then considered as an essential tool to further our understanding of the epidemiology of GY disease in this vineyard.

As a result of the clustering and correlation analysis we have been able to establish a chronological sequence of events that begins in autumn with the LSLC, is followed by out of season growth in winter and then by an early bud-burst in Spring. In summer, many of these vines then show the usual symptoms of GY diseases and in the following spring they often show a late bud-burst.

Spatial and temporal analysis of the distribution of the usual GY symptoms across the vineyard shows that their are "hot spots" or *foci* of infection seen in the second year of our observations that can be related to the presence of infected single vines at those sites in the vineyard in the first year. While our analysis clearly demonstrated that the vines that showed symptoms in the first year were not the vines showing symptoms in the second year, the symptoms in the second year are mostly seen on vines in the immediate neighbourhood of those vines that had shown symptoms in the first year. This provides the first evidence for spread of the infection from infected vines into the neighbouring vines, a situation we previously suspected, but had no evidence for. This limited spread of infection may also indicate that the vector does not move very far.

VIRUS DISEASES AFFECTING CULTIVATED GRAPEVINES IN CHILE

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Grapevines have been cultivated in Chile since the XVI th century, but not until 1850 commercial plantings with French cultivars were established in the central area of the country. During the last years the area cultivated with grapes for wine production has increased significantly, reaching about 56,000 hectares (SAG, 1996). Despite this, there are no comprehensive studies about the presence of virus-like diseases in grapevines for wine production including all the areas where grapes are grown. However, through studies carried out regionally there is enough evidence of the presence of three world-wide known viruses in Chilean vineyards. These are grape fanleaf virus (GFLV), grape leafroll virus (GLRV) and grapevine virus A (GVA) (Auger, 1994).

To establish the real importance of this diseases in wine grapes vineyards, a survey was carried out during the 1996/97 season in several fields in central and south-central Chile. The area cover in the survey was approximately 1,200 hectares. Fifty-eight leaf/shoot samples from plants showing symptoms resembling virus like diseases (mosaic, leaf deformation, leaf colour change), were taken at eight different localities. Ten different viruses were analysed using commercial ELISA kits (DAS-ELISA) and herbaceous indicator plants (*Chenopodium quinoa*, *Cucumis sativus*, *Phaseolus vulgaris* and *Nicotiana clevelandii*.). The viruses tested were Alfalfa Mosaic Virus, Arabis Mosaic Virus, Grape FanLeaf Virus, Grape Leaf Roll Virus, Grape Virus A, Peach Rossette Mosaic Virus, Strawberry Latent Ringspot Virus, Tobacco Mosaic Virus, Tobacco Ringspot Virus and Tomato Ringspot Virus. All these are described affecting grapes in other countries around the world (Frazier, 1970; Pearson and Goheen, 1988). At the same time, soil samples were taken to determine the presence of nematodes which could be associated to these diseases mainly *Longidorus* and *Xiphinema* genus. These samples (500 grams) were taken from the root area of the plants tested. For the analysis of the soil two techniques were used, one qualitative (Baermann) and the other quantitative (Coob) (Taylor, 1971). Also additional information, helpful to determine the epidemiology of the disease was collected from the field. This included age of the plants, the pattern of distribution of unhealthy plants and the presence of weeds and insects, in the surrounding.

The results show that the majority of the samples collected showed at least the presence of one of the viruses tested. On the other hand, *Xiphinema* was always associated with plants infected with nepoviruses. Additionally for those samples showing virus like symptoms, but did not tested positive for any of the viruses under study, the presence of other kind of organism (phytoplasma) is suspected. Further studies should be done to determine the presence of other pathogens associated.

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A SURVEY OF GRAPEVINE VIRUSES IN PALESTINE

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With a surface of about 9,000 Ha, the grapevine ranks second among the fruit crops of Palestine, being preceded only by olive. Table grape varieties are by far the most widely grown, being represented by both native (e.g., Dabouqi, Zaini, Beitoni, Halawani, Darawishi, Jandali, Marrawi, Romi and foreign (e.g. Early superior, Perlette, Flame seedless, Emperor) cultivars. Although scattered enclaves of self-rooted vines exist, the great majority of commercial vineyards are grafted on American rootstocks (e.g. 161/49, SO4, 1103P, 140Ru, 3309, 41B, *Vitis rupestris*).

The sanitary status of Palestinian viticulture is not totally unknown because of the similarity with the viticultural industries of the neighbouring countries (Israel and Jordan), which have been investigated in some detail. However, since there is no published record of a thorough survey of Palestinian vineyards for assessing the presence and incidence of virus infections, an investigation to this aim was initiated, the preliminary results of which are reported herein.

Field surveys for symptom observation were conducted in autumn 1996. Mature canes were collected at random from 635 individual vines (566 *Vitis vinifera* and 69 rootstocks) in 61 vineyards of 8 different areas (Betlehem, Gaza, Ramallah, Jerusalem, Jenin, Jericho, Nablus, and Hebron) at the time of the first survey and during winter 1997. All samples were brought to Bari to be analysed for the presence of the following viruses: GFLV, GFkV, GVA, GVB, GLRaV-1, -2, -3, -7. Tests were made on cortical scraping extracts by DAS-ELISA (GFLV, GLRaV-3), protein A DAS-ELISA (GVA), DASI-ELISA (GFkV, GVB), and biotin-streptavidin DASI-ELISA (GLRaV-1, -2, -7). Polyclonal antisera and monoclonal antibodies raised at the University of Bari were used as reagents.

Mechanical transmissions to the standard series of herbaceous hosts were made from about 200 samples chosen at random, and shoots from about 120 samples were green-grafted in spring 1997 onto *Vitis riparia*, 110R, and LN33 (1)

Due to the late season, the only symptoms that could be observed and identified with reasonable confidence in the field were those typical of leafroll disease (i.e. rolling and reddening of the leaves) in most of the red-fruited cultivars. Fanleaf symptoms were not obvious and rugose wood was only seen in the unfrequent cases in which there was a marked corky reaction of the bark at the base of the vines, or when pits and grooves were showing on the outer surface of the cortex.

The results of green-grafting, aimed at ascertaining the occurrence of vein mosaic, vein necrosis and corky bark are not yet available. With sap transmission tests, only GFLV was recovered from a few samples.

Serological assays were more informative (Tab. 1). About 82% of the European grape samples (463 infected out of 566) from commercial vineyards contained one or more viruses, whereas the infection rate of mother plants of American roostocks was as low as 20% (14 infected samples out of 55). As to individual viruses, there was a clear-cut prevalence of trichoviruses (GVA and GVB) and closteroviruses (GLRaV-1, -2, -3) including GLRaV-7, which however, was found in a single vine of cv. Sultanina. Surprisingly low (1.4%) was the incidence of GFLV, and GFkV was also less represented than in other Mediterranean areas. This may depend in part on the low infection rate of rootstocks, of which 41B and 161/49 were the only ones containing GFLV and GFkV, respectively.

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

Virus	EUROPEAN GRAPES (566 samples tested)		ROOTSTOCKS (69 samples tested)	
	Infected samples	Infection percentage	Infected samples	Infection percentage
GVA	374	66.1	4	5.8
GLRaV-1	258	45.6	0	0
GLRaV-3	123	21.7	7	10.1
GFkV	89	15.7	4	5.8
GLRaV-2	47	8.3	1	1.4
GVB	21	3.7	0	0
GFLV	7	1.2	1	1.4
GLRaV-7	1	0.2	0	0

From the above data it can be inferred that rugose wood (Kober stem grooving, in particular) and leafroll are by far the most widespread diseases in Palestine. This is not surprising considering that GVA and GLRaV-3, two of the most frequently encountered viruses, are agents of these diseases and are both transmitted by vectors (mealybugs) (2) that are very common in the area. With an average infection of 10%, rootstocks are a further, although not a major source of GLRaV-3. An unusual finding was the prevalence of GLRaV-1 over GLRaV-3, which is not in line with the notion that the reverse is true in warm climates. This relatively high incidence of GLRaV-1 is puzzling, considering that this virus was not detected in any of the rootstocks and that it is not known to be transmitted by mealybugs (2, 3).

The results of the present survey have shown that Palestinian viticulture is not in better sanitary conditions than the rest of the Mediterranean countries. The implementation of measures for its improvement should therefore be seriously considered.

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A SURVEY OF GRAPEVINE VIRUSES IN TURKEY

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Grapevine is one of major crops of Turkey, which according to recent FAO statistics, ranks third among the Mediterranean countries. Investigations carried out over the years, have shown that the Turkish viticultural industry is affected by many virus diseases (1 and references therein), but a country-wide survey of viruses has not yet been made. A study to this effect was therefore initiated, the preliminary results of which are reported hereafter.

In autumn 1996, samples were collected from vines showing symptoms of fanleaf (deformation of leaves and canes, poor fruit setting, reduced growth) or leafroll (reddening and rolling of the leaves) in six different viticultural areas, to be analysed for the presence of grapevine fanleaf virus (GFLV) and grapevine leafroll-associated viruses 1, 3 and 7 (GLRaV-1, GLRaV-3, GLRaV-7). Tests were carried out on cortical scraping extracts from mature canes by DAS-ELISA for GFLV, GLRaV-3 and -7, and by DASI-ELISA with biotinylated IgGs for GLRaV-1.

Out of 84 samples, 57 (68%) proved to be infected by one or more of the viruses looked for. The relative distribution of these viruses in the localities site of the survey was as reported in Tab. 1.

Table 1. Relative percent incidence of four grapevine viruses in six Turkish viticultural districts

Locality	GFLV	GLRaV-1	GLRaV-3	GLRaV-7
Izmir (Menemen)	12.5	0	50	0
Çannakale (Bozcaada)	0	100	0	25
Çannakale (Bayramçi)	8.3	50	25	8.3
Manisa	20	0	30	0
Tekirdag	35	25	30	0
Nevsheir	0	50	9	4.5

Multiple infections were rather frequent, consisting of mixtures of GLRaV-1 + GLRaV-7; GFLV + GLRaV-1; GFLV + GLRaV-3; GFLV + GLRaV-1 + GLRaV-3. GLRaV-1, found in 34 out 84 vines (average infection 40%) was the most common virus and, contrary to what currently thought for southern areas, it prevailed over GLRaV-3 (average infection 21%). An interesting result was the finding of GLRaV-7 in 5 vines (about 6% of the total), which confirms earlier unpublished records of the presence of this virus in Turkey.

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OCCURRENCE OF GRAPEVINE FANLEAF VIRUS (GFLV) AND GRAPEVINE LEAFROLL ASSOCIATED VIRUS 3 (GLRaV 3) IN THE AZOREAN ISLANDS PICO AND TERCEIRA

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A survey of the virological situation of local cultivars of *Vitis vinifera* L. in the Azorean islands Pico and Terceira was carried out to determine the degree of virus infection. In a first assay, vines from different vineyards were tested for the presence of GFLV, GLRaV1, GLRaV 3, RRV and GVA.

GFLV and GLRaV3 were detected in high concentrations by using TWO-STEP-DAS-ELISA with antisera from Bioreba (Switzerland), in all the cultivars used for the production of regional wines (Lopes 1977), which seems to be the first record of these viruses in the Azores. Considering the obtained results, it will be necessary, to establish a sanitation programme corresponding to international legislation. This is of major importance since the natural vectors, nematodes of the species *Xiphinema* have been recorded in the islands (Macara 1994).

Therefore, efforts were made first to find virus-free material to be chosen as mother plants and then to find plants with single infections for an *in vitro* reference collection of viruses to be further integrated in a programme of virus elimination.

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GRAPEVINE LEAFROLL (GLRaV), FLECK (GFkV) AND GRAPEVINE FANLEAF (GFLV)-*Xiphinema index* IN THE VINEYARDS OF THE GUADIANA BASIN, SPAIN.

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Guadiana basin consists in a plateau of 400 m of medium altitude, situated in the Mid southern of the Iberian Peninsula, bounded by Montes de Toledo at the North and Sierra Morena mountain in the South, it has an extension of more than 67.000 km², more than 80% belonged to Spain ant the rest to Portugal. Five sections can be distinguished in Guadian basin, three of then corresponding to Castilla- La Mancha, one to Extremadura and the last one to Portugal. Most important vineyards of Guadiana basin are localised in "La Mancha", in the central region, where the greatest vineyard in the World grown (more than 700.000 ha) and in "Tierra de Barros", Badajoz, in the Midwestern of Spain, nearby Portugal (some 85.000 ha). Soil in both zones has a characteristic petrocalcic deep horizon, that confer them a certain supressiveness against nematodes (2). Climate are continental Mediterranean , Supra-Mediterranean in La Mancha (7) with a temperature rate of 8°C-13°C, under 20° from October to May and higher than 40° along the summer season in La Mancha, while in Tierra de Barros Climate is Meso-mediterranean (7) with a temperature average of 13°C-17°C and more warm winters.

Grapevine fanleaf virus (GFLV) and its vector nematode *X.index* are known to be widespread in Spain, with a infection rate lower than previously estimated on the basis of visual diagnosis, and Grapevine Fleck Virus (GFkV) has been recently detected in La Mancha (1) in the 40% of Airén, Garnacha and Cencibel varieties (4), but until now they have not been studied in Tierra de Barros and neither other virus affecting vines. The low correlation between GFLV presence and symptomatology observed, as well as that of commercial vines affected by GFkV and the lack of information about Grapevine Leaf Roll Virus (GLRaV), reported as a widespread virus in the Mediterranean basin with infection rates higher than 30% (5), let us to do a comparative study of these viruses in both regions.

MATERIAL AND METHODS,

More than 1.000 samples from different vine plants tissues (leaves, shoot, cane, root, grape, seed, et.) and soil for GFLV detection and virus vector nematodes presence, 337 for GLFkV, GLRaV-1 and GVA (Trichovirus) were analysed in La Mancha, taken at random mainly in the varieties Airén, Garnacha and Cencivel,, and more than 1.000 and 110 respectively in Tierra de Barros, from varieties Pardina, Blanca Cayetana, Tempranillo and Macabeo among others. Presence of virus was determine by ELISA-DAS with mono and policlonal commercial antibodies. Nematodes were extracted from soil by Flegg's method (3).

RESULTS AND DISCUSSION

The figure 1 shows that the most widespread virus in La Mancha is GLFkV, in 32% of samples, GLRaV-3 appeared in the 13,5%, GFLV in the 7,4 and GLRaV-1 in the 2,4%. Correlation GFLV-*X.index* was of 5 to 10 % under traditional system crops, but is increasing in that vineyards where irrigation was introduced (1). In Tierra the Barros GLFkV was again the most widespread virus, appeared in the 43,6%, GLRaV-3 with the 37,3%, GLRaV-1 in 11,8% and finally GFLV in a 6,4%. The trichovirus (GVA) has not been found in any of the two studied areas.

GLFkV is reported for the first time in Spain, being the most widespread virus in both regions, followed by GLRaV-3, reported as the one of the most spread in the Mediterranean region (6), as was found also in Galicia (8) and in Jerez. vineyards (5). By the contrary, GLRaV-1 is the less frequent in La Mancha as reported in Galicia (8) in only a few old vines, however reach fair important levels in Tierra de Barros, where appeared more spread than GFLV. On the other hand, GFLV incidence in both areas is much less important than previously reported. The high incidence of GLFkV in both areas is pointed out and the interest of extended the study to other viticultural areas in the country is encouraged, as well as the study of the incidence of GLRaV- 1 and GLRaV-3 vectors in relation with the environmental characteristics in each area, that could explain the higher incidence of these virus in Tierra de Barros. The more superficial situation of the petrocalcic horizon in Tierra de Barros could explain the less incidence of GFLV, because its suppressive action on vector nematodes (2).
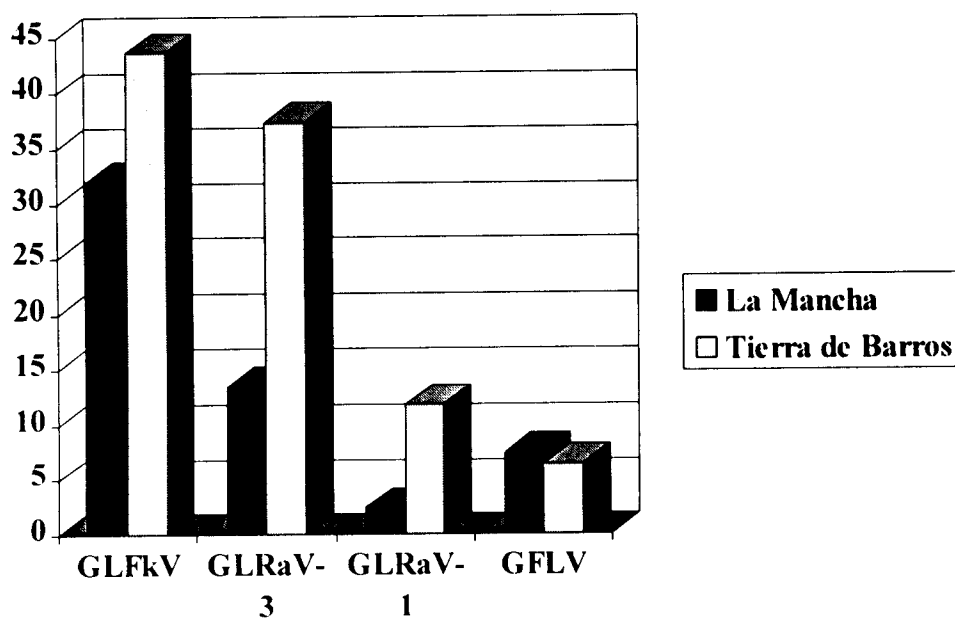
Figure 1.- Percentage of incidence of the four viruses in the two studied areas.

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ASSOCIATION OF CLOSTEROVIRUSES GLRaV 1 & GLRaV 3 WITH LEAFROLL SYMPTOMS IN GREEK VINEYARDS

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During 1994-1996 an extensive survey of leafroll disease of grapevine was carried out in the main viticultural areas of Greece, in order to: (a) estimate the occurrence, prevalence and distribution of GLRaV 1 and GLRaV 3, the most wide spread and consistently connected with leaf roll disease viruses (Boscia et al. 1995), and (b) investigate the relationship between these two Closteroviruses and the grapevine leafroll disease.

Based on visual observations in late summer and fall the diseased grapevines were noticed in commercial vineyards in the islands of Crete, Limnos and Samos, in Peloponessus, Central Greece, Thessaly and Macedonia. The varietal collection of the Faculty of Agriculture, Aristotle University of Thessaloniki was also inspected. Virus detection was done by direct double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark & Adams, 1977). Polyclonal antibodies and their conjugates of GLRaV 1 and GLRaV 3 were obtained from Agritest, Valenzano, Italy and Bioreba AG Basel, Switzerland, and procedures were according to the instructions provided with the kits. Dormant canes were collected from all branches of a leafroll-infected grapevine and phloem bark scrapings were taken from at least four canes/stock to eliminate probable sampling error due to the lack of uniform distribution of Closteroviruses (Rawhani & Gollino, 1995). Each grapevine was tested at least twice. Samples with absorbance readings (A_{405}) over three times the average of four wells/plate of healthy control were considered positive.

Typical leafroll symptoms (downward rolling of the leaves from the margins and red coloration on the interveinal areas, with the vein remaining green) were observed in almost all vineyards inspected. Some cultivars appeared to be very susceptible to the disease showing severe symptoms. Red-berries cultivars were heavily infected with an incidence varying according to the locality and the cultivar. In this study a total of 494 grapevine samples exhibiting leafroll symptoms were processed. An overall 97.4% of grapevines were infected by GLRaV 1 and GLRaV 3. Single GLRaV 1 infections were detected in 42.4% and GLRaV 3 in 47,8%. Double infections with GLRaV 1 + GLRaV 3 were found in 9.8% of the tested stocks (Table 1 and 2).

A constant association has been found with GLRaV 1 and GLRaV 3 and leafroll symptoms in Greek vineyards. With the exception of cv. Roditis (red berries) in all greek cultivars GLRaV 1 was the most frequent independently on whether grapevines were red or white berries. GLRaV 3 appeared to have a higher incidence in North Greece, while GLRaV 1 was clearly prevalent in the South Greece and islands (Table 1 and 2).

On the other hand the absence of positive reactions in a few samples (13/494) of leafroll-affected grapevines is not surprising, because other Closteroviruses may be also present (Boscia et al., 1995).

Table 1. Distribution of GLRaV 1 and GLRaV 3 in greek vineyards

Regions	GLRaV 1	GLRaV 3	GLRaV 1 + GLRaV 3	Infected/Total
North Greece (Thessaly & Macedonia)	10	210	16	236/242
South Greece & islands	194	20	31	245/252
Total	204	230	7	481/494
%	42.4	47.8	9.8	97.4

Table 2. Results of DAS-ELISA against GLRaV 1 and GLRaV 3 in grapevine cultivars grown in greek vineyards

Cultivars	N° vines	GLRaV 1	GLRaV 3	GLRaV ı + GLRaV 3
Roditis (red berries)	184	16	156	12
Korinthiaki (red)	39	24	7	7
Soultana (white)	33	21	6	0
Black of Nemeas	90	79	0	11
Sabatiano (white)	21	17	4	0
Moschofilero (white)	8	8	0	0
Muscat of Alexandrie	9	6	2	1
Bakouri (red)	9	9	0	0
Liatiko (red)	7	7	0	0
Others greek cvs	54	12	35	4
Imported cvs	40	5	20	12
TOTAL	494	204	230	47
		TOTAL INFECTED		481

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EPIDEMIOLOGICAL SURVEY OF THE GRAPEVINE LEAFROLL DISEASE IN FRENCH WINE GROWING REGIONS

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Grapevine leafroll-associated viruses (GLRaVs) are known or highly suspected to be naturally transmitted by pseudococcid and coccid mealybugs. In experimental conditions GLRaV-3 was successfully transmitted from vine to vine by mealybugs of the *Planococcus*, *Pseudococcus* and *Pulvinaria* genera, GLRaV-2 seems to have at least 2 *Pseudococcus* species as vectors (1, 2, 3).

In France there are very few reported data on the grapevine leafroll epidemiology, although mealybugs of the above-mentioned genera are present in the French vineyards. Serological surveys made in 1994/95 on grapevine samples from propagation material in 8 wine-growing regions (Alsace, Bordelais, Bourgogne, Languedoc-Roussillon, Midi-Pyrénées, Provence-Côte d'Azur, Pays de Loire, Rhône-Alpes) showed contamination rates of 0-2.5% and 0-8.5% for GLRaV-1 and 3 respectively. To have a better idea of the real situation in the vineyards of every region and of the potential risks of contamination of healthy plantations, especially of scion and rootstock mother plants, we have initiated in 1995 an observation network with the participation of the Office National Inter-professionnel des Vins (ONIVINS) and of regional partners (SICAREX Beaujolais, ATV Bourgogne, CIV Champagne, regional stations of the Plant Protection Service...). The task of the network is first to make a state of the art per region and then to follow in more details grapevine plots in which spread of the leafroll disease has been observed recently by symptom expression and/or detection of GLRaVs.

The first results come from Beaujolais and Bourgogne where the extension of leafroll symptoms was observed during the last decade on cvs. Gamay and Pinot noir, respectively, in propagation plots and in production plots. In 1995 samples collected randomly in these plots were tested by ELISA and revealed the presence of GLRaV-1 or GLRaV-3 in each symptom-showing vine, as in some symptomless vines. In 1996 four "model" plots were selected per region and samples were collected following a statistical distribution using a computer program named LOVE (Logiciel d'Observation de la Vigne en Europe), established for epidemiological surveys of eutypiose by F. Mimiague (Univ. Bordeaux) and which leads to a coarse value of the infection rate per plot. Based on ELISA the following table shows the incidence of GLRaV-1 and GLRaV-3 in the selected plots.

Region	BEAUJOLAIS				BOURGOGNE			
Locality	St Laurent	St Etienne	Odenas1	Odenas2	Aloxe C	Beaune U	Rully	Beaune B.
GLRaV-1	3%	17%	4%	4%	1%	3%	2%	30%
GLRaV-3	80%	20%	0	0	0.5%	11%	2%	8%

Except for the plots from Odenas which contain only GLRaV-1, both viruses are present at different levels in the plots which were investigated but almost never in the same vines, which could indicate that they were probably not transmitted together from vine to vine. Mealybugs collected in these vineyards were identified as *Pulvinaria vitis*, *Parthenolecanium corni* and *Heliococcus bohemicus*, a Pseudococcid species found mostly under temperate climate. In parallel mealybugs collected in vineyards of Languedoc-Roussillon, disregarding the presence or not of leafroll, were shown to belong essentially to the species *Planococcus ficus*, *P. citri* and *Pulvinaria vitis*.

The same work will be undertaken in 1997 and the following years for these plots to see any evolution of the disease and, if it is the case, the relationships with the mealybug populations present on the grapevines. The vectoring capacity of the mealybug species will be assessed either in natural conditions by replanting healthy vines in the heavily contaminated plots and in experimental conditions using mealybugs reared on potato tubers and controlled transmission parameters. A special attention is given to *H. bohemicus* in the case of the Beaujolais-Bourgogne conditions. For the other wine-growing regions the prospections are starting in 1997 according to the informations provided by the corresponding regional partners of the network.

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TRANSMISSION OF GRAPEVINE LEAFROLL VIRUS 1 (GLRV-1) AND GRAPEVINE VIRUS A (GVA) BY SCALE INSECTS

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Grapevine leafroll disease (LR) has been reported in all viticultural countries and causes reductions of yield and fruit quality in many cultivars of *Vitis vinifera* (7). Up to now, seven serologically different clostero-like viruses have been found associated with the disease and named "grapevine leafroll-associated viruses" (GLRaV -1 to -7) (5). Among these, GLRaV-1 and GLRaV-3 are the most widespread and were shown to be causal agents of the disease (2); therefore it has been proposed to drop for them the word "associated" (2, 4).

In recent works we found natural spread of grapevine leafroll in vineyards planted with virus-free clones of *V. vinifera*. The vines that exhibited LR symptoms resulted to be infected by GLRV-3 or GLRV-1 or by both viruses (3, 6). In those vineyards we found the presence of the scale insects *Neopulvinaria innumerabilis* Rathvon, *Parthenolecanium corni* Bouché, *P. persicae* Fabbre and *Pulvinaria vitis* L.. In a previous paper we reported the experimental transmission from grape to grape of GLRV-3 by *P. vitis* (1). In order to verify whether scale insects are also involved in spreading of GLRV-1, transmission trials were carried out using the former two species.

MATERIALS AND METHODS

The following *V. vinifera* accessions were used as inoculum sources:
-cv Barbera, accession MI-B7: symptomless but infected with GLRV-1;
-cv Barbera, accession F7 V18: naturally infected with GLRV-1 and GVA, and showing severe leafroll symptoms. Cuttings of these accessions were potted and grown in an insect-proof chamber at 22-24°C and 14h photoperiod. Seedlings of *Vitis vinifera* and, in some cases, cuttings of virus-free clones of the cultivars Pinot noir (5V-17) and Barbera (MI-B34), were used as test plants.

Crawlers of the two species of scale insects were allowed to feed separately for about a month on infected vines. The crawlers were then transferred to the healthy vines for an inoculation period of at least 14 days. In total, 19 seedlings and 4 healthy rooted cuttings were inoculated, as indicated in table 1. The plants were then sprayed with insecticide and transferred to an insect-proof greenhouse at 20-22°C. The healthy controls consisted of seedlings on which were transferred crawlers born and always fed on healthy seedlings.

Four months later the inoculated vines and the healthy controls were checked in double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for GLRV-1 and GVA using partially purified leaf extracts, according to Gugerli *et al.* (9).

RESULTS AND DISCUSSION

The results of ELISA tests are summarised in Table 1. They show that the scale insects *N. innumerabilis* and *P. corni* can transmit filamentous viruses from grape to grape. *P. corni* is clearly a vector of GLRV-1 but seems unable to transmit GVA. *N. innumerabilis* is able to transmit GVA and GLRV-1 (at least when associated with GVA). Though the low number of inoculated vines does not allow to draw definitive conclusions, some hypotheses can be proposed to explain the different behaviour of the two vector species used in these transmission trials:

a) - inoculum sources were infected by different GLRV-1 strains and *P. corni* transmitted only the strain present in accession MI-B7 while *N. innumerabilis* transmitted only the one present in accession F7 V18. In fact the two accessions have strong symptomatological differences: the accession MI-B7 is asymptomatic, in spite of the great sensitivity of cv Barbera to leafroll disease, and so may be infected by a mild GLRV-1 strain, whereas accession F7 V18 showed strong leafroll symptoms and seems to be infected by a GLRV-1 virulent strain (GVA should not be involved in leafroll aetiology). It is worthwhile to remember that different strains have already been found for GLRaV-2 (8);

b) - *P. corni* transmitted only the GLRV-1 strain present in accession MI-B7; *N. innumerabilis* transmitted only the GLRV-1 strain present in accession F7 V18 because apparently this insect species is a GVA vector and could have transmitted also GLRV-1 using a GVA helper component. More work is in progress to verify these hypotheses.

In any case, this seems to be the first report of insect transmission of GLRV-1 and scale insect transmission of GVA.

Table 1. Summarised results of the transmission tests.

Scale insect	Inoculum source	N° of inoculated plants	N° of positive ELISA	
			GLRV-1	GVA
N. innumerabilis	GLRV-1	5	0	0
N. innumerabilis	GLRV-1 + GVA	6	3	4*
N. innumerabilis	control	3	0	0
P. corni	GLRV-1	5	2	0
P. corni	GLRV-1 + GVA	7	0	0
P. corni	control	1	0	0

*) 3 out of 4 in mixed infection with GLRV-1.

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EPIDEMIOLOGY OF THE GRAPEVINE LEAFROLL-MEALYBUG COMPLEX IN CYPRUS

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Leafroll is the most widespread virus disease of grapevines in Cyprus with an average incidence of about 80% in introduced varieties and 45% in traditional ones. In recent years a number of leafroll-associated closteroviruses (GLRaV) have been implicated in the aetiology of this disease. Four such viruses, designated GLRaV-1,2,3 and 4, have been identified in Cyprus. Of these, GLRaV-3 appears to be the most common.

For many years leafroll was thought to be transmitted solely through infected propagating material. However, the widespread distribution of leafroll disease and GLRaV-3 in Cyprus led us to investigate the possibility of virus spread by other means, namely by the mealybug species *Planococcus ficus* (Signoret) and *P. citri* (Risso), which commonly infest vines under humid conditions. The first indication of natural GLRaV-3 spread in the island was obtained in the late 1980's, when clean stocks introduced from the University of California, Davis, or produced locally became infected after having grown for a few years in a greenhouse in Nicosia, or in variety collections in the open field. Further investigations to substantiate the natural spread of GLRaV-3 by mealybugs involved the following field and laboratory studies:

1. Monitoring the natural spread of GLRaV-3. Since 1991 the incidence of GLRaV-3 has been monitored by regular surveys and ELISA tests in four grapevine plots situated at Kornos, Zygi and Akhelia (old and new station). The results show significant differences among sites, with the highest rate of natural spread occurring at Zygi, where virus incidence reached 70.5% in 1996 compared to 19.7% in 1991. By contrast, there was hardly any spread at Kornos, where virus incidence remained at about 5% since 1991. During 1996, the virus was also detected (1.2%) for the first time at the new station at Akhelia, while virus incidence in the old station increased further, reaching 70% from 44% in 1991.

2. Virus incidence in different grapevine varieties. Levels of infection detected in American rootstock varieties were very low in all plots under study. Thus, in the Akhelia plot the level of infection in five *Vitis vinifera* varieties varied from 73% to 94%, compared to only 7% in *V. rupestris*. This difference could be attributed either to a difference in susceptibility between the two groups of varieties or to a difference in detectability of the virus by ELISA in their phloem tissues. In order to resolve this question, all plants of American rootstock varieties in the Zygi plantation were grafted with a leafroll indicator (Cabernet Franc), from which GLRaV-3 was detected by ELISA. Virus detection was improved with this method (7/85 positive plants) compared to direct detection in phloem tissue from the rootstock variety (3/85 positive plants). Even this higher level of infection, however, was much below respective levels in vinifera varieties (73-94%), suggesting a lower susceptibility of rootstock varieties to the mealybug-GLRaV-3 complex. Similar results were obtained with duplicate tests carried out in the greenhouse on cuttings of rootstock varieties from Zygi.

3. Exposure of potted indicator plants to natural air-borne infection. In order to exclude the possibility of the virus being transmitted to neighbouring vines via natural root grafting (suggested by the pattern of virus spread in the field), virus-free indicator plants, grown in pots in the greenhouse were transferred to the grapevine plot at Zygi and placed under GLRaV-3 infected vinifera vines, taking all necessary precautions to prevent root contact. In these tests, 4 out of 20 exposed test plants became infected, thus substantiating the occurrence of natural air-borne transmission of the virus under field conditions.

4. Population dynamics of mealybugs on grapevines. Beginning in June 1996 surveys were carried out periodically in the Zygi and Kornos plantations to determine mealybug populations on wine, table and rootstock varieties. The results show that populations at Kornos were much lower, in agreement with the lower virus incidence observed in this plot. Likewise, in agreement with virus incidence data, populations on rootstock varieties were generally lower than those on table and wine grape varieties.

5. Laboratory transmission tests with wild mealybugs. Mealybugs collected from the field were used to inoculate healthy vine plants growing in pots in the greenhouse. The virus was successfully transmitted to 17 out of 22 inoculated plants, verifying the role of mealybugs in the natural spread of GLRaV-3. Furthermore, GLRaV-3 was detected in 3 out of 8 mealybug collections from Zygi, which were tested by polymerase chain reaction (PCR) at the University of Bari.

6. Transmission tests with laboratory-reared mealybugs. Two mealybug species were identified on grapevines, i.e. *Planococcus ficus* and *P. citri*. Both species were able to transmit GLRaV-3 under controlled laboratory conditions, but the former was much more efficient than the latter. In tests with different numbers of insects per plant, 75 larvae of *P. ficus* resulted in 100% successful transmission of GLRaV-3 to healthy Cabernet Franc test plants, whereas the same number of *P. citri* larvae resulted in only 45% transmission. Similar results were obtained using the hybrid LN-33 as test variety. Further trials to determine the detailed virus-vector relationships, i.e. acquisition and inoculation feeding periods, retention, latent period etc., were inconclusive due to cross-contamination problems, and will be repeated in 1997.

7. Reaction of different varieties. A series of laboratory transmission tests were carried out to investigate further the apparent lack or very low levels of GLRaV-3 infection observed in American rootstock varieties under field conditions. In these tests, plants of the vinifera variety Cabernet Franc, the hybrid LN-33 and the American rootstock varieties *V. rupestris* and 110R were inoculated with different mealybug numbers of the species *P. ficus*. Results were in complete agreement with those obtained in the field, and showed that the varieties Cabernet Franc and LN-33 were readily infected (100% infection with 75 insects per plant), whereas the apparent levels of infection determined with ELISA in *V. rupestris* and 110R were only 10% and 30%, respectively. Following these tests, *V. rupestris* and 110R plants were top-grafted with Cabernet Franc (leafroll indicator) and the plants were tested again by ELISA, using tissue from both the rootstock and the scion. The results show considerable improvement in virus detection using Cabernet Franc as indicator, but levels of infection were always lower than those determined on vinifera varieties. These results are in complete agreement with those obtained in the field. Tests are currently in progress to evaluate different procedural modifications aiming to improve the sensitivity of ELISA in detecting GLRaV-3 in American rootstock varieties.

8. Tests with other possible vectors of GLRaV-3 and with other closterovirus-related diseases. In addition to mealybugs, other insects are also investigated as possible vectors of GLRaV-3, including *Ceroplastes rusci* and *Zygina ramni*, but results so far are inconclusive. Epidemiological studies and mealybug transmission tests in the laboratory are also conducted with two other closterovirus-related diseases, rupestris stem pitting and grapevine corky bark. No conclusive results have been obtained so far largely because of the unavailability of a quick and reliable laboratory assay method for these diseases. Rupestris stem pitting, however, appears to be spreading naturally under field conditions in a pattern similar to that of leafroll.

In conclusion, results presented in this paper indicate that GLRaV-3 and possibly other grapevine closteroviruses are spreading naturally in Cyprus by means other than infected propagating material. The role of mealybugs in the transmission of GLRaV-3 has been established while for other closterovirus-related diseases, such as rupestris stem pitting and corky bark, is still under investigation.

The rate of leafroll spread in some areas is alarmingly high whereas in other areas natural spread is hardly detectable. This points out the importance of micro-climate in the development of mealybug infestation and virus spread. Relative humidity appears to be a decisive factor and in this respect considerable virus spread is expected to occur in the irrigated table grapes grown along the south and south-western coastal plain of the island, rather than in the rainfed wine grapes grown in mountainous areas. Severe mealybug infestation associated with natural spread of GLRaV-3 was observed in several greenhouses and nurseries possibly due to prevailing high humidity. In as much as mealybugs are implicated as natural vectors of grapevine viruses, it is essential to study their ecology and population dynamics and to relate the results of such studies to the regional variability observed in virus spread. Preliminary data indicate a close correlation between mealybug infestation and virus spread.

Another decisive factor in the natural spread of GLRaV-3 appears to be the sanitary status of planting material. The use of clean propagating material produced under conditions that prevent its infection in the nursery, as practised in the case of the Akhelia new station plot, appears to be of paramount importance for the control of virus spread in the vineyard. Further, the excessive use of insecticides against other insect pests, notably the grape berry moth, is known to encourage mealybug infestation and thus the natural spread of GLRaV-3.

The leafroll-mealybug complex and associated epidemiological factors should be seriously considered in the implementation of certification programs for the production, maintenance and distribution of healthy propagating material of grapevines.

The difference in virus spread between European varieties and American rootstock varieties is an observation of particular interest and should be pursued further. To a certain extent this difference is an artifact due to the difficult detection of GLRaV-3 by ELISA in rootstock tissue. However, it appears that American rootstock varieties possess also a mechanism of resistance to GLRaV-3 or its mealybug vector, which deserves further investigation.

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INFLUENCE OF AGRONOMIC TECHNIQUES ON THE EPIDEMIOLOGY OF GFLV.

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Agro-environmental values of Castilla-La Mancha are mainly centred in its geographic characteristics, that avoid the introduction of pathogens from other areas. Such a values are mostly due to its climatic conditions, with temperature rates under 20°C from October to May and more than 40°C in summer season, its soil characteristics and, over all, the traditional agricultural techniques of high environmental quality. However, the recent changes introduced, because the economic pressure, in these agrarian systems, mostly those dealing with irrigation, can increase the risks of pathogens incidence (3). On the other hand, it is well known that environmental factors, mainly soil temperature and moisture, influenced the development of *Xiphinema index*. Therefore, the study of the influence that such changes could be on the development of this nematode and, consequently, in the GFLV transmission and spread, is carried out comparing size populations and its vector capability in both, dry farming and irrigation systems.

MATERIAL AND METHODS

Some 1.000 soil samples from vineyards under dry farming system were analysed for nematodes presence, and plant tissues (leaves, shoot, cane, root, grape, seed, et.) for GFLV detection since 1989 in La Mancha. Some 100 samples of soil and plant were studied along 1995-96 in infected vineyards under deep irrigation method. Presence of virus was determine by ELISA-DAS and nematodes were extracted from soil by Flegg's method (3). Soil moisture (WW) in every sample point of the irrigated field was also determine, according its weight before and after desiccation in chamber at 4°C. Nematodes were extracted from soil by Flegg's method (4).

RESULTS AND DISCUSSION

X.index populations seem to be tolerant to the soil texture and pH in the region, the nematode survive the summer season by remaining in deeper argilic horizons, where moisture is preserved, having only a generation per year from March to May, with a difference of a month in juveniles hatching, between zones with 1°C of temperature difference (1,2). Under traditional agrarian techniques, in which less yield comport a higher grape quality (3), nematode populations are often low (10-15 ind./Kg soil) only in the most infected focus reached 100 ind./Kg soil, and the incidence of GFLV is of the 11%, being its correlation with the virus vector nematodes of 5-15% in random samples and 58-100% in the infected foci. Such infected foci appeared isolates, very localised and the infection spread is extremely slow along years.

In 1993 in a vineyard, GFLV symptoms appeared in one corner and (Fig.1) deep irrigation was introduced in order to increase yield. In 1995 symptoms of vein-banding, yellow mosaic and wood deformation appeared widespread all along the parcel. Sixty samples of plant and soil were analysed as well as the soil moisture (WW) in each sampling point. *X.index* appeared in the 80% of the samples with higher populations than 500 ind/Kg soil, GFLV was in the 54% of samples and correlation virus-vector was of 66% (Fig.1). The study of correlation between soil moisture (WW) and nematode populations shown that nematode populations increase in points with a soil moisture (WW) between 4% and 10%, being the higher population between the 5%-9%. After 10% of soil moisture (WW) nematode populations decreased drastically. On the other hand, nematode spread is much easier in moist soil and consequently GFLV is quickly spread all along the field as shown in Fig.1.

This observations show the importance of the agronomic techniques in the epidemiology of the disease, since of the vineyard yield increase with irrigation, could be a negative practice when the vector nematodes are in the soil, as nematode populations and their dispersal greatly increase with soil moisture. In such a way disease is quickly spread and replanting of death vines is impracticable.

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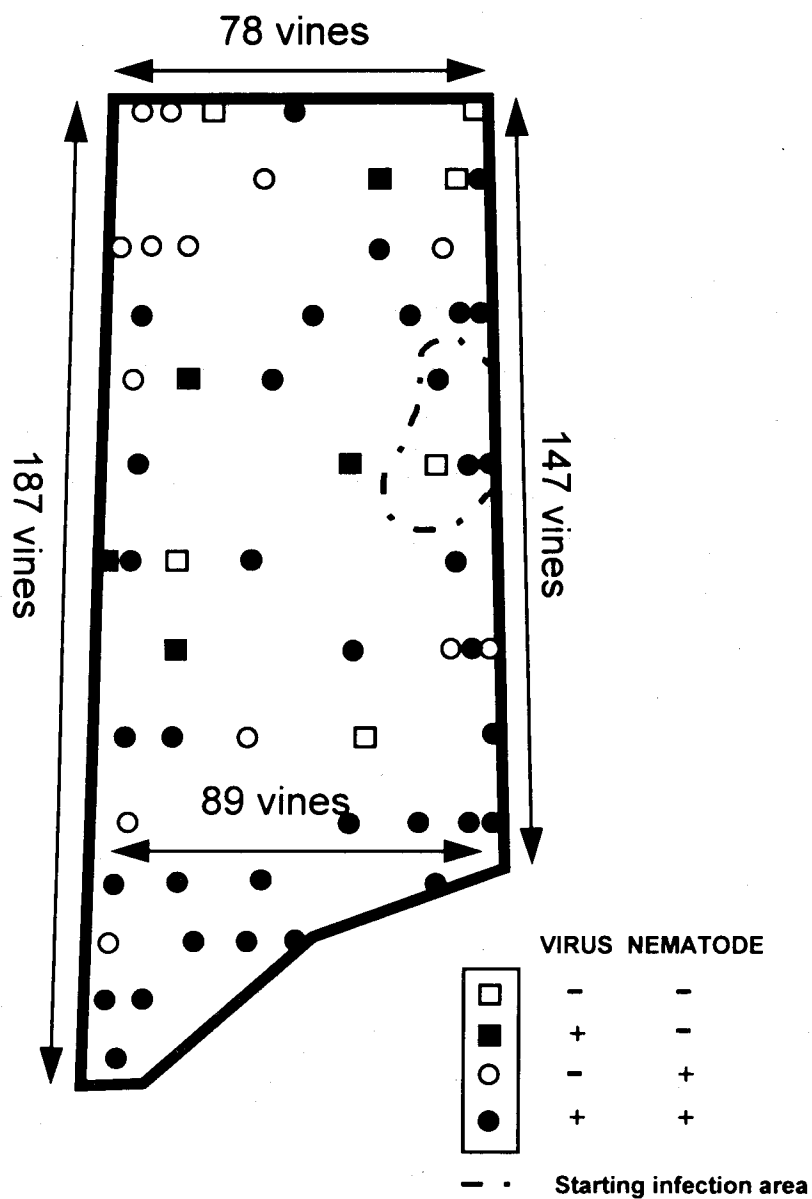
Figure 1.- Spread and correlation between GFLV and *X.index* in a grapevine under deep irrigation.

EPIDEMIOLOGY, SPREAD, ROOTSTOCK SENSITIVITY AND ECONOMICAL IMPACT OF CORKY-BARK DISEASE IN GRAPEVINES

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Grapevine corky-bark is one of the major diseases affecting grapevines in many countries, causing substantial damage, especially in certain rootstock-variety combinations (9). Although several closterovirus-like particles have been isolated from infected plants and associated with the disease, such as grapevine virus A (GVA), grapevine virus B (GVB), grapevine leafroll-associated virus 2 (GLRaV 2 = CB-100, and more recently, grapevine viruses C and D (GVC, GVD) and capillovirus-like particles (1,2,3,4,8,12,13, 14). The aetiology of this disease is still unclear. Natural field spread was first reported in Mexico 1980 (20) and later (1990) in South Africa (5) and (1996) in Israel (19). Controlled transmission trials carried out in Italy, Israel, South Africa and the USA using different species of mealybugs, demonstrated that GVA, GVB and GLRaV 2 (CB 100) are transmitted by these vectors (5,9,10,11,16,17).

A study was initiated to determine differences in sensitivity to corky-bark of Thompson-Seedless grafted on 4 different rootstocks, evaluate the economic impact and confirm the field spread of this disease (18).

The experimental plot consisted of healthy Thompson-Seedless grafted onto Richter-110, Paulsen 1103, 41B and 16-13. CB infected Thompson-Seedless were grafted on the same rootstocks and planted at random. Both healthy and affected plants on their own roots were included. Indicator plants (Rupestris St.George, LN 33, Mission and Baco 22A) were inter-planted among them. Thompson-Seedless grapevines were monitored twice yearly for symptoms and samples were grafted on indicator plants to verify disease incidence. From the 3rd year on the yield of each plant was collected and weighed and recorded. The indicator plants were monitored for symptoms and tested by ELISA.

The most sensitive combination was that grafted on 16-13. The first typical symptoms appeared as early as one year after planting and some of the plants died 2-3 years later. The lowest yield was on 16-13, R-110 and Paulsen 1103 were the better combinations and 41B was intermediate. Yield reduction was quantitative and qualitative as well.

Indicator plants demonstrated typical corky-bark symptoms on LN 33 after 5 years at least. Some RSG plants did not show typical symptoms. Indexing by grafting and ELISA tests confirmed that corky-bark was present in these plants.

This work indicates the economic impact of corky-bark disease on yield of infected grapevines, and the differences of various rootstock cultivar combinations as to their disease resistance or tolerance. Results obtained with the inter-planted indicators demonstrates the spread among grapevines in the vineyard.

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INDUCED RESISTANCE OF GRAPEVINE - PRELIMINARY RESULTS ON HOST REACTION IN VIRUS INFECTED GRAPEVINES

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INTRODUCTION

Pathogens induce a defence response within host plants that protects against challenge by the same or another infectious agent. The response on challenge infection is systemically spread in the plant and causes a systemic acquired resistance (SAR) (7). Elicitors e.g. polysaccharides of fungal and bacterial cell-wall trigger the SAR as well as virus infections. SAR response is probably based on multiple mechanisms, which can lead to long term and broad spectrum disease control (4). The induction of phenylpropanoid pathways appears to play a crucial role in resistance response (3, 5). The activation causes an accumulation of phenolic metabolites which might possess antiviral activity (6). Moreover challenge of plants by elicitors causes the *de novo* synthesis of pathogenesis-related proteins (PRP´s) in tissue of affected plants. The concept of SAR has been inferred from model studies on *Vitis* in our group (1, 2). More recently studies have been started to investigate the response mechanisms in *Vitis* challenged by viruses.
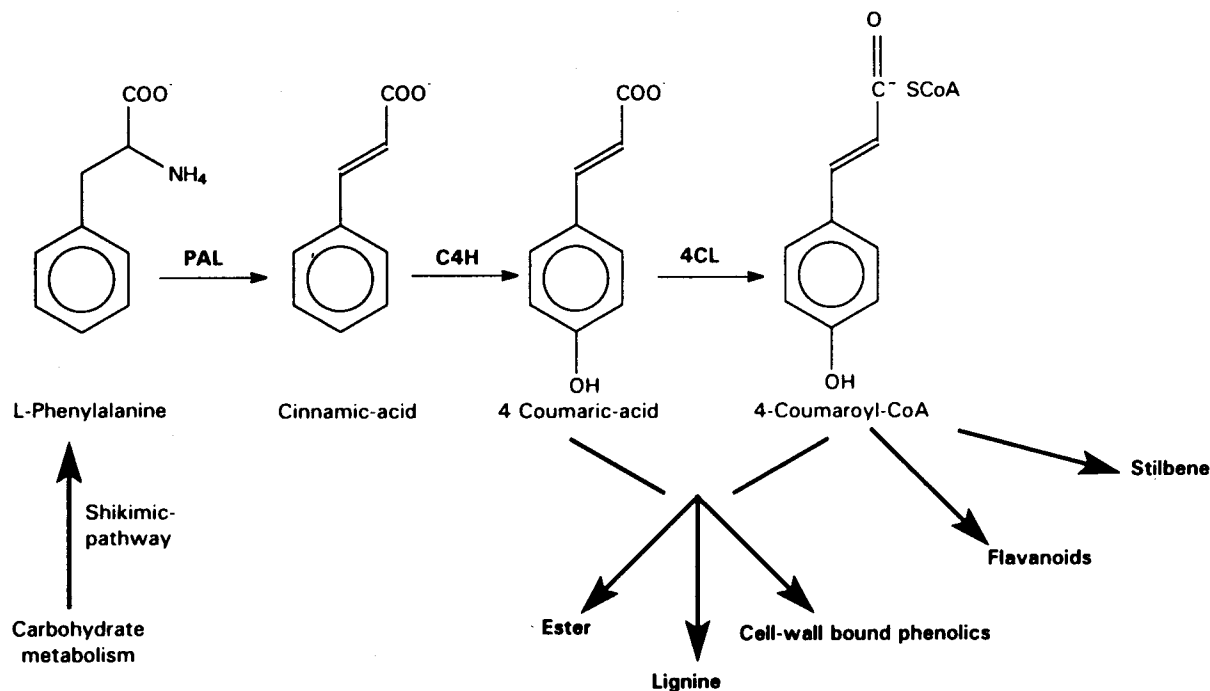
Figure 1. Phenylpropanoid pathway (2)

METHODS

Cell suspension culture of *Vitis vinifera* L. cv. Pinot noir clone FR 54-86 virus tested and cv. Pinot noir FR-LR601 GLRaV infected was established and used as a model system for the investigation of the response mechanisms. Studies on plants were carried out on cuttings from both accessions (Pinot noir clone FR 54-86 virus tested and Pinot noir FR-LR 601). From the healthy cell culture, elicited by yeast extract, cDNA from SAM:caffeoyl-CoA 3-O-methyltransferase (CCoAOMT), probably involved in phenolic ester formation, was cloned and sequenced. A highly active grapevine CCoAOMT was expressed in *E. coli* and used to raise specific antibodies. Specific antibodies and cDNA for stilbenesynthase and phenylalanine-ammonia-lyase (PAL) were available too.

Additionally cDNAs were cloned from the elicited cell culture and sequenced, encoding a basic class I (VCHIT1b) and an acid class II (VCH3) chitinase respectively. As well antibodies for these chitinases were available. In a further experiment a PR1 from *Vitis* was cloned. Transcripts of the enzymes were detected by Northern blot hybridisation. The expression of enzymes involved in host-pathogen interaction

were revealed by means of western blot. The products of the phenylpropanoid pathways, e.g. stilbens, flavanoids, were assayed chromatographically.

RESULTS AND DISCUSSION

Elicitation by pathogens induced the *PAL*-mRNA and the activity of this enzyme. PAL is the key-enzyme of the phenylpropanoid pathway and this step provides the precursors for the synthesis of phytoalexins (resveratrol and other stilbens), flavanoids and other pathogen inducible phenolics like lignine and cell-wall bound esters. The stilbensynthase (STS) is catalysing the synthesis of resveratrol which may play a role as antibiotic product in grapevine cells. Northern blot analysis of induced cells showed an increase of the *STS*-transcript. Elicitors raised the abundance of *CCoAOMT*-mRNA. For the methylation of coumaric and ferulic esters the caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) is responsible. Coumaric and Ferulic esters are lignin precursors and accumulate to the cell-wall. They may act as antibiotics. Especially in plants infected by GLRaV 1 an induction of the enzymes of the phenylpropanoid pathways was detected. The activation of precursors of the flavanoid biosynthesis may be responsible for the reddening of leafroll infected leaves.

The transcript abundance of the two *Vitis* chitinases was measured in response to pathogen infection in cells and leaves of *Vitis*. Different induction kinetics of the two PRP´s were revealed. Additionally a systemic induction of *class III chitinase*-mRNA was found. The expression kinetics and the role of the PR1 are still unknown.

CONCLUSION

Now molecular and biochemical tools are available to investigate the possible role of virus induced resistance response in the phenomenon of cross protection. Further experiments concerning the induction of virus resistance by elicitors are still under investigation.

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GRAPEVINE GENETICALLY TRANSFORMED WITH THE COAT PROTEIN GENE OF GRAPEVINE FANLEAF VIRUS: AN ANALYSIS OF TRANSFORMANTS.

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In order to test the usefulness of coat protein (CP)-mediated protection against the fanleaf disease in grapevine, the CP gene of GFLV (grapevine fanleaf virus) was transferred to several rootstock varieties. This paper presents results obtained with *Vitis berlandieri* x *V. rupestris* 110 Richter and *Vitis rupestris*, concerning
- (1) the characterisation of transgene integration and expression,
- (2) resistance tests to the infection by GFLV.

The construct used was produced by L. Pinck, IBMP/CNRS, Strasbourg, as explained in Bardonnet *et al.* (1994). The CP gene of GFLV strain F13 is expressed in a P35S promoter/ NOS terminator cassette. A GUS-intron gene, under the same p35S promoter, was introduced for grapevine transformation.

The transformation was performed by coculture of anther-derived embryogenic calli with *Agrobacterium tumefaciens* strain LBA4404. Selection of transgenic cells on kanamycin- containing solid medium, and regeneration of plantlets were done as described in Krastanova *et al.* (1995).

For analysis of the transformants, total DNA was extracted from leaves of vitroplantlets or greenhouse-grown plants (young leaves), according to the protocol of Lodhi *et al.* (1994) modified by P. Coutos-Thévenot (LVMH Recherche, Colombes). Independent transformants were identified by PCR and Southern analyses and selected for further characterisation on basis of the following criteria:
- -actual integration of the CP gene in the grapevine genome and stability of the integration in vegetatively propagated plantlets,
- -no integration of plasmid sequences located outside of the T-DNA, such as those functional in bacteria.

The number of copies of the CP gene varied between 1 and more than 5.

The transgenic expression of the CP gene, as evidenced by ELISA, using a polyclonal antiserum against purified GFLV, was generally low. But some transformants consistently exhibited a significant level of CP production. Consistent differences were also found between transformants in the expression of the GUS protein in roots, assayed by a colorimetric test.

Immuno-gold decorated virus-like aggregates were observed by electronic microscopy in some *Nicotiana benthamiana* transformants (produced by Bardonnet *et al.*) expressing the GFLV CP.

Protection tests against GFLV infection were performed using three methods: green-grafting of greenhouse-acclimated transformants, micrografting of vitroplantlets, and transmission of the GFLV by its natural nematode vector *Xiphinema index*. The nematodes used for those tests were first obtained from a contaminated vineyard (Frejus, France) and subsequently from nematode rearings (collaboration of D.ÊEsmenjaud, INRA Antibes).

All green-grafted transformants tested proved to be infected by GFLV after one month of contact with the inoculum (introduced as rootstock or graft). The *in vitro*-micrografted transformants tested were also infected, but a follow-up of cuttings derived from those transformants after inoculation showed an apparent recovery in two 110R.

Twenty transformants of each variety were tested by nematode transmission (3 to 8 months according to the assay), and further kept under observation after the inoculation period was finished. Among those, differences in the spread of systemic infection or in symptomatology could be observed, but none of them exhibited immunity to GFLV.

Several alternative constructs, based on the viral polymerase, Vpgn + protease or untranslatable CP genes of GFLV, have been used to transform 110R, with a view of further testing pathogen - derived resistance in grapevine.

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BIOTECHNOLOGICAL APPROACHES TO GRAPEVINE VIRUS RESISTANCE BREEDING

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Grapevine fanleaf disease and rugose wood complex are widely spread viral diseases,of grapevine, the world's most widely spread viral grown fruit crop. Grapevine fanleaf virus (GFLV) and Arabis mosaic virus (ArMV) are soil-borne nepoviruses, which - together with other nepoviruses are the causing agents of grapevine fanleaf disease. on the other hand we chose the two tentative trichoviruses, namely grapevine virus A (GVA) and grapevine virus B (GVB) (Minafra et al. 1994), likely to be involved in the aetiology of Kober stem grooving and corky bark, respectively, both diseases of the rugose wood complex.

Since the first report of the use of pathogen derived sequences to confer resistance (Sanford and Johnston 1985) there has been produced a large number of transgenic plants. Many of these contain sequences of the viral coat protein gene. There are, however, also reports of a range of other sequences of viral origin, e.g. replicase, protease and movement protein genes. Several different approaches to engineer pathogen derived resistance to viruses in grapevine have already been reported (Steinkellner *et al.* 1991, Brault *et al.* 1993, Le Gall *et al.* 1994, Krastanova *et al.* 1995, Mauro *et al.* 1995, Goelles *et al.* 1997).

Although the use of this type of resistance may bring enormous potential benefits, its use is being delayed because of concerns about potential hazards to agriculture and environment. Taking such safety considerations into account, in this paper the virus resistance breeding program of the IAM will be discussed, focusing on the following topics:

-presentation and justification of the constructs used (Goelles 1994)

-optimisation of the transformation procedure for herbaceous model and woody host plants by using different explants (Goelles et al. this volume)

-selection of putative transformants of *Vitis sp.* (Goelles et al. this volume)

-challenging of transgenic lines and protection observed (Moser 1997)

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ELECTROPORATION OF GRAPEVINE PROTOPLASTS: INOCULATION OF GFLV INTO GRAPEVINE FOR THE SCREENING OF TRANSGENIC PLANTS

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Fanleaf degeneration, one of the most serious viral diseases in grape, is caused by the Grapevine fanleaf virus (GFLV). This virus belongs to the *nepovirus* group and is spread from root to root by a dagging nematode, *Xiphinema index* (Thorne and Allen). The use of nematicides has failed to control the vector. Another way of controlling fanleaf degeneration could be the use of resistant rootstocks obtained either by traditional breeding or by gene transfer methods. But, because direct GFLV inoculation to grapevine is unfeasible, screening for fanleaf resistance is tedious and requires either testing in infected soil over several years (1) or grafting under certain circumstances (2, 3). We have developed a new way of inoculating GFLV into grapevine based on protoplast electroporation in order to identify GFLV resistant clones.

MATERIAL AND METHODS

Protoplasts were obtained from *in vitro* grown leaves of the 41B rootstock variety and from embryogenic cell suspension of 41B. Overnight digestion was performed in respectively mannitol, KCl 5 mM, $CaCl_2$, $2H_2O$ 2 mM and $MgCl_2$, $6H_2O$ 0.4 mM with cellulase 2% (w/v)(FLUCKA) and pectinase 1% (w/v) (FLUCKA) for mesophyll protoplasts (osmotic pressure: 0.54 osm/kg) and in CPW medium with cellulase 2%, macerozyme 1% and driselase 0.5% (FLUCKA) for suspension protoplasts (osmotic pressure : 0.45 osm/kg). Protoplasts were then cultured in half-strength MS medium.

Two electroporation media were used : medium A = $CaCl_2$, $2H_2O$ 0.1 mM, pH 5.6 (4) adjusted with mannitol to the proper osmotic pressure; medium B = Hepes 10mM, NaCl 150 mM, $CaCl_2$, $2H_2O$ 5 mM, pH 7.1 adjusted with mannitol (5). Pre-chilled protoplasts at a density of 500,000 protoplasts/ml were electroporated with the Bio-Rad gene pulser apparatus.

In order to establish protoplast permeation, calcein uptake was monitored: 5mM calcein in electroporation medium was mixed to the protoplasts before electroporation. Following electroporation and 2 rinses, the permeabilized protoplasts were observed under UV light. The frequency of permeation was estimated by counting the number of totally green fluorescent protoplasts out of 100 viable protoplasts. Viability was estimated using aceto-carmin stain.

For infection protoplasts were electroporated with either RNA or viral particles. Virus replication was detected 72 hours after electroporation by Western blotting using antiserum raised against the 38kda protein (P38), which is the viral movement protein (6).

RESULTS

We first monitored calcein uptake to determine appropriate electric field strength and pulse duration. Calcein uptake did not occur in non-electroporated protoplasts in A medium but green fluorescent protoplasts were observed in B medium, even without electroporation: 15-20% of the mesophyll protoplasts exhibited some yellowish fluorescence in the negative controls.

For both protoplast populations, and both electroporation media, calcein uptake increased with the voltage and the capacitance. In A medium, best permeation conditions for mesophyll protoplasts were 300 Volts and 175 μF: over 35% protoplasts showed bright fluorescent cytoplasm but the viability decreased to 28%. In B medium, when taking into account the high fluorescent background in the negative control, the permeation was less important, even at 175 μF and 250-300 Volts which were the best experimental conditions in this buffer : barely 20% protoplasts were permeabilised. Therefore, for further experiments, A medium was used.

This study allowed us to select electroporation conditions suitable for grapevine mesophyll protoplasts : one pulse at 150 or 175 μF and between 150 and 200 Volts yielded 30% bright green fluorescent protoplasts and a viability of about 70% after electroporation. For embryogenic suspension protoplasts, the best conditions were : 150 μF and 200 Volts. About 30% protoplasts showed a bright green fluorescence due to intense calcein uptake.

Two μg of purified virus were then used for inoculation. We were not able to detect any virus replication in non-electroporated protoplasts mixed with GFLV particles, either from mesophyll or from cell suspension. For mesophyll protoplasts, several electrical conditions were tested: 175 μF and 124, 150, 174 and 200 Volts for virus inoculation. At 174 and 200 Volts, we could detect a significant amount of P38 protein in the protoplasts indicating that the virus penetration and replication had occurred. When using 10 ng of viral RNA, optimal GFLV replication was observed following electroporation at 150 μF and 174 Volts (figure 1.a).

In embryogenic suspension protoplasts, at 150 µF and 200 Volts, replication was clearly observed with the virus and at 150 µF and 200 Volts with viral RNA (figure 1.b).

These conditions were successfully applied to electroporate transgenic 41B protoplasts and will be discussed.
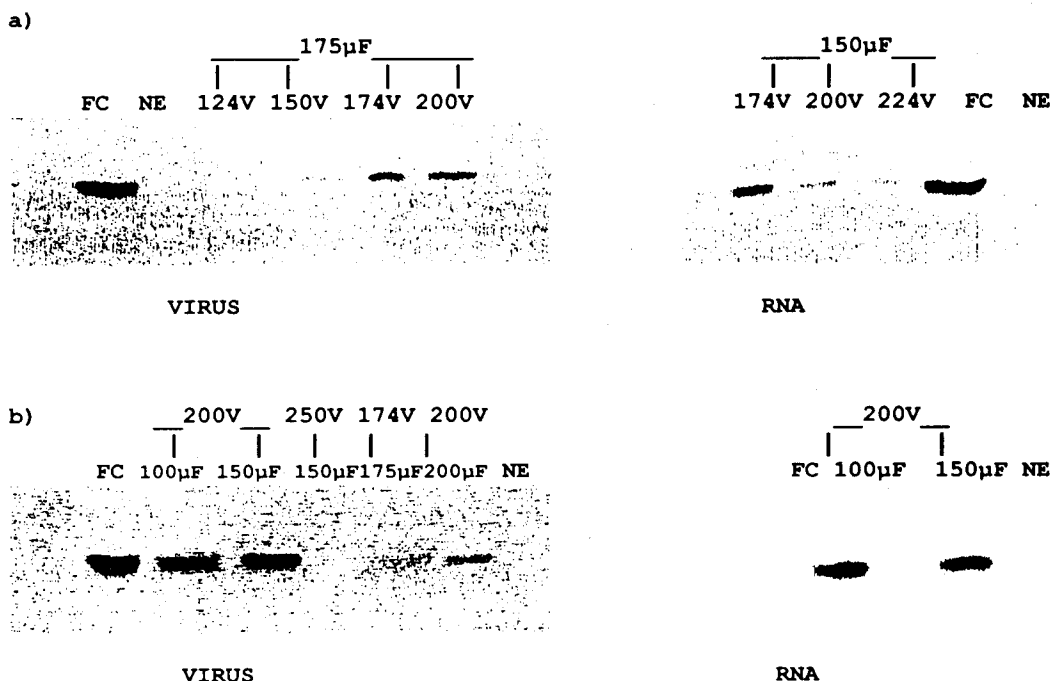
a)

b)

Figure 1: Detection of the P38 GFLV protein by Western blotting
    a) in 41B mesophyll protoplasts
    b) in 41B embryogenic cell suspension protoplasts after electroporation at different conditions of capacitance and voltage with 2 µg of virus or 10 ng of viral RNA. FC: infected leaves NE: non-electroporated protoplasts

CONCLUSION

Until now, it was not possible to inoculate GFLV to the grapevine without the help of a biological vector. The results presented in this paper indicate that electroporation is an efficient mean of introducing viral materials into grapevine and this is the first example of direct GFLV infection into a *Vitis* species. Based on these results, we are designing a screening essay for fanleaf resistance that is rapid, efficient and reproducible.

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PROTECTION AGAINST GRAPEVINE FANLEAF NEPOVIRUS IN TRANSGENIC TOBACCO EXPRESSING THE VPG-PROTEINASE OR THE VIRAL REPLICASE GENE.

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Grapevine fanleaf nepovirus (GFLV) causes the widespread "court-noué" disease. We reported previously that infection with GFLV in transgenic tobacco plants expressing GFLV coat protein (CP) was delayed and reduced (1) as also observed in CP-mediated protection assays with many viruses (2). Different other strategies based on genetic engineered pathogen-derived resistance have been investigated, they mainly concerned movement protein and viral replicase genes (2). We report here results on protection against GFLV infection obtained with *Nicotiana benthamiana* transformed with the VPg-proteinase gene or the replicase gene of GFLV, in which the catalytic site GDD was mutated into RDD.

The virus and the recombinant plasmids. GFLV RNA-1 and RNA-2 code for polyproteins P1 and P2 respectively which are processed into structural or functional proteins by the viral proteinase coded by RNA-1 (3-4). The VPg-proteinase (VPgPro) gene was amplified by PCR using primer A to introduce a *Bam*HI cloning site, the leader sequence of GFLV RNA3 and an ATG codon to permit expression of this gene and primer B to introduce a stop codon, a polyA sequence and a *Bam*HI site (Fig.1). The PCR product was cloned in the *Bam*HI site of pROKI and transformation of *N. benthamiana* was as reported previously for CP(1).

The RNA polymerase or replicase gene is located downstream of the proteinase cistron (3). A chimeric replicase gene was constructed by introducing a *Bam*HI cloning site, a Lütcke leader sequence and an ATG start codon in front of the Val^{1463} codon (polyprotein P1 numbering) of plasmid pPol2 (5). The catalytic GDD motif of replicase was mutated to RDD in which a *Nru*I site was introduced to control the resulting mutation. The entire chimeric replicase gene was cloned and the *Bam*HI fragments bearing replicase gene in pPolRDD and in the non-mutated pPolGDD were cloned in the *Bam*HI site of pROKI vector between a CaMV 35S promoter and the NOS terminator signal (Fig.1) to yield pRPGDD and pRPRDD respectively which were mobilised into *A. tumefaciens* LBA4404 . The transformation of 8-10 weeks old *N. benthamiana* was as described previously (1).

Identification of transformed plants. The transformed plants were first selected on the basis of their resistance to kanamycin. The selected VPgProteinase or "VP", the replicase "GDD" and the "RDD" transformed plants were further tested for the presence of the gene of interest and the corresponding messenger RNA. Total RNAs containing the mRNA of the inserted gene, if expressed, were reverse transcribed to produce a cDNA encompassing the VPgPro or the replicase gene. The cDNA was amplified by PCR and part of the PCR products were digested with *Sst*I for VP and *Nru*I for RDD. Specificity of the PCR products, was tested by hybridisation with specific VPgPro or replicase probes.
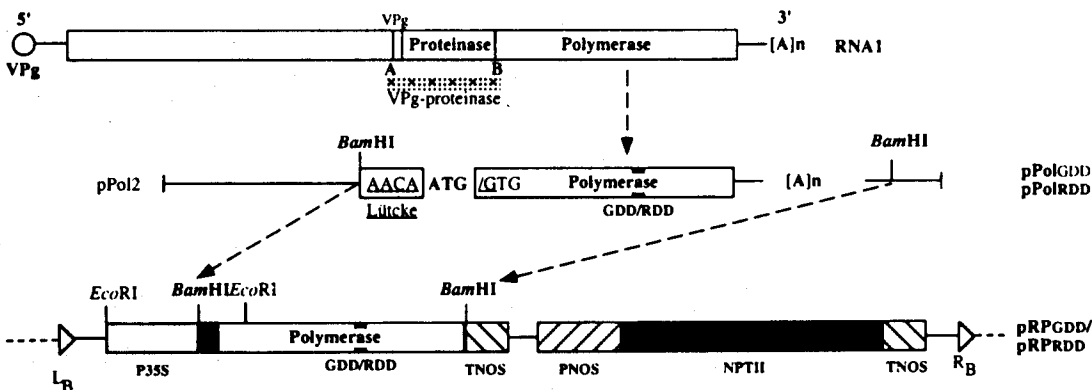

Figure 1. Schematic representation of GFLV-RNA1 and of the replicase gene inserted in plasmid pROKI (Constructs pRPGDD and pRPRDD). The positions of the primers A and B used for PCR amplification of the VPg-proteinase gene are indicated under the RNA1.

Infectivity assay. Ten *N. benthamiana* plants of F1 generation of each transformant line were used for protection assays. Two basal leaves were inoculated with 50 μl of virus at 2 or 20 μg/ml.

Evaluation of protection. Since *N. benthamiana* produced no symptoms upon infection, protection against GFLV was evaluated from total RNA by VPg-northern immunoblotting (6) or by northern blot

analysis with dioxygenin-labelled probes of total RNA from the upper systemic leaves. In systemic leaves of plants at 45 and 60 days post infection (dpi) the amount of viral RNA was evaluated by RT-PCR.

RESULTS AND DISCUSSION

Protection with VPgPro. The protection level reached against an inoculum of 2 μg/ml virus in VPgPro-transformed plants can be compared to the effects observed in our previous CP-mediated protection assays (1). Comparison of the level of viral RNA at 12, 18 and 36 dpi indicated that infection level was only reduced (Fig. 2a). At 36 dpi all plants are infected but the amount of viral RNA in these plants remained still lower than in the non-transformed control.
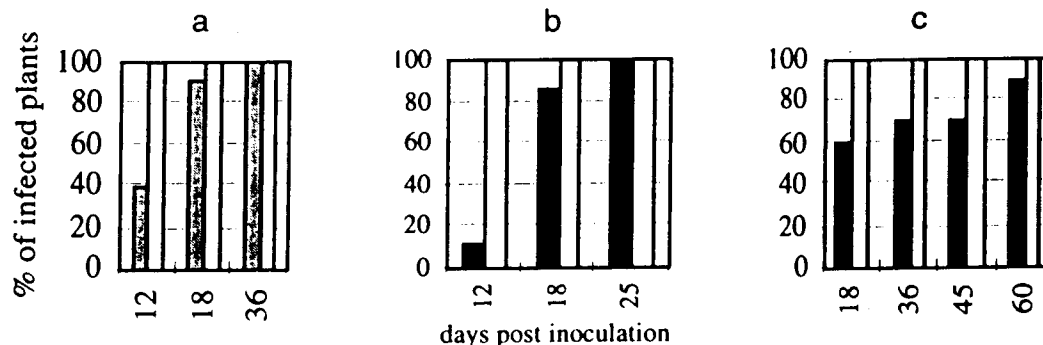
Figure 2. Histograms indicating the percentage of infected transgenic plants transformed with (a) VPgPro, (b) GDD replicase (wild type) and (c) RDD mutated replicase. The concentration of virus in the inoculum is: in a 2 μg/ml, in b and c 20 μg/ml. The non-transgenic controls are indicated by empty bars.

Protection with replicase. GDD-transformed plants show an effective but not long-lasting protection effect against high virus (20 μg/ml) infection since after 12 dpi, when all the non transgenic plants used as control were infected, 14% of the GDD transformed plants were detectably infected. This percentage reached 86% at 18 dpi and all plants were infected at 25 dpi (Fig. 2b). For RDD-transformed plants, at 18 dpi only 60% of each line were infected. A 30% protection ratio was still obtained after 45 dpi (Fig.2c) and no virus was detected in systemic leaves of 2 lines of transformed plants at 60 dpi. RT-PCR detection of viral RNA after 60 dpi confirmed that no virus was present in systemic leaves of these plants. This clearly indicates that transformation of tobacco with the mutated replicase gene can induce a complete resistance to high strength of GFLV inoculum (20 μg/ml). For the other lines the infection level is reduced and delayed but no complete protection was observed.

CONCLUSION

If a similar protection mechanism seems to exist for GDD-transformed plants the delay of systemic invasion is shorter than for the RDD lines for a same inoculum strength. After 18 days near 90% of the GDD plants tested were infected against 60% for the RDD-transformed plants and no resistant plants were obtained. Thus, protection obtained by the expression of modified polymerase in *N. benthamiana* appears very efficient since some plants were totally resistant at 60 dpi to high concentration of virus and others show significant delay in virus infection cycle. This protection level might be sufficient for the inoculum delivered by the nematodes in the roots. Such a long-lasting protection is particularly important in the case of perennial crops such as grapevine for which GFLV is one of the principal viral pathogen.

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TRANSFORMATION OF GRAPEVINE ROOTSTOCKS CONTAINING GENES FROM GRAPEVINE FANLEAF VIRUS AND GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUS 2 AND 3

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Grapevine fanleaf and leafroll are the two most important and widespread virus diseases of grapes. Grapevine fanleaf virus (GFLV) is transmitted by a soil-borne nematode. Grapevine leafroll associated closterovirus 3 (GLRaV 3) is transmitted by mealybugs, however, there is no evidence on vector transmission of GLRaV 2. At present, effective insecticide and nematecide are either not available or cause environment pollution. Availability of genetic engineered virus-resistant grapevines should provide an alternative solution to control these important virus diseases. In the present study, we would like to use viral genes to introduce resistance to a nepovirus (GFLV) and two closteroviruses (GLRaV 2 and 3) on transgenic grapevine rootstocks.

Embryogenic callus cultures were obtained from anthers of rootstocks (3309 C, Riparia Gloire, 5C, 110 Richter, SO4 and MGT 101-14) which, except for Riparia Gloire, were previously indexed by woody indexing to be free of viruses. Induction of embryogenic calli was obtained on MS basic medium containing 2,4 D and BA. Depending on the genotype, percentage of embryogenic callus formation from anthers of rootstocks was between 5 and 18%. Early stage embryogenic calli were cocultivated with *Agrobacterium tumefaciens* strain (C58Z707 or LBA4404) containing different constructs of virus genes. These gene constructs included sense translatable, antisense and nontranslatable coat protein genes from GFLV and GLRaV 3 and the sense translatable coat protein gene of GLRaV 2. Over 17 independent transformation experiments were performed with embryogenic cultures from four rootstocks. After 3-4 months under selection, secondary embryogenic *calli* were recovered and plantlets were regenerated in MS basic medium without hormones. Regenerated plantlets were then transferred to rooting medium. So far, numerous putative transgenic lines of Riparia Gloire, 3309 C, 110 Richter, and MGT101-14 have been developed. Initial tests with NPT-II ELISA were positive. Characterisation of transgenic plants is being carried out using different techniques, including PCR, GUS assay, NPTII ELISA and DAS-ELISA for coat protein expression. Analysis of plants expressing the sense translatable coat protein gene of GFLV showed various levels of expression ranging from low (OD405nm <0.1), medium (0.1-1.0) to high (>1.0). Resistance to GFLV is being evaluated by different inoculation techniques including micrografting, heterografting with GFLV infected *Chenopodium quinoa*, in vitro green grafting and woody grafting.

VIRAL RESISTANCE IN *NICOTIANA BENTHAMIANA* EXPRESSING ALTERED FORMS OF THE COAT PROTEIN GENE OF GRAPEVINE FANLEAF VIRUS

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Grapevine fanleaf virus (GFLV), a soil-borne nepovirus, together with other nepoviruses causes grapevine fanleaf disease, one of the most damaging and widespread viral diseases affecting grapevine. Several different approaches to engineer pathogen-derived resistance to viruses in grapevine have already been reported (Steinkellner *et al.* 1991; Brault *et al.* 1993; Le Gall *et al.* 1994; Krastanova *et al.* 1995; Mauro *et al.* 1995; Gölles *et al.* 1997).

For *Agrobacterium*-mediated transformation we constructed six plasmids containing different chimeric GFLV coat protein (CP) genes: constructs containing either the full-length CP, nontranslatable forms of the gene (in both sense and antisense orientation), truncated forms of the CP gene (5' or 3' truncated), or a CP gene with a 15 nt deletion within the molecule (Gölles 1994; Gölles et al. 1997) were used to transform leaf discs of *Nicotiana benthamiana*. After the selection of transformed tissue and the regeneration of putatively transformed plantlets they were analysed for the expression of the particular CP gene at the transcriptional and, as in the case of the full-length CP, translational level. Original transformants (R_0) were selfed. Following the germination *in vivo* on kanamycine solution in order to eliminate non transgenic seedlings (Moser 1997) first generation populations (R_1) were challenged by mechanical inoculation with infected plant sap. Some plants transgenic for the full-length CP gene were fully resistant to virus infection. After virus inoculation these lines remained symptomless and no virus could be detected in leaves even 60 dpi (Moser 1997). Challenge infection experiments with plants transgenic for altered forms of the CP gene are currently being carried out and comparative results will be presented.

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REGENERATION OF *VITIS* SP. TRANSFORMED WITH COAT PROTEIN GENE SEQUENCES OF FOUR DIFFERENT GRAPEVINE VIRUSES

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Grapevine fanleaf disease and rugose wood complex are widely spread viral diseases of grapevines, the world's most widely-grown fruit crop. Grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV) are soil-borne nepoviruses, both causing together with other nepoviruses grapevine fanleaf disease. The tentative trichoviruses grapevine virus A (GVA) and grapevine virus B (GVB) (Minafra *et al*. 1994) are likely involved in the aetiology of Kober stem grooving and corky bark, respectively, diseases of the rugose wood complex.

In an attempt to induce virus resistance (Gölles *et al*. 1997b) we introduced chimeric CP genes of GFLV (including nontranslatable and truncated forms of the CP genes), ArMV (Gölles *et al*. 1997a), GVA, and GVB into embryogenic cultures of *Vitis vinifera* (Russalka cv. - selfpollinated) and 110 Richter (*Vitis rupestris x Vitis Berlandieri*) via *Agrobacterium*-mediated transformation. Putàtively transgenic embryos were selected and germinated. Transgenic plants were analysed both at the transcriptional and, as in the case of the full-length CP constructs, translational level. The plant lines which demonstrate the highest expression level will be propagated *in vitro* and used for challenge infection experiments in order to determine the protection against the homologous and related viruses.

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COAT PROTEIN-MEDIATED RESISTANCE AGAINST GRAPEVINE VIRUS A AND GRAPEVINE VIRUS B IN NICOTIANA BENTHAMIANA AND NICOTIANA OCCIDENTALIS

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The rugose wood complex of grapevine occurs in most if not all viticultural countries of the world. The tentative trichoviruses grapevine virus A (GVA) and grapevine virus B (GVB) are involved in the aetiology of Kober stem grooving and corky bark, respectively, two of the syndromes of the complex (1). As with many other grapevine viruses, efficient natural resistance to GVA and GVB may not occur in Vitis. This prompted a study for the introduction of pathogen-derived resistance in grapevines.

In the attempt to induce coat protein-mediated resistance two transformation vectors were constructed in a pBin19 plasmid containing an expression cassette with the coat protein genes of GVA and GVB, respectively, under the trascriptional control of the 35S CaMV promoter and a nos polyA signal. These plasmids were used for Agrobacterium-mediated transformation of leaf discs of Nicotiana benthamiana and N. occidentalis, the experimental hosts of the two viruses. Original transformants (R^0) were tested for the correct insertion of the transgenes by PCR amplification of total DNA and Northern blot for the transcribed mRNAs. Eleven lines of N. benthamiana and 15 of N. occidentalis were selfed and their seeds screened in vivo on kanamycine to eliminate non transgenic seedlings (2).

Notwithstanding mRNAs for both genes were expressed in vivo, coat protein did not seem to accumulate either in R^0 or R^1 plants, since it was not detected by Western blots, using polyclonal antisera or monoclonal antibodies. Challenge infection experiments were carried out in seedlings of the R^1 generation by mechanical inoculation with purified virus suspension (1 mg/ml). The number of kanamycin-resistant R^1 seedlings that became visibly infected ranged from 20 to 40%. Virus accumulation was monitored with ELISA from 3 to 15 days post inoculation. Several lines showed a consistent reduction in virus accumulation (about 0.1 O.D. vs. 0.6 O.D. in non-transgenic control) while symptoms development was delayed (3 to 4 days) and the responses were somewhat milder than in the control.

These preliminary experiments suggest that partial resistance to GVA and GVB infection, probably mediated by the expression of mRNAs, can be obtained in Nicotiana by the insertion of the coat protein genes of these viruses. Transformation of Vitis is now being attempted.

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TRANSGENIC *NICOTIANA BENTHAMIANA* PLANTS RESISTANT TO GRAPEVINE VIRUS A

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Grapevine virus A (GVA) has been associated with rugose wood diseased grapevines (2, 4) as well as with leafroll infected ones (1, 5, 7, 10). The virus is transmitted mainly by propagation material, and to a lower extent, by mealybugs (6, 8, 9). Some isolates can be mechanically transmitted to herbaceous hosts (1, 3).

The use of virus-free propagation material is the most applied (and important) tool to growing healthy vineyards. Recent advances in molecular techniques and genetic engineering enable the production of transgenic plants (3). The introduction of foreign genes, such as the viral coat protein, were intended to produce virus-resistant plants.

In this work we describe trials to transform *Nicotiana benthamiana* plants using the GVA coat protein gene, and the engendering of GVA resistance in some lines. Transformation of grapevine with the same gene is currently in progress.

We have been sequencing the genome of grapevine virus A (GVA) in Israel, exchanging material and information with Minafra's group in Bari, Italy, when the full GVA sequence was accomplished by Minafra *et al.*, (1).

We have generated a number of GVA clones (in the phagemid vector *Lambda* Zap) constituting partial genome segments of GVA. We have transformed *N. benthamiana* plants with some of these clones and followed up the possible development of resistance. So far we have checked out two coat protein (CP) constructs: one comprising the full-length CP cistron (bases 6401-7021) and another comprising a truncated gene (bases 6426-6817).

Double-stranded GVA-RNA was isolated from GVA-infected *N. benthamiana* plants. Each construct has been amplified by RT-PCR from the viral double-stranded RNA and was cloned into the plasmid PCRII (TA-cloning). The inserts were sequenced, and were transferred (following cleavage with NdeI and BamHI) to the plasmid pjd-330 between the 35S promoter and the nos terminator (replacing the GUS gene). The construct 35S-CP-nos (or 35S-CP$_{truncated}$-nos) was cleaved out with XbaI and ligated into the binary, hygromycine (Hg) resistant, plasmid pGPTV-HPT. The TA-cloned constructs were checked for correct expression by the transcription/translation assay Tnt (Promega). Western blot analyses indeed indicated the expression of the correct size protein for each case.

Table 1: ELISA readings* of GVA in several CP-transgenic lines of *N. benthamiana*

Weeks after inoculation	Non transgenic	line 1	line 2	line 3	line 4	line 5	line 6	line 7
4	0.283	0.131	0.162	0.169	0.094	0.076	0.050	0.160
6	0.160	0.153	0.201	---	0.077	0.184	0.092	0.153
8	0.220	0.143	0.270	0.345	---	0.271	0.098	0.105
10	0.215	0.686	0:091	0.174	0.097	0.114	0.097	0.073

* Average blank value from non-infected CP-transgenic plant = 0.057

Following agrotransformation, the developed *N. benthamiana* shoots were allowed to grow without selection. Hg-selection was performed only at a later stage. After hardening and transfer to the greenhouse, the plants were inoculated with GVA. Symptom development and virus titers (by ELISA) were followed up for 2 months (Table 1). The truncated CP construct did not engender any resistance. Seven lines of plants carrying the full-CP transgene were analysed. Only plant #1 exhibited any symptoms. Three other symptomless lines (#2, #3 & #7) were shown by ELISA to be normally infected. Lines # 4 & #6 did not support GVA infection and line #5 displayed delayed infection. However, a remission was observed with lines #2, #7 and possibly #5, reminiscent of the co-suppression phenomenon described for some other plants carrying a viral transgene.

In conclusion, GVA-resistant *N. benthamiana* lines were obtained following transformation with GVA-CP. Delayed infection and remission from infection were also observed. The CP transgene did not affect the ELISA readings significantly. Following these results, transformation of grapevine has been initiated. The transgenic plants will be further checked for resistance by movement protein antibodies and by PCR.

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RESISTANCE TO NEPOVIRUSES IN GRAPEVINE: EXPRESSION OF SEVERAL PUTATIVE RESISTANCE GENES IN TRANSGENIC PLANTS

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INTRODUCTION

The most important soil-borne grapevine viruses are transmitted from the roots of infected vines to the neighbouring healthy ones by nematodes (nepoviruses). Three such viruses are known to occur in Swiss vineyards: grapevine fanleaf virus (GFLV), arabis mosaic virus (ArMV) and raspberry ringspot virus (RRSV). The nepoviruses cause quick destruction of young plants or a gradual decline over several years (infectious degeneration). GFLV is the most widespread virus in grapevines and is the nepovirus of greatest economic importance. Until recently it was possible to control the vector population by disinfecting the soil with specific nematicides. However, the use of the main efficient chemical (dichlorpropen) is no longer permitted in Switzerland since the substance was shown to have cancerogenic properties and contamination of the ground water cannot be excluded. Important areas of Western and Southern Switzerland are contaminated by nepoviruses and its corresponding vectors. Therefore new approaches for introducing virus resistance to our local vine varieties must be explored.

Over the past decade, several strategies have been used to engineer plants for virus resistance, including antisense strategy, use of virus-derived genes, such as the coat protein (CP) or the replicase (RNA dependent RNA polymerase) genes, or expression of mammalian genes having antiviral properties (2,5 oligoadenylate synthase, RNAse L). Strategies based on the expression of virus-derived genes in the plant cells have been reported to be the most successful way to confer resistance against virus infection. This concept is referred to pathogen (or parasite)-derived resistance (PDR).

Therefore the goal of this work is to use similar strategies to introduce resistance to nepoviruses in grapevine rootstock species grown in Swiss vineyards (3309 Couderc, *V. rupestris x V. riparia*) and in a model species St-George du Lot (*V. rupestris*). A number of chimeric genes made of various coding sequences from the GFLV or ArMV genomes, as well as two mammalian genes having antiviral properties were constructed under the control of two constitutive promoters (CaMV 35S or soybean translation elongation factor 1α) (figure 1) and used to transform *N. benthamiana* or grapevine plants.

RESULTS

A. Transgenic *N. benthamiana* plants

Transgenic *N. benthamiana* plants transformed with a grapevine fanleaf virus coat protein gene (GFLV-CP) or an arabis mosaic virus CP gene (ArMV-CP), under the control of two distinct constitutive promoters (CaMV 35S or soybean translation elongation factor 1α) were regenerated. Molecular analyses (Southern blots, PCR) confirmed the presence of the foreign sequences. Expression of the transgenes was verified by RT-PCR and accumulation of the CP product was assayed by ELISA or Western blots. Results with the GFLF-CP transgenes showed that, although the CP-protein RNA transcripts were produced in all plants tested, no CP could be detected by several immunological techniques. When transgenic *N. benthamiana* plants were infected with GFLV, they all showed a delay in symptoms development compared to the untransformed control *N. benthamiana* plants.

In contrast, results with the ArMV-CP transgenes showed that not only the CP-protein RNA transcripts were produced in all plants tested, but in addition some transgenic lines expressed at very high level the ArMV-CP protein; in some cases, analysis by electron microscopy revealed the presence of virus-like isometrical particles, mostly of the penetrated type, typical for empty virus shells, as it was reported previously (1). Upon mechanical inoculation with ArMV, they all showed a delay in symptoms development compared to the untransformed control *N. benthamiana* plants.

B. Transgenic grapevine plants

A number of transgenic grapevine plants (St- George du Lot and the rootstock 3309 Couderc) transformed with one of the transgenes described in figure 1 were regenerated. Except for one variegated plant, all the regenerated transformed plants were phenotypically normal. Confirmation of the transgenic state was accomplished by rooting assay on kanamycin, GUS assay (when applicable), and molecular analyses (PCR and/or Southern blots).

As for the *N. benthamiana*, grapevine plants transformed with the GFLV-CP gene did not accumulate the GFLV-CP product at detectable level by several immunological techniques. Moreover, in

contrast to the results obtained for the transgenic *N. benthamiana*, grapevine plants transformed with the ArMV-CP gene did not accumulate the ArMV-CP product.

Protection experiments were performed by grafting putative resistance transgenic plants onto infected grapevine rootstock plants. Both micrografting (grafting of vitroplants) and green-grafting were performed for a number of independent transgenic lines. So far, all the transgenic lines transformed with the GFLV-CP genes appeared to be sensitive to GFLV. Infection experiments of transgenic grapevine plants by viruliferous nematodes are currently under way.

Only two independent transgenic *V. rupestris* plants transformed with the GFLV replicase gene could be regenerated. Molecular analysis of these lines showed that one line, called 030711, contains 5 intact copies of the transgene. Protection experiments by micrografting 030711 shoots onto GFLV-infected *V. rupestris* rootstock showed that more than 80% of the transgenic grafted shoots were infected by the virus, demonstrating that this line was not resistant to GFLV infection under these conditions. Surprisingly, a green-grafting experiment started in May 1996 between an GFLV-infected 3309 rootstock and the 030711 line showed that, although the rootstock 3309 was still infected with the GFLV, no virus could be detected in the 030711 scion, even after being grafted for almost one year. However, data from other more recent green-grafting experiments between GFLV-infected 3309 plants and 030711 scions resulted in infection of the scion. The reason for this discrepancy is unclear, but the resistance phenotype may be due to some complex gene silencing mechanisms, which could be turned on and off depending on the environmental conditions and/or the physiological state of the plant (reviewed in 2). This sequence homology dependent resistance hypothesis is currently under investigation. To our knowledge, this is the first report of a transgenic grapevine plant protected against virus infection by genetic engineering.

Although additional experiments are needed to confirm these data (especially infection by viruliferous nematodes), introduction of virus resistance traits into grapevine plants by genetic transformation looks already very promising.

Figure 1 : T-DNA structure of the binary vectors carrying the various putative viral resistance transgenes
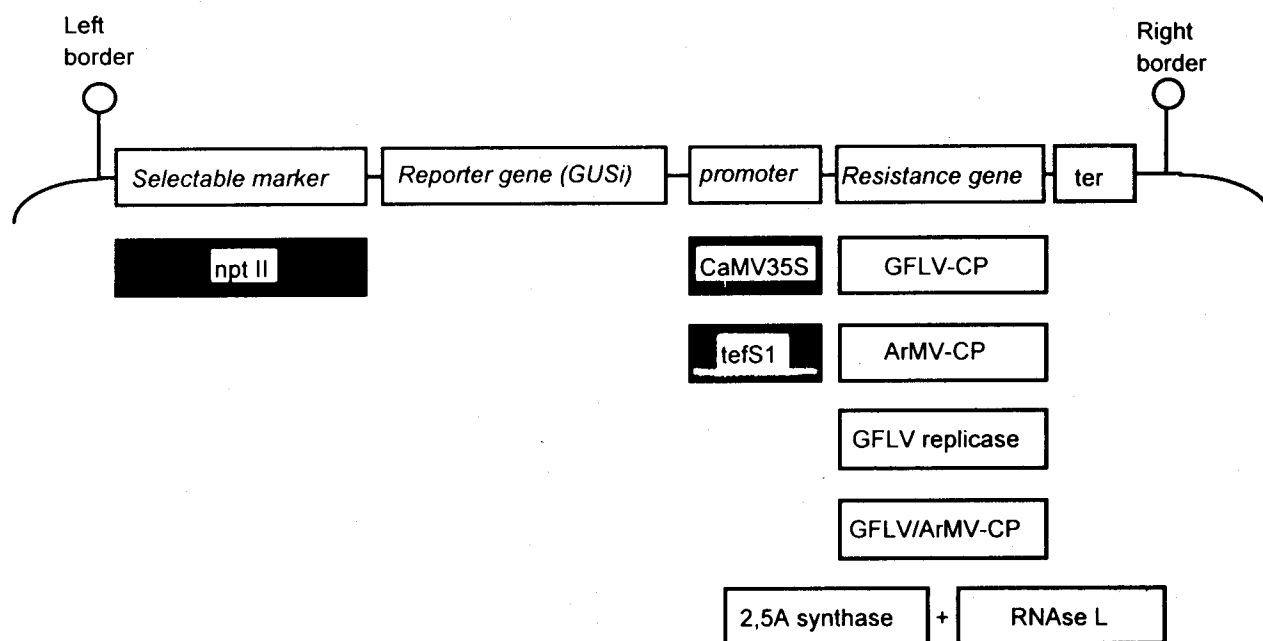
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PUTATIVE MOVEMENT PROTEINS OF GRAPEVINE VIRUSES A AND B: IMMUNODETECTION *IN VIVO* AND USE FOR TRANSFORMATION OF *NICOTIANA* PLANTS.

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Grapevine virus A (GVA) and grapevine virus B (GVB), both belonging to the newly established genus *Vitivirus*, are the putative agents of Kober stem grooving and corky bark, respectively, two economically relevant diseases of the rugose wood complex (1). Because no natural resistance to either of these diseases may occur in *Vitis*, it was decided to address this problem transgenically, by introducing coat protein-mediated (2) or, as reported herein, movement protein-mediated resistance. In this study, *Nicotiana benthamiana* and *N. occidentalis* were used as a model for identifying the sites of intracellular localisation of the non-structural proteins expressed by ORF3 of both viruses and found to be related to the 30K movement protein (MP) family (3), and for genetic transformation.

MP genes of GVA and GVB were amplified by PCR using specific primer sets and GST-translational fusion products were expressed in *Escherichia coli* with a pGEX vector. MP-specific polyclonal antisera were raised in rabbits immunised with gel-purified fusion proteins. Western blots from subcellular fractions of infected *Nicotiana* plants extracted from 6 to 18 days post-inoculation, showed the transient presence of MPs in a cytoplasmatic membrane fraction and their more stable accumulation in cell wall fractions. The observation of colloidal gold-labelled thin sections of infected tissues substantially confirmed these findings.

Agrobacterium tumefaciens-mediated genetic transformation of *N. benthamiana* and *N.occidentalis* was attempted with the MP of GVA and GVB, respectively. Constructs were prepared in a pRT103 intermediate vector and sense- and antisense-oriented genes (MP+ and MP-) were cloned in pGA482. The assembled vectors were inserted in the LBA4404 strain of *A. tumefaciens* by triparental mating and used to transform *Nicotiana* leaf discs. Total RNA extracted from R° transformant lines was assayed by digoxigenin-labelled riboprobe hybridisation or by RT-PCR to verify insertion of the transgenes. *Nicotiana* lines expressing sense and antisense MP RNA transcripts of both GVA and GVB were obtained but serological detection by Western blots of translated proteins was unsuccessful. Screening of transgenic lines for resistance to kanamycin and the evaluation of the protective effect of transcribed MP+ and MP-RNAs following challenge inoculation is currently under way.

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THE USE OF TISSUE CULTURE IN THE CONTROL OF GRAPEVINE VIRUSES

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Tissue culture techniques developed and have been exploited immensely in the last thirty to forty years. The first successful culturing of plant embryos on artificial media was performed by Henning in 1904 (18). In later years the use of tissue culture tools in various aspects of agricultural research was studied, developed and applied. The culturing of cherry embryos by Tukey 1933 (24), was a milestone in the research of woody plants with wide implications for embryo culturing in general. The first successful culture of meristem tips was performed by Ball in Nasturtium in 1946 (1), a technique which was soon after adapted for herbaceous and woody plants, and proved to be of use in different areas of research. In the 1950s to the 1970s research of plant tissue culture was further developed, to the point enabling the regeneration of whole plants from cultured single cells, protoplasts, anthers and microspores.

These technologies are now applied in four different categories:
1. Micropropagation and rapid clonal multiplication.
2. Virus elimination.
3. Germplasm conservation.
4. Genetic manipulation.

1. Many woody plants in general and grapevine in particular are propagated vegetatively, using cuttings of rootstocks or cultivars and graftings, to establish new vineyards. Preliminary sources are either introductions, local existing material, new breedings or certified virus free material. These exist as single or very few source plants and have to be propagated rapidly to large scale. The use of *in vitro* methods for rapid propagation proved to be an important tool , capable of producing a large number of plant in a relative short time. (12,15).

2. The use of virus-free propagation material is instrumental to establish healthy, long-lasting vineyards. The conventional method of producing virus-free propagation material was for many years thermotherapy (9,10). First attempts to harness *in vitro* methods for this purpose were initiated in the 1960s by Glaze (7). The advances of tissue culture technology enable now production of virus-free material via meristem tip (2,3,19 21), fragmented apexes (4,5),and *in vitro* somatic embryogenesis (8). In some cases *in vitro* methods are combined with chemotherapy or thermotherapy (2). Plants acquired using these methods can be rapidly propagated using *in vitro* methods. As mentioned above, tissue culture methods may also be used to: a. increase virus titer in order to facilitate their detection (17). b. as source plants for mechanical inoculation to herbaceous plants, in order to enable purification and characterisation of viruses (18). c. as a virus reservoir for study purposes. d. rapid in vitro indexing by mimicking in vivo indexing on indicator plants, shortening the time for symptom appearance from 2-3 years to 3 months (22). The application of stress inducing agents on plantlets grown in vitro can be used too for indexing purposes (23).

3. Germplasm conservation is essential for maintaining genetic diversity and gene pools for crop improvement. Field maintenance of woody plants which can not be stored as seeds is expensive, demands huge areas and labour and encounters many pest problems. Using conservation in tissue culture applying various slow growth techniques, cryopreservation are of great benefit to these crops (13, 20).

4. Genetic manipulations and transformation open a new era in plant breeding. Conventional breeding uses *in vitro* techniques such as embryo cultivation and regeneration, protoplast fusion and anther culturing. The ability to introduce foreign genes into plant cells using efficient vectors and regenerating viable plants, opens new opportunities for breeding improved plants, modifications etc.(6). These methods are now experimented aiming to produce virus resistant plants by introducing of different virus genes.

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OCCURRENCE OF HEALTHY GREEN SHOOT TIPS FROM VIRUS-INFECTED INDICATOR VINES.

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Several investigators - from 1906 to 1995 - have observed healthy shoot tips of naturally grown stems of vegetatively propagated plants previously infected with systemic pathogens.(1), (2), (3), (4), (5), (7), (8), (9), (11).

From a practical standpoint, rooting and propagating stem tips of rapidly grown mother plants possibly infected with systemic pathogens remains to be developed to a level of commercial usefulness. The intention of our experiments was to exonerate small and medium clone-breeding enterprises from time consuming techniques , in particular "long term indexing" and from the very expensive techniques of "short term indexing" (6). Indications will be given here how our work is overcoming this notion.

Candidate vines for pathogen elimination are from many sources. For studies with specific viruses, infected indicator vines are used wherever possible. Thus the final plants do not need to be re-indexed; self-indexing was expected.

The potted plants are placed into the growth chamber and forced as rapidly as possible. Forcing of potted grapevines is most effective when a productive root system is „working" in a well aerated and fertilised potting substrate. Therefore new growth may start immediately. Under any circumstances, the young stems have to be tied for strict vertical growth. The tips of the „telescoped" stems made up internodes, - more or less long depending on the growth rate. Green shoot tips are cut under non-sterile conditions (a simpler and cheaper option than cutting under sterile conditions) and placed in aerated water for root formation (10).

Partially etiolated softwood stem tips
- of the nepovirus-indicator „Siegfriedrebe" infected with raspberry ringspot virus,
- of the leafroll-indicator „Blauer Spätburgunder" infected with Grapevine leafroll associated virus type I or type III,
- of the corky bark-indicator „LN 33" showing symptoms of corky bark,

6-10 cm long after forcing in the growth chamber at 30° C +/- 2° C, were rooted in aerated water. Tanks were equipped with aquarium aerators and placed in the growth chamber which was thermostatically controlled at 30° C +/- 2° C and artificially lighted. The cuttings were suspended by a rack of styropor which was perforated by 1 cm holes. After the tips rooted (usually 14-21 days) the young plants were transplanted into perlite without mineral nutrients, and placed in a moderately heated room (20° C +/- 2° C) with natural day light. After one week the plants along with their containers were transferred to the greenhouse and fertilising was started. After the young vines were very well established, six of them were planted into steam-sterilised potting soil in 80-litre containers and placed in the greenhouse. The vines are then held in containers until symptoms develop.

We rooted and propagated
- healthy vines from the nepovirus-indicator „Siegfriedrebe" previously infected with raspberry ringspot virus,
- healthy vines from the corky bark-indicator „LN 33" previously corky bark-affected (probably caused by a trichovirus),
- healthy vines from the leafroll-indicator „Blauer Spätburgunder" previously infected with Grapevine leafroll-associated virus type I or type III.

Infected indicator vines treated in our experiments have remained healthy during three growing seasons up to now.

The results in elimination of virus diseases from grapevines by propagation of rapidly grown green tip cuttings reported here have to be critically discussed: Was the delay between treatment and self-indexing long enough ? Is anyone - scientist or breeder - going to take the risk of not real indexing after treatment for all systemic pathogens that might initially be present? Instead of a binding answer: In serological GFLV-tests with young leaves, grown at 32° C in a walk-in growth chamber with permanent lighting for ten days and then transferred into the greenhouse, no serological reaction has been found after ten weeks. This means that no reinfection of the young leaves occurred coming from the basal parts of the treated plant (3). Shoot tips of nepovirus-infected potted vines, taken after forcing mother plants at 30°C +/- 2° C mist propagating the explants near 30° C and establishing them in greenhouse containers after rooting, did not show any symptom of infection and were negative by ELISA for seven years (11).

The method used at Leiwen seems to be yet adequate for eliminating virus diseases that may be carried in nursery stock. We see little progress in any system for virus elimination from grapevines requiring „long term indexing" and „short term indexing" a l o n e for use in small and medium private enterprises. Note: Hard wood indexing for the rugose wood complex (stemgrooving/stempitting) takes 4 years! (KRIEL, pers. comm., 1994). Rooting of green shoot tips grown very rapidly is a propagation technique which

works self-acting for eliminating systemic pathogens, does not need high qualified personnel and the care for running successfully may be limited.

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VIRUS ELIMINATION BY THERMOTHERAPY - EARLY SCREENING AND IMPROVED DIAGNOSIS WITH IC-PCR

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Elimination of viruses in infected grapevine by thermotherapy and regeneration of new plants out of the apical meristem is state of the art for the phytosanitary sanitation of clones and genotypes. The behaviour of Nepoviruses,Closteroviruses and others (Trichoviruses) differs especially in successful elimination rate. While the Nepoviruses GFLV, ArMV and GCMV could be eliminated without escapes (100% elimination rate), sanitation for GLRV I and III failed in some cases. Primarily the success of elimination was confirmed by ELISA testing. Symptoms of virus disease observed at the leaves of ELISA negativ clones in the greenhouse demanded improvements in sensitivity of viral diagnosis.

GLRV III infested vines of the cultivar Roesler (new bred cultivar, *Vitis vinifera*) were sanitated by heat treatment linked with meristem preparation. Regenerated plants showed symptoms of viral infection first reappearing after two years of cultivation. ELISA tests at the end of the second vegetation period confirmed a weak infection (double value of healthy control plant) with GLRaV III, which had been the reason for heat treatment procedure. By directly comparing the sensitivity of ELISA and IC-PCR, results showed that ELISA does not detect mild infection in juvenile (almost 2 years after acclimatization) material therefore IC-PCR indicated the presence of GLRV III in young plantlets leaving the acclimatization box free of any symptom. Therefore elimination of escaped grapevine plants could take place immediately after regeneration.

Due to the opportunity of using IC-PCR for diagnosis we performed some field-testing. Grapevine with visual decline and symptoms could be detected as virus infested by ELISA as well as by IC-PCR. Plants with weak symptoms (latent incidence) and generally rootstocks could not sufficiently be diagnosed by ELISA test. As IC-PCR is a highly sensitive method it is specifically appropriate for diagnosis of tissue culture plants and juvenile regenerants, for rootstocks also in the fields, for young tissue by testing Clostero- and Trichovirus and for detecting Nepoviruses in old tissue or during hot summer time.

Since our demands for diagnosis could not be fullfilled by ELISA we established IC-PCR diagnosis for GFLV, ArMV, GCMV (Brandt et al.) and GLRaV III (Habili et al.) as well as for GVA (Minafra and Hadidi). Furthermore we developed an IC-PCR diagnosis test for Strawberry Latent Ringspot Virus (SLRV).

Sample preparation and extraction is performed in the same way as for ELISA. Coating IgGs for capturing the antigene is done in PCR tubes instead of microtiter plates. The crucial step of the procedure is the transcription of RNA to DNA by the Reverse Transcriptase. Finally the amplification of a specific DNA related to the viral sequence records the infestation with the virus.

Controlling the reliability of the method we sequenced our amplification products and found some differences of the SLRV grapevine-isolate. Our method is suitable to detect SLRV of isolates from grapevine, strawberry, other small fruits and chestnut. Despite the heterogeneous sequence of SLRV and their lacking homology to Nepoviruses all involved isolates of SLRV could be detected. Therefore this procedure is a detection method for broad host range. Amplified DNA of GLRV III showed a high degree of homology to prior sequenced isolates. Currently we are working on improving our test system for GLRaV I.

As far as available success in virus elimination can be confirmed by IC-PCR. The advantages of IC-PCR are the improved sensitivity and the possible use for all tissues during the whole season. Disadvantages are the requirements of high technical skills and the lacking automatization for a large number of samples.

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THE EUROPEAN COLLECTION OF GRAPEVINE VIRUS DISEASES

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In the framework of an European scientific network for the harmonisation of the sanitary certification schemes in the wine-growing countries of the European Union a reference collection of grapevine viruses and virus-like diseases is being established at Colmar. The aim of this collection is to dispose of reference source material for the diagnosis of grapevine viroses which can be provided to services/institutions in charge of sanitary selection, certification and/or quarantine in the EU countries. It will also constitute a good panel of virus isolates for the study of biological, serological and genetic variability using the current investigation tools (grapevine indicator varieties, herbaceous hosts, vectors, poly- and monoclonal antibodies, PCR primers) for fundamental as well as applied - i.e. routine diagnosis - purposes. The collection is going to be progressively opened to other European and Mediterranean wine-growing countries to have an enlarged view of the variability, if any existing, among the selected isolates of a given virus.

In practice we have started in 1994 to compile grapevines infected by various isolates of the known grapevine viruses or virus-like agents coming from each participating EU country as from other countries since 1996. The grapevines are grown on rockwool, own-rooted and grafted on the corresponding disease indicator variety, in concrete containers located in an insect-proof greenhouse. Their sanitary status is currently assessed by indexing and ELISA/PCR for the major diseases and viruses, first to check the presence of the virus or disease for which the grapevine accession is given as a positive control, but also to know (or verify, if the information has been provided) the other viruses/virus-like agents eventually co-infecting each grapevine accession. A descriptive data base, the frame of which will be presented, is being created for the storage and the updating of all the informations provided and acquired for every grapevine accession.

Table 1: List of the viruses/diseases included in the collection and origin of the donor laboratories (spring 1997)

Origin	nepo-viruses	GFkV	GLRaV	GVA	GVB	leafroll	RSF	LNSG	Enations	vein necrosis	vein mosaic
Italy											
North	x	x	x	x			x	x		x	x
South	x	x	x	x	x		x	x	x	x	
Sardinia		x	x	x	x		x	x	x	x	
France											
North	x	x	x	x	x		x			x	x
South	x	x	x		x		x			x	
Germany	x	x									
Spain		x	x		x						
Portugal		x	x		x						
Greece	x					x	x				
Hungary	x										
Turkey	x					x					

Nepoviruses: GFLV, ArMV, GCMV, RRV, SLRV, TBRV
GLRaVs: GLRaV-1, -2, -3, -4, -5

PHOTOSYNTHESIS IN GRAPEVINES INFECTED WITH LEAFROLL VIRUS (GLRaV-3)

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Leafroll is prevalent in the vineyards of N.W. of Spain. The commonest of the leafroll associated viruses in this region GLRaV-3, is present in about 40% of vines (1). Leafroll can affect the longevity of vines, their yield, and the ripening of their grapes, but the severity of these effects depends on the environmental conditions, the rootstock and graft varieties, and other factors (6), and their economic importance also depends on the use to which the grapes will be put. Caló (2) and Walter (5) reviewed the effects of viruses on numerous aspects of viticulture including metabolic and nutritional parameters. In this work we investigated the relationship between the presence of GLRaV-3 and/or leafroll symptoms and certain physiological parameters (including net photosynthesis) during the development of the grape.

At roughly 3-week intervals between mid-July and late August, an ADC LCA-3 Infrared Gas Analyser (IRGA) equipped with a PLC-2 chamber was used to measure the transpiration rate (E, in mmol/m^2), stomatic conductance (GS, in mol/m^2.s^1) and net photosynthesis (P, in μmol/m^2.s) of well-exposed leaves arising from the third and/or fourth nodes of three randomly chosen healthy vines and three randomly chosen GLRaV-3-infected vines in each of two vineyards (Beluso and Meaño); measurements were made on four leaves on each healthy vine and on four symptom-free and four diseased leaves on each infected vine, and GLRaV-3 status was determined by DAS-ELISA. In addition, on five days in July and August, measurements were made several times between dawn and dusk on four marked leaves of each of three GLRaV-3-positive and three GLRaV-3-negative potted vines; the leaves from the infected plants were symptomless.

There were no significant differences between healthy and infected potted plants as regards any of the three measured variables, which all exhibited great variability (with apparent contradictions between the results obtained in July and August and diurnal patterns that were independents of GLRaV-3 status. The field measurements of net photosynthesis all showed significant differences between leaves on healthy plants and diseased leaves (which exhibited rolling, stiffening and yellowing); on two occasions there were also significant differences in transpiration rate, and on one a significant difference in stomatic conductance (Tables 1 and 2). Net photosynthesis in symptom-free leaves of infected plants was less than in uninfected plants, but the difference was only statistically significant on one occasion.

Table 1. Transpiration rate (E, in mmol/m^2), stomatic conductance (GS, in mol/m^2.s^1) and net photosynthesis (P, in μmol/m^2 in leaves with (HCS) or without (HSS) symptoms of leafroll on grapevines with (+) or without (-) GLRaV-3, at the Beluso vineyard. Values are means of measurements on 4 leaves on each of three plants

		GLRaV-3(-)	GLRaV-3(+)		
	Date	HSS	HSS	HCS	F
E	14/7/93	3.292 a	3.925 b	3.783 b	10.9 ***
	7/8/93	2.833 a	2.75 a	2.233 b	10.2 ***
	24/8/93	1.233	1.317	1.308	0.25 ns
GS	14/7/93	0.44	0.607	0.448	2.2 ns
	7/8/93	0.22 a	0.222 a	0.108 b	11.2 ***
	24/8/93	0.09	0.161	0.101	1.4 ns
P	14/7/93	11.32 a	9.533 a	3.95 b	24.4 ***
	7/8/93	8.567 a	7.767 a	3.95 b	21.9 ***
	24/8/93	5.783 a	5.575 a	4.267 b	5.4 **

ns, not significant; **, ***, significant at the $p < 0.01$ and $p < 0.001$ levels respectively. Values associated with different letters in the same row are significantly different at the $p < 0.05$ level

Table 2. Transpiration rate (E, in mmol/m^2), stomatic conductance (GS, in mol/m^2.s^1) and net photosynthesis (P, in μmol/m^2 in leaves with (HCS) or without (HSS) symptoms of leafroll on grapevines with (+) or without (-) GLRaV-3, at the Beluso vineyard. Values are means of measurements on 4 leaves on each of three plants.

	Date	GLRaV-3(-) HSS	GLRaV-3(+) HSS	HCS	F
E	14/7/93	4.73	4.67	4.65	0.29 ns
	8/8/93	2.45	2.22	2.02	2.3 ns
	30/8/93	2.03	2.22	1.85	1.6 ns
GS	14/7/93	0.59	0.65	0.59	0.44 ns
	8/8/93	0.16	0.16	0.09	2.6 ns
	30/8/93	0.07	0.07	0.05	2.5 ns
P	14/7/93	8.29 a	7.64 a	4.96 b	5.3 **
	8/8/93	6.11 a	4.72 b	2.89 b	12.8 ***
	30/8/93	4.18 a	4 a	2.11 b	9.2 ***

ns,not significant; **, ***, significant at the $p < 0.01$ and $p < 0.001$ levels respectively. Values associated with different letters in the same row are significantly different at the $p < 0.05$ level

These findings may be interpreted as showing that one of the factors responsible for the difference in performance between healthy and GLRaV-3-infected vines may be reduced photosynthesis by leaves with symptoms of leafroll. This suggests that the deleterious consequences of infection will be greater in varieties or conditions in which the symptoms of leafroll develop early. It will be of interest to investigate further the progressive decline of photosynthetic capacity as symptoms develop.

In both the vineyards studied in this work, the white variety Albariño was trained on a horizontal trellis, a system that appears to minimise the adverse effects of infection by GLRaV-3 (1). Since the symptoms of leafroll in this variety, though unmistakable, are relatively mild, appear late in the season and chiefly affect old leaves it seems plausible that the beneficial effect of the horizontal training system may be due to its keeping old leaves in the shade, where the radiation they receive is less than the 400 μmol/m^2 s required for normal photosynthesis (4). It is possible that differences in training practices may be partly other reported differences between different regions as regards the severity of the effects of leafroll in the same varieties.

Finally, we point out that although the measurement of photosynthetic activity by means of IRGAs has hitherto been used largely to compare training systems and evaluate the effects of water stress (3,4), it is coming to be applied in most areas of grapevine research, and may prove to be of great value for determination of the effects of viral disease.

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PHOTOSYNTHESIS AND GRAPE COMPOSITION OF A *VITIS VINIFERA* CLONE AFTER VIRUS SANITATION

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Grapevine leafroll (GLR) and rugose wood (RW) are harmful and widespread viral diseases. At the present GLRaV-1 and GLRaV-3 (grapevine leafroll associated closterovirus 1 and 3) are considered the main pathogenic agents associated to GLR whereas GVA (grapevine trichovirus A) is reported to be associated with Kober stem grooving, one of the rugose wood complex syndromes. Delayed maturity, reduced vegetative growth and reduced berry pigmentation are long known as GLR and RW detrimental effects in grapevine, however, only recently these effects have been specifically examined by studying the behaviour of the same genotype (i.e. clone) with or without the presence of specific viruses. Recent studies (1), carried out on the same genotype, showed that the phenolic compounds are deeply involved in the vine-virus relationships.

The purpose of this paper is to discuss the influence exerted by GLRaV-3 and GVA on leaf and berry phenolic composition and on some agronomical and physiological aptitudes of the same grapevine genotype.

The experiment was carried out in a vineyard established in NorthwEst Italy in 1988. Infected (MP) and heat-treated (HT) healthy progenies (30 vines each treatment) of Nebbiolo clone 415 were grafted on healthy Kober 5BB rootstocks. All the vines were repeatedly tested by ELISA for GFLV (grapevine fanleaf virus), GFkV (grapevine fleck virus), GVA, GLRaV-1 and GLRaV-3 using commercial coating and conjugate antibody preparations. The virological status of infected and heat-treated vines is shown in table 1.

The main agronomical parameters and juice composition were evaluated over the period 1992-95. Leaf photosynthetic rate was measured by means of ADC LCA-3 gas analyser at four different dates during the vegetative season of 1994. Evaluations of phenolic compounds were carried out the same year on both leaves and berries (2).

The association of GLRaV-3 and GVA in the vines had no effect on the yield but induced a reduction in vegetative vigour, juice soluble solids and acidity (table 2).

Photosynthetic measurements (fig. 1) already showed a reduction in leaf photosynthetic rate in the MP compared to the HT vines at the time of fruit set and then before the appearance of leaf reddening and rolling, typical GLR symptoms. The efficiency of MP vine leaves decreased dramatically over the season with the appearance of the symptoms.

Leaf blade anthocyanins composition in MP and HT vines did not show any qualitative differences whereas quantitative differences were evident (fig. 2). In MP vines anthocyanins were detectable early in the season (before symptoms) and were very high later, whereas in HT vines their quantity was still very low at harvest. The higher amount of catechins, not coloured phenols, found in the MP leaves might also be a plant reaction to viral infection (fig. 3).

Among anthocyanins, peonidin 3-glucoside dominated other anthocyanins and its relative importance increased as the season progressed. The anthocyanin accumulation rate in berry skin was opposite to the one in leaves and in HT vines it was constantly higher than in MP vines (fig. 4).

Heat-treatment generated healthy progeny with wider and greener canopy able to achieve a better photosynthetic activity which resulted in better grape maturity. Viral symptoms are the consequence and not the cause of the reduction of leaf efficiency. Leaf and berry phenolic contents, anthocyanins and catechins in particular, are deeply modified by the virus infection.

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Table 1. Results of the ELISA test on Nebbiolo clone 415 vines before (PM) and after (HT) heat-treatment.

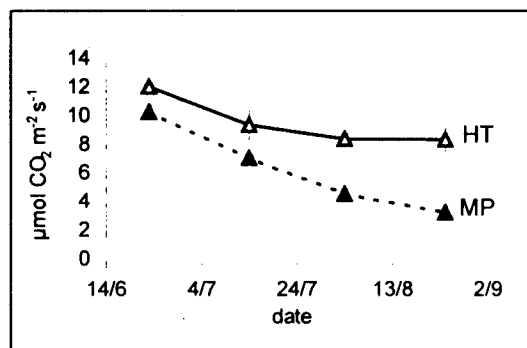| CLONE	GLRaV-1	GLRaV-3	GVA	GFLV	GFkV
MP	-	+	+	-	-
HT	-	-	-	-	-

(averages 1992-95; ns = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001).

DATA	MP	HT	Signif.
Pruning weight (g/vine)	905	1058	***
Yield (kg/vine)	1.67	1.52	ns
Average cluster weight (g)	199	212	**
Berry weight (g)	2.10	2.10	ns
Soluble solids (°Brix)	20.8	21.3	**
Titratable acidity (g/l)	9.19	8.82	**
pH	3.00	3.01	**
Tartaric acid (g/l)	6.91	7.08	ns
Malic acid (g/l)	3.55	3.30	**
K+ content (%)	1.36	1.35	ns

Fig.1. Net photosynthesis trend in the leaves of virus-infected (MP) and heat-treated (HT) vines of Nebbiolo clone 415 (means ± standard error).
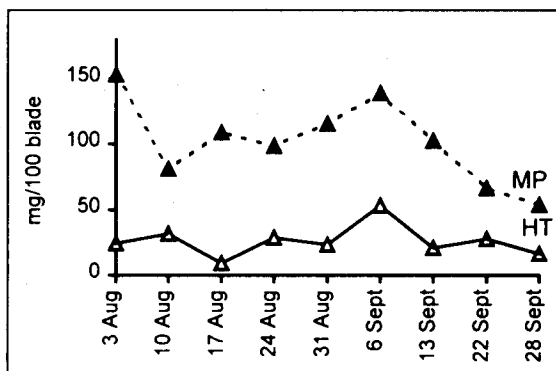
Fig. 3. Accumulation trend of blade catechins of virus-infected (MP) and heat-treated (HT) vines of Nebbiolo clone 415.

Fig. 2. Accumulation trend of total anthocyanidin index in the blades of Nebbiolo clone 415 virus-infected (MP) and heat-treated (HT) vines.

Fig. 4. Accumulation trend of the total anthocyanin index (- - - - MP; ——— HT) and of the main anthocyanidin (peonidin 3-glucoside = Pn) content in the berry skin of Nebbiolo clone 415 virus-infected (MP) and heat-treated (HT) vines.

Table 2. Agronomical and enological parameters of original GLRaV-3 and GVA virus-infected (MP) and heat-treated (HT) vines of Nebbiolo clone 415

SANITARY CHECK-UP OF GRAPEVINE MOTHER PLANTS IN FRANCE

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Implementing national and European phytosanitary regulations, ONIVINS has in charge, since 1968, the control of the multiplication of planting material from clonal selection.

Until 1989, the technical controls were based only on visual observations. From 1990, it was decided to check by ELISA for the presence of ArMV and GFLV in the whole of the pre-multiplication (basis material) and multiplication (mother vines). Some of these plots were planted for longer than 30 years.

The sanitary check-up is being performed in a two-step procedure. In a first time, the 100 ha of basis material were systematically analysed, plant after plant, grouping leaf samples from 20 plants per test. The results of tests done from 1990 to 1992 -summarised at the ICVG Meeting in Montreux, 1993 - revealed positive samples and led to relegation or uprooting of the corresponding plots.

In order to quicken the process, ONIVINS appealed to six approved laboratories which met a series of pre-established constraints relative to qualification of staff, minimal equipment and mainly application of harmonised procedure for the analyses. In this way, the successive steps of the protocol have been detailed, the extraction method of the antigens (grinding) and the method for interpreting the OD readings (threshold calculated from the average of negative controls, using a software). The six laboratories are using the same batch of regents simultaneously detecting ArMV and GFLV; positive samples are analysed again with reagents specifically detecting ArMV or GFLV. Blind inter-lab controls and ring tests are periodically done.

For the control of the mother vines, the sampling strategy is lighter than the plant after plant sampling of basis material : a sampling table is used which takes into consideration the surface of the plot and the planting density. In order to lengthen the period during which samples can be taken in the fields and to provide best conditions for sending and keeping them, an important part of the analyses are done on woody canes from November to February. Cane samples are grouped by 12; leaf samples, harvested in May and June, are grouped by 20. Samples are labelled with a code and blind tested.

* The results are reported in the following table :

Season	spring 1990	spring 1991	spring 1992	spring 1993	spring 1994	spring 1995	winter 95/16	spring 1996	winter 96/97	TOTAL
Samples	leaves	leaves	leaves	leaves	leaves	leaves	woody canes	leaves	woody canes	
N° of tests	1556	7104	7424	4248	7273	6661	26800	27000	22200	110226
Surface (ha) of tested :										
- rootstock basis material	-	28,6	7,9	0,6	3,3	-	-	-	-	40,4
- vinifera basis material	8,8	21,9	23,6	2,1	1,6	-	-	-	-	58
- rootstock mother vines	-	-	9,2	92,2	138	88	37	434	307	1005,6
- vinifera mother vines	8,7	12,8	20,6	30,5	109,8	184	563	462	173	1564,66
TOTAL	17,5	63,2	61,4	125,4	252,5	272	600	896	480	2768,66

Taking into account the grouping of the samples. It is difficult to evaluate the rate of contamination. In addition, after detecting positive samples in a plot, additional sampling is done in the same plot in order to specify extend and position of the contaminated parts in the plot. In this case, more samples are taken per surface unit and positive results are more numerous because the samples come from suspicious areas. All these considerations make it difficult to have a statistical interpretation of the results.

In other respects, the total results at the national level cannot reflect geographical disparity. Though the check-up is not yet complete, it can be noted that comparatively the level of contamination is low in some regions whereas, in other regions, it reaches a level which is not negligible from an economic point of view. A detailed examination of the result reveals that in some plots the contamination is undeniable because of the high number of positive samples ; in other plots, the low number of positives (1 to 3) makes it difficult to understand the way of contamination.

In any case, the sanitary check-up is continuing and the final assessment will be drawn at the end of the process in 1998. It can already be underlined that the use of ELISA for large scale analysis is reliable provided that some precautions are taken (sampling, preparation of extracts, reading of results). The results obtained until now show that more than 90 % of the propagation plant material so far tested is not infected by ArMV and GFLV. However, one has to note that despite the regulations applied for planting mother vines, viral contamination has occurred year after year.

The main factors which can explain these results are :
- the plant species grown in the field before planting mother-vines and the duration of the fallow ;

- environmental conditions such as the presence of grapevines or other virus/vector hosts in the vicinity; isolation of the plots, topography, running of water, inundation ;
- human factors such as substitution of plants, deposit of soil, error in handling.

These considerations prompt to remain vigilant in the future and to reinforce the regulations ; better define the criterions for the choice of the fields, define the period of the first ELISA control after planting and the periodicity of the test.

Whatever, this improvement of the sanitary status of propagating plant material will allow to distribute certified plants with an increased guarantee for the viticulturists.

METHODS FOR THE EVALUATION OF VIRUS EFFECTS ON GRAPEVINES

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Knowledge of virus effects on grapevines is a very important matter when we are engaged on taking decisions about the opportunity and rigor of sanitary selection of varieties. But, at present, knowledge on this subject remains quite insufficient (2, 4), probably due to weakness of methods currently used to evaluate the effects of the virus on the plant. The available results are frequently very heterogeneous, meaning also that they are probably obtained under unique genetic and environmental conditions, so they could not apply to the broad range of different situations that we can find in European viticulture.

Meanwhile, by using a new method to evaluate the effects of leafroll on near 30 ancient Portuguese varieties, we are obtaining results that simultaneously reveal a satisfactory stability and quite unexpected small yield losses due to virus infection. So, our main objective is to discuss weaknesses of current methods and to present a new one that can bypass the difficulties above referred

Data used for the analysis refers to leafroll associated virus (GLRaV-3) effect on four years mean yield (1991,92,94,95) observed in an experimental population of clones of Portuguese cv. Camarate. This population was grafted in 1990, under an experimental design of 4 replicates (blocs) X 4 plants. Total number of clones was 234, being 106 infected with GLRaV-3 and 128 virus free. Broad sense heritability of mean yield was 0.661.

Three classes of methods are currently used to evaluate virus effects on vine plants:
- comparison of a clone naturally infected (or a small number of clones) with a different clone (or a small number) free of the virus.
- comparison between a clone naturally infected and the same clone after virus elimination treatment (e.g. thermotherapy)
- comparison between a virus free clone and the same clone after artificial inoculation with the virus concerned.

All these methods suffer from serious weaknesses. First method is influenced by genetic variation of clones. Virus free and virus infected clones that are taken for the comparison may differ greatly in their genetic determinism, so this difference can entirely mask the virus effect. This risk is supported by data from our selection work as illustrated in figure 1. When we compare an isolated clone extracted at random from the sub-population of 106 infected clones with other extracted from the sub-population of 128 virus free the difference is fairly high, but it doesn't corresponds to the true difference represented by the dashed lines. This is also indicated by the variance of differences between 40 pairs extracted, meaning that this difference is highly unstable, depending of which clones are randomly extracted each time. This result indicates that such a difference can apply strictly to the pair of clones concerned but don't allow generalisation to the whole variety. Perhaps such a generalisation will be only acceptable in a few number of situations, when the variety and the virus population are genetically homogeneous, under these conditions small samples of clones became more representative. However these situations must be rare. High levels of genetic variability is strongly confirmed by our selection work (1). Moreover, we must expect also for the existence of genetic variability of the virus and for interaction plant X virus.

The second method above referred is weakened by impossibility of virus elimination process to be directed to a particular virus, so the possible results to be obtained can't be imputed to that virus.
Moreover the problem of genetic variability of the plant and of the virus remains present and doesn't allow generalisation.

The third method faces to the difficulty of isolation and of mechanical transmission of virus. Transmission is currently done by grafting, unfortunately this method allows the transmission of other pathological agents that can carry other undesirable influences and interactions.

View these difficulties, which methods can we use for the evaluation of virus effects on plants?

The methodology we use in Portugal for vine selection is based on great experimental populations frequently containing many clones infected and many clones free of a particular virus. Under these conditions it becomes possible to compare the mean performance of clones infected and virus free in a way like that of the former method above referred, with the difference resulting from the use of great samples of clones (hence, more representatives) in place of one or a small number. Such samples tends to be representatives (that is, equals) concerning all factors not under control and to show a difference that we can impute to the unique factor under control: the presence-absence of the virus. As we can see in fig. 1, mean yield values for infected and virus free clones tend to a good stability when size of samples reaches 20 clones. The same can be seen throughout variance of differences between infected and virus free clones that narrow when samples grow from 1 till near 20 and stabilises above this number.

We have utilised this methodology for the evaluation of leafroll associated virus 3 effect on yield of 27 Portuguese varieties. Results (not presented) regarding yield losses due to the virus range from -17% to

+14% with a mean of about -5% (2, 3). These values for the range and mean, are moderate when compared to others commonly found in bibliography. The range is yet slightly large but this may result from differences in susceptibility of varieties to the virus or from environmental influences (mainly distinct temperatures in experimental fields situated near the Littoral or in continental regions).

Coherence of these results plays in favour of high strength of this method when compared to others that have been used for the same objectives in the near past.
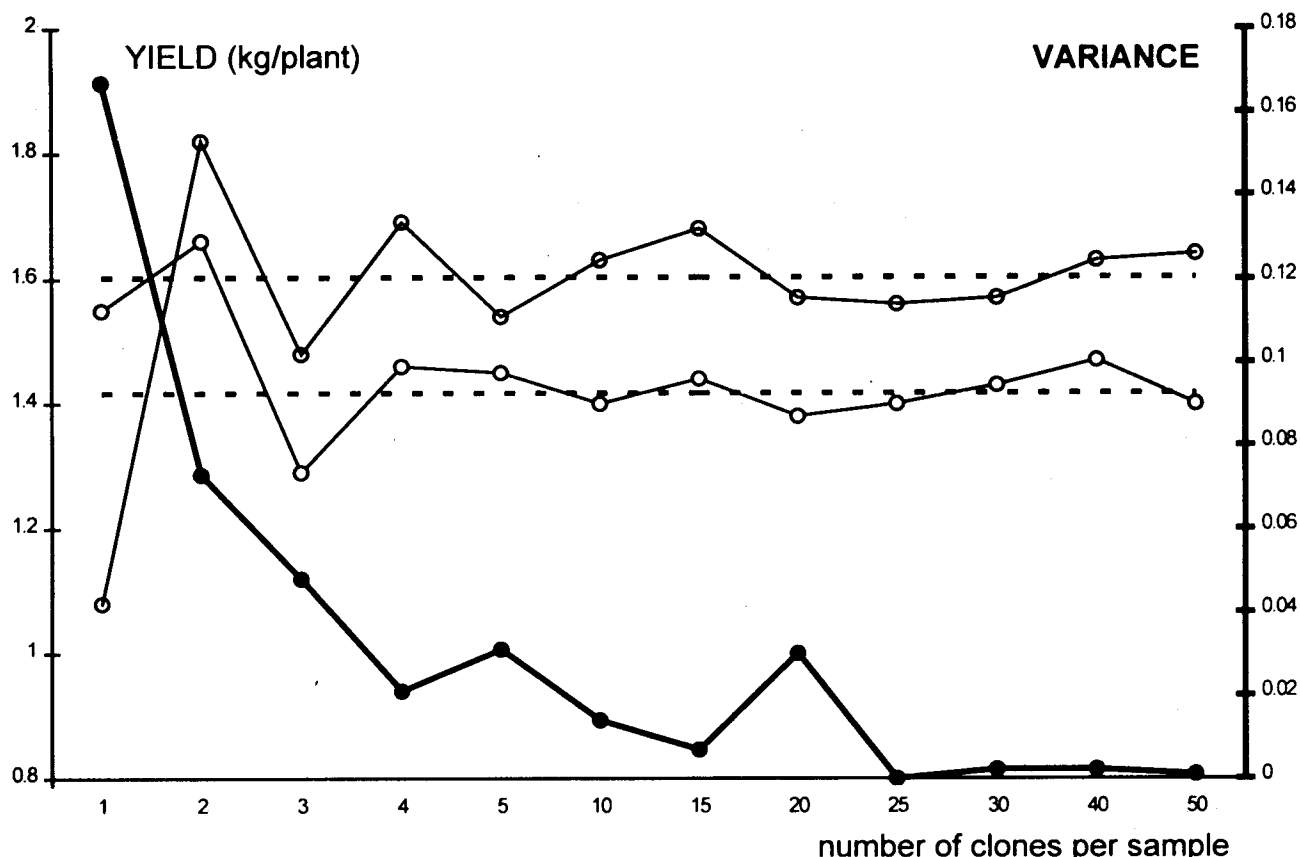
Figure 1 - Mean yield of samples composed from 1 to 50 clones, extracted at random from sub-populations of 106 infected and 128 virus free of cv. Camarate (left scale, light lines). And variance of 40 differences between sample means for each sample size (right scale. dark line). Dashed lines represent the mean of 106 clones infected and of 128 leafroll free.

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CONSIDERATIONS ON GRAPEVINE SELECTION AND CERTIFICATION

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The control of infectious diseases of the grapevine relies very little on natural resistance, for the majority of cultivated varieties are susceptible to varying extents to most of the virus-induced disorders. Protection induced by cross-protection or gene transfer is still under investigation, and the control of vectors (nematodes, mealybugs, leafhoppers) by chemical treatments is under progressive restraint for environment safety reasons. Thus, preventive measures such as sanitary selection remain the most effective way at our disposal for limiting the detrimental impact of virus infections. Novel acquisitions on the aetiology and epidemiology of major diseases and the development of new advanced detection techniques, have a bearing on the improvement of sanitary selection and its efficient application to the expanding world-wide exchange of propagating material.

To this effect, the following questions need to be addressed :
- what is the impact of single specific viruses (*e.g.* any given GLRaV *vs* the leafroll complex as a whole) ?
- which viruses and virus diseases are to be considered in a certification scheme ?
- which techniques are best suited for sensitive detection of viruses for sanitary selection and certification purposes ?
- when is the most appropriate time for virus testing ?
- can sanitary selection lead to a genetic erosion of the cultivars ?
- what is the bearing of the sanitary status on clonal variability ?

Recently, the Group of Experts "Sélection" of the OIV discussed the "clonal selection of the grapevine : balance between genetic and sanitary selection", underlying the "lack of precise evaluations of the impact of given viruses on the crop".

The nature and severity of the effects of virus vary in function of :
- virus species
- virulence of isolates of a given virus species
- *Vitis* species, variety, (clone ?)
- age of the infected vine
- interaction with other viruses or pathogens
- interaction with agro-climatic conditions
- spread by vectors

Plenty of evidence indicates that fanleaf, leafroll, rugose wood, fleck and other virus diseases can be very detrimental to grapevines and their products (1). There are cases, however, of milder effects induced by hypovirulent virus strains (2).

In the past, the effects on the crop were estimated by comparing the performance of symptomatic *vs* symptomless vines or, at the most, using clones indexed for the presence of a given disease. We now know that some diseases (*e.g.* leafroll and rugose wood) are complex. However, because the evaluation of the effects of single components (*e.g.* Rupestris stem pitting *vs* Kober stem grooving or corky bark) or of single viruses (*e.g.* GLRaVs) of these complexes is possible, experiments can be designed for answering the recurrent questions raised by viticulturists and breeders : are all the components of a complex equally harmful ?

The OIV Group of Experts "Sélection" will elaborate an internationally accepted protocol for evaluating the impact of virus infections.

The outcome of surveys of viruses and virus diseases made the world over, have unanimously shown that they are widely spread. Cases have been found of varieties totally infected. In these instances valuable clones can be recovered by eliminating infectious agents by heat therapy or *in vitro* meristem tip culture, or a combination of the two. In general, there is enough genetic variability in grape populations, even if heavily infected, that a genotypic or phenotypic erosion caused by sanitary selection can hardly be claimed. On the other hand, pomological selection is much more restrictive. Schöffling and Deroo (3) report that in Germany only 0.05% of the candidate clones that undergo selection for agronomic and qualitative characters are registered and propagated. Thus, the conservative breeders engaged in clonal selection must make a point of preserving the widest possible genotypic/phenotypic diversity.

Real differences between clones of the same cultivar are not always obvious. Sometimes, the impression is that the difference is more in the label that identifies the clone than in anything else. By converse, for interspecific roostocks it is generally accepted that the major, if not the only difference between two clones may lie in the relative sanitary status. Thus, the role of virus infections in the clonal variability of some *Vitis vinifera* varieties needs to be studied in more detail.

An important issue is the time at which, during the clonal selection process, it is best to proceed with sanitary evaluations (4). In our mind, vines that underwent field selection, prior to planting in performance plots for clonal evaluation, should be hot water-treated, to eliminate phytoplasmas, and must be checked for disease freedom with the following tests (5) :

- indexing on *P.n.*, *C.f.*, or other red berry varieties for LR.
- indexing on *V. rupestris* (fanleaf, *Rupestris* stem pitting, fleck)
- indexing on Kober 5BB (Kober stem grooving, graft incompatibility)
- indexing on LN33 (corky bark, LN33 stem grooving)
- ELISA for nepoviruses present in the surveyed region
- ELISA for mealybug-transmitted closteroviruses
- ELISA for mealybug-transmitted trichoviruses.

Foundation blocks for the production of basic and certified material must be kept under continuous surveillance, especially for contamination by vector-transmitted viruses and phytoplasmas. For large-scale surveys, sensitive techniques such as ELISA and PCR must be made increasingly reliable and user's friendly.

Virus infections often result in lower yield and quality (i.e. sugar content and other traits) of the crop (1). Even when the outcome of certain experiments can lead to the conclusion that virus infections induce an increase of sugar content, a better evaluation of the experimental data may show that the conclusion reached is incorrect. For example, Balthazard (6) demonstrated that the decrease in sugar content registered in a cv. Savagnin clone freed from leafroll by heat treatment was only due to the difference in the yield between infected and sanitised plants. When regression coefficients were calculated using the same data, the alleged difference in sugar content was no longer significant.

In conclusion, there is a compelling demand from professional organisations and control services to : (i) dispose of the simplest possible scheme for sanitary selection and certification ; (ii) to have ultimate information on whether all single incitants of complex disorders (*e.g.* closteroviruses with leafroll or trichoviruses and the like with rugose wood) are equally detrimental ; (iii) dispose of sensitive, reliable, easy-to-use and harmonised protocols for laboratory diagnosis. These are the issues to be addressed and the challenge for the years to come.

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IMPROVEMENTS IN THE *IN VITRO* CULTURE OF MERISTEM SHOOT TIPS FOR SANITATION AND ESTABLISHMENT OF ROOTED EXPLANTS.

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The main obstacles met during *in vitro* meristem tip culture for freeing grapevine from viruses reside in the: (i) low percentage of explant stabilisation (i.e. survival after 30-40 days of culture); (ii) reduced multiplication rate after stabilisation, especially after the first subcultures; (iii) difficulty of adaptation of certain cultivars to the *in vitro* culture.

To address these problems tests were carried out using 211 accessions from 89 different cultivars selected in three different southern Italian regions and identified as putative clones. In particular, 90 clones of 36 different cultivar were from Apulia, 42 clones of 21 cultivars from Abruzzo, and 76 clones of 5 cultivars from Calabria. Cold-stored mature canes of alla accessions were rooted in sand in a growth chamber at 28-30 °C, or in a greenhouse at 25 °C. Meristem tips 0.4-0.6 mm in size were excised from vigorously growing apical shoots 3-4 cm long that had been surface- sterilised with 10% sodium hypochloride and thoroughly washed. The explants were plated and grown at 24 °C with a 16 h photoperiod (5000 lux).

Of the different growth media tested two denoted 1A and ML, respectively, proved the best for explant stabilisation. Medium the 1A contained half strength Murashige and Skoog (MS) (1) macro-elements, full strength MS micro-elements, Z_4 vitamins (2), 50 mg/l ferrous sulphate, 55 mg/l sodium EDTA, 0.6 mg/l BAP, 0.01 mg/l NAA, 30g/l sucrose, 7 g/l agar, pH 5.7-5.8. Medium ML differed from the above because it contained Quoirin and Lepoivre (3) macro-elements instead of MS. The real discriminating difference with the respect to medium 1A was the lack of calcium chloride.

For optimising multiplication rate after stabilisation besides the ML medium, a third medium, denoted M, was found suitable. Medium M had the same composition as 1A but contained full strength MS macro-elements.

The cumulative results of explant growth and stabilisation on medium 1A (Tab.1) clearly indicated that the explants from Apulia, regardless of the cultivar, had no difficulty in becoming established within 30-40 days. An the exception was cv. Corniola, a table grape variety, whose explants, prior to surface sterilisation had to be dipped in a 1g/l citric-ascorbic acid solution. Some of the explants of cultivars from Calabria (3 of 5) and most of those from Abruzzo (12 of 21) did not adapt well to *in vitro* culture and did not survive past the first 2-4 weeks. These recalcitrant cultivars, however, grew satisfactorily in the ML medium as shown in Tab. 2.

Table 1. Establishment and survival (stabilisation) in medium 1A of explants from different southern Italian grape cultivars

Geographical origin	Cultivars/clones in culture	Cultivars/clones stabilised	Stabilisation time (days)
Apulia	36/90	36/90	30-40
Calabria	5/76	2/27	30
Abruzzo	21/42	9/19	40

Table. 2 Establishment and survival of explants from recalcitrant cultivars from Calabria and Abruzzo in ML medium

Geographical origin	Cultivars/clones in culture	Cultivars/clones stabilised	Stabilisation time (days)
Calabria	3/49	2/45	30
Abruzzo	12/23	9/12	40

The adaptation/multiplication phase, characterised by multiple (3 to 4) subcultures every 15-21 days, was not always successfully accomplished. A certain number of cultivars which had grown through the stabilisation phase without no apparent problems, did not adapt to subculturing, their explants turned brown and died. This problem was largely overcome by the use of different media as shown in Tab. 3.

Table 3. Adaptation of grape explants to *in vitro* culture past the stabilisation phase

Geographical origin	Stabilised cvs/adapted cvs	Multiplication time (days)	Medium
Apulia	36/29	60-90	M
Calabria	2/2	90	ML
Abruzzo	9/8	60-120	M/ML

The final steps of the *in vitro* culture process are rooting of explants and transplanting. Very satisfactory results were obtained by transplanting vigorously growing non rooted explants at the end of the

multiplication phase directly into pre-compressed peat disks (Jiffy pots) soaked in 1.5% IBA solution, then potting them in a greenhouse after three weeks. Less developed explants were first rooted *in vitro* in a ML medium with the addition of 30 mg/l adenine sulphate and 1.5 mg/l IBA, then transplanted directly in pots containing a soil mix.

The above described procedure besides facilitating the *in vitro* culture of recalcitrant cultivars, expedites the whole process by shortening in average by 50% the time needed to go from the meristem tip explant to the rooted greenhouse-grown plantlet.

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VIRUS ELIMINATION IN GRAPEVINE CULTIVARS OF NORTH-WESTERN ITALY THROUGH MERISTEM CULTURE AND *IN VITRO* THERMOTHERAPY

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Production of virus-free propagation material has become very important. The official protocol for clonal selection in Italy includes phytosanitary certification for at least 9 virus or virus-like diseases. Virus elimination becomes essential when no virus-free clone is available (this sometimes happens to local cultivars) or when qualitatively very good biotypes are affected by asymptomatic virus diseases.

The Centro Miglioramento genetico e Biologia Vite of the Italian Consiglio Nazionale delle Ricerche has been operating in the field of grapevine clonal selection for more than 20 years. Recently, phloemic virus eradication through meristem culture has been routinely performed (1), while *in vitro* thermotherapy, mainly for GFLV (grapevine fanleaf virus) elimination, has recently started. Most of the results obtained in these years are summarised in this paper.

A total of 13 clones out of 6 different Italian wine grapevine varieties were subjected to meristem culture. They were originally infected by closteroviruses and/or trichovirus (GLRaV type 1 and 3, GVA). Additional 2 clones of 2 different cultivars, 'Moscato bianco' clone Loazzolo IIIB14 and 'Favorita' clone 105, infected by GFLV, were treated with *in vitro* thermotherapy.

Woody cuttings of the infected mother plants were collected during the winter, cleaned and stored at 4°C. To start the cultures, the cuttings were forced to sprout at room temperature.

For meristem culture, the apical buds were collected after 2 to 3 weeks and the surface was sterilised in a 30% commercial bleach solution. Meristems (about 0.5 mm) were excised and cultured on a Murashige and Skoog (2) medium modified reducing the mineral salts to half the strength and sucrose to 20 $g \cdot l^{-1}$. BAP (Benzyl Amino Purine, 9 µM) was added to the medium; pH was adjusted to 5.6 before autoclaving. Cultures were grown at 23°C with a 16 h photoperiod and 50 $\mu mol \cdot s^{-1} \cdot m^{2}$ light intensity.

For *in vitro* thermotherapy, the shoots sprouting from the cuttings were collected after about 6 weeks. After surface sterilisation, the axillary buds were excised and cultured on a growth regulator-free medium. After 2 weeks, the growing buds were transferred to a growth chamber at 34°C. After a 9 to 11 week heat treatment, the axillary and apical buds from each green shoot were excised and cultured on a medium containing 9 µM BAP (3).

Shoots 1-2 cm long obtained by meristem culture or by thermotherapy were transferred to a rooting medium containing 2.5 µM Naphthalene Acetic Acid and 2.5 µM Indol Butyric Acid for 5 days, and then to a growth regulator-free medium. After rooting, plants were micropropagated. One or more subclones were obtained from each infected mother plant. During micropropagation, samples were taken repeatedly and tested for virus presence by ELISA (4).

CULTIVAR	CLONE label	Sanitary status ([1])	No. of meristem-originated subclones	% totally ELISA-negative
'Albarola'	3	GVA, GLRaV-1 and 3	3	66.7
	17	GLR, RW ([2])	1	100
'Bosco'	3	GVA, GLRaV-3	8	100
	18	GLR, RW ([2])	1	100
	101	GVA, GLRaV-3	12	91.7
	103	GLR, RW ([2])	3	100
	131	GLR, RW([2])	1	100
'Rossese'	37	GVA, GLRaV-1	23	82.6
'Pigato'	121	GVA, GLRaV-1 and 3	4	100
'Vermentino'	78	GVA, GLRaV-1	3	100
	111	GVA, GLRaV-3	1	100
'Grignolino'	79	GVA	4	75
	87	GVA, GLRaV-1	24	54.2

Table 1. Effect of meristem culture on virus eradication from grapevine cultivars from north-western Italy. ([1]) GVA = grapevine virus A; GLRaV-1 and -3 = grapevine leafroll associated closterovirus type 1 and 3; GLR = grapevine leafroll symptoms; RW = rugose wood symptoms. ([2]) Mother plants not checked by ELISA.

On the whole, ELISA tests were negative for GVA, GLRaV-1 and 3 in 79.3% of the growing explants from meristem culture. The percentage of virus-free plants varied according to the cultivar and the original sanitary status (table 1). It has been previously shown that cultivar and virus type can influence the virus elimination rate (5). Different cultivar response could be related to the difficulty in isolating the

meristem and consequently to the meristem wholeness. Besides, in our case we cannot exclude the fact that the differences are due to different skills and experience of the operators: 4 people performed the meristem excision, each one dealing with different cultivars.

GVA and GLRaV-1 seem more difficult to be eradicated compared to GLRaV-3 (table 2), although the rates of ELISA-negative subclones are quite high in all the serological tests. All the subclones were also ELISA-negative for GFkV (grapevine fleck virus) and GFLV.

Plants originated from *in vitro* thermotherapy were ELISA-negative for GFLV in both treated clones. We are planning to extend this procedure to more clones in order to get additional data. A modification in the protocol could be the reduction of the length of the heat period to 6 or 8 weeks, thus avoiding strong damages to the treated explants.

Virus	No. of sub-clones tested	% ELISA-negative
GVA	84	80.5
GLRaV-I	56	86
GLRaV-III	28	100

Table 2. Percentage of ELISA-negative subclones as a function of virus and virus-like disease.

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HEAT-THERAPY OF VIRUS-INFECTED *VITIS VINIFERA* CULTIVARS IN EMILIA ROMAGNA (NORTHERN ITALY)

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Control of grapevine graft-transmissible virus and virus-like diseases depends on the use of disease-free plant material for nursery stock production. Attempts to select healthy clones from old vine populations of *Vitis vinifera* L. cultivars carried out in the Italian region of Emilia Romagna have been partially successful. Most of the cultivars selected in a joint program, with the Dipartimento di Colture Arboree university of Bologna, were found to be virus-infected. Therefore it was necessary to adopt some therapeutic method to sanitise those clones that have good viticultural traits for commercial production.

Heat therapy provides a very useful tool to obtain virus-free plants (3). This technique has also been successfully applied for grapevine sanitising. The conventional method of rooting the terminal tips, from shoots growing on exposed vines, in a mist bed has proved to be highly ineffective (1). Thus an *in vitro* shoot tip rooting technique was adopted to improve the therapeutic efficacy (4).

Various clones of different locally grown wine grape cultivars were subjected to heat-treatment. These had been previously found to be infected by various combinations of grapevine fanleaf nepovirus (GFLV), grapevine fleck virus (GFkV), grapevine leafroll-associated closterovirus 1 and 3 (GLRaV-1 and 3) and grapevine trichovirus A (GVA).

Two-year old potted parent vines were grown in a heat chamber at $37\,^{\circ}C \pm 2\,^{\circ}C$ for 60-200 days. Shoot tips, about 0.5 cm, were periodically removed and aseptically transferred to test tubes with a suitable rooting medium. The medium contained the macronutrients, micronutrients and vitamins reported by Murashige and Skoog (2), supplemented with 3-indol butyric acid (0.3 mg/l), sucrose (2%), agar (0.5%) and active charcoal (3 mg/l). Regenerated plantlets were subsequently tested with ELISA for the viruses concerned, using commercial antiserum preparations (Agritest, Valenzano, Italy). Serological tests were repeated 2-3 times using bark scrapings of dormant canes, starting one year after transplanting to the soil.

This method of combined heat-therapy and *in vitro* rooting of the shoot tips was successful. Currently, 281 out of 427 regenerated plantlets of 26 clones from 12 different cultivars (Albana, Alionza, Ancellotta, Biancale, Canino, Cargarello, Lambrusco, Montuni, Moscato, Ribolla, Sangiovese, Trebbiano) are virus-free. Results indicated an average viral eradication efficiency of 65.8% (range 19.5%-100%). The effectiveness of this therapy is probably the result of the long heat treatment period applied in many cases but most of all to the reduced size of the tips collected from the virus-infected parent vines. This confirms the previous observations of other investigators (1, 4).

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CLONAL SELECTION OF THE MOST REPRESENTATIVE GRAPE VARIETIES IN CAMPANIA REGION: SANITARY ASPECTS,

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Campania is one of the most important region of Italy concerning the number of local grape varieties. Each year 2,5 million new root cuttings are planted, but, until now, only certified propagation material of Aglianico variety is available.

On account of the presence of 17 local D.O.C. wines, obtained from Greco, Fiano, Falanghina, Aglianico, Piedirosso and Biancolella varieties, and the constant increase of production, a joint program among the local Administration, the Institute of Fruit Tree, the Department of Food Science (both University of Naples) and the Institute of Plant Pathology of Rome started four years ago with the aim to select the most representative grape varieties.

Concerning sanitary selection, out of 3 000 remarkable samples, 660 putative clones, belonging to 27 different varieties, were analysed. Samples showed weak or absent viral symptoms.

88 samples resulted negative to the serological assays against the main grapevine viruses (GVA, GFLV, GLRaV 1,2,3, GFkV, ArMV). Virus and virus-like diseases diffusion was different according to the variety and the growing areas.

At the end of biological assays, 20 putative clones, belonging to Aglianico, Coda di Volpe, Falanghina and Piedirosso varieties, resulted virus-free and ready to be submitted to Certification procedure.

Plants of six different varieties (Forastera infected by ArMV + GLRaV3, Biancolella by GFLV + GLRaV3, Falanghina by GLRaV3, Piedirosso by GLRaV2 + GLRaV3, Fiano by GFLV and Greco by GLRaV3) were in vitro micrografted to eliminate the viral infection. After one year, all micrografted plants resulted virus-free by ELISA tests.

This experience confirms that the grapevine virus and virus-like diseases are widespread mainly in areas where sanitary selection has never been performed.

Anyway, a reliable certification scheme allowed the individuation of grapevine virus-free putative clones.

DETRIMENTAL EFFECTS OF FILAMENTOUS VIRUSES TO TABLE GRAPE VARIETIES NEWLY INTRODUCED IN SOUTHERN ITALY

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To widen the offer for an increasingly demanding market, a number of new table grape varieties have been introduced in the last few years in Apulia, the leading table grape growing region in Italy. In many areas young arbors of cvs Red Globe and King's Ruby, two of the most popular new introductions, began to show a disease condition that caused much concern to the growers. Affected vines delayed bud break by a couple of weeks and exhibited extensive bud failure. The vegetation was less vigorous than normal and the crop visibly reduced. The leaves displayed early reddening and some rolling of the blades, but the bunches were pale coloured, failing to ripen properly. Decline and death of the vines was not uncommon in cv. Red Globe.

Although the field symptoms clearly indicated that this was a case of leafroll, their severity suggested to investigate the disease in more detail to identify the viruses involved. To this aim, in autumn 1995 a vineyard each of cv. Red Globe and King's Ruby were selected 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. Grapevine fanleaf virus (GFLV) and grapevine leafroll-associated virus 1, 2, 3, and 7 were detected with polyclonal antisera but monoclonal antibodies and the relative protocols (1, 2, 3) were used for grapevine fleck virus (GFkV) and grapevine viruses A (GVA) and B (GVB). Biotinilated antibodies were used for GLRaV-1, -2, and -7. Samples giving doubtful ELISA responses were checked by immunoelectron microscopy or, limited to GLRaV-2, also by polymerase chain reaction.

As shown in Tab. 1, the presence of symptoms was associated in both varieties with a remarkable reduction of the yield (c. 22% and 24% in Red Globe and King's Ruby, respectively) and a lower sugar content of the bunches (minus 5 %). Because of the pale colouring of the berries 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 of the crop and number of clusters. With cv. Red Globe, additional damage derived from the death of vines, about 50% of which (36 of 78) were uprooted in two years.

Table 1. Yield and sugar content of symptomatic (S) and symptomless (H) vines of cv. Red Globe and King's Ruby

Cultivars		Vines (n°)	Mean yield (Kg/plant)	Bunches (n°)	Sugar cont. (%)	Dead vines (n°)
Red globe	H	51	24	30	14	0
Red globe	S	78	19	23	9	36
King's R.	H	38	29	58	15	0
King's R.	S	51	22	45	10	0

Only four viruses (GFkV, GLRaV-2, GLRaV-3, and GVA) were detected by ELISA in the whole of the 218 plants examined. Symptomatic vines of both cultivars had the highest infection rate, i.e. 85% and 8% in Red Globe and King's Ruby, versus 12% and 55% of symptomless vines. The surprisingly high infection value in symptomless King's Ruby was due to GFkV (Tab. 2). In symptomatic Red Globe vines the most common viruses were GLRaV-2 (76%), GLRaV-3 (54%), and GVA (51%), whereas in King's Ruby GLRaV-3 (55%) and GFkV (53%) prevailed. GLRaV-2 and GLRaV-3 not found in symptomless King's Ruby and Red Globe, respectively (Tab.2).

Table 2. Results of ELISA tests

Cultivars		Total vines	Infected vines (%)	GLRaV-2 (%)	GLRaV-3 (%)	GVA (%)	GFkV (%)
Red G.	H[a]	51	12	12	0	0	2
Red G.	S	78	85	76	54	51	10
King's	H	38	55	0	5	11	53
King's	S	51	78	24	55	24	53

[a]H = symptomless; S = symptomatic

The pooled data of GLRaV-2, GLRaV-3 and GVA incidence in diseases and symptomless vines (Tab. 3) suggest that: (i) closteroviruses are the major responsible for the leafroll syndrome and related

detrimental effects on the crop; (ii) GVA has a bearing in the induction of cv. Red Globe decline, as this virus was present in over 50% of the symptomatic vines and in more than 2/3 of those that died.

Table 3. Pooled data of ELISA and IEM tests for detection of closteroviruses (GLRaV-2 and GLRaV-3) and GVA

Cultivars		Total vines	Closterovirus infections (%)	GVA infections (%)
Red Globe	H[a]	51	14	0
Red Globe	S	78	100	51
King's Ruby	H	38	5	11
King's Ruby	S	51	75	24

[a]H = symptomless; S = symptomatic

The origin of such a heavy inoculum of filamentous viruses is unknown, but is likely that it came from budwood and/or roostocks rather than mealybug vectors. If so, the present case represents a further emblematic 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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EFFECT OF VIRUS INFECTION ON OWN-ROOTED CLONES OF DIFFERENT WINE GRAPES CULTIVARS FROM SARDINIA

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The detrimental effect of virus infections on the quantity and quality of grapevine crops is well established (1). However, most of the available data come from assessments made on grafted vines from commercial vineyards. In Sardinia (Southern Italy), a number of plants of different wine grapes varieties were identified in the course of a clonal and sanitary selection programme, most of which had pomological and bearing characteristics above the average and showed no apparent signs of virus diseases. The best selections were clonally propagated and five plants from each of the candidate clones were grown on their own roots in the a repository established in the late 70s.

A survey of candidate clones of cvs Cannonau (CN), Malvasia di Bosa (MLV), Pascale di Cagliari (PSC), Vermentino (VRM), and Vernaccia (VRN) was made by ELISA for the presence of grapevine leafroll-associated viruses 1, 2 and 3 (GLRaV-1, -2 and -3), grapevine virus A (GVA), and grapevine fleck virus (GFkV) using mostly commercial antisera. These tests revealed that, except for cv Malvasia di Bosa, which was totally infected, some of the selections of the other cultivars were free from all the above viruses, whereas others were not, and exhibited different types of mixed infections (Tab. 1).

The availability of own-rooted *Vitis vinifera* varieties with a different sanitary status suggested to investigate whether and to what extent infection by certain given viruses could affect the performance of the vines in absence of rootstocks.

To this effect, 25 virus free clones of four varieties (Cannonau, Pascale di Cagliari, Vermentino and Vernaccia) and 56 virus-infected clones of the same cultivars plus Malvasia di Bosa, where kept under observations for 9 years (1988-1996) determining yield, sugar content and titratable acidity. The data were worked out statistically according to Duncan's test (2).

Although a certain variation was observed between cultivar responses to infection in different years. The means of the 9-year data reported in Tab. 1 indicate quite clearly that the presence of viruses did not induce a statistically significant reduction of the yield, except with cv. Cannonau which registered an average decrease of 25%. Likewise, viruses did not seem to affect the quality of the crop (sugar content and titratable acidity) of any of the varieties.

Table 1. Performance of own-rooted clonal selections of different wine grape cultivars in presence or not viruses in different combination.

Cultivar		GVA	GLV-1	GLV-2	GLV-3	GFkV	Clones (n°)	Yield (q/Ha)	Sugar (%)	Acidity (g/l)
CN	H						8	121.50a	23.33a	7.01a
	I	+	+	-	+	+	12	95.25b	22.31a	6.98a
MLV	H						0	0	0	0
	I	-	-	+	. -	+	10	60.69a	22.90a	8.31a
	I	-	-	-	+	+	6	65.76a	22.29a	7.89a
PSC	H						4	145.38a	18.98a	6.50a
	I	+	-	-	+	+	9	141.86a	19.63a	6.83a
VRM	H						9	112.48a	21.21a	8.01a
	I	+	-	-	+	+	12	112.42a	20.96a	7.94a
VRN	H						4	44.59a	23.89a	6.75a
	I	+	+	-	+	+	7	42.80a	22.58a	6.84a

From the above, it would appear that of the five cultivars under study, only cv. Cannonau is intrinsically susceptible to virus infection which interferes with its bearing characteristics. At first sight, this may look surprising and in contrast with what reported in the literature but, in fact, is much less so if one takes into consideration that: (i) all selection were practically symptomless and retained this condition throughout the period of observation, which may be taken as an indication that the infecting viruses strains were mild. If so, it would be reasonable to expect a limited negative impact on the host; (ii) in general, self-rooted vines are more tolerant to virus infections. At least, they escape the adverse influence exerted by certain rootstocks in the presence of viruses like GVA and GLRaV-2, which are agents of disease of combination [i.e., Kober stem grooving (3) and graft incompatibility (4)] of GFkV (see also 5 and 6).

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NEVER ENDING STORY OF GRAPEVINE CLONAL SELECTION

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INTRODUCTION

Slovenia is traditional viticulture country with two distinct wine growing regions, the continental ant the littoral one. More than 70 *vinifera* cultivars are grown there, among them 28 local ones to be perspective for commercial growing or to be saved as grapevine germplasm.

Clonal selection originate back to the 1956 when the first clones of 'Laški rizling' ('Welschriesling') and 'Sauvignon' were selected. For more than 30 years the standard positive mass selection has been proposed for all the mother block vineyards. This selection comprehend the five year individual pomological evaluation of all the tentative mother plants. The population of mother vines consist of circa 90.000 *vinifera* and 35.000 rootstock plants, for 240.000 tentative mother plants the selection will be finished in next two years. The work is centralised and supported by Ministry of Agriculture. Five years in row all the vines are inspected for the known symptoms of virus diseases, (infectious degeneration, leaf roll and rugose wood), and lately also for "Flavescence dorée" the phytoplasma disease.

In preliminary ELISA screening (1) we have stated that circa 12% of infectious degeneration and up to 38% of leafroll and similar diseases were not detected in field inspections due to their latency. In order to estimate the real sanitary status of grapevine propagating material in Slovenia, the complete sanitary selection with indexing and ELISA was organised for all the planting material in clonal selection. Its procedure has been renewed in 1990/91. Therefore two selection stations - nuclei were formed (Ormo for the continental and Vipava for the littoral part of the vineyards). Their program is based on the international guidelines proposed by ICVG and O.I.V. (2, 3, 4, 5).

MATERIAL AND METHODS

In indexing procedures the traditional grape green-grafting was used, the grafts were enhanced in mist chamber. Standard indicator vines were used as well as some of our local *vinifera* varieties (' ametovka'). The virus free status of our own indicators was obtained by meristem culture (6). In ELISA leaf samples (for nepoviruses only) were collected in May, or wood shavings were used during winter dormancy. Sanofi -Plantest and Bioreba ELISA test diagnostic kits for GFV, ArMV, GLRaV I and III, and GVA were used. According to pomological selection and origin of the planting material the following vines were tested:

- local Slovene varieties for special wine making programs and conservation of vine genetic resources;
- elite vines or clonal candidates issued out of five year positive selection (167 plants of 17 cvs);
- old clonal vines from conservation - collection mother blocks (230 plants, 2 cvs and 4 clones);
- vines of first and second multiplication step (5.400 plants, 14 cvs and 90 clonal candidates);
- imported rootstocks.

For each clone a mother plant and all the offsprings were tested whenever possible, to trace the route and the source of the infection. In last three years we made from 2.000 to 3.500 tests/year, the detectability and reliability of the tests is high enough (96%) to use the ELISA as a routine test in clonal selection. Polyclonal antigens gave more dubious readings as the monoclonal ones, where more than 10% of tests had to be repeated. 20% of vines tested with ELISA were included in indexing and were observed for two years.

RESULTS

Local white cultivars ('Zelen', 'Glera', 'Rebula', 'Vitovska grganja', 'Danijela', 'Poljšakica', 'Volovnik', 'Cividin') expressed high incidence of GFV (38%) and lower (8%) for closteroviruses. The drop of potential clone candidates for some varieties is more than 90%. The source of GFV is likely to be in pre-phylloxera time. The infections are very often connected with poor flower set and staminate flowers. The economic important cultivars were submitted to further meristem culture to avoid risk and long lasting search for one pomological and sanitary suitable plant of a variety. No ArMV infections were detected with old varieties. The oldest Slovene grapevine plant, the 400 years old vine of cv. ' ametovka' was free of the listed viruses.

For the Karst red cv. 'Refošk' with 52 clonal candidates, 5% were GLRaV I and 10% were GLRaV III positive. Mother plants (some of them over 70 years old) were infected only with one or the other virus. With a mother plant to be viral all the subclones were diseased, but there some mixed infections were found. It suggests the diseased rootstock as a source of the virus. In last five years no natural spread of the leafroll in the clonal vineyard has been observed, mealybugs have not been found in the area.

Two of the clonal candidates with 20 offspring each, expressed a severe rugose wood in the field. The one with symptoms only on the *vinifera* part of the trunk gave complete GLRaV I positive reaction, the other one with the rugose wood on the rootstock (SO4) gave negative ELISA. There was no positive reaction in indexing, too. From previous research we know that the cv.' Refošk' was heavily infected with all forms of rugose wood which cause the severe decline of the vines. From 2850 vines inspected in the vineyards in 1996, there were 22% of diseased plants. In that group 40% of the vines had no positive

reaction in ELISA or in conventional indexing procedures. There are strong evidences that the well known incompatibility of the rootstock 420A and 'Refošk' originate in a virus infection.

The oldest clones and subclones of 'Laški rizling' (cl. 178 and 22) 'Sauvignon' (cl.43 and 20), kept in collections for last 25 years, were healthy expect for one subclone (178/29) which was 758% infected with GLRaV III. This infection was complete latent with no morphological or quality effect on the vines and was the first one to be detected in the continental part of Slovenia. The vines of the second multiplication step in clonal selection were free of above listed viruses, except for two series of vines grafted on rootstock of unknown origin. The diseased vines were dismissed.

Sanitary monitoring of the imported rootstock (base and certificate status) in mother blocks showed 2-5% of either GLRaV I or III. The diseased vines were destroyed. The rootstock plantings are under further control for the threat of the natural spread of disease.

CONCLUSIONS

Pomological part of the clonal selection gave a lot of improvement to the sanitary status of the planting material but it is not sufficient. The improved diagnostic methods demands to test the clones over and over again. The grape people are asking: to which extend, in what time intervals, at which expense?

The populations of local varieties which were propagated for long time in a small closed circle expressed a high incidence of some viruses and demand a specific national sanitary control programme. Are we the good ones to decide whether or not the bad grapes (i. e. virus diseased) should be destroyed? Are we neglecting some valuable genetic resources?

The importance of the latent infections especially on rootstocks was underestimated. Are all the European countries where grapes are grown, protected against the uncontrolled trade of the planting material at the same level?

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EVOLUTION OF LEAFROLL (GLRaV-3) EFFECT ON GRAPEVINE YIELD AND POTENTIAL ETHANOL

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INTRODUCTION

Grapevine leafroll, associated to a complex of at least 7 closterovirus, is considered a very important component of the sanitary clonal selection because of its effects on the cultivars behaviour causing intense reddening of the leaves with decrease chlorophyll pigmentation and influence on the maturation process (4). In Portugal, for all grapevine cultivars in the clonal selection program, the predominant closterovirus present is GLRaV-3 (grapevine leafroll associated virus-serotype 3) being the percentage very high in some cultivars (e.g. 90% for cv. Tinta Francisca, 93% for cv. Alvarinho, 98% for cv. Esgana Cão (1; 3).

Frequently, however, expression of leafroll symptoms is moderate, because it is dependent on the environment and other factors. The influence of temperature and radiation in the symptom intensity of GLRaV-3 has been consistently observed in Portugal. For example infected clones of cv. Periquita show typical leafroll symptoms in Oeste region (mild climate) but the same clones in a more eastern region (with very hot and dry summers) have no symptoms (3; 5).

The purpose of this study is to evaluate how GLRaV-3 influences agronomic and aenological parameters as grapevine yield and potential ethanol, under the hot environment of Douro region and if those influences grow or remain stable along the years, when no symptoms are developed.

MATERIALS AND METHODS

Data were collected from two experimental populations of clones, one with 166 clones of cv. Tinto Cão and other with 198 clones of cv. Touriga Nacional, established in the Douro region. The region has a typical Mediterranean climate with very high summer temperatures associated to a high water deficit - Thorntwaite DB'3sa' - (2). The study was based on the 66 more productive clones (25 infected with GLRaV-3) of cv. Tinta Cão and on the 73 more productive clones (21 infected with GLRaV-3) of cv. Touriga Nacional. No symptoms of disease were detected. The infection by GLRaV-3 was detected by DAS-ELISA. All clones were free from GLRaV-1 and GFLV (grapevine fanleaf virus).

The comparison among healthy and infected clones was done by Student's t test for 1989, 1990, 1993 and 1995 for Tinto Cão and from 1989 up to 1996 for Touriga Nacional.

The broad sense heritability (h^2) was estimated in order to quantify the degree of genetic influence on the yield and on potential ethanol.

RESULTS AND DISCUSSION

The productivity of cv. Tinto Cão for the 4-year study showed no significant differences between the 41 healthy and the 25 GLRaV-3 infected clones (Table 1).

Table 1. Grape yield (kg/plant) of healthy and infected clones of cv. Tinto Cão

	1989	1990	1993	1995
Infected	0.90	1.93	1.85	1.55
Healthy	0.81	1.80	1.80	1.53
Significance	n.s.	n.s.	n.s.	n.s.
Heritability (h^2)	52%	33%	54%	18%

For the cultivar Touriga Nacional the results of mean productivity (= yield)   for the group of healthy *vs* infected clones in the 7-year study showed a high heritability value ($h^2 = 0.87$) and no significant difference between the two groups (1.18 kg/plant for the GLRaV-3 infected group and 1.20 kg/plant for the healthy group). When each individual year is considered no significant difference is also evident for the all period tested (Table 2).

Table 2 - Grape yield (kg/plant) of healthy and infected clones of cv. Touriga Nacional

	1989	1990	1992	1993	1994	1995	1996
Infected	0.96	1.12	1.21	0.60	1.55	1.32	1.78
Healthy	0.96	1.19	1.26	0.69	1.59	1.25	1.71
Significance	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Analysis of the potential ethanol for cv. Touriga Nacional, showed also no significant difference between the infected (11.4°) and the healthy (11.6°) groups of clones on the overall 4-year period. However, when each year is individually observed an irregular behaviour is detected on the two groups for the period tested. The deleterious effect of GLRaV-3 in the potential ethanol is clear in 1995 (Table 3).

Table 3 - Potential ethanol of healthy and infected clones of cv. Touriga Nacional

	1992	1993	1994	1995
Infected	12.8	10.1	11.1	12.9
Healthy	12.8	10.4	11.1	13.5
Significance	ns	*	ns	* * *
Heritability (h^2)	67%	77%	75%	82%

Therefore, more data must be collected to understand if the deleterious effect of GLRaV-3 on the potential ethanol is an isolated event or will continue to increase over time, as with degenerative type of viruses. Otherwise, studies done in 1994 and 1995 on the same clones showed no significative differences for other quality parameters like total acidity, pH, polyphenol content, anthocian content and corant intensity of the must (5; 6).

CONCLUSIONS

GLRaV-3 did not induce deleterious effect on yield of cv. Tinto Cão and cv. Touriga Nacional, on each individual year and over time, for the several years assayed.

The effect of GLRaV-3 on potential ethanol for cv. Touriga Nacional was not detected in the global 4-year study. However, for the last year of this study the deleterious effect of the virus was highly significant. Concerning these results on the quality parameters, and others presented in these Extended Abstracts (Martins et al.) it is evident the importance of further knowledge on the effects of viruses, namely on maturation, over time.

However, if we can confirm the moderate and non degenerative effect of virus the conservation of high productivity clones even if associated with them, will be important for maintenance of genetic biodiversity of grapevines.

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EFFECT OF LEAFROLL ASSOCIATED VIRUS GLRaV-3 ON YIELD OF GRAPEVINES: NEW RESULTS, NEW PERSPECTIVES

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INTRODUCTION

Some recent studies about leafroll effect on vine plants have led to the conclusion that virus infection is responsible for important yield losses (1,2,3,4). Accuracy of methods currently used for those studies is not well established (mainly due to low number of clones to be compared), however selectors generally agree that results originated this way are good enough to justify a sanitary selection fairly intense and with complete priority over genetic selection. In this work we present results on yield of leafroll infected and healthy clones of 6 ancient Portuguese cultivars that contradict most results found in the correspondent bibliography. We also discuss the erroneous signification frequently ascribed to mean differences between infected and healthy clones, as a basis for selection strategies.

MATERIALS AND METHODS

Results were obtained in 6 experimental populations of clones from each of cvs. Rabo de Ovelha, Alvarinho, Arinto, Crato Branco, Moscatel de Setúbal and Vital, planted under experimental designs of 78 to 266 clones x 4 replicates (blocs) x 4 plants. Sanitary status was determined by DAS-ELISA. Data are 2-3 years mean yields, depending on cultivars. Environmental variation in experimental fields was fairly reduced, as indicated by broad sense heritability estimates ranging from 0.649 (Moscatel de Setúbal) to 0.867 (Alvarinho).

RESULTS AND DISCUSSION

As show in figure 1, differences between healthy and infected clones are quite moderate when compared to those found in bibliography. Only two cultivars show high yield losses due to GLRaV-3, three others show very low effects, one of them even shows a yield gain associated with infection (however, this difference is not statistical significant). These results suggest that leafroll must not be considered a serious problem just in all agronomic situations. Probably it will be more dangerous only under specific contexts resulting from cultivar, environment and virus population. Among the 6 studied cultivars Arinto and Vital were grown under a mild summer climate and showed the greater differences, so higher virus effects seems to be a result of lower summer temperatures.

The observation of differences between mean values of healthy and infected clones has greatly influenced thinking of selectors in the near past, leading them to emphasise excessively the importance of sanitary selection and to forget the potential of genetic selection. We think this is an erroneous idea, not in accordance with selection objectives or with experimental data now available, neither with theorical foundations of selection. Mean differences between healthy and infected clones can authorise conclusion about the effect of leafroll on vineyards that are already growing over a concrete area, but no relation exists between those differences and the gains to be obtained by new selections of superior clones from now on.

A key concept in selection work is selection differential, because the gain depends directly from it. But the mean difference healthy-infected clones don't count to differential, it depends only from mean superiority of clones situated near the top of yield ranking when related to general mean. If we regard precisely the two cultivars with higher losses associated with virus infection (Arinto and Vital) we can conclude that selection differential may be quite higher (depending on number of clones to be selected) than the mean difference between healthy and infected clones. In these situations selection with priority to virus status leads us to a reduced gain when, however, it would be possible to gain much more. In other situations (e.g. Crato Branco) sanitary priority can becomes even a negative selection, in respect to yield. So, in both situations, a conflict becomes apparent between sanitary selection and genetic selection.

Some people may think that an easy way to bypass this conflict is to put a higher number of healthy clones into experimental populations. Under a theoretical view this may be a more safe way to undergo selection. However, in practice, it is not a realistic solution, because leafroll free genotypes are scarce within some cultivars (e.g. Alvarinho, Moscatel de Setúbal and others not referred in this work). Moreover, even if we can find a safe number of leafroll free clones they may be infected with other virus and the problem will remain continuously unsolved.
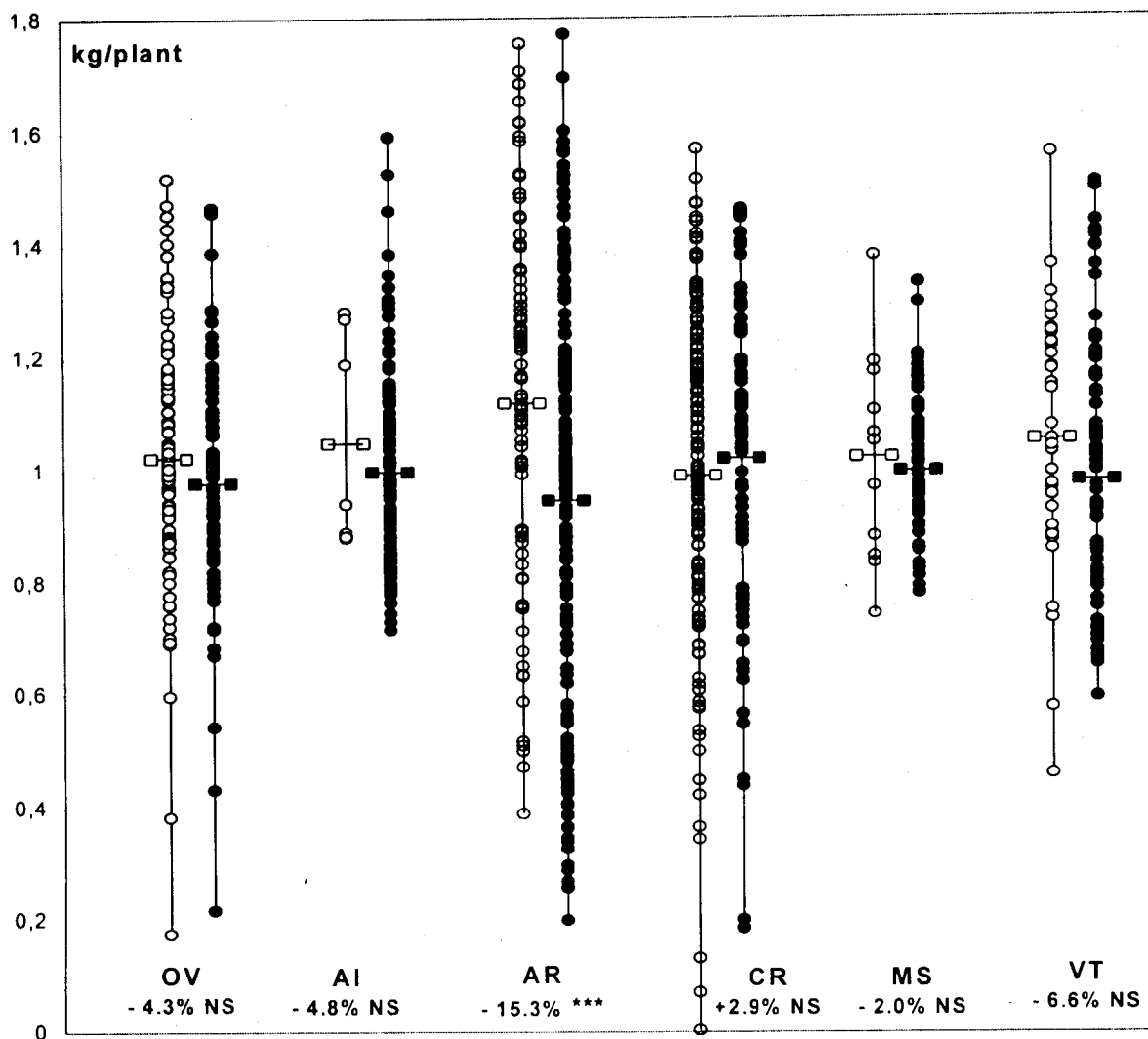
Figure 1 - Graphic representation of yield of clones healthy (o) and infected ( ) with leafroll associated virus 3 from 6 ancient Portuguese cultivars: OV - Rabo de Ovelha; AI - Alvarinho; AR - Arinto; CR - Crato Branco; MS - Moscatel de Setúbal; VT - Vital. In order to narrow the scale, all values were devised by the population mean. Under cultivar symbols are indicated mean differences of yield between healthy and GLRaV-3 infected clones (NS - no significant; * significant at 5%; *** significant at 0.1%).   - mean of leafroll free clones;   - mean of infected clones.

CONCLUSIONS

Yield differences between leafroll infected and healthy clones revealed to be moderate. Differences between the more productive clones in each population (both healthy and infected ones) and the correspondent mean are much higher, allowing selections with high genetic gains. So, priority to sanitary selection conflicts with genetic selection, avoiding utilisation of high performance genotypes and obtention of the best results.

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THE PRODUCTION OF CERTIFIED PLANTING MATERIAL OF GRAPEVINE IN THE UKRAINE

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Viticulture is the important branch of agriculture in the Ukraine. Vineyards are especially concentrated in the South and the Western part of the Ukraine. In other regions there are also some vineyards, but they do not play an important role in the Ukrainian economy.

Tairov Research Institute of Viticulture and Oenology is currently working on the clonal evaluation of 186 clones of 38 European cultivars and 82 clones of 6 rootstock cultivars. Among the cultivars that have been studied in the Ukraine are the old cultivars, such as Cabernet Sauvignon, Riesling, Rcatciteli, Muscat Hamburg and others. The cultivars of Ukrainian selection and the perspective but not in current use cultivars, include Muscat tairovskij, Marseljskij black early, Odesskij souvenir, Sucholimanskij belij, Irschai oliver and others. Clonal selection is conducted in 3 stages. In the first year plants are selected from the vineyards which are looked healthy , 10-20 years old are chosen and examined (Po). The second and third stages (P1 and P2): the heritability and stability of their productivity and quality are determined.
But the 1 and 2 vegetative generation are studied on the clone collection vineyard already.
Control - the average indices among of the studied grape plants and clones.

Every year the phytosanitary selection is conducted in all of the Ukrainian regions. It proved the possibility to isolate the plants that are visually free of virus and crown gall diseases. The following diagnosis of virus diseases in laboratory conditions proved the rational to reject diseased plants. For latent infection diagnosis we apply the graftings on the woody indicator plants, ELISA-test, dsRNA method and molecular hybridisation with radio-labelled and non radio-labelled marked dsRNA probes.

The dsRNA method is used for the preliminary screening of plants. For dsRNA isolation, differentmethods, including extraction with phenol were applied. Currently we used the method of dsRNA extraction without the organic solvents. Instead of these compounds a mixture of potassium acetate and SDS is used. For dsRNA purification, chromatography on CF-11 cellulose is used.

The I 125 marked dsRNA probe was used for testing the grapevine plants for the presence of GFLV, GLRaV 1, GLRaV 3, GVA and GFkV. We have also used the biotinilated probes. The last probe was applied for testing the grapevine viruses directly from plants extracts or from partially purified extracts.
For crown gall disease diagnosis, the probe we used was prepared from a part of TI-plasmid and labelled by I 125 or digoxigenin.

For cultivars totally infected by viruses, heat treatment at +38 C together with subsequent apex cultivation in vitro was used.

In our tissue culture laboratory a biotechnological of grapevine propagation system was used. It is based on the utilisation of ionic substrates. Greenhouse grown grapevine plants are selected and they are grown in on a mineral substrate that exclude the secondary infection by virus- and crown gall diseases. In April the apices of the green shoots of 5-6 cm length are cut and 2 buds are left for the secondary shoots development. The apices of the forced shoots are subsequently used for in vitro cultivation. This procedure provides the possibility to obtain the initial planting material for in vitro cultivation during the entire year. It is very important because the selection of initial planting material from the vineyard may take place only until the middle of July. The apices are planted in vitro on agar medium. In the first stages of this procedure the media composition was varied according to the cultivars peculiarity. Thus a special medium was developed for Muscat belij cultivar which is very difficult to root and propagate in vitro.

At the stage of micro-cuttings and micro-clone cultivation the artificial medium "Biona" was used instead of agar. This substrate was prepared with the ion-exchange resins and the nutrients enter the plants by an ion exchange process. With this procedure the possibility of "salt burn" almost totally excluded and the plants obtain their nutrients at each stage of development by ion exchange. The "Biona" may be used many times and if necessary it may be regenerated. The micro-clones grow and develop on "Biona" media better then on the agar media.

The plants with 4-5 internodes and 5 cm length are planted from in vitro cultivation to the greenhouse conditions for adaptation. For this purpose the ceolite is used. The ceolite is the natural mineral that posses the same characteristic as the "Biona" and contains high amounts of potassium. Before using the ceolite was charged by some mineral salts solution the composition of which was developed at Tairov Research Institute of Viticulture and Oenology.

The similarity of the substrates ("Biona" and ceolite) physico-chemical composition for in vitro and in vivo micro-clone cultivation is very good for the plants adaptation which reached 85-90%. After the adaptation period the plants are left in the same containers with the ceolite without the additional replanting; until they reach standard plants length.

For optimisation of the light conditions special phytolamps were used with the spectrum composition of 12,5% blue and 87,5% of red illumination. In this conditions the micro-clones adaptation was even 95-100%.

Above mentioned technology was used for 50 clones of 30 grapevine cultivars propagation. The high level of micro-clone propagation (300 plants from one bud) gave the possibility to receive the certified planting material rapidly in required quantities.