

**11th Meeting  
of the  
International Council for the  
Study of Viruses and  
Virus Diseases  
of the Grapevine**

**ICVG**

**Extended Abstracts**

**Montreux, Switzerland**

**6 - 9 September 1993**

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**Edited by P. Gugerli**



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## PREFACE

These extended abstracts will be distributed to the participants attending the 11th Meeting of ICVG at the beginning of the conference in Montreux. Texts have been copied from the original documents either by electronic scanning and text recognition, by direct transfer from the computer disk file or by manual acquisition. Electronic text recognition may have added a few errors, but we have done our best to eliminate them, together with others, by checking spelling and doing a partial editing on some texts to standardize the final presentation of the papers. Due to the short time available, we were not able to send copies of the final texts for proofreading to all authors concerned. Nevertheless, we hope that these printed extended abstracts summarize correctly the interesting information, which will be presented during the meeting.

P. Gugerli

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## ADVANCES IN GRAPEVINE VIROLOGY: 1991-93

Martelli, G.P.

Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italy

This presentation does not represent a comprehensive review of all contributions to grapevine virology published since the 10th Meeting of ICVG (Volos, 1990), for it does not cover papers on viroids and intracellular prokaryotes, nor on epidemiology and control. It outlines noteworthy advances made in the recovery and description of new viruses, the molecular biology of known viruses, virus-host relationships, and detection methods by advanced serological and molecular techniques. A brief account is given of significant studies on these subjects and their results are critically discussed.

### New viruses and new data on known diseases

#### A. Isometric viruses

The phloem-limited non mechanically transmissible virus that was reported to be associated with fleck (2) was proven to be the agent of this disease and, accordingly, it was named grapevine fleck virus (GFkV) (3). Fulfilment of Koch's postulates was as close as possible, considering that preparations of this virus are not infectious, and could not be reintroduced in purified form into the specific indicator *Vitis rupestris*. The availability of a polyclonal antiserum to GFkV allowed the initiation of large scale surveys, that showed this virus to be of common occurrence in the Mediterranean, with infection rates up and above 30% (25 and unpublished information).

Grapevine ajinashika disease-associated virus (GAaV) is another non mechanically transmissible icosahedral virus recovered from Japanese vines of cv. Koshu affected by a disorder from which its name originates (33). GAaV has properties somewhat resembling those of GFkV, but is serologically unrelated to it and is considered to be a possible luteovirus (33). As yet, no ultimate evidence has been provided that GAaV is the agent of ajinashika disease.

The characterization of grapevine Tunisian ringspot virus (GTRV) was completed. Properties of this virus were such that its classification as a definitive species of the *Nepovirus* genus was proposed (4). Lack of further recovery of GTRV from Tunisian vines confirmed this virus to be an occasional parasite of no economic relevance.

Grapevine line pattern virus (GLPV), the agent of a minor disease of Hungarian vines, was transmitted through the host's seeds (22), thus corroborating the contention that it is a species of the *Ilarvirus* genus.

#### B. Rod-shaped and filamentous viruses

A novel virus with anisometric particles referred to as grapevine labile rod-shaped virus (GLRSV) was recovered from a symptomless vine of cv. Merlot in Italy (9). GLRSV is not mechanically transmissible, has rigid particles of two predominant lengths (150-175 and 275-300 nm) exhibiting an evident surface substructure and a central canal. Although the size and type of nucleic acid of GLRSV were not determined, the morphology and size of the virions suggest its possible inclusion in the genus *Furovirus*.

Closteroviruses, i.e. filamentous, flexuous virions with prominent transverse striations, open particle structure, and length ranging from 750 to 2000 nm and above, were the object of intensive investigations.

According to the latest taxonomical views, viruses with the above particle morphology may be classified in three distinct genera: *Closterovirus* (particles 1400-2200 nm long), *Capillovirus* and *Trichovirus*, both with particles not exceeding 800 nm in length (24). Closteroviruses from grapevines will ultimately be assigned to the appropriate genus on the basis of adequate physico-chemical and molecular information. However, it seems likely that most, if not all, viruses currently referred to as "leafroll-associated" belong to the *Closterovirus* genus, whereas viruses with shorter particles and a polyadenylated 3' end, such as grapevine virus A (GVA) and grapevine virus B (GVB), will be classified as trichoviruses.

Important new additions to the list of closteroviruses were:

(i) Grapevine corky bark-associated virus (GCBaV), a possible closterovirus with particles 1400-2000 nm long, coat protein subunits with mol. wt of 24 kDa, serologically distinct from grapevine leafroll-associated viruses II (GLRaV II) and III (GLRaV III) (34). The serological relationships of GCBaV with other long grapevine closteroviruses was not investigated, nor its etiological relationship with corky bark disease established for certain. Thus, the contention that GCBaV may be the agent of corky bark (34) rests on somewhat shaky

ground.

(iii) Grapevine virus B (GVB), a mechanically transmissible possible *Trichovirus* species with particles about 800 nm long. GVB was characterized biologically, physico-chemically, serologically and, in part, epidemiologically (5) and molecularly (41). It has a (+)sense ssRNA genome c. 7600 nucleotide in size, and coat protein subunits of c. 23 kDa. It is serologically (5) and molecularly (41) unrelated to GVA but, like GVA, is transmitted by pseudococcid mealybugs (5). GVB has natural variants that can be distinguished biologically and molecularly (41), one of which was recovered in USA from a leafroll-infected vine and reported as a distinct virus (19). The fair association of GVB (more than 60% recovery) with vines indexing positive for corky bark, prompted the suggestion that it may be implicated in the etiology of this disease (5).

An unnamed closterolike virus with particles 725 nm long was isolated in Canada from *in vitro* grown explants of cv. Semillon affected by corky bark, and multiplied in *Nicotiana* species (28). A culture of this virus was studied in Italy (Bari) and found to contain particles c. 775 nm in length biologically and serologically indistinguishable from GVB (5). A partial comparison of this GVB isolate with GVA made in Canada (29), showed that there was no serological relation between the two and that the mol. wt of their coat protein subunits differed. In particular, values calculated in Canada (29) were higher than those reported from Italy (5), i.e. 27 and 25.7 kDa versus 22.5 and 23 kDa for GVA and GVB, respectively. These differences lie more or less within the range of variation that can be expected for determinations made in different laboratories. However, it may be worth mentioning that apple chlorotic leafspot virus, the type species of the *Trichovirus* genus in which GVA and GVB may belong, has a coat protein with mol. wt of c. 22 kDa (13).

## Molecular biology

Although the molecular study of some closterolike viruses (GVA, GVB, GLRaV III), is under way in different laboratories no detailed report on these investigations has been published. Thus, the bulk of contributions on the molecular biology of grapevine viruses rests on investigations carried out on nepoviruses, primarily in France.

The whole nucleotide sequence of grapevine fanleaf virus (GFLV) was determined, thus completing the molecular knowledge of the viral genome (38). The genomic organization and strategy of replication of the same virus were also elucidated and found to be in total agreement with those typical of the genus *Nepovirus* (15). The series of molecular investigations on GFLV was wound up by the production of infectious transcripts of the satellite RNA, capable of replicating in a protoplast system (17). This represents a further step towards the understanding of the mechanism whereby a RNA molecule (the satellite) parasitizes another (the viral genome), thus becoming a potential tool for virus control.

The maturation map of the proteins encoded by RNA 2 of grapevine chrome mosaic (GCMV) and tomato black ring (TBRV) viruses was determined with *in vitro* translation experiments. The monocistronic GCMV RNA 2 molecule was translated in a polypeptide of c. 150 kDa which was then cleaved by a protease encoded by RNA 1 of the same virus into two mature non structural proteins of 44 and 46 kDa, respectively, plus the 56 kDa coat protein (7). All three proteins were also detected *in vivo* in infected *Chenopodium quinoa* extracts, thus validating the maturation map deduced from *in vitro* experiments (18).

These molecular investigations, however theoretical as they may appear, have an outcome that is already finding the way into practical applications. It is thanks to the basic knowledge gathered with such studies, that transgenic vines expressing the coat protein gene of GFLV (B. Walter, personal communication) and GCMV (T. Candresse, personal communication) were produced in France. This represents the first example of successful genetic manipulation of *Vitis* for the introduction of possible resistance to viruses, which projects grapevine virology in the molecular era, and opens to novel, still unexplored control strategies.

## Virus-host relationships

### A. Ultrastructural studies

Vines of different cultivars affected by rupestris stem pitting or GLRaV III were studied ultrastructurally in China (44) and Italy (10), respectively. In the Chinese material, profiles of virus particles were observed in phloem parenchyma cells forming aggregates deprived of the clustered vesicular elements typical of closterovirus infections (44). The virus present in the Chinese samples was not identified, whereas the bundles of filamentous structures present in phloem elements of five Italian grapevine cultivars, were shown to be GLRaV III particles by immunogold labelling (10). Infected cells contained plenty of membranous vesicles with fine fibrils which appeared to derive from deranged mitochondria. These mitochondrial vesicles were single-membraned, which distinguished them from the comparable double-membraned structures elicited in mitochondria by GFLV infections (6, 11). Based on similarities in particle length, size of coat protein subunits and cytopathological features (i.e. mitochondrial vesiculation) Faoro and coworkers (10) suggested that GLRaV

I, GLRaV III and dendrobium vein necrosis virus, constitute a separate subgroup in the *Closterovirus* genus. Although this may as well prove to be so following the obtention of additional data, it should be kept in mind that subdivisions within the *Closterovirus* genus will likely be dictated by differences in genome organization and expression more than anything else.

A detailed study of GVA-induced cytopathology in *N. benthamiana* leaves was carried out in Canada (30), whose results do not appear to add much to what is already known on the subject, except for the observation that vein border cells may play a prominent role in the multiplication and movement of virions through the leaf. Source material for this study was from plants that had been inoculated with purified GVA preparations. According to the authors (30) this made their observations more reliable than those reported earlier (39) because of the possibility that the mealybugs used in the latter work for transferring GVA from grapes to *N. clevelandii* had inoculated more than one virus.

Although theoretically tenable, this claim appears largely unsubstantiated in the specific instance, for two orders of reasons:

- (i) it is now known that, in addition to GVA, mealybugs can transmit GLRaV III (but not to herbaceous hosts) and GVB, neither of which occurred in the material studied by Rosciglione and coworkers (39);
- (ii) differences in the cytopathology of *N. benthamiana* (30) and *N. clevelandii* (39) are so minor that they hardly support the contention that two diverse viral systems were investigated.

#### B. dsRNA profiles

Electrophoretic patterns of dsRNAs from diseased vine extracts are becoming an increasing popular subject of study, especially for disorders known or suspected to be elicited by closteroviruses. dsRNAs are molecules produced during viral replication either on full-length genomic (-)RNA templates, or by premature termination of (+)strand synthesis at subgenomic RNA promoters (14). This leads to formation of dsRNA molecules of different size which, in the case of closteroviruses can be rather numerous. The subgenomic nature of smaller than genomic dsRNAs of GVA, GVB and GLRaV III was demonstrated by the presence of sequence homology between them and the respective genomic RNAs (26, 41, 43).

dsRNAs accumulate in infected tissues from which they can be extracted efficiently if adequate techniques are applied (37). Electrophoretic separation of dsRNA preparations yields patterns that, in principle, could be specific for any given virus, or virus strain (8, 32). With grapevines, however, there are conflicting reports, as exemplified by data on dsRNAs from rupestris stem pitting-diseased plants. According to papers from different laboratories dsRNAs associated with this disease are: two, with mol. wt  $5.3$  and  $4.4 \times 10^6$  daltons (1); one, with mol. wt  $5.5 \times 10^6$  daltons (45); one, with a mol. wt of c. 240,000 daltons (31). Furthermore, dsRNA patterns vary with the season (20) or the type of donor (J. Wadjianny and A. Minafra, personal communication), which makes their use as a tool for specific identification of grapevine diseases or viruses unreliable, at least at the present status of knowledge. dsRNAs, however, can be usefully utilized as infection markers for quarantine introductions and for evaluating the outcome of sanitation treatments (16).

#### Diagnosis

The experience of the past three years has confirmed that ELISA is by far the most utilized technique not only for virus detection and identification, but also for studying sampling strategies (40). Extracts from cortical tissues (bark scrapings) remain a most dependable source of antigen for ELISA testing although, for certain viruses, actively growing tissues or petioles from mature leaves may give better readings (3, 20). The widespread use of ELISA is favoured by the availability of an ever growing series of commercial kits and the production of monoclonal antibodies, which improve detection protocols (4). Biotin-avidin ELISA (20) and chemiluminescent immunoassays (36) were applied for detection of grapevine leafroll associated closteroviruses I, II, III and IV, with satisfactory results. In particular, the chemiluminescent assay was reported to be more sensitive than other conventional immunoenzymatic systems, it was used with very high antibody dilutions, and yielded negligible non specific signals when applied to crude sap preparations (36).

The potentialities of molecular hybridization are being increasingly exploited as a result of the greater attention paid to molecular biology studies. The very contents of these Proceedings support this contention, which is also corroborated by the many papers published in the last couple of years or so. Thus, cloned cDNA probes with radioactive labelling were successfully utilized for the specific detection of GFLV (12) and its satellite RNA (42), GVA (26), GVB (41) and GLRaV III (23, 43). Most of these cDNA probes were synthesized on templates consisting of genomic RNA extracted from purified virus. For GLRaV III, however, the template was denatured dsRNA (23, 43), a technique that opens the way to production of specific probes for non mechanically transmissible viruses whose dsRNAs can be recovered from infected tissues.

A drawback of molecular hybridization tests is that they can be applied with difficulty to grapevine crude sap extracts, either because of the low titre of target nucleic acid, or because of the presence of interfering

substances that generate a high background or false positive signals. Some sort of manipulation is therefore necessary, which may consist either of using purified total nucleic acid extracts as test samples, or amplifying target nucleic acid through reverse transcription-polymerase chain reaction. This latter technique which is now very fashionable, was successfully used for the detection of arabis mosaic virus (21), GVA (27) and GLRaV III (23), and is expected to have an increasingly wider application in the immediate future.

#### Concluding remarks

Improved technology is the major single factor responsible for the remarkable advances registered in grapevine virology in the last three years.

The results of studies on leafroll and rugose wood, two diseases whose intricate nature is being unravelled, are flowing in at a rate that opens to optimistic predictions for the future. Likewise, alternative control methods based on biotechnological approaches are being pursued, once again with exciting perspectives.

Molecular biology and advanced diagnostics are the areas where progresses of great consequence can reasonably be expected in the short term.

All this will further increase the level of knowledge on various aspects of infectious diseases of *Vitis*, and will generate and make available new advanced technology. It would be a shameful loss if this technology were not transferred as efficiently and rapidly as possible to the whole scientific community. To this effect, fostering international cooperation in any of its multiple aspects, could represent the winning strategy of a design that ICVG should be willing and ready to pursue.

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## CYTOPATHOLOGY OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS I (GLRaV-I)

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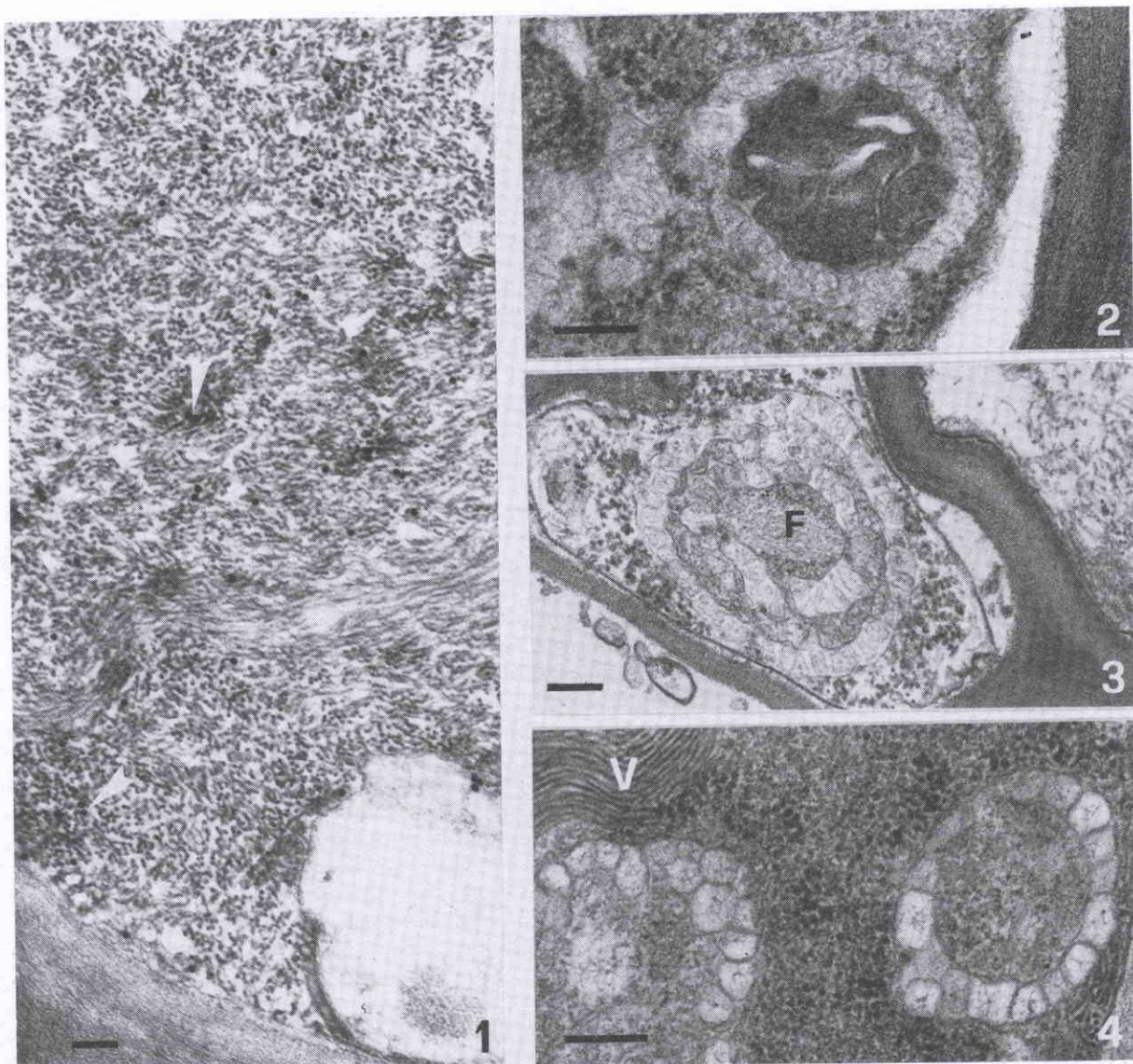
GLRaV-I and GLRaV-III are the most frequently detected closteroviruses in leafroll-diseased grapevines (1,2,3). Though serologically unrelated, they have been tentatively classified in the same closterovirus subgroup because of their particle size (12 x 2200-1800 nm) and coat protein weight (39-43 kDa) (4).

To find out other possible relationships between the two viruses we have carried out a cytopathological study on GLRaV-I infected grapevines and the results have been compared with those previously obtained studying GLRaV-III infected plants (5). Furthermore we have investigated grapevine tissues infected with both viruses to verify the possibility of distinguishing the cytopathic effects produced by each closterovirus.

Leafroll-affected grapevine plants, belonging to the cultivars Räuschling and Merlot and infected with GLRaV-I and GLRaV-I+GLRaV-III respectively have been used in this study. All plants, grown either in controlled experimental fields or *in vitro*, have been tested by ISEM and ELISA for the presence of the above viruses and the absence of other known grapevine closteroviruses such as GLRaV-II, GLRaV-IV, GLRaV-V, GVA and GVB. The absence of GFKV has also been assessed. Virus-free plants of the same cultivars were used as controls. Leaf main veins and surrounding tissues, including small parts of the adjacent lamina, were excised and cross cut in 0.5-1mm pieces, while dipped in a mixture of 2% glutaraldehyde + 2.5% paraformaldehyde in 0.1 phosphate buffer, pH 7.2. Samples were left in the fixative for 1/2 h under slight depression at room temperature and for 2 h at 4°C, then carefully washed for 1-2 h in the same buffer and postfixed in 1% osmium tetroxide for 2 h. Dehydration was carried out in an ethanol series and embedding in Epon-Araldite or London Resin White. Part of the samples were not osmicated to allow immunogold labelling experiments, which were carried out following a previously reported protocol (6).

The ultrastructural examinations of GLRaV-I infected samples showed clearly that the alterations due to the infection were restricted to the phloem tissue. The main cytopathic effects observed were the presence of long, flexuous filaments, 12 nm in dia, and vesiculated mitochondria. Flexuous filaments were recognized as GLRaV-I particles by the gold probe. They were organized in bundles which rarely occupied the whole sieve tube lumen (Fig.1). Small bundles of virions were also present in the cytoplasm of parenchyma phloem cells, often associated with vesiculated mitochondria. These structures were visible in different stages of the vesiculating process that started with the formation of 50-100 nm vesicles between the inner and outer mitochondrial envelope to form a characteristic "crown" of stacked vesicles around the organelle (Fig.2). Cristae were also involved in the vesiculating process while stroma degraded (Fig.3). Finally, mitochondria were hardly recognizable as they appeared as electron transparent organelles with membranes lined by rows of vesicles. Crowned mitochondria and bundles of filaments were also observed in some parenchyma phloem cells of the Merlot clone (Fig.4) doubly infected with GLRaV-I and GLRaV-III. In other phloem cells of the same clone other vesiculated mitochondria in early stages of degeneration did not show the characteristic crown of staked vesicles. Their peripheral vesicles appeared loose as the ones already observed in numerous grapevine cultivars infected by GLRaV-III alone (5). Therefore it seems likely that the vesiculation of these mitochondria had been induced by GLRaV-III.

From the comparison of these results with those previously obtained from GLRaV-III infected grapevines (5) it can be concluded that the cytopathic effects produced by both GLRaV-I and GLRaV-III are very similar and characterized by the presence of vesiculated mitochondria. This supports the hypothesis of a taxonomic relationship between the two viruses and suggests to include GLRaV-I in the new closterovirus subgroup typified by GLRaV-III. However it must be noted that the crowned form of vesiculated mitochondria has never been observed in GLRaV-III infected plants, thus this peculiar cytopathic effect could be used as diagnostic marker of GLRaV-I infection.



Figs. 1-3. Phloem cells of cv. Räusching infected with GLRaV-I (all bars = 200 nm).

Fig. 1. Sieve tube filled with virus particles labelled by the gold probe (arrows); Fig. 2. "crowned" mitochondrion in an early stage of degeneration; Fig. 3. a more deranged vesiculating mitochondrion with filaments (F) in the stroma.

Fig. 4. Phloem cell of cv. Merlot infected with GLRaV-I and GLRaV-III together; crowned mitochondria are associated with bundles of filaments which are presumably GLRaV-I virions (V) (bar = 200 nm).

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# MOLECULAR CLONING OF dsRNA ISOLATED FROM TISSUE INFECTED WITH GRAPEVINE LEAFROLL VIRUS TYPE III.

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Grapevine leafroll is one of the most widespread and economically important viral disease of grapevines in the world. At least four serologically distinct types, designated I, II, III, and IV, of grapevine leafroll associated closteroviruses (GLRaV) have been described (1,2,4). A New York isolate, previously designated as NY-1, is the type member of GLRaV type III and antisera have been produced against isolated virions. Although type III antigens in disease vines are effectively detected by ELISA with homologous antisera, the serological distinctness of the GLRaV types limits the usefulness of type III antisera for detecting the other types of GLRaV (2). Thus, a better approach for detecting a range of GLRaV types may be in the use of nucleic acid probes that react to portions of the genome that have homology among the GLRaV types.

The objectives of this research on GLRaV type III are to establish a broad spectrum detection method for GLRaV using nucleic acid probes, to determine the genome organization of GLRaV type III, and to clone and engineer its coat protein gene.

Double stranded RNA was isolated from batches of Pinot noir infected with the NY-1 isolate of GLRaV type III and electrophoresed in acrylamide gels. A dsRNA of about 20 kilobase pairs (kbp) was extracted and further purified in low melting agarose. A portion of the synthesized cDNA (3) was cloned in Lambda ZAPII vector (Stratagene, CA) in order to establish a cDNA library, while another portion of synthesized cDNA was amplified by PCR with Uni-Amp adapters and primers (Clontech Laboratories, Inc., CA). The PCR amplified cDNA was used as probes to identify lambda phages with cDNA inserts that were specific for GLRaV dsRNA.

About 200 plasmid clones with cDNA inserts ranging from several hundred bp to 3 kbp were identified. Northern blot hybridization showed that selected inserts were specific to GLRaV dsRNA (Fig 1). Furthermore, using custom synthesized primers generated from partial sequences of cDNA clones, a specific DNA fragment was amplified by PCR from templates of cloned cDNA, dsRNA from infected tissue, and RNA isolated from partially purified virus preparations (Fig 2). A restriction enzyme map of cDNA clones has been derived for 20% of the 20 kbp dsRNA and a stretch of 3 kbp has been sequenced.

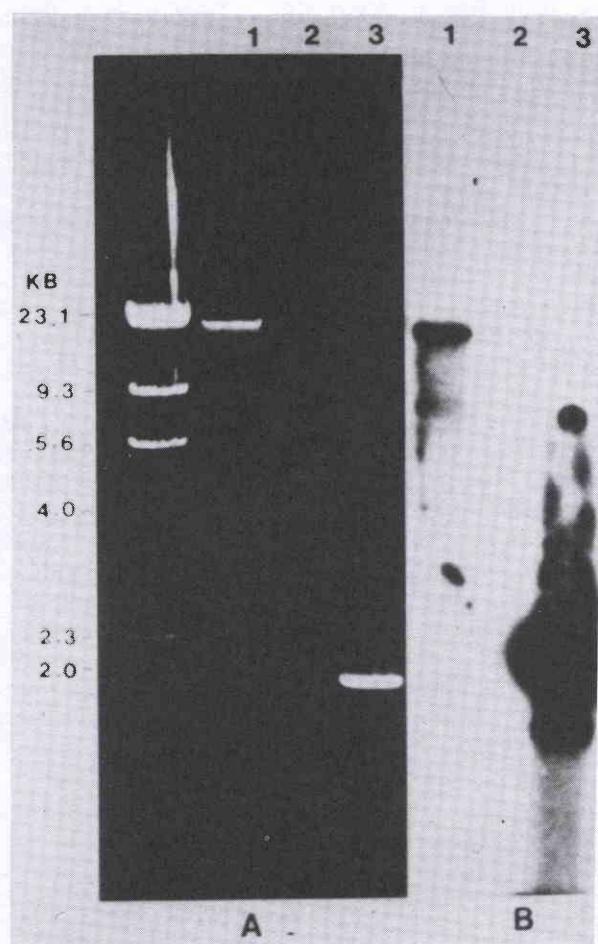


Figure 1

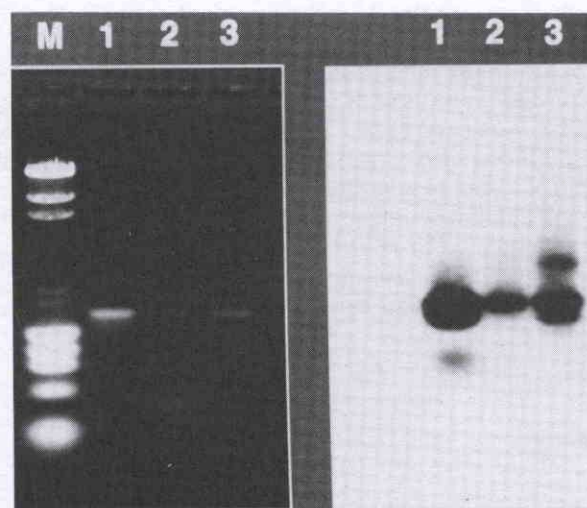


Figure 2

Figure 1A shows ethidium bromide stained dsRNA purified from GLRaV infected tissue (lane 1) and healthy tissue (lane 2), and cloned cDNA from which the probe was made (lane 3). Fig. 1B shows hybridization of the cDNA probe to transferred material from Fig. 1A.

Figure 2 shows ethidium bromide stained PCR products (left) and reaction of the products by Southern hybridization with cDNA clone that is specific to dsRNA from GLRaV type III infected tissue (right). Lanes are: (1) cloned cDNA, purified dsRNA, (2) purified dsRNA, and (3) RNA isolated from partially virus preparations.

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## GRAPEVINE LEAFROLL ASSOCIATED VIRUS II ANALYZED BY MONOCLONAL ANTIBODIES

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Grapevine leafroll associated virus II (GLRaV II) was first identified in extracts of leafroll diseased grapevines cv. Chasselas grown in Western Switzerland (3). Its existence was put forward because partially purified closterovirus-like particles, with a frequent maximum particle length of 1800 nm, from leafroll diseased Chasselas vines, did not react with antiserum to grapevine leafroll associated virus I (GLRaV I). Its distinct serological identity was further confirmed with homologous antiserum, which did not cross-react with either GLRaV I nor with a third grapevine leafroll associated virus (GLRaV III) from cv. Frappato (7). Leafroll symptoms on Gamay Rouge de Loire associated with GLRaV II were distinct from those of GLRaV I and GLRaV III infections: the reddening included not only the tissue between the main veins but the entire leaf blade (2).

Electron microscopy of immuno-precipitated GLRaV II particles occasionally revealed a variable degree of decoration, pointing out a possible mixed infection. The publication of two conflicting molecular weights for the coat protein of GLRaV II (1; 8), identified elsewhere with our antiserum, further backed up this hypothesis. However, low virus concentration and the inherent lack of specificity of the available antiserum hindered us to prove this hypothesis. Therefore we have now developed monospecific antibodies, i.e., monoclonal antibodies, to GLRaV II to distinguish unequivocally different virus particles in the hypothetic mixed infection. We tried to develop monoclonal antibodies of which each could be used by three different techniques to identify without ambiguity the same antigen, i.e., virus particle type: ELISA, electron microscopy of immuno-precipitates (IPEM) and Western analysis. Suitability for ELISA simplifies large-scale investigations. Antibodies that either precipitate, aggregate or decorate virus particles are useful for the physical identification of the antigen by electron microscopy. Activity in Western analysis allows further identification of the virus by the characteristic molecular weight of its coat protein. In order to compare GLRaV II serologically with other GLRaV isolates, we also developed antisera and monoclonal antibodies to two GLRaV's from leafroll diseased Thompson Seedless and Emperor grapevines imported in 1985 from North America (C. F. Luhn, Davis, California). They were maintained on their own roots in the grapevine virus collection vineyard at Nyon to avoid contamination through grafting. In analogy with data of HU *et al.* (1990) (5), we called the corresponding virus isolates GLRaV IV and GLRaV V (table 1). The production of MCA's to GLRaV I, GLRaV III and GVA 784 were reported earlier (4).

Purification of virus particles, immunization and production of monoclonal antibodies, antibody processing, ELISA, IPEM and Western analysis were done as specified elsewhere in these extended abstracts (6).

Particles of GLRaV II of Chasselas 8/22 analyzed by ELISA, IPEM and Western immunoblots reacted with the homologous MCA's 29-1 but with none of the other MCA's listed in table 1. The apparent molecular weight of the coat protein was unexpectedly low: 26500 daltons. These results were essentially in agreement with those obtained with the analogous antisera (Gugerli, unpublished data). However, we observed, as expected, decorated and some non-decorated long closterovirus particles in GLRaV II preparations exposed to MCA 29-1 (Fig. 1). This is evidence for the complex composition of GLRaV II and for the existence of a further closterovirus-like entity in leafroll diseased cv. Chasselas. We called the component recognized by MCA 29-1 'GLRaV IIb' and the non-identified component 'GLRaV IIa'. Curiously, we not only observed non-decorated particles but also very frequently particles of which only one end, 50 to 100 nm long, remained non-decorated. Comparative serological investigations showed that the French antiserum to GLRaV II, prepared by Zimmermann *et al.* (4) (kindly provided by B. Walter, INRA, Colmar), also reacted with an antigen of the same size on Western immunoblots (in agreement with their results), whereas an American antiserum to GLRaV II (kindly provided by D. Gonsalves, New York State Agric. Exp. Station, Geneva) recognized in GLRaV II preparations of Chasselas 8/22 dominantly two antigens with molecular weights of 32800 and 54000 daltons. This latter result disagrees with the value given for the coat protein of the homologous American isolate (1).

GLRaV IIb was not only detected with MCA 29-1 in leafroll infected plants but also in all corky bark infected vines maintained in the grapevine virus collection at Nyon: Chasselas 8/7, Ln33, Gamay Rouge de la Loire and Semillon (V.C.A3v7, kindly provided by C.F. Luhn, Davis, California). This is some evidence for an association between GLRaV IIb and corky bark disease. The symptoms associated with GLRaV II infections on Gamay Rouge de la Loire, mentioned above, might also support this hypothesis.

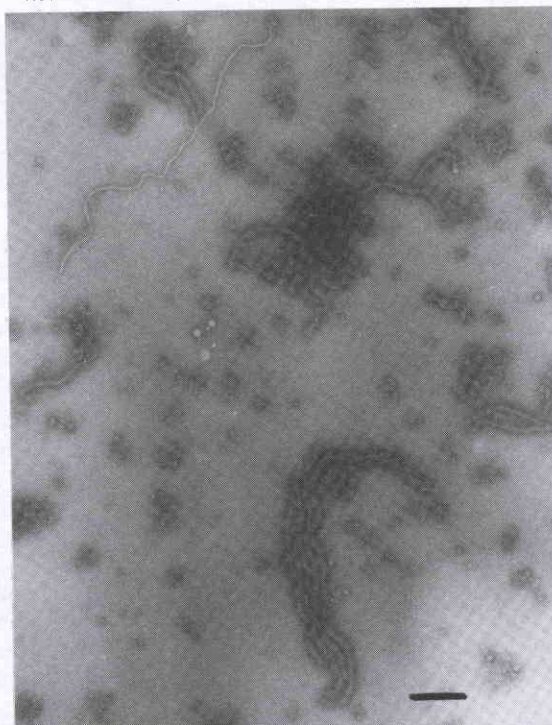
In conclusion, these results partially clarify the conflicting results about GLRaV II. The original GLRaV II was in fact not a pure virus isolate. The homologous antiserum could therefore react with more than one virus, which is a great disadvantage of many presently available 'polyclonal' antisera. The French antiserum to GLRaV II reacts strongly with the Swiss GLRaV IIb isolate, which is characterized by the low molecular weight of its coat protein and a very visible cross-banding seen by electron microscopy (Gugerli, unpublished results). This virus might not only be associated with leafroll on some cultivars but also with corky bark

disease. GLRaV IIa, the other component of GLRaV II of Chasselas 8/22 needs still to be characterized. The monoclonal antibodies developed during this study not only improve future diagnosis but also confirmed more precisely the characteristic molecular weights of GLRaV's I to V.

Table 1. Monoclonal antibodies to GLRaV's and molecular weights of their coat proteins determined by Western analysis.

No MCA	Source of immunogen (cv., clone, origin)	Closterovirus	Mol. wt of coat protein (daltons)
2-4	Räuschling 24, Switzerland	GLRaV I	38800
29-1	Chasselas 8/22, Switzerland	GLRaV IIb	26500
8	Frappato Mortillo, Italy	GLRaV III	44300
3-1	Thompson Seedless V.C.A2v22 (LDS clone from B7v9) Davis, Calif.	GLRaV IV	34700
43	Emperor V.C.A2v18 (White Emperor from Fresno v2) Davis, California	GLRaV V	35600
14-9	Italia, Italy	GVA 784	25100

Figure 1. Immuno-precipitated GLRaV II particles: GLRaV IIb decorated with MCA 29-1 and GLRaV IIa non-decorated (bar = 100 nm).



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## A COMPARISON OF GRAPEVINE VIRUS B ISOLATES

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Grapevine virus B (GVB) is a newly described mechanically transmissible virus, associated with corky bark disease (1). Its particles are very flexuous filaments about 800 nm long, morphologically similar to, and showing the conspicuous cross banding of, closteroviruses. However, the presence of a polyadenylated 3' end in the genomic RNA (P. Saldarelli, unpublished data) suggests that GVB does not belong in the *Closterovirus* genus.

An investigation is in progress, in which nine GVB isolates of different geographical origins are being comparatively studied among themselves and with an isolate of grapevine virus A (GVA). Viral isolates are: GVB-BA and GVB-LN33 (Apulia, Italy); GVB-SS, GVB-SS-A, GVB-SS-B, GVB-SS-C, GVB-SS-D (Sardinia, Italy); GVB-Se and GV-LRNOV (USA), GVB-M (Canada).

The preliminary results of these investigations have shown that:

(i) GVB was recovered by mechanical inoculation from slightly more than 60% of the vines indexing positive for corky bark on LN33 indicators;

(ii) All isolates were readily transmitted to a range of herbaceous hosts restricted to a few *Nicotiana* species. Based on host responses, two biological variants were identified: isolates that induce a more or less severe necrotic reaction in *Nicotiana occidentalis* and *N. cavicola* (GVB-SS-D and GV-LRNOV), and isolates (all others) that in the same hosts elicit primarily chlorosis and vein clearing. Local and systemic infections were obtained also in *N. benthamiana*, but the extent of successful transmission and the intensity of symptomatological responses varied with the season;

(iii) In immunoelectron microscopy tests, all isolates were recognized and decorated by antisera to GVB-Se and GVB-BA, including isolate GVB-SS-D and GV-LRNOV. However, the impression was that the decoration of the particles of the latter two viruses was rather patchy. None of the GVB isolates was recognized by GVA antisera, nor GVB antisera decorated GVA particles;

(iv) All isolates had a single type of coat protein subunits which migrated at the same rate in 12% SDS-PAGE (Mol. wt about 23 kDa), except for GVB-SS-D, whose subunits showed a slightly faster migration, comparable with that of GVA coat protein (Mol. wt about 22.5 kDa).

(v) Electrophoretic dsRNA patterns from infected *Nicotiana* species were the same for all GVB isolates. These patterns were clearly distinguishable from those of GVA both in electrophoresis and by molecular hybridization (Northern blots).

(vi) No differences were observed in the cytopathology of *Nicotiana* species infected with the different GVB isolates;

(vii) A cloned DNA probe complementary to genomic RNA sequences of isolate GVB-Se (2) hybridized equally well with the homologous template and all other isolates that induced chlorosis and vein clearing in *Nicotiana* species. The much lighter hybridization signal previously observed with GVB-SS-D (2) was confirmed, but repeated tests in lower stringency conditions, showed that the probe hybridizes also with isolate GV-LRNOV. The same probe did not recognize GVA RNA sequences in any of the experiments performed.

The comparative studies have confirmed that GVA and GVB, which are likely to belong to the same genus because of the similar particle morphology and comparable biological and epidemiological behaviour, are serologically and molecularly distinct. However, whereas GVA isolates appear to be relatively uniform biologically and, in part, serologically (only minor serological variants have been identified), GVB exhibits a wider variability. In the present investigations, this variability was clear-cut in the reaction of herbaceous hosts and at the molecular level, but it may be expected to appear also at the serological level, when better reagents (high titre polyclonal antisera and monoclonal antibodies) will be available.

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# ASSOCIATION OF A NON MECHANICALLY TRANSMISSIBLE ISOMETRIC VIRUS WITH ASTEROID MOSAIC OF GRAPEVINE.

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Asteroid mosaic is a long known graft-transmissible virus-like disease of the grapevine that elicits characteristic localized clearing of the main and secondary veins on the leaves of the indicator *Vitis rupestris* (2). This disease was originally described in California (3) from where it is now disappearing (A.C. Goheen, personal communication). In *Vitis vinifera*, asteroid mosaic-like symptoms were repeatedly observed in Europe and South Africa (6) but no published experimental evidence was provided of their graft transmissibility to *V. rupestris*. New symptomatological observations from Greece (4) and successful graft transmission trials (P. E. Kyriakopoulou, unpublished results) constitute the first convincing proof of the occurrence of asteroid mosaic outside USA.

In the assumption that the agent of asteroid mosaic is a virus, investigations were initiated with the authentic Californian source. Infected *V. rupestris*, cuttings were propagated in a glasshouse in Bari and used for transmission and extraction of the putative virus.

Whereas the typical symptoms of the disease were readily reproduced in *V. rupestris* by chip bud grafting, no success was obtained in repeated attempts to inoculate a herbaceous host range with expressed sap from symptomatic leaves or young roots, in the presence of various types of extraction buffers.

Extensive electron microscope observations of thin sectioned tissues excised from roots and leaves of infected *V. rupestris* were equally negative. Nevertheless virus purification was attempted from *V. rupestris* applying slightly modified versions of the technique used for the recovery of grapevine fleck virus (GFkV) (1). Donor materials were whole symptomatic leaves, main veins freed from adjacent tissues, petioles, cortical scrapings or young roots, which were processed separately. Tissues were either homogenized in an extraction buffer prior to enzyme treatment (1% pectinase and 2% cellulase) or were ground in liquid nitrogen and homogenized with no subsequent enzymic treatment. Alternate cycles of low- and high-speed centrifugation and sucrose density gradient centrifugation followed.

Concentrated preparations before or after density gradient centrifugation contained isometric virus-like particles of two types: empty shells penetrated by the stain and apparently intact particles. Empty shells were significantly more numerous when tissues had been frozen in liquid nitrogen. The particles had a rounded outline, a diameter of about 30 nm, and resembled very much those of GFkV. The source material, however, was not infected by fleck nor by any of the known grapevine nepoviruses, as ascertained serologically (ELISA) and by mechanical transmissions. Concentrated, partially purified virus preparations did not react in ELISA with monoclonal antibodies or a polyclonal antiserum to GFkV, nor were they decorated by the same antiserum. The same preparations were not decorated nor did they react in immunodiffusion tests with an antiserum to a virus associated with grapevine Ajinashika disease (5).

These results, however preliminary as they are, indicate that a non mechanically transmissible, novel isometric virus is associated with asteroid mosaic. This virus resembles morphologically GFkV but is serologically distinct from it. In this connection it is worth noting that fleck and asteroid mosaic are the only two diseases of *Vitis* that have *V. rupestris* as the sole indicator, which reacts to both with somewhat similar responses.

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## POTATO VIRUS X IN TUNISIAN GRAPEVINES

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Two biologically distinct strains of potato virus X (PVX) were recovered by inoculation of sap from vines of cv. Carignan and Grenache in two different Tunisian localities, north (Ain-Ghellal) and south (Sidi Messaud) of Tunis, respectively. Infected vines were either apparently symptomless, or showed vein banding, sometimes accompanied by reddish rings.

The two viral isolates differed from one another because of the symptomatological reactions induced in some herbaceous hosts, i.e. chlorotic-necrotic ringspots versus chlorotic mottling in *Nicotiana* species, but had comparable physicochemical properties, serological and intracellular behaviour. They were compared with, and found indistinguishable from, an authentic PVX strain from potato.

PVX was detected by ELISA in about 4% of 180 samples collected at Ain-Ghellal and was reinoculated successfully into grapevine rootlings by subcortical injections, but not by mechanical inoculation to seedlings.

PVX had already been recorded from grapevine in Italy, but it was an occasional isolation from a single plant of cv. Barbera (1). As in the present instance, the Italian PVX isolate could not be reinoculated to grapevine seedlings. Although this may be taken as an indication that PVX is little pathogenic to *Vitis*, the Tunisian data on virus distribution in the field seem to support the notion that PVX infections in grapevines may no longer be regarded as a mere scientific curiosity.

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# A REVISED ESTIMATE OF THE SIZE OF THE CAPSID PROTEIN OF GVA

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Grapevine virus A (GVA) was initially isolated from a vine of *Vitis vinifera* L. cv. Pigato showing stem pitting symptoms (2). The virus was propagated in *Nicotiana clevelandii* Gray, from which it was purified and characterized (1). The size of the capsid protein (CP) of GVA was reported to be about 22 kDa (1), but this value has not been independently verified. The objective of the experiments reported here was to estimate the size of the CP of three GVA isolates.

The GVA-infected grapevines used in this study were *V. vinifera* 'Limberger', 'Müller-Thurgau' (1/1 GM) and 'Kadarka'. In vitro shoot tip cultures of these grapevines were established and maintained as described elsewhere (6). ELISA and ISEM were used to verify that the cultures were GVA-infected. GVA was then transmitted from cultures of each vine to *N. benthamiana* seedlings, using a procedure already reported (9). In vitro node cultures of the symptomatic *N. benthamiana*, established and maintained as previously described (7, 8), were used as starting material for the purification of the GVA isolates (7, 8). Purified virus was resuspended in 0.02 M phosphate buffer, pH 7.2. CP was extracted from the purified virus preparations by an SDS-KCl method (5).

Samples consisting of either SDS-KCl extracted CP or purified virus were analyzed by SDS-PAGE on 15% polyacrylamide gels using the method of Laemmli (4) or a modified Neville method (3). The gels were fixed and stained using a Bio-Rad silver stain kit. Silver Stain SDS-PAGE Standards (Bio-Rad) were used to estimate the molecular weight (MW) of the GVA CP. Replicate gels were electroblotted onto nitrocellulose membranes for Western analysis (11) using rabbit anti-GVA serum prepared in our lab (8).

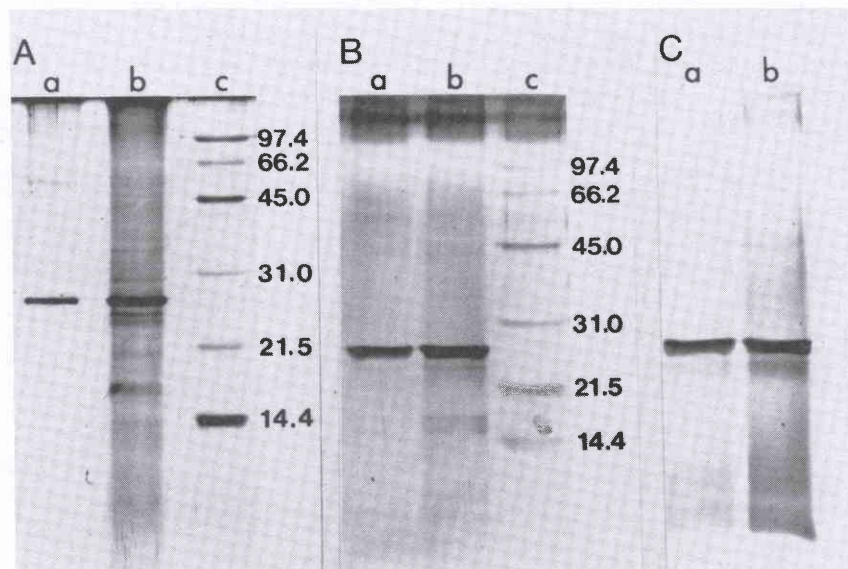


Figure 1. SDS-PAGE in 15 % polyacrylamide gels, using the method of Laemmli (A) or a modified Neville method (B,C), of SDS-KCl extracted capsid proteins (lane a) and purified virions (lane b) of GVA isolated from *V. vinifera* 'Limberger'. Gels in A and B were silver stained. Lane c contains Bio-Rad molecular weight marker proteins, with sizes (kDa) indicated on the right. Gel C shows a Western blot using rabbit anti-GVA serum. Lane a contains SDS-KCl extracted GVA CP. Lane b contains purified GVA.

The SDS-KCl extract from purified GVA contained only one major band (Fig. 1A and B, lane a), which consistently had a MW of about 26.4-26.5 kDa, using either electrophoresis method. This band corresponded in size to the major band detected in the purified GVA preparation (Fig. 1A and B, lane b). It was definitively identified as GVA CP, as it was clearly detected in both the SDS-KCl extract and in the purified virus

preparation by Western analysis of replicate gels using our anti-GVA serum (Fig. 1C). Fainter bands detected in the SDS-KCl extract by silver staining or Western analysis may be degradation products and aggregates of the GVA CP, or they may represent host contaminants. All three isolates of GVA had the same size CP.

Boccardo and d'Aquilio (1) reported a MW of about 22 kDa for the CP of GVA. They used two different SDS-PAGE methods for their determinations, one of which was that of Laemmli (4). They conducted sufficient replications to ensure the statistical validity of their results. The difference between their value and ours might be due to the different procedures used to extract CP from the purified virus, or it could be due to the use of different protein MW markers. The sources of the proteins used as MW markers by Boccardo and D'Aquilio were not indicated in their report. The MW value reported here was based on the relative migration in our gels of some widely utilized markers. The size of the viral CP is an important criterion for assigning viruses to subgroups (10). Commercial formulations of protein MW markers provide a convenient means of standardizing viral CP MW determinations from lab to lab. Their use would greatly facilitate the classification of closteroviruses infecting grapevines.

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# GENOME DIVERSITY OF FIELD ISOLATES OF GRAPEVINE FANLEAF VIRUS (GFLV) ANALYZED BY SINGLE STRANDED CONFORMATION (SSCP) AND RESTRICTION FRAGMENT LENGTH (RFLP) POLYMORPHISMS.

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The existence of a certain degree of variability between isolates of plant viruses is well known. However, due to difficulties encountered in defining criteria and methodologies suitable for screening a high enough number of isolates, diversity has not been sufficiently studied. In the last years the development of nucleic acid methodologies enabled studies on variability at the molecular level. From these studies, the concept of a particular virus as a "quasi species", i. e., a population of distinct genome sequences, is emerging (1). Most of these studies have been confined to "laboratory plants".

For viruses of woody hosts, studies at the genome level are hindered by the laborious protocols needed to extract intact viral RNA and, as a consequence, very little has been done in this field. In the case of Grapevine Fanleaf Virus (GFLV), Fuchs *et al.* (2) attempted to distinguish between isolates of distinct geographic origins using cDNA probes. This methodology did not prove to be sufficiently discriminating; it was suggested a significant homology between the nucleotide sequences of the analyzed isolates. On the other hand, the nucleotide sequence of the coat protein gene of two isolates showed a 90 % homology (3).

Due to the high number of copies of the viral genome that can be obtained by the Polymerase Chain Reaction (PCR), this technique is predestined for the preparation of viral genomes. Moreover, if preceded and coupled by an immunocapture step (IC/PCR), no laborious nucleic acid extractions are needed and a large number of samples can be analyzed (4, see companion paper in these abstracts). We combined IC/PCR with Restriction Fragment Length Polymorphism (RFLP) analysis and Single Stranded Conformation Polymorphism (SSCP) analysis, since this last technique is most suitable for the detection of point mutations (5). By using this approach we tried to assess the diversity of GFLV isolates from a vineyard subjected to infection in natural conditions.

## Material and methods.

The grapevine samples used in this analyzed by the IC/PCR technique in this study are described in a companion paper in these abstracts. SSCP analysis was performed according to Orita *et al.* (5); however DNA was detected by silver staining with no need of radioactive labelling. For RFLP analysis, the amplified product was digested with HAE III, MSP I and Hinf I. Each digestion was done with 5 units of enzyme and one fifth of the amplified product, according to the manufacturer's instructions. The data obtained from restriction profiles was analyzed by means of the NTSYS package. Similarity between samples was computed by the Sorensen's coefficient. The cluster analysis was performed by the UPGMA method.

## Results and discussion

SSCP analysis was performed to estimate the diversity of the nucleotide sequence of GFLV isolates collected in the same vineyard. The results, presented in Fig. 1, show that for most of them the nucleotide sequence was distinct from the nearby isolates. It is conceivable that in each plant coexists a mixture of strains irregularly distributed. If this is true, then the variability we noticed could be an illusory effect of picking distinct strains from a mixture that could be similarly in nearby plants. The uniformity of strain distribution within the same grapevine plant was tested by collecting small tissue pieces (about 30 mg) in distinct canes of each of a few plants and amplified separately. SSCP analysis revealed that all the samples from each plant had the same electrophoretic profile, thus reinforcing the idea of the existence of distinct strains in each plant.

As SSCP analysis can not quantify the extent of the genomic differences between isolates, RFLP analysis was assayed. Digestion of the amplified product with restriction enzymes confirmed the results already obtained with SSCP. A more precise idea of the relationship between the isolates was obtained from cluster analysis of the restriction profiles (Fig. 2). It is rather surprising to observe so much diversity between isolates of GFLV inside the same vineyard, and subjected to natural infection. On the other hand, some of the isolates showed a closer relationship to isolates from a distinct geographic region than to the nearby ones. Within the sampling area in the vineyard we did not find any correlation between the matrix of Sorensen's similarity coefficients and the matrix of Euclidean distances between the plants from where the samples were collected, suggesting little or no effect of the nematode transmission on the homogenization of strains of GFLV in the field.

Variability is one of the most important aspects of plant virology; it needs simplified methodology for its

assessment. SSCP and RFLP analysis can contribute to fill the gap and, to our knowledge, the former one has not been used in the comparison of virus isolates at the genomic level. Coupled to IC/PCR they proved to be very convenient methods for quick identification of virus strains.

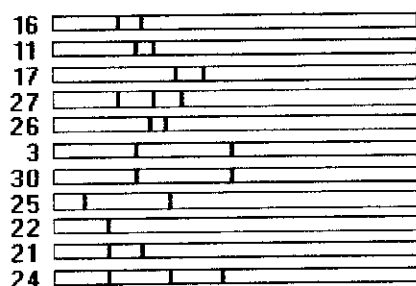


Fig. 1. Single Stranded Conformation Polymorphism. Scheme of the location of bands in the polyacrylamide electrophoresis gel. The electrophoresis was from left to right. Numbers correspond to different samples collected in the same vineyard, inside a 20 m x 12 m area.

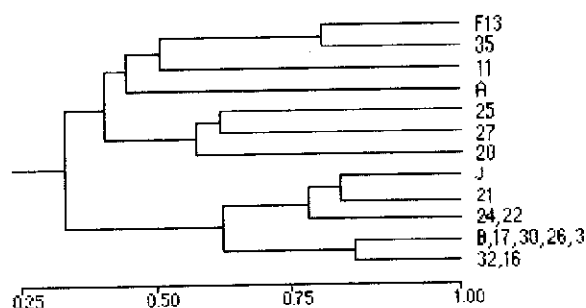


Fig. 2. Dendrogram constructed from RFLP data, representing the degree of relationship between GFLV isolates. All numbered samples are from the same vineyard located in Algarve, Portugal. Samples A and B are from Estação Agronómica Nacional (Oeiras, Portugal). Sample J is a cloned coat protein gene from an isolate of Davis, California (3). F13 is an isolate sequenced by Serghini et al. (6); in this case the restriction profile was deduced from the nucleotide sequence.

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# SEROLOGICAL COMPARISON OF SOME ARABIS MOSAIC VIRUS AND GRAPEVINE FANLEAF VIRUS ISOLATES

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Grapevine isolates of arabis mosaic (ArMV) and grapevine fanleaf (GFLV) virus are serologically closely related viruses, showing no or little differences (3). ArMV has a wide natural host range and usually shows a wide array of serological variants (5), while GFLV has a strict adaption to a single host which explains the serological similarity among GFLV isolates.

Recently Savino *et al.* (6) isolated a natural serological variant of grapevine fanleaf virus from Tunisia, indicating that a serological variant of this virus may arise and become established. Serological variants of ArMV and GFLV - like in other cases, tobamoviruses, cucumoviruses etc. - may influence the reliability of detection by ELISA technique.

To study serological relationship and serological variants of arabis mosaic virus and grapevine fanleaf virus some isolates (originated from Bulgaria, France, Hungary and Slovakia) were compared.

The viruses - all originally isolated from naturally infected *Vitis vinifera* L. - were as follows: ArMV-TYV-1, ArMV-AbYV-2 and GFLV-BBFL-1 isolates from Hungary, ArMV-Bra from Slovakia (G. Vanek), ArMV-862 from Bulgaria (M. Yankulova) and ArMV-T69 Colmar from France. (All the isolates, except ArMV-862, were from collection of Lehoczy J.).

All the isolates were propagated in *Chenopodium quinoa* L. plants. Antisera to ArMV-TYV-1 and GFLV-BBFL-1 were prepared. Antiserum to GFLV-PFL-16 isolates was prepared previously (1).

Immuno-diffusion tests were performed in 0.8 % agarose in 40 m sodium barbital buffer pH 8.6. ELISA tests were performed as described by Clark and Adams (2) (DAS-ELISA) or Edwards and Cooper (4) (PAS-ELISA).

Homologous and heterologous titers in double-diffusion tests obtained with antisera GFLV-PFL-16, GFLV-BBFL-1 and ArMV-TYV-1 are presented in table 1. For each antiserum-antigen combination the serological differentiation index (SDI) was calculated.

Table 1 Antisera titers and serological differentiation indices (number in brackets) among GFLV and ArMV isolates.

Antisera	Antigen					
	GFLV-BBFL-1	ArMV-TYV-1	ArMV-T69	ArMV-Bra	ArMV-AbYV-2	ArMV-862
GFLV-PFL-16	64 (1)	8 (4)	16 (3)	16 (3)	8 (4)	8 (4)
GFLV-BBFL-1	1024 (0)	128 (3)	32 (5)	32 (5)	64 (4)	8 (7)
ArMV-TYV-1	128 (3)	1024 (0)	256 (2)	512 (1)	256 (2)	256 (2)

The results showed that GFLV and ArMV isolates could be differentiated on the basis of immuno-diffusion test. The differences were dependent on virus isolates.

In DAS-ELISA ArMV-TYV-1 and GFLV-BBFL-1 could not detect the heterologous virus (average SDI = 3). In other cases - ArMV-TYV-1 conjugate and ArMV-T69, ArMV-Bra, ArMV-AbYV-2 and ArMV-862 antigens - the reliability of detection in some circumstances (low virus titer etc.) could be uncertain.

In PAS-ELISA heterologous ArMV isolates could be detected more reliably.

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# CURRENT STATUS OF RESEARCH ON GRAPEVINE VIROIDS

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Viroids were first identified through biological expression as the causal agents of plant disease. However, the physical-chemical aspects of grapevine viroid detection, isolation and characterization has advanced more rapidly than the understanding of the intrinsic biological activity of viroids and the consequences on vine performance. This fact has been due, in part, to the lack of alternate herbaceous hosts and the initial lack of viroid-free control vines.

The organizational scheme for grapevine viroids first proposed and accepted in 1990 at the 10th Meeting of the ICVG (1) has gained general acceptance in the viroid literature (2) as presented in Table 1.

TABLE 1. TOWARD A CONSENSUS NOMENCLATURE FOR THE GRAPEVINE VIROIDS

Group	Synonymous Designations	Nucleotide Number	Disease	Grapevine Viroid
1	GVd-s, CEVd-g	369	(NR)	CEVd-g
2	AGVd GVd-g	369 (NR)	(NR) (NR)	AGVd GVd-g
3	GVd-f, GVd-1, GYSVd GVd-2, GVd-1B	367 363	YELLOW SPECKLE YELLOW SPECKLE	GYSVd-1 GYSVd-2
4	HSVd-g (Riesling), GVd-3 NSVd-g (Japan), GVd-3	298 297	(NR) (NR)	HSVd-g

(NR) = no report

The relationships originally proposed from physical properties and molecular hybridization reactions with viroid-specific probe, have been reinforced by additional comparative studies (3) employing the polymerase chain reaction (PCR) analysis (Table 2).

TABLE 2. COMMON NUCLEOTIDE SEQUENCES OF GRAPEVINE VIROIDS IDENTIFIED BY POLYMERASE CHAIN REACTION (PCR) ANALYSES AND VIROID SPECIFIC PROBES

Cloned Viroid Probes*						
Group	Grapevine Viroid	Viroid Residues	PCR Product* Residues	GYSVd-1	HSVd-g	AGVd
1	CEVd-g	369	---	--	--	--
2	AGVd	369	375	--	--	+
3	GYSVd-1	367	223	+	--	--
	GYSVd-2	363	363	+	--	--
4	HSVd-g	297	297	--	+	--

\* Data reported by Rezaian *et al.*, 1992, Intervirology 34, 38-43.

Although the viroids as a group have elicited much interest as unique, small RNA molecules with unusual structural features, the biological activity of viroids to alter the "normal" plant growth and development remains

the most dramatic and definitive property of the viroid molecule. It has become evident that viroid replication is not inexorably tied to viroid pathogenesis and the production of disease symptoms.

The grapevine viroids (GVd) occupy an unusual niche among the viroids in that:

- 1) the GVd's are spread worldwide in a virtual ubiquitous manner (4),
- 2) only yellow speckle (YS) has been confirmed as a viroid induced disease of vines which is expressed principally in Australia (5).

Nevertheless, this nominal disease threat attributed to viroids does not obviate the fact that, **every vine response including viticultural characters, disease expression, indexing reaction, and even wine quality is observed through a viroid background.** Acknowledgement of this statement supports the importance of the clarification of the biological potential of grapevine viroids. This remains as the primary challenge on the scene of grapevine viroid research today.

Analysis of 24 cDNA clones of GYSVd-1 indicates a high frequency of sequence variation which occur principally in the pathogenic (P) domain (6). A comparison of clones of GYSVd-2, which also induces yellow speckle symptoms, did not display a similar sequence variability centered in the P domain. A review of the properties of GYSVd-1 and GYSVd-2 (Table 3) and particularly the common ability to induce YS symptoms between two viroids sharing only 73% sequence homology challenges the implied relationship as "strains". By convention it is generally accepted that related strains share a 90% sequence homology.

TABLE 3. COMPARISON OF GRAPEVINE VIROIDS INDUCING YELLOW SPECKLE SYMPTOMS

	GYSVd-1	GYSVd-2
1) Synonyms:	GV-f; GV-1; GYSV	GV-2; GV-1b
2) Residues:	367	363
3) Homology for GYSVd-1:	100%	73%
4) Symptom Expression:		
a) grapevines:	yellow speckle	yellow speckle
b) other host:	none	none
5) Occurrence in vines:		
a) frequency all vines:	85%	13%
b) predominant vines:	wine	table

This review prompts a closer examination on the subject of the "variable" and "erratic" expression of yellow speckle symptoms which has frequently been repeated in the YS literature. "One of the most remarkable features of GYS is the extreme variability in symptom expression" which is noted "...following an exceptionally hot summer" (7). It has been reported that 38% of the cultivars are YS positive by self expression and 24% more by indexing, yet approximately 85% of the vines from California and European sources carry one of the two viroids implicated in the yellow speckle disease.

Contributing to this perspective is the statement that "...the leaf symptom associated with vein banding disease are due to a yellow speckle infection, INTENSIFIED by co-infection with fanleaf virus" (8).

What then exactly is the YELLOW SPECKLE disease? And what is the relationship of yellow speckle to the VEIN BANDING syndrome?

- a) Has the YS syndrome been accurately described as a simple disease expression induced by two different viroids?
- b) Does a complex situation exist with multiple disease expressions described under the single generic term "yellow speckle"?
- c) Does GYSVd-1 comprise a population of size related viroids only some of which are competent to induce YS or VB symptoms?
- d) Is YS an acceptable symptom expression induced by GYSVd or a "stress condition" aggravated by the presence of viroids? Is the prime factor in the expression of YS the viroid or climate?
- e) Are "yellow speckle" and "vein banding" (VB) distinct diseases or degrees of severity?

In any discussion of YS and VB as expressions of physiological modifications heightened by the presence of viroids, the consideration can be introduced that viroids might not constitute consummate pathogens but offer a potential for controlling some aspect of vine growth and development (9). Since the viroid is not integrated into host genome and does not contribute genetic information which is translated into protein, the viroid apparently acts by influencing the expression of the normal host genome. In essence, this process may involve exploitation of the inherent potential for variation that resides in the vine. The fact that viroids are currently being used to dwarf citrus in commercial plantings suggests that this procedure may provide a practical tool for "customizing" vine growth.

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# PERFORMANCE OF VIROID-FREE CABERNET SAUVIGNON VINES

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California grapevine varieties and rootstocks contain one or more of the three principal grapevine viroids (GYSVd-1, GYSVd-2, and HSVd-g). The effects of viroids on vine performance and wine quality has been impeded by the lack of viroid-free true to type varieties. Performance of the first field trials in the world with viroid-free grape varieties is continuing into the sixth growing season at the Oakville Experiment Station in the Napa Valley, California

In this study, own-rooted Cabernet Sauvignon vines with three different viroid profiles were tested for vine growth, yield, fruit maturity and wine quality. Vines were planted in 1988 on a spacing of 2.4 m X 3.6 m (vine X row) and trained to a bi-lateral cordon, spur-pruned system.

Viroid-free vines were obtained by shoot-tip culture (STC). Vines propagated from this viroid-free source were then inoculated with GYSVd-1, GYSVd-2, and HSVdg. Cabernet Sauvignon Selection #4, certified tested from the Foundation Plant Materials Service, U.C. Davis containing naturally-occurring HSVd-g was used as reference material.

Table 1. Effect of viroid content on growth and yield of own-rooted Cabernet Sauvignon grapevines, Oakville, Napa Valley, Ca, 1992

Viroid status	Prunings (kg/vine)	Shoot numbers	Shoot wt. (g)	Yield (kg/vine)	Yield/ prunings
1) STC (viroid-free)	3.72 a	33.4 a	112 a	9.97 a	2.73 b
2) STC + GYSVd-1 + GYSVd-2 + HSVd-g	3.16 ab	34.0 a	93 a	9.95 a	3.16 ab
3) Selection #4 + HSVd-g	2.88 b	32.9 a	87 a	10.96 a	3.83 a

Mean separation by Duncan's multiple range,  $p < 0.05$ .

TABLE 2. Effect of viroid content on yield components of own-rooted Cabernet Sauvignon grapevines. Oakville. Napa Valley. Ca. 1992

Viroid status	Clusters/vine	Cluster wt (g)	Berries/cluster	Berry wt (g)
1) STC (viroid-free)	64.4 a	155 ab	133 ab	1.16 a
2) STC + GYSVd-1 + GYSVd-2 + HSVd-g	67.4 a	147 b	122 b	1.21 a
3) Selection #4 + HSVd-g	63.0 a	174 a	141 a	1.24 a

Table 3. Effect of viroid content on juice maturity indices of own-rooted Cabernet Sauvignon grapevines, Oakville, Napa Valley, CA, 1992

Viroid status	°Brix	Titrateable acid (g/l <sup>-1</sup> )	pH
1) STC (viroid-free)	22.7 a	8.8 a	3.10 ab
2) STC + GYSVd-1 + GYSVd-2 + HSVd-g	22.7 a	8.4 a	3.15 a
3) Selection #4 + HSVd-g	22.5 a	9.0 a	3.06 b

In both 1991 and 1992, measurements were made of vine yield components, juice maturity indices, and dormant season cane pruning weights. In 1991, vine harvest data showed that reference material had the greatest yield (12.2 lbs/vine) when compared to the viroid-free (8.9 lbs/vine) and the triple inoculated vines (10.7 lbs/vine). The low yield of viroid-free material was a result of fewer berries/cluster. The reference vines were slower to mature resulting in a harvest date several days later than the other two treatments. In the 1992 harvest, the crop weights were not significantly different. No disease symptoms of yellow speckle or vein banding have been observed to date. Test wines were made from the harvest of each treatment and are being subjected to chemical and sensory evaluation as well as a vertical comparison to the test wine of 1991.

## THE ROLE OF GRAPEVINE VIROIDS IN YELLOW SPECKLE AND VEIN BANDING DISEASES

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The role of viroids as agents of plant diseases is well established. The ubiquitous distribution of viroids in vines throughout the world, however, raises questions as to the role of viroids as primary causal agents or as factors in disease expression. Although the two viroids GYSVd-1 and GYSVd-2 have been so designated for inducing yellow speckle (YS) symptoms on grapevines in Australia, it has not yet been possible to confirm a simple cause-effect relationship between YS symptoms and a single viroid for vines in California. Experiments have been initiated to evaluate the role of grapevine viroids as the causal agent of yellow speckle disease and the expression of vein banding (VB) symptoms in association with grapevine fanleaf virus.

With these circumstances and a review of the properties of yellow speckle and vein banding diseases, a more complex etiology for viroid induced grapevine diseases might be entertained. The extreme variability in YS and VB symptom expression plus the erratic occurrence of the diseases, which usually appear following an exceptionally hot period, might indicate primarily a stress condition which is only aggravated by the presence of the viroid. Is then the climate or viroids the prime factor inducing yellow speckle symptoms? A controlled environment regime to promote YS symptom development has been used to test the following materials.

Cultivar	Viroid content	Yellow speckle
Cabernet Sauvignon	viroid-free	unknown
" "	GYSV-1, GYSV-2, HSV-g	"
" "	GYSV-1	"
" "	GYSV-2	"
Sauvignon blanc	viroid-free	"
" "	GYSV-1, GYSV-2, HSV-g	"
Cabernet franc	GYSV-1	"
039-16	GYSV-2	"
Zinfandel	GYSV-1 and HSV-g	"
Mission	GYSV-1 and HSV-g	+ + + +

Synergism among pathogenic agents is not an uncommon occurrence. It is important to recognize that because of the virtual ubiquitous occurrence of viroids in grapevines, all vine responses including disease expressions are viewed against a viroid background and potentially modulated by them. The origin of the vein banding symptom has evoked some differences of opinion and has been recognized as either a response to fanleaf alone or as the result of a mixed infection of yellow speckle with the presence of fanleaf.

To test the hypothesis that grapevine fanleaf virus interacts with viroids to induce vein banding, a field trial using miniplots has been established. *Xiphinema index* from GFLV infected vines have been inoculated to both Cabernet Sauvignon and Sauvignon blanc in the presence and absence of the major grapevine viroids, GYSV-1, GYSV-2, and HSV-g.

With these tests in place a number of considerations can be discussed for the definition of grapevine viroids and the role of viroids in expression of 'disease' symptoms.

(1) Is yellow speckle a single disease caused by GYSVd-1 or GYSVd-2 which is present in over 85% of all grapevines or an amalgam of different diseases, the distinct symptoms of which have not yet been adequately defined?

(2) Is the vein banding symptom induced by yellow speckle viroid in the presence of fanleaf disease or by a yet undefined component of the GYSVd-1 population, i.e., a putative grapevine vein banding viroid or GVBVd?

Since every viroid population is comprised of variants it is difficult to establish the presence of physically

distinct sub-populations which may be responsible for the different symptom expression. Nevertheless, analysis of viroids derived from tissue expressing YS and VB symptoms was undertaken to investigate the presence of population heterogeneity which might be linked to disease expression.

A difference in the titer of the 'GYSVd-1 like' viroid was noted between non-symptomatic and YS or VB expressing tissues. A GYSVd-2 like viroid was never found in association with YS expressing tissues. No molecular distinctions could be detected by sPAGE, however, heterogeneity was displayed in apparent full-length cDNA products from PCR amplification with GYSVd-1 specific primers.

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## GRAPEVINE ASTEROID MOSAIC IN GREECE: POSITIVE INDEXING RESULTS AND VIROIDS ASSOCIATED

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Symptoms of grapevine asteroid mosaic (Hewitt 1954) (2) were reported in Greece in 1990 (Kyriakopoulou, 1991) (3). They were observed in Soultanina variety in mountainous Corinthia, close to Ancient Nemea. Indexing studies followed in the years 1991 and 1992. Canes collected from affected vines were indexed, either by grafting the indicator *Vitis rupestris* St. George on them, or by letting them grow in the greenhouse and observing symptoms on their leaves (self indexing). The grafting trials were performed both, in the spring of 1991 with indicator material from Colmar, France, and in 1992 with indicator material from Bari, Italy; grafting was performed in March and the grafted canes were put to root and sprout in the greenhouse. Self indexing consisted of rooting the affected Soultanina canes in the greenhouse and observing in the field symptoms on their own growth throughout the 2 years. Both types of indexing trials gave consistently results positive for grapevine asteroid mosaic (Kyriakopoulou, 1992) (4). St. George indicator gave the typical reaction, i.e. vein clearing-yellowing on its first new growth in May, both in the 1991 and in the 1992 trials. Self-indexed Soultanina gave consistently the typical symptoms of the disease on its leaves during both growing seasons, 1991 and 1992. These data are considered as showing the existence of the disease of grapevine asteroid mosaic in Greece.

Leaves with typical symptoms from self-indexed Soultanina were examined for the presence of viroids after analysis of total nucleic acid extracts. Two methods were used:

a) bidirectional electrophoresis (Schumacher *et al.*, 1983) (5) followed by silver staining and

b) hybridization on RNA blots using radioactive probes specific for the viroids HSVd, PSTVd, CEVd, GYSVd (Tsagris *et al.*, 1990) (6). Strong bands showing hybridization were detected with the HSVd and GYSVd probes at high stringency, while no signals could be detected by the CEVd and PSTVd probes. The correlation of the two viroids found in the asteroid mosaic affected grapevine tissues with the respective symptomatology is not known.

Authentic grapevine asteroid mosaic from California has recently been found associated with a non mechanically transmissible isometric virus (Boscia *et al.*, 1993) (1). The Greek material is to be subjected to the same examination.

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## INFLUENCE OF VIRUS AND VIRUS-LIKE DISEASES OF GRAPEVINE IN SHOOT CULTURES.

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### Introduction

*In vitro* culture of various species and cultivars of *Vitis* has become a well established practice in commercial industry (1). A good sanitary status of initial explants is the prerequisite either for producing large scale clonal propagated healthy plantlets or for minimizing complications during rooting, acclimatization and establishment of plantlets in the greenhouse. The purpose of this work was to evaluate how virus and virus-like diseases could influence *Vitis vinifera* micropropagation.

### Materials and methods

Grape material source: Dormant cuttings were collected from infected and healthy vines (table 1) and ten plants of each were rooted and grown in a greenhouse. All tissue culture explants were obtained from these plants. The sanitary status of initially selected material was ascertained by woody indexing, ELISA and ISEM.

Table 1. Grapevine clones grown *in vitro*

Variety	Clone	Sanitary status	Diagnostic method
Trebbiano toscano	LRF	RW, GFLV	woody test, ELISA
Trebbiano toscano	ENC	GLRaV-II, III, GFLV	woody test, ELISA, ISEM
Trebbiano toscano	ENC-H	Healthy	woody test, ELISA, ISEM
Unknown	J1	GFLV	woody test, ELISA
Unknown	J1-H	Healthy	woody test, ELISA

Tissue culture. Single-node explants comprising an axillary bud were surface disinfected and placed in culture tubes containing Murashige and Skoog (1962) medium supplemented with 6-benzylaminopurine (BAP) (1.0 mg/l), sucrose (30 g/l) (medium A). Proliferating shoot cultures were subcultured onto fresh medium A at 4 week intervals. J1 and J1-H were let to grow and subcultured at 8 week intervals also on MS medium modified by Galzy (1964) and supplemented with sucrose (20.0 g/l) (medium B).

Both media were adjusted to pH 5.7 and solidified with 7 g/l Difco Bacto agar.

The effect of viral infection on plantlets was evaluated by determining the following parameters: percentage of survived shoots and number of internodes at the end of each subculture.

Monitoring of GFLV: ELISA was used to monitor the presence of GFLV on J1 shoots, grown on media A and B, at different stages.

### Results

Tissue culture: The morphological characters of infected plantlets maintained in culture were different when compared with those of healthy ones. In fact, the leaves were malformed and reduced in size and the whole plantlet had a bushy aspect. Yet, the tissues were highly vitrified and the callus production was increased. The survival and the number of internodes at the end of each subculture were different according to the sanitary status of shoots (table 2). In fact, the healthy control showed almost 100% of survived shoots at the end of each subculture, whereas diseased ones, as LRF, were reduced to less than 50%. Unexpectedly, GFLV reduced only the number of internodes and did not affect heavily the survival during the three subcultures. Similar growth was observed on healthy and infected J1 cultured on medium B.

Monitoring of GFLV: All J1 shoots, grown on media A and B, were infected. The virus was clearly detectable throughout the entire period examined. On both media the uninfected control gave an  $A_{405}$  value of 0.170. No marked differences in the virus content were observed in shoots grown on medium A (Fig. 3) and the ratio ' $A_{405}$  sample/ $A_{405}$  uninfected' was 5.6 for all samples. On the contrary, the virus content in shoots grown on medium B was not constant. The ratio ' $A_{405}$  sample/ $A_{405}$  uninfected' was 8.5 at the end of the first subculture and only 1.3 at the third one (Fig 3).

Table 2. Shoots and number of internodes obtained at the end of each subculture.

Clone	No (and %) of survived shoots			No of internodes		
	I sub.	II sub.	III sub.	I sub.	II sub.	III sub.
LRF	15/25 (60)	11/15 (73.3)	5/11 (45.4)	3.6	1.8	2.4
ENC	20/28 (71.4)	16/20 (80)	10/16 (62.5)	5.2	3.2	3.5
ENC-H	18/20 (90)	18/18 (100)	18/18 (100)	5.0	5.2	5.5
J1	21/21 (100)	21/21 (100)	19/21 (90.4)	4.5	3.4	2.9
J1-H	25/25 (100)	25/25 (100)	25/25 (100)	5.3	4.5	4.4

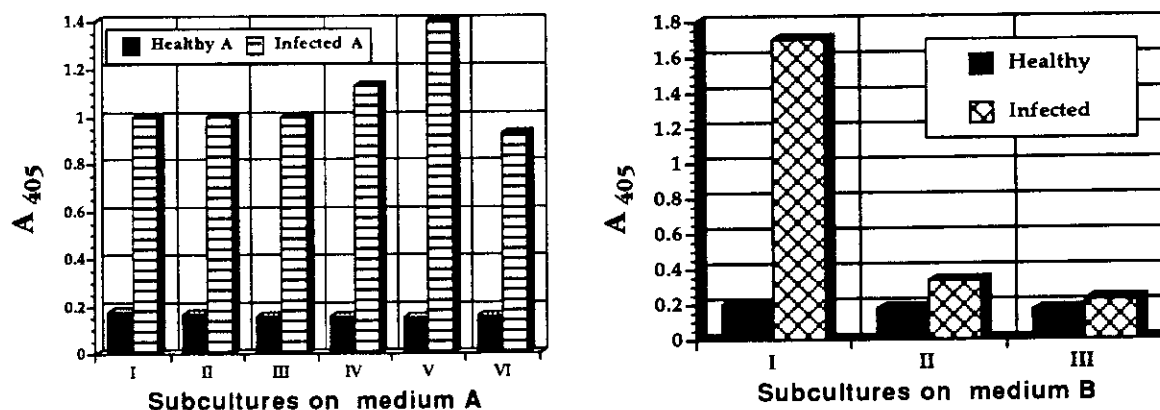


Figure 3. Virus content in J1 shoots grown on medium A and B.

#### Conclusion

Our preliminary results confirm the negative effect that viruses cause in grapevine micropropagation. In fact, infected plantlets are reduced in size, have malformed leaves and a reduced number of internodes. The survival is differently affected according to the virus.

GFLV concentration was constant during the six subcultures on medium A, confirming the results of Monette (2). On the contrary, the reduction of GFLV in shoots grown on medium B is a phenomenon not well understood and could be explained by the lack of cytokinin (B.A.P.). This aspect is still under study.

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## ON THE CORRELATION BETWEEN GRAPEVINE VIRUS A (GVA) AND RUGOSE WOOD

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A study was carried out in Apulian vineyards to assess the distribution and incidence of the closterolike viruses grapevine virus A (GVA), grapevine leaf roll associated virus I (GLRaV I) and grapevine leaf roll associated virus III (GLRaV III), and to investigate their possible correlation with rugose wood (RW) disease. The search for viruses was also extended to the agents of grapevine fanleaf (GFLV) and grapevine fleck (GFkV) diseases.

The survey was made on a relatively high number of different cultivars, some of which of new introduction, like Perlon, Chardonnay, Matilde and Superior seedless. Mature canes were collected from 10% of vines from each of 17 vineyards surveyed in different Apulian areas in late autumn 1991 and stored at 4°C.

In late spring the presence of rugose wood (RW) symptoms was investigated by removing a strip of bark at the graft union level of all vines under observation.

Virus detection and identification were by ELISA on extracts from cortical scrapings, using, except for GLRaV I, kits prepared with locally produced antisera, including a recently developed monoclonal antibody to GVA (1).

Of 1828 ELISA-tested vines, 88.3% proved to be infected by at least one of the five viruses. Only 2 vineyards (cv. Matilde and Chardonnay) out of the 17 surveyed had a level of infection lower than 50%, whereas 6 (cv. Michele Palieri, Primus, Sangiovese, Pampanuto, Primitivo and Perlon) were totally infected. Particularly widespread were GLRaV III and GVA (77% and 56%, respectively) and GFkV (59%). Less widespread were GLRaV I (12%) and GFLV (7%), the latter being more frequent in older vineyards.

A possible explanation for the high level of infection by the former three viruses (GVA, GLRaV III and GFkV), in absolute values and by comparison with figures obtained in previous surveys, may lie in: (i) the epidemiological behaviour of GLRaV III and GVA, which are both transmitted by Pseudococcid mealybugs (2), the symptomless presence of GFkV in rootstocks and the little attention paid to fleck disease in certification schemes, and (ii) improved detection technology with utilization of antisera that give more reliable responses in ELISA. On the other hand, the increased use of certified material and the lack of natural spread of GLRaV I, which apparently is not transmitted by mealybugs, is perhaps at the base of the low infection levels detected for GFLV and GLRaV I.

About 24% (432 out of 1828) of the vines surveyed had rugose wood (RW) symptoms on the scion, rootstock or both. Of these vines, only 8% did not appear to contain closterolike viruses.

Of the vines positive for GVA (alone or in mixture with GFLV and/or GFkV), 17% had RW, and comparable figures were obtained for single infections by GLRaV III (19%) or GLRaV I (12%). These data could hardly suggest a preferential association of any of the three closterolike viruses with RW. However, if mixed infection were taken into consideration, a clear-cut trend towards a remarkably high association as a function of the type of virus combination, became evident: 23% in the case of joint infection by GVA and GLRaV I, 31% when the viruses were GVA and GLRaV III, and 50% when the three closterolike viruses (GVA, GLRaV I and GLRaV III) occurred together.

Thus, it appears that the presence of GVA has a bearing on the occurrence of wood abnormalities in grapevines, and that the frequency of RW symptoms is enhanced by the presence of GLRaV I and GLRaV III (this latter virus in particular). Similar patterns of association between closterolike viruses and RW were observed elsewhere in Italy, in the course of studies in which a possible relationship of GVA with Kober stem grooving was hypothesized (3). The present data are not backed by graft transmission trials, and therefore consider RW as a whole, without distinction between the single diseases that constitute the complex. This, however, does not lessen the potential interest of these observations which seem to indicate the existence of some sort of synergism between different closterolike viruses, which may influence the symptomatological response of the vines. If this is so, and admitting the alleged role played by GLRaV I and GLRaV III in the etiology of leafroll disease, it would not be surprising if in the end, the conclusion would be reached that leafroll and rugose wood are facets of the same pathological complex, and that shifting from leafrolling to stem pitting (or grooving) and viceversa could be a function of the interaction between different closterolike viruses and perhaps the grapevine variety and/or the scion/rootstock combination.

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## GRAPEVINE VIRUS B IN SARDINIA

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Grapevine Virus B (GVB) is a 800 nm long closterolike virus recently isolated from corky bark-infected vines, and characterized (1). Preliminary investigations indicated that GVB occurs in Sardinia (3) where corky bark has been detected in about 2% of the vines indexed during sanitary selection for certification (2).

We now report further data on the presence of GVB and of its association with LN-33 indicators showing differential symptomatological responses. The investigation concerned two distinct groups of donors that included *Vitis vinifera* cultivars and American rootstock hybrids, which had both elicited corky bark-like response in LN-33.

The first group consisted of 16 donors, only part of which showed symptoms. In particular, symptomatic donors were seven cv Italia vines grafted on 1103 P or *V. rupestris* that were visibly affected by rugose wood on the scion and/or rootstock; one Pascale di Cagliari vine with no rugose wood, but exhibiting a generalized reddening of the foliage of a type differing from that ordinarily induced by leafroll; two LN-33 indicators that had been graft-inoculated from Italia donors other than the seven mentioned above; one naturally infected LN33 vine. Symptomless donors were two vines of cv Malvasia di Bosa, one of cv Vernaccia and two of *V. rupestris*. All donors of the first group induced in LN-33 consistent and severe symptomatological responses such as yellowish spots and mild deformation of the leaves, swelling and cracking of the basal internodes, stunting and stem grooving. These symptoms recurred regularly in the years following graft inoculation.

The second group was made up of six donors, three of which of cv Italia and one each of cv Pascale di Cagliari, *V. rupestris* and LN-33 that had been graft-inoculated from an Italia vine differing from the above. All accessions of this group induced in LN-33 reactions which milder than usual, incomplete, and erratic for they did not develop on all grafted indicators throughout the years.

Mechanical transmissions were made from both donors to *Nicotiana occidentalis* using sap expressed from young roots of greenhouse-forced cuttings. Infected *N. occidentalis* plants were used, in turn, for sap inoculating *N. cavicola*, *N. rotundifolia* and *N. benthamiana*. Dodder (*Cuscuta campestris*) transmissions were also attempted by bridging directly potgrown infected vines or herbaceous hosts to healthy *N. occidentalis*, or by inoculating herbaceous hosts with sap expressed from dodder that had been growing on infected vines and/or *Nicotiana* species.

Mechanical transmissions were successfully obtained from 10 out of 16 donors (about 63%) of the first group, but from none of the donors of the second group. The overall recovery of GVB was therefore of nearly 50%.

Two types of symptomatological reactions were observed in *N. occidentalis*. Seven viral isolates out of 10 induced chlorotic local lesions followed by a striking systemic vein clearing, whereas the remaining three isolates elicited, in addition, mild leaf deformation and a more or less extensive necrosis of the top leaves.

The necrotic reactions were stronger in *N. cavicola*, which expressed necrotic stippling and rings on both inoculated and systemically infected leaves. No differentiation between isolates could be detected in *N. rotundifolia*, which reacted in all cases with chlorotic local lesions and systemic vein clearing, and in *N. benthamiana*, which showed mild and erratic reactions.

No direct transmission was obtained through dodder, although this plant was able to acquire the virus as shown by the positive results obtained when *Nicotiana* species were inoculated with dodder tissue extracts.

All virus isolates, regardless of the origin (grapevine or dodder) and the symptoms induced in *Nicotiana* species were equally well decorated by an antiserum to GVB raised with in an authentic isolate of this virus (1).

Based on the above results, two sets of conclusion can be drawn:

a) GVB is common in Sardinia where it shows a fair level of association with vines indexing positive for corky bark. The over 60 % recovery from the group of donors with strong and consistent reactions tallies with the figure reported from Apulia (1). The reasons for the consistently negative results of transmissions from donors with irregular reactions are difficult to interpret on the basis of the current information. Whether this behaviour depends on the virus (i.e. strain with differential distribution and/or replication power) or on the host cannot be said. Similarly obscure are the reasons by which dodder acquires GVB but is unable to translocate it successfully to another host.

b) There are GVB variants which can be distinguished biologically because of the reactions induced on herbaceous hosts. There are indications that other aspects (e.g. molecular) are perhaps linked with this differential biological behaviour (3), but other possible discriminating traits like, for instance, serological properties remain to be ascertained.

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# INVESTIGATIONS ON THE AETIOLOGY OF KOBER STEM GROOVING

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The role of grapevine virus A (GVA) in the aetiology of Kober stem grooving (KSG) has been investigated by a survey of several leafroll- and KSG-infected *Vitis vinifera* cultivars from French vineyards and from the Vassal grapevine collection, as by the follow-up of the progeny clones from heat-treated Servant and Klevener plants initially infected by leafroll and KSG.

Among 130 *V. vinifera* cultivars infected by leafroll, 60 were shown to carry latently Kober stem grooving by indexing on Kober 5BB or SO4. Whereas all of the plants contained at least one of the five grapevine leafroll-associated closteroviruses, GVA was detected by ELISA in 53 out of the 60 KSG-infected cultivars. Virus purification from 40 g samples of a dozen of plants infected by leafroll and Rupestris stem pitting, but not KSG, did not reveal the presence of GVA. Results are now being confirmed or completed by RT-PCR using GVA-specific oligonucleotide primers (1). GVA has never been found alone, nor without inducing KSG, and was predominantly combined with GLRaV I (>80%) in the studied population.

Two varieties originally affected by leafroll and KSG, and containing either GLRaV I + GVA (Klevener de Heiligenstein) or GLRaV I + III + GVA (Servant), have been treated by thermotherapy (37°C for more than 100 days) followed by apex-grafting on Vialla hypocotyls. Table 1 shows that separation of the two diseases could be obtained in some progeny clones and that GVA was clearly associated with KSG.

Table 1: Viral content of clones issued from heat-treated Klevener and Servant initially infected by leafroll and KSG

Variety	Indexing	Nb clones	GLRaV	GVA
Klevener	Roll + KSG +	6	I	+
	Roll- KSG +	10	-	+
	Roll + KSG-	1	I	-
	Roll- KSG-	3	-	-
Servant	Roll + KSG +	2	I, III	+
	Roll- KSG +	2	-	+
	Roll + KSG-	-	-	-
	Roll- KSG-	3	-	-

These results give new arguments for the straight involvement of GVA in Kober stem grooving, rather than in Rupestris stem pitting as previously suspected. We have started transmission experiments by heterografting from GVA-infected *Nicotiana benthamiana* to Kober 5BB which should bring us further evidence of the Kober stem grooving aetiology. Study of the close association of GVA with GLRaVs in natural conditions is also undertaken in order to know if it only relies on the natural spread of the diseases or on the multiplication of GVA in grapevine tissues as well.

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## ELECTRON MICROSCOPE OBSERVATION OF GRAPEVINE LEAFROLL VIRUS

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Grapevine leafroll is common in the People's Republic of China. In Zhengzhou, central region of China, ninety percent of vines showed symptoms as observed during a recent survey. Leafroll is therefore a severe problem. Leaves of infected vines reddened and rolled downward in red cultivars and were pale green in white cultivars. In both cases, the infected berries were smaller than normal ones and had a lower sugar content and less flavour.

Since leafroll pathogens were not identified in China we worked on the isolation, purification and identification of the leafroll associated viruses. The process and results are summarized as follows:

Leaves of diseased ZF-1, the most susceptible cultivar of grapevine in China, were kept over night in a refrigerator, cut into small pieces with scissors, homogenized (2:1 ratio - v/w) in 0.5 M Tris-HCl buffer containing 2% polyvinylpyrrolidone, 0.2% ethanolsulphydryl and 0.01 M MgCl<sub>2</sub>, pH 8.2. The homogenate was filtered through a double layer cheesecloth and the filtrate clarified by centrifugation in a 55 p-72 Hitachi centrifuge at 8000 rpm for 25 min. The clarified supernatant was completed with 0.5% Tween-20 and 5% Triton-100, stirred for 30 min and clarified again by centrifugation at 8000 rpm for 25 min. The clarified supernatant was then centrifuged at high speed, at 30000 rpm, for two hours. The resuspended pellet was again clarified by centrifugation at 8000 rpm for 25 min and virus particles were finally negatively stained with potassium phosphotungstate for three min and observed and photographed with a Hitachi H 600 electron microscope at a magnification of 30000. Curled filamentous virus-like particles were clearly seen. Their size was 12 nm x 2.000 nm (figure 1) and were similar to the particles of closteroviruses.

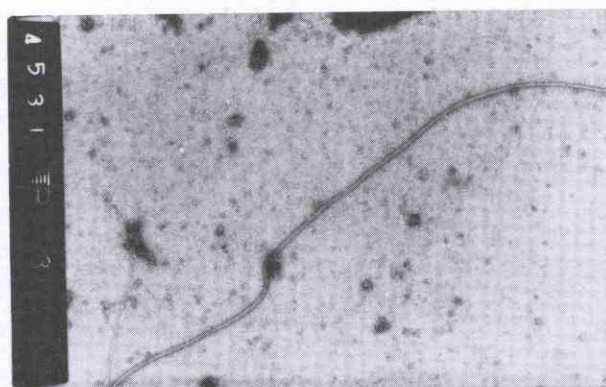


Figure 1: Grapevine leafroll associated virus (magnification: 30.000 x)

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## RUGOSE WOOD OF GRAPEVINE IN YEMEN

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Grapevine (*Vitis vinifera*) is grown in Yemen since time immemorial. The great majority of commercial vineyards is located in the central highlands of North Yemen in the provinces of Sana'a and Saada where some 18 cultivars for fresh consumption and raisin, all apparently of local origin, are cultivated. No foreign varieties (especially European) are commercially grown in the country and little, if any, grapevine germplasm was introduced from abroad, also in recent times. Due to the absence of phylloxera, all grapes are self rooted.

A survey carried out in spring 1992 showed that Yemeni viticulture enjoys a remarkably healthy status. The only disease detected for certain was a stem grooving condition of a single cultivar (cv. Asimi) in the Sana'a area. Symptoms were typical of rugose wood: slight depressions were visible on the surface of the cortex after removal of outer bark and, when the cortex was peeled, mild but distinct grooves appeared on the woody cylinder with the corresponding ridge-like protrusions on the cambial face of the cortex. There were no symptoms on the leaves, and in general, the vigour and yield of the vines seemed to be little affected. The disorder was not observed in any other cultivar grown in the area, or anywhere else in the country.

Canes and leaves collected from over 130 individual vines of cvs Asimi, Aswad Adi, Artraf, Bayadi Adi, Irki, Jawfi, Jubari, Raziki and Zeitun were brought to Bari and analyzed for virus presence. ELISA and mechanical transmissions to herbaceous hosts were consistently negative for grapevine fanleaf (GFLV), grapevine fleck (Gfkv) and grapevine leafroll associated virus I (GLRaV I). Two vines of cv. Bayadi Adi and one each of cv. Aswad Adi and Asimi, were positive for grapevine leafroll associated virus III (GLRaV III), but all vines of cv. Asimi with stem grooving were found to contain grapevine virus A (GVA). The same virus was occasionally found in cvs Aswad Adi and Bayadi Adi, both of which did not exhibit stem grooving symptoms.

*In vitro* cultures were established from 2-3 mm long shoot tips excised from cv. Asimi vines. Mechanical transmissions were attempted from established explants grown in culture for several months and were repeated later on directly from young leaves of glasshouse-grown rootlings. Symptoms were readily obtained in *Nicotiana benthamiana* from both sources and GVA was identified in these plants by ELISA and immunoelectron microscopy (IEM). Virus particles were plentiful and were specifically decorated by polyclonal antisera and monoclonal antibodies to GVA (1). However, the same preparations contained also filamentous particles much longer and thinner than those of GVA, which were not decorated by antisera to GVA or GLRaV I and GLRaV III. These filaments did not show the cross banding and open structure typical of closterolike viruses.

When subcultures were made from infected *N. benthamiana* to other herbaceous hosts, symptoms (i.e. vein clearing and mild leaf deformations) developed in *N. clelandii*, *N. occidentalis*, *N. cavicola*, *N. megalosiphon*, and, unexpectedly, in *Gomphrena globosa*, *Chenopodium quinoa* and *Chenopodium amaranticolor*, all of which reacted with chlorotic/necrotic local lesions. *G. globosa* and *Datura stramonium* were also infected systemically without showing symptoms. The presence of both types of particles in all the above hosts was experimentally ascertained.

Massive accumulations of filamentous structures occurred in the cytoplasm of parenchyma cells and sieve tubes of *N. benthamiana*, but the two types of particles could hardly be distinguished.

The nature of the long filaments isolated from Yemeni vines is still unknown. At this stage it is not possible to establish whether they are plant constituents whose production was triggered or enhanced by viral infection, or are virus-related products, or particles of an undetermined virus.

The Yemeni findings not only confirm the relationship of GVA with rugose wood, but strongly support the notion that closterolike viruses (GVA and GLRaV III) had a long association with *V. vinifera* and may have travelled with it from the centre(s) of origin to far away places.

### Acknowledgements

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## DISEASE SYMPTOM EXPRESSION IN LN 33 AND SO4 ROOTSTOCKS GRAFTED WITH A CORKY BARK-AFFECTED 'SEMILLON' INTERSTEM AND A 'CABERNET SAUVIGNON' SCION

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Grapevine corky bark (CB) disease is a serious problem in many parts of the world (1). Certain countries will allow the importation of grapevine material only if it can be certified free of the disease. A number of closterovirus-like particles (CVLPs) of various modal lengths have recently been isolated from CB-affected grapevines (2, 4, 5), but the causal agent of the disease has not yet been definitively identified. CB does not produce leaf or cane symptoms on most *Vitis vinifera* varieties. On the *V. vinifera* varieties which develop leaf symptoms (1), these are difficult to distinguish from the symptoms of grapevine leafroll (GLR) disease. Current indexing procedures for CB disease rely on the use of woody indicator varieties. CB can be clearly distinguished from GLR by using the hybrid LN 33 (Couderc 1613 X Thompson Seedless) as an indicator. CB induces severe cane symptoms on LN 33 but GLR does not.

Tanne et al. (6) have reported that the rootstock utilized influences the severity of symptoms in CB-affected grapevines. Climate might also influence CB disease symptom expression. While CB disease symptoms can readily be detected on LN 33 rootstock in the mild climate of Sidney, British Columbia, Canada, they might not be apparent in colder climates. A collaborative experiment to evaluate the influence of both rootstock and climate on CB disease symptom expression was initiated recently by the authors, under the Canada-Germany Science and Technology Agreement. Although the collaboration is still in its preliminary stages, some interesting observations can already be reported.

In preparation for a comparative field trial in Germany and Canada, the following grapevines were micropropagated (3): virus-free *V. vinifera* cv. Cabernet Sauvignon, virus-free and CB-affected *V. vinifera* cv. Semillon and virus-free rootstocks of the varieties LN 33 and SO4. The use of in vitro shoot tip culture for the production of the grapevines ensures uniformity of the plants to be compared between sites. Plantlets were regenerated from the grapevine shoot tip cultures. Virus-free or CB-affected 'Semillon' was grafted as an interstem on both LN 33 and SO4 rootstocks and virus-free 'Cabernet Sauvignon' was grafted as a scion on all four combinations. Grafted vines were kept in pots in a greenhouse. Disease symptoms developed on vines with the CB-affected 'Semillon' as an interstem within two months of grafting four-month-old regenerated plantlets.

On vines consisting of 'Cabernet Sauvignon'/CB-affected 'Semillon'/LN 33 (Fig. 1, right), the rootstock developed both leaf and cane symptoms typical of CB disease. The wood was swollen and split longitudinally. The leaves, including the veins, were red. The 'Cabernet Sauvignon' on these vines also developed symptoms. Their leaves exhibited an interveinal reddening and marginal rolling similar to the symptoms of GLR disease. The vines consisting of 'Cabernet Sauvignon'/virus-free 'Semillon'/LN 33 showed no disease symptoms. On vines consisting of 'Cabernet Sauvignon'/CB-affected 'Semillon'/SO4 (Fig. 1, left), the rootstock developed no stem symptoms, but the leaves of the SO4 developed an interveinal chlorotic flecking. The leaves of the 'Cabernet Sauvignon' scion on these plants developed the reddening and rolling mentioned above.

The CB-affected 'Semillon' used as inoculum (interstem) in this experiment appeared to be free of GLR, based on the results of conventional woody indexing tests conducted both in Sidney and in California (5). This CB-affected 'Semillon' has recently been found by immunosorbent electron microscopy to contain at least five grapevine CVLPs, including GLRaV III (manuscript in preparation). In addition to monitoring the development of CB disease symptoms in the field under different climatic conditions, we also plan to probe the scion and rootstocks of the grafted vines for the presence of these CVLPs.

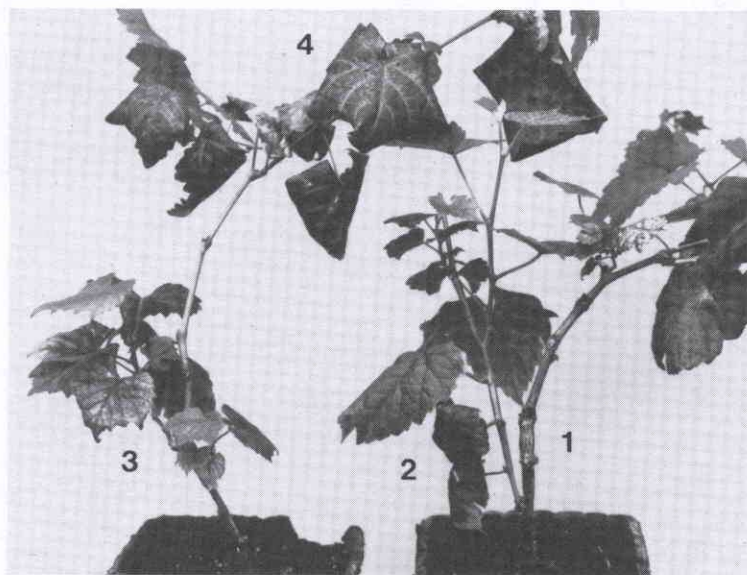


Figure 1. LN 33 cane (1) and leaf (2) symptoms typical of corky bark disease on a grafted vine consisting of virus-free 'Cabernet Sauvignon'/CB-affected 'Semillon'/virus-free LN 33 (right). Interveinal chlorotic flecking on the leaves of SO4 (3) and GLR-like symptoms on the leaves of 'Cabernet Sauvignon' (4) on a grafted vine consisting of virus-free 'Cabernet Sauvignon'/CB-affected 'Semillon'/virus-free SO4 (left). Symptoms were observed two months after grafting four-month-old micropropagated plantlets.

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# KOBER STEM GROOVING AND GRAPEVINE VIRUS A: A POSSIBLE RELATIONSHIP

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Rugose wood (RW) is widely spread in the island of Sardinia (southern Italy), where the four diseases of the complex, i.e. Rupestris stem pitting (RSP), Kober stem grooving (KSG), Corky bark (CB) and LN stem grooving (LNSG), were experimentally identified (2, 3).

There is mounting evidence that RW has a viral etiology in which two closteroviruses, namely grapevine virus A (GVA) and grapevine virus B (GVB) may be implicated. However, neither of these viruses has been associated for certain with any of the four RW diseases. An analysis of the results of indexing trials on 84 vines of different cultivars subjected to sanitary selection and of the relative presence of GVA, grapevine leafroll associated viruses I (GLRaV I) and III (GLRaV III), has revealed a preferential association of GVA with KSG, as reported in this paper.

The 84 donor vines were candidate clones belonging to 11 *Vitis vinifera* cultivars as follows: 20 clones of cv Vermentino, 16 of Malvasia di Bosa; 11 each of Pascale di Cagliari and Vernaccia; 10 of Cannonau; 4 each of Italia, Monica and Nieddera; 2 of Torbato; one each of Aleatico and Trebbiano. All donors were indexed by omega grafting on *V. rupestris*, Kober 5BB and LN-33 and the symptoms (stem pitting and/or stem grooving) read three years afterwards on indicators that had been uprooted, autoclaved and peeled clean of cortex. Individual donors were checked serologically (ELISA) for the presence of GVA, GLRaV I and GLRaV III using either commercial kits (Bioreba, Basel), or antisera (polyclonal and monoclonal) raised at Bari (3). The tests were repeated several times using petioles from mature leaves and/or cortical scrapings from dormant canes as antigen source.

None of the 84 donor vines indexed positive for corky bark or LN stem grooving, whereas 44 (slightly more than 52%) proved to be affected by RSP and 17 (about 20%) by KSG. Of these, however, 11 had also RSP infections. Apparently disease-free vines (no reactions on any of the indicators) were 34 (about 41%).

The distribution of diseases and viruses, expressed as percent values, was as reported below:

Diseases	No virus detected	Viruses						
		GLRaV			GVA			
		I	III	I + III	alone	GLRaV I	GLRaV III	GLRaV I + III
RSP	73	0	15	3	6	3	0	0
RSP + KSG	9	0	18	0	18	18	18	18
KSG	0	0	0	0	33	33	0	33
No symptoms	73	6	12	0	3	0	6	0

An equal proportion of the donor vines (73%) that proved to be apparently free from diseases or were affected by RSP alone, were also apparently deprived of viruses. GVA alone or in mixed infections with GLRaV I was found in 9% of the donors with RSP alone and in 9% of the apparently disease-free donors (3% in single infection and 6% in association with GLRaV III).

Higher percentages of GVA were detected in donors indexing positive for RSP and KSG, totalling 18% in single infection and 54% in mixed infections with GLRaV I and GLRaV III.

An even higher incidence of GVA was ascertained in donors indexing positive for KSG alone. In this case, single infections were 33% and mixed infections with GLRaV I and GLRaV III were 66%. Interestingly, none of GVA-free vines positive for KSG contained GLRaV I and III alone or in mixture.

In summary, whereas a high percentage (73%) of donors inducing RSP symptoms only were apparently deprived of viruses, this proportion dropped considerably (9%) when the donor vines were affected by both RSP and KSG and became nil when the affecting disease was KSG alone.

Furthermore, GVA either by itself or together with leafroll-associated closteroviruses was present in 9% of the donors apparently affected by RSP alone, 72% of the donors reacting positive for RSP and KSG, and

the totality (100%) of the donors inducing symptoms of KSG alone.

These data, together with the lack of detection of leafroll-associated closteroviruses in GVA-free KSG-affected candidate clones, are strongly indicative of the remarkably close association of GVA with Kober stem grooving.

On the other hand it seems plausible to conclude that none of the viruses considered in the present study shows any consistent association with Rupestris stem pitting. It is, however, unclear whether Kober stem grooving symptoms are influenced, and to what extent, by the presence of leafroll-associated closteroviruses I and III.

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# OCCURRENCE OF SHIRAZ DISEASE IN SOUTH AFRICA

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In South Africa Shiraz disease (SD), which resembles Corky bark (CB) on the cultivars Malbec, Shiraz and Merlot is highly destructive. This disease is latent in other cultivars with respect to the corky bark-like symptoms. Findings on the spread, symptomatology, serology and field indexing are reported. The disease spreads very slowly. It is believed that *Planococcus ficus* might play a role in being a vector. Over a twelve year period only 24 Merlot plants out of a total of 5 886 showed natural reinfection in the field. This reinfection occurred during the past three years. No reinfection pattern could be observed.

Leaf symptoms is leafroll-like, however leaves may also change completely to a wine-red colour. SD leaf coloration starts later in the season than leafroll leaf coloration. Canes remain immature and droopy while budburst is either markedly delayed or does not occur at all. The SD-infected Shiraz, Merlot and Malbec plants shatter their leaves later than leafroll infected plants of the same cultivars.

Concluded from ISEM and ELISA tests performed on SD-infected plants, they were all infected with GLRaV-III in combination with either/or GLRaV-I, II and GVA. Corky bark material did not give in all cases positive reactions for GLRaV-III. Based on field indexing (on LN-33) SD-infected material does not always reveal symptoms associated with Corky bark infection (Table 1), but in all cases serological tests were positive for leafroll infection on Cabernet franc. On the contrary Corky bark infected material does not always show SD with field indexing trials (Table 1).

We are of the opinion that SD is probably serologically related to GLRaV-III, or a synergistic effect of multiple virus infection.

It may, however, be a completely unknown agent. Our indexing results confirmed that it is not Corky bark. Elimination of the pathogen is easily obtained by means of thermotherapy and apical meristem culture *in vitro*.

Extensive serological studies and field indexing are now underway which will be later accompanied by RNA studies of isolated GLRaV-III from leafroll infected as well as SD-infected Merlot plants.

Table 1: Field indexing results of infected vines

Cultivar tested	Field indexing results			
	Leafroll (Cabernet franc)	Corky bark (LN 33)	Shiraz disease (Shiraz)	Stem pitting (St George)
Sauvignon blanc	-	+	-	+
Tinta Roriz	+	+	+	+
Chenin blanc	+	-	+	-
Verdelho	+	+	-	N/A
Cabernet Sauvignon	+	-	+	-
Chardonnay	N/A	+	-	+
Souzao	+	+	+	-
Pontac	+	-	+	+
Waltham Cross	+	-	+	N/A

N/A = not available

## RESISTANCE TO TRANSMISSION OF GRAPEVINE FANLEAF VIRUS BY *XIPHINEMA INDEX*

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### INTRODUCTION

Nematodes often cause serious problems in viticulture, either by injuring plants directly by feeding, or by transmitting viruses which give rise to various diseases. Since chemical control of nematodes in vineyards becomes more and more restricted in several countries, breeding of resistant varieties and/or rootstocks is an important goal in grape breeding. Resistance to nematodes, defined as the capability of plants to limit the growth rate of a certain nematode species does not sufficiently solve the problem. The plants to be bred in Germany, therefore, should show first of all resistance to transmission of viruses by nematodes.

The aim of our investigations was 1. to broaden our knowledge about the mechanism of resistance to nematode transmission of viruses and 2. the evaluation of genetic resources for that type of resistance.

In order to test a great many of accessions, a quick test has been elaborated which enabled us to investigate accessions for nematode resistance and resistance to transmission of viruses by nematodes within three month (1, 2). A strain of *X. index*, kindly received from INRA, Colmar, was used for the transmission studies of Grapevine Fanleaf Virus strain Ortenberg, Badenia.

### RESULTS

The investigation of 13 accessions of *Vitis rotundifolia* and *V. munsoniana* has shown a high degree of resistance to nematodes as well as for virus transmission by nematodes. Starting from 200 *X. index* inoculated per pot, the population of the nematodes had increased in the highly sensitive control plants of cv. Siegfried up to a maximum of 20.800 per pot after three months of cultivation. In *V. rotundifolia* and *V. munsoniana* on the other hand, the number of *X. index* were almost the same as at the time of inoculation.

Roots of *V. rotundifolia* attacked by *X. index* never produced hypertrophied multinucleate cells as observed in normal host plants. This and the hypersensitivity reaction found after feeding by *X. index* on *V. rotundifolia* roots might be the reason for preventing nematodes extracting enough food necessary for surviving and reproduction (3).

Roots of *V. rotundifolia* and *V. munsoniana* never showed any swellings or galls after inoculation with *X. index*. Both species, therefore, can be considered as tolerant to nematode feeding.

Virus particles injected during salivation of *X. index* might be prevented from surviving and spreading into the neighbouring root cells by the hypersensitivity reaction. This mechanism of resistance does not exclude any infections. Therefore, a certain amount of transmission could be expected. In fact, transmission was observed in several experiments ranging from 0 - 13 %. Supposing the resistance to transmission of viruses can be traced back to the same genetical factors we can consider all genotypes as resistant which demonstrate an average of about 3 - 5 %, may be up to 10 %, transmission.

In order to introduce genes for both types of resistance to new varieties further knowledge was needed about the genetic resources.

120 accessions were tested in the last years including wild growing species, hybrids with *V. rotundifolia* and different clones originated from various breeding programs. In the average 20 plants each were tested of all accessions. In the experiments in which no transmission was observed for the first time the test was repeated. In 42 of the accessions either no transmission or transmission up to 5 or 10 % was observed (Table 1).

Of the 42 resistant accessions 11 belong to *V. rotundifolia* or *V. munsoniana*, the muscadine group. These species seem to be still the best source for genes of resistance to nematodes and virus transmission by nematodes. Nine accessions represented F<sub>1</sub>-hybrids *V. vinifera* x *V. rotundifolia*, which were kindly provided by Dr. Bouquet, Montpellier and Dr. Walker, Davis. All resistant hybrids showed about the same level of resistance while the susceptible hybrids, on the other hand, all showed a similar level of transmission. From these results it may be concluded that the genetic basis of resistance seems to be rather simple. So far known from former investigations hybrids with *V. rotundifolia* mostly display serious problems regarding their fertility and general growth. Therefore, great stress was laid upon the investigations of bunch grapes from the southeastern parts of the United States, which all have 2n = 38 chromosomes and can easily be crossed with other bunch grapes. From former investigations it could be assumed that the desired traits may be found within the species from these regions (4, 5). Especially *Vitis rotundifolia*, which is synonym with *V. aestivalis*, was mentioned as a valuable source of resistance. As listed in table 1, several species showed resistant accessions



ut in the same species there occurred also susceptible accessions. None of the species could therefore be designated as entirely resistant. Several breeding stocks showed sufficient resistance. Consequently it can be concluded that further investigations in searching for factors of resistance for nematodes and virus transmission by nematodes is worthwhile.

Table 1 : Number of resistant accessions which showed transmission of GFV by *Xiphinema index* from 0 to 10 %

Accessions	0	5	10
<i>V. rotundifolia</i>	3	2	1
<i>V. munsoniana</i>	5		
F 1 hybrids <i>V. vinifera</i> x <i>V. munsoniana</i>	9		
<i>Y. aestivalis</i>	1		
<i>V. candicans</i>	1		
<i>V. cinerea</i>	1		
<i>V. cordifolia</i>	1		
<i>V. longii</i>		1	
<i>V. shuttleworthii</i>	2	1	
<i>V. solonis</i>		1	
undetermined spec.	1		
breeding stocks	6	2	2

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# MEALYBUG TRANSMISSION OF CORKY BARK DISEASE AND AN ASSOCIATED VIRUS TO HEALTHY GRAPEVINE

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The causative agent of the corky-bark disease is considered to be of viral etiology due to its graft-transmissibility (1). In recent years a few clostero-like virus particles have been observed in infected phloem and purified therefrom (3, 4, 5). However, the etiology of the disease is yet unknown and no vector for this disease has been reported so far.

Reports giving evidence for the spread of the disease in vineyards came from Mexico and Israel (10, 11). Therefore, a study looking for a possible corky-bark-transmitting vector has been initiated. Since mealybugs were found to transmit a number of leafroll-associated closteroviruses (2, 6, 7, 8, 9), this insect was selected for the corky-bark transmission studies. Field experiments were also carried out.

Two species of mealybugs, *Pseudococcus longispinus* and *Planococcus ficus* were used for controlled transmission trials in the greenhouse. Cultures of mealybug crawlers were maintained on potato tubers. Groups (5-10) of crawlers were transmitted to donor grapevines which had been index as corky-bark infected. The crawlers were allowed an acquisition period of 48-72 hours. The crawlers were then transferred to healthy LN-33 indicator grapevines for an inoculation access feeding of 3-7 days, in an insect-proof chamber at 25°C. The plants were then fumigated and transferred to an insect-proof greenhouse for observation.

ELISA tests (10) were carried out with antibodies prepared against a corky-bark-associated closterovirus (1400 nm; kindly provided by Dr. D. Gonsalves). ISEM tests were also carried out.

In addition to the controlled transmission assays, a field test was also initiated. Healthy LN-33 indicator vines were interplanted in a commercial vineyard affected by the corky-bark disease, and inspected for several years for the appearance of the disease symptoms.

Mealybug inoculated LN-33 vines exhibited typical corky-bark symptoms on leaves and canes, approximately five months after inoculation. ELISA tests (Table 1) were carried out with symptom showing, as well as with symptomless grapevines. These tests demonstrated the incidence of a closterovirus in the mealybug-inoculated LN-33 plants, and not in the symptomless plants. Furthermore, the LN-33 indicator vines planted in the field also gradually exhibited corky-bark symptoms.

Based on these results, it can be concluded that *P. longispinus* and *P. ficus* transmitted a closterovirus along with disease symptoms from diseased grapevines to healthy ones. The reported spread of corky-bark in the vineyard was confirmed experimentally, and the vector transmissibility of corky-bark was thereby established.

Table 1: ELISA of grapevines inoculated by mealybugs (*Planococcus ficus*) with a closterovirus associated with grapevine corky-bark disease.

Plant No.	OD <sub>405</sub> Reading (30'- 60')
MBF-Gamay	0.641 (0.060)
MBF-3-3	1.151 (0.006)
MBF-1-1	0.370 (0.015)
MBF-4-6	0.406 (0.060)
MBF-5-10	0.347 (0.060)
MBF-12	0.397 (0.060)
MBF-13	1.034 (0.102)
MBF-14	0.234 (0.060)
MBF-17	0.514 (0.084)
MBF-6	0.605 (0.084)

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## RUGOSE WOOD COMPLEX OF GRAPEVINE IN NORTHEASTERN ITALY: OCCURRENCE OF RUPESTRIS STEM PITTING AND KOBER STEM GROOVING

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The study of rugose wood, one of the major diseases of grapevine, is important in order to establish the plant-virus relationship, definition of the causal agent, identification of specific symptoms and to develop fast and reliable diagnostic methods. The disease is often latent in most of the *Vitis* varieties, in rootstocks as well as in European grapes. Specific symptoms are apparent on only a few varieties, especially when the plants have been grafted: pits and/or grooves show up on the woody cylinder of the rootstock or the scion or in both.

ELISA is regarded as a complementary test in the search for closteroviruses, of which grapevine virus A (GVA) could be associated with this kind of disease (1). The correct testing requires the use of indicators: *Vitis rupestris*, Kober 5BB (*V. berlandieri* x *V. riparia*) and LN33 (Couderc 1613 x *V. berlandieri*). These indicators enable the assessment of Rupestris Stem Pitting (RSP), Kober Stem Grooving (KSG) and LN 33 Stem Grooving (LNSG) (3).

The complex syndrome of rugose wood has been studied on material cultivated in northeastern Italy since 1988. Preliminary results are reported in this paper.

Biotype and different cultivars scions have been grafted with a machine (5 grafts per candidate plant) on the three indicators *V. rupestris*, Kober 5BB and LN33. A scion of healthy Merlot was grafted between the *V. rupestris* and the Kober 5BB when rootstock candidates were indexed. Controls were carried out in the third year by stripping off the bark from field-grown vines.

Rugose wood incidence on the different indicators are displayed in Table 1. The amount of rugose wood on rootstocks is not high (6.7%) and contains only RSP. KSG and LNSG were absent.

Instead, the percentage of plants affected by rugose wood on European grapes was very high: RSP was dominant (52.2%) compared to the KSG (0.9%), while the LNSG itself was absent. The association of RSP and KSG was observed only on 4 candidate plants, one of which also had LNSG and the other had LNSG and corky bark too.

The symptom typology on the woody cylinder of the *V. rupestris* indicator did not always show up univocally: some plants showed slight pits which were distributed in strips on the woody cylinder, especially under the grafting point; other plants showed grooves on the whole rootstock wood instead. On the Kober 5BB or LN33 indicators the woody cylinder showed evidence of extended grooves.

The low incidence of KSG is definitely due to the adopted pre-selection protocol. In the commercial vineyards of northeastern Italy, to which this report refers, most of the European grapes have been grafted on Kober 5BB rootstocks. Diseased vines may have been directly detected by debarking the plants in the field. The presence of RSP, however, may only be ascertained by indexing on *V. rupestris*.

In as far as the high incidence of rugose wood on *V. vinifera* is concerned, the appearance of light pittings like RSP may be due to the type of grafting used during this study - in this mechanical graft procedure the candidates are placed on the top of the indicator - by inducing this pitting type on the woody cylinder near the grafting point because of lack of affinity (2).

In conclusion, it appears that the differentiation of different forms of rugose wood by indexing on *Vitis* indicators is clear: KSP may be diagnosed during pre-selection, once the cultivated plants have been grafted on Kober 5BB, whereas RSP and LNSG require indexing on specific indicators. The occurrence of mixed infections is possible, as found in some plants in this study.

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Table 1. Detection of rugose wood complex on plants of different varieties of rootstocks and European grapes (*Vitis vinifera*)

Origin of samples	Years	Culti- vars	Bio- type	Candi- date plants	Healthy	Infected			
						RSP	KSP	LNSG	RSP + KSP
		nr.	nr.	nr.	nr.	nr.	nr.	nr.	nr.
Rootstocks									
Ampelographic collection	1988-90	40	40	40	35	5	0	0	0
Mother plant collection	1989-91	12	12	92	90	2	0	0	0
Mother plant collection	1990-92	25	36	93	85	8	0	0	0
	Total	77	88	225	210	15	0	0	0
	%				93.3	6.7	0	0	0
European grapes									
Ampelographic collection	1988-90	26	26	26	12	13	0	0	1
Selected varieties	1988-90	23	83	108	42	66	0	0	0
Selected varieties	1989-91	41	44	134	70	61	1	0	2*
Selected varieties	1990-92	53	86	188	86	98	3	0	1
	Total	143	239	456	210	239	4	0	4
	%				46.0	52.2	0.9	0	0.9

\* = One candidate with only LNSG and the other with LNSG and corky bark

## DIFFERENTIAL INDEXING TRIALS ON GRAPEVINE RUGOSE WOOD SYNDROMES

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Grapevine rugose wood is now considered to be a complex disease. Four apparently different syndromes can be identified using graft transmission to proper selective indicators. These are: *Rupestris* stem pitting (RSP), Kober stem grooving (KSG), LN33 stem grooving (LSG) and Corky bark (CB). Their causal agents are still unknown, but closterovirus-like particles have been recently found in vines showing wood alterations (1).

During a sanitary clonal selection program, several candidate clones of *Vitis vinifera* L. cultivars were found to be carriers of an agent (s) inducing pitting and/or grooving of the woody cylinder (2). On the basis of the availability of these infected materials and the recent findings on this topic (1), indexing trials were carried out to compare indicator responses to graft-inoculation of various different sources of rugose wood.

### Material and methods

For the study, 22 infected clones of 15 different cultivars were used. A series of standard grapevine indicators, including *V. rupestris* Sheele cv St. George, *V. berlandieri* x *V. riparia* Michaux cv Kober 5BB and hybrid LN33 were inoculated in 1988.

Dormant cuttings of the indicators were whip-grafted and chip-budded with bud-scions from the donor vines, according to the bench-grafting technique. After callusing and waxing, a minimum of three successful graft combinations were planted in a nursery plot and then symptoms of graft-transmitted diseases were recorded. Rugose wood was finally checked on indicators at the end of the third season after planting, by uprooting the single plants and removing the bark from the stem.

### Results and conclusions

Results of biological indexing are summarized in Table 1. Apparent single infections of RSP, KSG and LSG were recognized. However, the majority of the clones tested showed different probable combinations of these rugose wood components. Corky bark (CB) occurrence was not definitely ascertained, due to the absence of swollen and cracked internode symptoms on LN33 specific indicator plants.

The findings corroborate the information published by other authors (1).

Tab. 1 - Indicator reaction and incidence of graft-transmitted rugose wood syndromes in a sample of different infected *V. vinifera* clones

Rugose wood syndromes	Indicators (a)			No. of infected clones
	St. George	KS BB	LN33	
Rupestris stem pitting (RSP)	+	-	-	6
Kober stem growing (KSG)	-	+	-	2
LN33 stem growing (LSG)	-	-	+	1
RSP + KSG	+	+	-	8
KSG + LSG	-	+	+	1
RSP + KSG + LSG	+	+	+	4

(a) + = positive reaction; - = negative reaction

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# INVOLVEMENT OF TWO GRAPEVINE LEAFROLL-ASSOCIATED VIRUSES IN SYNDROMES DISTINCT FROM LEAFROLL OF *VITIS VINIFERA*

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A survey of 125 leafroll-infected *V. vinifera* cultivars collected either in the French grapevine collection at Vassal or in French vineyards was made to correlate indexing results, especially those concerning leafroll of *V. riparia* Gloire ("leafroll II")(1) and graft incompatibility on Kober 5BB (2), with the presence of GLRaV I, II, III and V.

Indexing was made by the traditional method using Pinot Noir for leafroll, *V. riparia* Gloire for vein mosaic and "leafroll II", and Kober 5BB for Kober stem grooving. Results were in some cases confirmed by green cutting-graft indexing using Cabernet franc for leafroll and 44 Laquenexy for "leafroll II". Presence of GLRaVs was assessed by ELISA using polyclonal antisera.

Table 1 shows that all the 31 *V. vinifera* varieties which transmitted leafroll symptoms to *V. riparia* Gloire contained GLRaV I, whereas none of the 55 varieties without GLRaV I was carrying this syndrome. Moreover, in the case of two varieties, Servant and Klevener de Heiligenstein, both types of leafroll were eliminated in parallel by heat treatment, coinciding with disappearing of GLRaV I. Nevertheless, 39 plants with GLRaV I did not transmit leafroll to *V. riparia*, nor to 44 Laquenexy.

Table 1: Correlation between GLRaVs and leafroll on *V. riparia* (leafroll II)

Virus detected by ELISA	Nb of clones leafroll + /leafroll II +	Nb of clones leafroll + /leafroll II -
GLRaV I	25	32
GLRaV I + II, III and/or V	6	7
GLRaV II, III and/or V	-	55

Table 2 shows that almost all the plants inducing graft incompatibility when indexed on Kober 5BB were infected with GLRaV II. In the opposite to GLRaV I, GLRaV II has never been detected without induction of graft incompatibility.

Table 2: Correlation between GLRaVs and graft incompatibility on Kober 5BB

Virus detected by ELISA	Nb of clones incompatible	Nb of clones non incompatible
GLRaV II	11	-
GLRaV II + I, III and/or V	7	-
GLRaV I, III and/or V	1	107

From these results we can conclude, first that the so-called "leafroll II" is not a distinct leafroll disease, but rather corresponds to a particular strain of GLRaV I or to the interaction of GLRaV I with an unknown factor. It would be interesting to check these hypotheses using monoclonal antibodies or specific nucleic probes when available. Concerning graft incompatibility on Kober 5BB, it seems likely that GLRaV II is involved in the expression of the phenomenon, either directly or by interaction with a factor present in the rootstock (2). Preceding results with incompatible Chardonnay clones already showed that leafroll and incompatibility are linked, as they were eliminated at the same time by heat treatment. It is therefore expected that elimination of GLRaVs by sanitary selection and by sanitation, as by the control of their natural spread, will also reduce, for at least some, the risks of graft incompatibility of pathologic origin.

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# ASSOCIATION OF CORKY BARK WITH A PECULIAR FORM OF VEIN MOSAIC, YELLOW BLOTCH MOSAIC, DETECTED BY INDEXING ON *VITIS RIPARIA* GLOIRE

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In the course of indexing series made in 1990-92 on grape clones issued from vein yellowing leafroll-infected Chardonnay (1, 2) treated by thermotherapy and apex-grafting, unusually marked vein mosaic symptoms were noticed in parallel on *Vitis riparia* Gloire, 11OR and Rupestris du Lot for 30 out of 108 clones. On *V. riparia* symptoms were more pronounced than those of the known vein mosaic (3), as growth reduction, leaf distortion and "oily" yellow blotches covering the secondary veins of the indicator leaves. On Rupestris du Lot confusion was in some cases possible with fleck symptoms, although the mother plants were initially not infected by fleck.

Indeed, the originating Chardonnay were infected by leafroll, corky bark, vein necrosis, vein mosaic and Rupestris stem pitting (2) and, according to indexing tests made in 1983-85 after the curing treatments, none of the 30 yellow blotch mosaic sources was free from corky bark. The nature of the agent responsible of the corky bark disease in these clones has not been determined as yet. Grafting on a panel of rootstock species showed that yellow blotches could also be expressed by Kober 5BB, 5C, 44 Laquenexy, 101-14 Mgt, 161-49 C, 99R or 40 10 Cl.

In order to estimate yellow blotch mosaic effects in controlled climatic conditions, *V. riparia* Gloire, 11OR and Rupestris du Lot were used in a comparison assay by green cutting-graft indexing of two Chardonnay clones and a fleck-infected Pinot Noir. Symptoms were observed six weeks after grafting and are described on the following table 1.

Table 1

Indicator (9 plants)	Chardonnay TE 155 Ms + /YBM + /FI-/Nc + /CB +	Chardonnay TG 855 Ms + /YBM-/FI-/Nc-/CB-	Pinot Noir Ms + /YBM/FI + /Nc + /CB-
<i>V. riparia</i> Gloire	irregular growth oily yellow blotches leaf distortion	normal growth clear vein mosaic	normal growth strong vein mosaic symptoms similar to YBM on old leaves
Rupestris du Lot	growth reduction yellow spots similar to fleck on young leaves	normal growth healthy aspect or very light mosaic	normal growth clear fleck
110 R	growth reduction scattered yellow spots light leaf distortion	normal growth healthy aspect	normal growth clear vein necrosis

Ms: vein mosaic, YBM: yellow blotch mosaic, FI: fleck, Nc: vein necrosis, CB: corky bark

These results show that yellow blotch mosaic, probably related to the early leaf distorting mosaic due to corky bark on LN33 (3), may be a source of mistakes by confusion with (or masking of) fleck, or even court-noué, on Rupestris du Lot especially in favourable climatic conditions. These observations strengthen the arguments for the replacement, or the systematic complementation, of indexing by reliable immunological or biochemical diagnosis assays as soon as available.

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## ELISA DETECTION OF VIRUSES IN 'MOSCATO BIANCO' GRAPEVINES SHOWING DIFFERING CLUSTER MORPHOLOGY.

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White Muscat ('Moscato bianco') is a grape cultivar, grown in various parts of Italy from which a sweet, sparkling wine is produced. In the Loazzolo area (Asti, northern Italy), this cultivar is used to produce a 'Passito' wine of fine quality and old tradition, highly remunerative to the growers. The Passito is made with clusters dried before fermentation; those of shot-berry (SB) or loose (L) morphology, with high sugar content, are most suitable to this practice. SB- and L-type clusters are most frequently found on old or very old plants while younger plants bear generally tight (T) clusters. The present study was undertaken to check whether the SB or L morphology of clusters might be correlated with virus infection.

Three 30-year old vineyards were chosen at Loazzolo, and ten vines bearing clusters typical of the SB, L and T type were selected in each of them during the 1991 harvesting season. Diagnosis was done basically by DAS-ELISA on wood material scraped from pieces of dormant canes collected during winter pruning. The same cuttings were then grown in the glasshouse to produce fresh vegetation which was used as source of inoculum to herbaceous indicator plants. Phosphate buffer, 0.1 M pH 7, containing 2.5% nicotine and 2% polyvinylpyrrolidone (PVP) was used for sap extraction. The ELISA reagents were to the following viruses: grapevine virus A (GVA) Istituto di Fitovirologia Applicata, I.F.A.), grapevine fanleaf virus (GFLV, I.F.A. - Loewe), grapevine leafroll-associated virus I (GLRaV-I, Bioreba), and grapevine leafroll-associated virus III (GLRaV-III, Bioreba). Antigen samples were extracted from the tissue in 1/10 (w:v) 0.1 M Tris-HCl buffer pH 8 containing 2% PVP and 0.1% thioglycolic acid (I), and each tested in two wells in ELISA plates. The test was performed according to Clark and Adams (2) using as controls sap extracted as above from either healthy plants or plants known to be infected with the viruses investigated. Sap inoculation tests, performed in the glasshouse, were done using the following indicator plants: *Chenopodium amaranticolor*, *C. quinoa*, *Gomphrena globosa*, *Nicotiana benthamiana*, *N. clevelandii* and *N. megalosiphon*.

GFLV was detected by ELISA in 27 of 29 plants of the SB type, 19 of 29 plants of the L type, and 1 of 30 plants of the T type (Table 1). Positive reactions to the other viruses tested were as follows:

- of 29 SB plants: GVA in 26, GLRaV-I in 19, GLRaV-III in 3;
- of 29 L plants: GVA in 25, GLRaV-I in 24, GLRaV-III in 2;
- of 30 T plants: GVA in 26, GLRaV-I in 24, GLRaV-III in 5.

Single virus infections were detected in 3 cases for GFLV and in 1 case each for GVA, GLRaV-I and GLRaV-III. No viruses were detected in 4 plants. The remaining 78 plants were affected by two or more viruses in different combinations (Table 1).

Sap inoculation experiments were done during winter 1992-1993. GFLV was isolated from 12/15 plants of the SB type and 14/22 plants of the L type. It was not isolated from 18 plants of the T type. Infections were confirmed serologically by ELISA or by the agar gel double diffusion test. No viruses other than GFLV were transmitted to the indicator plants.

The presence of closteroviruses was on the whole equally high in the three groups of plants tested while GFLV was detected much more frequently in vines with SB-type clusters (93%) or L-type (65%) than in those with T-type (3%). Plants with either the SB- or the L-type clusters contained most frequently three viruses (GFLV, GVA, GLRaV-I), while those with T-type clusters were prevalently affected by two of these viruses (GVA, GLRaV-I) (cf. Table 1).

We conclude that, in the Loazzolo area, the SB and L morphology of 'Moscato bianco' clusters, favourable for production of the Passito wine, is correlated with GFLV infection.

Table 1: Viruses detected by ELISA in 'Moscato bianco' vines with different cluster morphology.

	Shot berries	Loose	Tight
	(SB)	(L)	(T)
GFLV	3	-	-
GFLV GLRaV-I	-	1	-
GFLV GLRaV-I GLRaV-III	-	1	-
GFLV GLRaV-III	1	-	-
GFLV GVA	5	2	-
GFLV GVA GLRaV-I	16	15	1
GFLV GVA GLRaV-III	1	-	-
GFLV GVA GLRaV-I GLRaV-III	1	-	-
GLRaV-I	-	1	-
GLRaV-III	-	-	1
GVA	-	1	-
GVA GLRaV-I	2	6	21
GVA GLRaV-I GLRaV-III	-	1	1
GVA GLRaV-III	-	-	3
NEGATIVE	-	1	3
DEAD	1	1	-
<b>TOTAL</b>	<b>30</b>	<b>30</b>	<b>30</b>

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## EFFECT OF HEAT THERAPY ON AGRONOMICAL AND ENOLOGICAL APTITUDES OF GRAPEVINE CLONES

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Piedmont is regarded as one of the best Italian grape growing areas. Unfortunately virus diseases are spread within the grapevine population. Symptom observation and virus indexing intensively carried out during a clonal selection project indicated the wide presence of grapevine leafroll (GLR) in clones of the cultivars 'Dolcetto' and 'Nebbiolo'. In addition the occurrence of grapevine fanleaf virus (GFV) infection was detected in all the 'Nebbiolo Michet' selections.

Growers are concerned about the damage the viruses may cause to vine performances but they are also doubtful about the possible change in terms of wine quality the use of very healthy propagation material may induce. It is to be remembered that the 100 % infected 'Nebbiolo Michet' is however locally regarded as one of the cultivars of highest quality for wine production. In addition, data on this subject obtained in European environments are very scarce (1; 2).

In order to better clarify the influence the presence or the absence of GFV and GLR may have on vine field performances and grape juice quality, trials have been carried out comparing the behaviour of original and heat treated clones of the previously cited cultivars.

### Materials and methods

Since 1981, well grown two year old potted vines of clones of 'Dolcetto', 'Nebbiolo' and 'Nebbiolo Michet' have been placed in a thermotherapy chamber with artificial lighting at about 37°C for periods varying between 70 and 140 days. Terminal shoot tips about 0.5 cm long were excised from single vines and rooted under mist or in an agarized nutrient medium under sterile conditions (3). The plantlets were then grown in a greenhouse prior to transplanting in the field. Established daughter vines were indexed for infectious diseases by grafting to known indicator varieties and tested by ELISA for GFV and GLR-associated viruses (GLRaVs). None of the indexings so far performed has shown any evidence of virus diseases.

Both original and heat treated material has been propagated on certified rootstocks. Experimental vineyards with a complete randomized block design were established in the years 1988-90. Morphological, agronomical and enological data were collected as soon as the vineyards began to produce and the preliminary results are discussed in this report.

### Results

The heat treated material performances compared to the ones of original material varied greatly depending on the virus infection formerly present. GFV elimination in 'Nebbiolo Michet' resulted in a dramatic increase of vigour and yield in the first year of production, so far the only one controlled (fig. 1). Despite the much heavier crop only the juice acidic parameters were increased by the treatment (data not shown), meanwhile the differences in sugars were not statistically different.

When the sanitation involved GLR-associated closteroviruses (types I and III), changes in vine performances were much less evident as shown by 'Nebbiolo' and 'Dolcetto' results (table 1 and 2). In this case only the increased vigour in both the cultivars and the higher berry weight only in 'Dolcetto', were statistically significant in heat treated material. Nevertheless, the general trend of heat treated material in both the cultivars is an increase in crop, cluster weight and juice titratable acidity and a reduction in soluble solids.

### Conclusions

The preliminary field results obtained comparing the performances of original and heat treated clones show that GFV elimination induces dramatic changes in vine behaviour. On the contrary, the effects of GLRaVs sanitation are less evident.

In general the heat treated progenies seem to be more vigorous and higher yielding and the quality of their grape juices not improved.

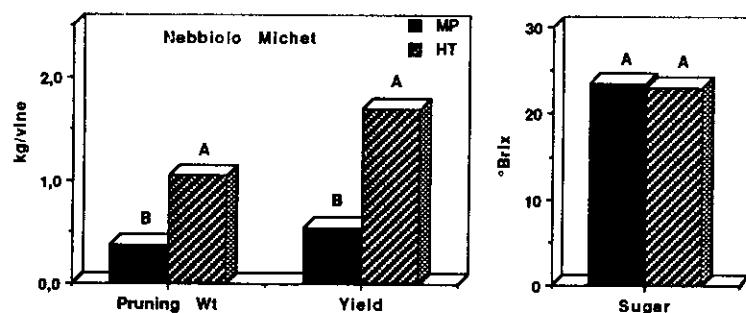


Figure 1. Histograms showing field performance of original (MP) and heat treated (HT) 'Nebbiolo Michet' clones (1992). Capital letters indicate Duncan test significance.

Data	MP	HT	Signif. F
Pruning Wt g/vine	833	923	*
Yield kg/vine	3,38	3,49	n.s.
Cluster Wt g	313	399	n.s.
Cluster/vine	9,6	10,1	n.s.
Berry Wt g	1,79	1,90	*
°Brix	20,6	20,3	n.s.
pH	3,46	3,46	n.s.
Tit. Acidity ‰	5,63	5,71	n.s.
Tartaric Ac. ‰	6,64	6,58	n.s.
Malic Ac. ‰	2,08	2,17	n.s.

Table 1. Agronomical data and must composition of original (MP) and heat treated (HT) 'Dolcetto' clones (average 1990-1992).

Data	MP	HT	Signif. F
Pruning Wt g/vine	856	1008	*
Yield kg/vine	2,29	2,58	n.s.
Cluster Wt g	234	270	n.s.
Cluster/vine	9,5	9,5	n.s.
°Brix	21,8	21,3	n.s.
pH	3,08	3,09	n.s.
Tit. Acidity ‰	7,51	7,72	n.s.

Table 2. Agronomical data and must composition of original (MP) and heat treated (HT) 'Nebbiolo' clones (average 1991-1992).

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# THE EFFECT OF VIRUS DISEASES ON GRAPE PRODUCTION IN KAHRAMANMARAS REGION IN TURKEY

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Kahramanmaras region is an important grapevine production area for table grape varieties since centuries. Nowadays, these varieties show drastic yield reduction year after year due to virus diseases (2).

This research was conducted to determine the viruses and the yield losses they induce. ELISA was used for virus detection (1). Infected samples were collected from the intensive grapevine production provinces of the region. 31 out of 70 samples were found to be infected with one or more viruses. Grapevine leafroll virus (GLRaV) (type I and type III), grapevine fleck virus (GFV), grapevine fanleaf (GFLV) and arabis mosaic (AMV) viruses were found. Most of the samples were infected by GLRaV I and GLRaV III. GFV was detected randomly. GFLV and AMV were also found to be associated to yield losses. In table 1 we represent viruses detected by ELISA in grapevines of the Kahramanmaras region.

Table 1: Viruses of grapevine in Kahramanmaras region in Turkey.

Table 1. Viruses of grapevine in Karahamamhara region in Turkey							
Location	Number of samples	Number of infected plants					Total number of infected plants
Name of province		GFLV	GFV	AMV	GLRaV		
					type I	type III	
Central	20	2	1	5	5	5	6
Türkoglu	5	1	0	2	4	2	4
Pazarcik	10	1	1	2	1	3	4
Karabiyikli	5	2	0	1	3	3	3
Afsin	10	2	1	2	0	1	3
Dereli	10	4	2	5	5	6	8
Elbistan	10	1	0	1	2	2	3
Total	70	13	5	18	20	22	31

After the identification of the viruses by ELISA, yield losses (2) were estimated by the comparison of the weight of total clusters, weight of total berries in a average cluster, the average weight of one berry and the number on berries in an average cluster from infected and healthy plants grown under the same conditions in the same vineyard (table 2).

Table 2: The effect of viruses of grapevine on the grape production in Kahramanmaraş region in Turkey

Location		WC (gr)	NB	WTB (gr)	10BW (gr)	WAB (gr)
Merkez	H	391.42	203	218.00	14.70	1.49
	I	132.14	168	64.85	5.07	0.50
Pazarcık	H	378.00	207	285.08	14.67	1.79
	I	138.20	185	59.00	3.04	0.30
Türkoglu	H	261.00	156	208.13	13.92	1.62
	I	167.00	182	91.78	4.97	0.49
Afsin	H	353.70	224	229.03	12.28	1.53
	I	149.50	196	78.64	4.07	0.40
Dereli	H	289.30	196	164.05	10.38	1.25
	I	123.60	172	56.89	3.73	0.37
Karabiyikli	H	286.00	156	195.80	15.52	1.55
	I	171.00	157	92.04	5.78	0.57
<b>Total</b>	<b>H</b>	<b>323.57</b>	<b>190</b>	<b>216.68</b>	<b>13.62</b>	<b>1.55</b>
	<b>I</b>	<b>146.91</b>	<b>177</b>	<b>73.87</b>	<b>4.44</b>	<b>0.45</b>

**WC:** Weight of an average cluster; **NB:** Total number of berries on an average cluster; **WTB:** Weight of total berries on an average cluster; **10BW:** Weight of 10 berries of an average cluster; **WAB:** Average weight of one berry; **H:** Healthy samples; **I:** Infected samples.

Results showed that viruses caused yield losses of approximately 45% in the surveyed areas.

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## FURTHER STUDIES ON CORKY BARK IN SARDINIA.

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Corky bark (CB) is one of the four diseases of the rugose wood complex (3). In fieldgrown *Vitis vinifera* cultivars it may not be clearly identified, whereas in the indicator LN-33 it induces highly characteristic modifications of the leaves (yellowish spots), shoots (swelling and cracking of the basal internodes) and stem (marked grooving).

In Sardinia, CB was the object of investigations that led first to its discovery (2), and now to further observations carried out in the framework of sanitary improvement programmes. The identification of CB was always based on the response of LN-33 indicators that, following inoculation by omega grafting, were grown in the field and observed for no less than three years.

Infected sources (i.e. either commercial varieties or symptomatic LN-33 indicators) were used for further graft transmission trials in which *V. rupestris*, a set of *Vitis* hybrids (Baco 22A, Kober 5BB, 140Ru, 420A and 157.11) and a set of *V. vinifera* cultivars (Cabernet franc, Cabernet sauvignon, Merlot and Pinot noir) were inoculated by omega grafting or chip budding, and transplanted in the field for symptom expression and reading.

### Field observations

All candidate clones of cv Italia indexing positive for CB, exhibited an intense grooving of the woody cylinder, often accompanied by exceedingly rough and spongy cortex of the scions, next to the graft union. These vines had also a reduced vigour and, occasionally, a mild rolling and chlorosis of the leaves. No alteration of the foliage or the wood was observed in cvs Alloppu, Argo mannu, Olivetta and Semidano, whereas the red fruited cv Pascale di Cagliari showed a peculiar diffuse pinkish discoloration of the leaves, and the white-fruited cvs Malvasia di Bosa and Vernaccia, a sporadic and mild leaf rolling and chlorosis.

### Transmission trials

All the above *V. vinifera* cultivars induced reactions in LN-33 which varied in type, severity and consistency of expression. On these bases, two groups of donors were identified:

- i) strong donors (about 75% of the tested clones) that induced the whole range of symptomatic responses on leaves, shoots and woody cylinder of each individual indicator, consistently (up 100%) and recurrently (several years in a row);
- ii) weak donors (about 25% of the tested clones) that induced mild, incomplete, inconsistent (reactions were not shown by all LN-33 individuals inoculated) and erratic (symptoms did not recur on the same individuals year after year) responses.

As reported elsewhere in these Proceedings (1), grapevine virus B was recovered (63% association) only from donors of the first group. Kober 5BB, 140Ru, 420A and 157.11 reacted with inconsistent and variously intense discolorations of the small veins and occasional alterations of the wood. Foliar discoloration were stronger in Kober 5BB where they turned into scattered chlorotic spots similar to those shown by LN-33. Baco 22A was always totally symptomless. *V. rupestris* showed no foliar reactions, whereas high (over 80%) and rather consistent were the symptoms on the wood, regardless of the type of grafting, i.e: omega or chip budding (Table 1). A fairly high (67 %) symptomatological response was obtained with chip budding also when the graft did not take. In any case, the alterations of the wood consisted in a generalized rugosity, quite different from the symptom induced in the same indicator by *Rupestris* stem pitting.

Cabernet franc, Cabernet sauvignon, Merlot and Pinot noir reacted with atypical reddening of mature leaves and stem grooving which, however, was not shown by all inoculated plants.

In any case, back inoculation to LN-33 from all grafted indicators, demonstrated that the disease had been transmitted regardless of the type of reaction shown.

TABLE 1. Reaction of *V.rupestris* to inoculation with CB-positive donors.

Type of grafting	Positive reactions %			Negative reactions
	Constant	Erratic	Totals	
Omega grafting	47	40	87	13
Chip budding:				
graft-take successful	83	0	83	17
graft-take unsuccessful	50	17	67	33

#### Concluding remarks

Based on the data derived from this complex series of field observations and transmission trials, the following conclusion can be drawn.

- i) The presence of corky bark in Sardinia is confirmed, although the disease has an overall low incidence, varying with the cultivar.
- ii) Diagnosis based on symptom expression in the field is risky, if not impossible. In fact, for cv Italia, there is no guarantee that the outward manifestations seen in the vineyards may not be caused by other diseases of the rugose wood complex (e.g. Kober stem grooving) which are often associated with CB. On the other hand, the pinkish leaf discolorations consistently occurring in infected cv Pascale di Cagliari, were also sporadically seen in CB-free vines.
- iii) CB is often latent in *V.vinifera* as demonstrated by its positive identification in more than 2% of symptomless candidate clones selected and indexed in Sardinia, and by the lack of visible reactions in graft-inoculated *V.vinifera* cultivars. Thus, there is little hope to eliminate CB from vines under selection through simple, visual observation, which makes indexing mandatory.
- iv) CB can be transmitted by grafting to several American *Vitis* species and rootstock hybrids in which it induces symptoms on leaves and stems that disclose its presence, but in a manner that is not reproducible enough, so as to represent a reliable alternative to LN-33.
- v) LN-33 remains the main and, by far, the most satisfactory indicator. However, even this hybrid reacts in a certain number of cases with erratic and inconsistent responses, the significance of which is obscure and may warrant additional investigations.

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## A STUNTING FACTOR IN *VITIS VINIFERA* TRANSMITTED BY GRAFTING TO KOBER 5BB.

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It is known that certain graft combination of *Vitis* may display striking expression of "incompatibility" leading to reduced growth and/or death of the scion. In France, for instance, negative reactions induced in rootstocks (especially Kober 5BB) by some clones of rootstocks and *V. vinifera* varieties were repeatedly reported (2, 3, 4, 7, 9), and in Italy a factor causing stunting and bushy growth of European grapes was recently identified in 140Ru (8).

As reported in the present paper, comparable situations were encountered in Sardinia in the course of indexing for sanitary selection, when different *V. vinifera* varieties were grafted with Kober 5BB scions. All donors were also omega-grafted with *V. rupestris*, LN33 and cv Mission, transplanted in the field and observed for three vegetative seasons.

The following viruses were searched for by ELISA as previously described (6), using mostly commercial kits, and/or by immunoelectron microscopy: grapevine fanleaf virus (GFLV), grapevine fleck virus (GFkV), grapevine virus A (GVA), grapevine virus B (GVB), grapevine leafroll associated viruses I (GLRaV I) and III (GLRaV III).

In field-grown grafted vines, the symptoms appeared during the first flush of vegetation. They consisted in delayed bud break (10-15 days later than in asymptomatic graft combinations), slow and reduced growth of shoots, small, chlorotic and, sometimes, puckered leaves. Furthermore, the contemporary development of many shoots from the adventitious buds at the base of the main shoots conferred compact and bushy appearance upon the plant. Occasionally, there was a remission of symptoms in the course of the season with resumption of normal growth. In most cases, however, the vines remained stunted, with small and weak canes, and reproduced the symptomatology in the following years. Out of 82 candidate clones assayed, 32 belonging to cv Malvasia di Bosa, Cannonau, Vermentino, Pascale di Cagliari, Monica, Aleatico and Italia proved to contain the graft-transmissible factor that elicited stunting in Kober 5BB. It prevailed in Malvasia di Bosa (over 70% of positive donors) followed by Pascale di Cagliari and Monica (about 40%) and by Aleatico, Cannonau and Vermentino in which the infection ranged between 15 and 20%. In all these clones, it persisted throughout the years and was transmitted, expressing symptoms with high consistency, except for a few cases, whenever Kober 5BB was grafted onto material from these sources. No reactions were obtained in *V. rupestris*, LN-33 and Mission, but material taken from these apparently normal donors reproduced the stunting syndrome when grafted with Kober 5BB. No stunting factor was apparently detected in 20 candidate clones of cv Vernaccia, Torbato, Nieddera, Nuragus, Bovale and Trebbiano.

The results of indexing did not reveal any specific association of the stunting factor with any of the diseases of the rugose wood complex or with leafroll. Rupestris stem pitting (RSP), for instance, was detected in 60% of the stunt-containing donors and in 40% of those that were stunt-free. The reverse was true for leafroll, which occurred in about 40 and 60% of stunt-positive and stunt-negative sources, respectively. Furthermore, 25 clones of different varieties, free from known virus and virus-like diseases, proved to contain the stunting factor. ELISA tests were also inconclusive. If it is true that GFkV was found in about 64% of the stunt-positive donors, it was also present in about 40% of the stunt-negative sources, two figures which are in fairly good agreement with data on GFkV incidence elsewhere in southern Italy (1). Likewise, closteroviruses (GVA, GVB, GLRaV I and GLRaV III) were more or less equally represented in both sets of donors, regardless of whether they were stunt-positive or negative.

Stunting and bushy growth conditions of Kober 5BB and Baco 22A had already been recorded from Sardinia (5) in the course of previous studies with cv Italia donors affected by enations, rugose wood, leafroll and fleck. The highly degraded sanitary status of those source materials and the less advanced knowledge of the virological scenario of grapevines, did not allow, at that time, to draw conclusion on the nature of these abnormalities.

Now however it seems plausible to reckon that a number of *V. vinifera* cultivars grown in Sardinia contain an infectious factor differing from viruses that cause ordinary grapevine diseases. This factor is latent in *V. vinifera*, *V. rupestris* and the hybrid LN 33, but it is perpetuated in the wood and is readily and consistently transmitted by grafting. Whether it is comparable with other infectious factors with a similar behaviour reported from France (9) and Italy (8) is unknown, and similarly unknown is its nature.

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## FURTHER INVESTIGATIONS ON 'RODITIS LEAF DISCOLORATION' DISEASE

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For the first time in October 1981 an apparently new virus disease of the grapevine, for which temporarily the name 'Roditis Leaf Discoloration' (RLD) was proposed, was observed on the red wine cultivar Roditis in Magnesia area, Central Greece, exhibiting a diversity of yellow and/or red discolorations of the leaf blade as well as clusters with small berries of no commercial value (2). The disease was transmissible to *Vitis vinifera* cv. Mission and to some herbaceous plants. Carnation mottle Carmovirus (CarMV) and grapevine fanleaf Nepovirus (GFLV) were consistently isolated by mechanical inoculation to herbaceous plants from diseased Roditis plants, as well as from a Mission indicator plant inoculated by grafting (1).

After the destruction of the initially observed infected vineyard, from which CarMV and GFLV were isolated, the only source left to us for further investigations were two diseased 'Roditis' plants and a Mission vine inoculated by grafting, planted both in the Institutes of Volos and Heraklion. A survey made during the period 1982-87 revealed that several other vineyards of the same area and the same cultivar presented stocks with similar symptoms. Three of them were selected in order to study the incidence and the spread of the disease during the next years, as well as to study the possible causal agent of the disease.

As an example of the incidence and the spread of the disease, we observed in one of the three vineyards in 1988 eight diseased stocks, in 1989 ten (two new infected vines), in 1990 sixteen (six new), in 1991 sixteen and in 1992 twenty-six (10 new).

Every year during the period 1989-1992 transmission experiments through mechanical inoculation of herbaceous test plants were carried out on *Chenopodium quinoa* Willd., *Gomphrena globosa* L., *Cucumis sativus* L., *Phaseolus vulgaris* L. cv "Bountiful", *Nicotiana benthamiana* Domin. and *Dianthus barbatus* L. using virus sources from 36 diseased plants from the three vineyards mentioned above, as well as from the initial material used for the description of the 'Roditis Leaf Discoloration' disease (two Roditis and one Mission vines).

Furthermore, during the same period ELISA was applied to detect GFLV, CarMV and GLRaV1 in the plants described above.

The results showed that all mechanical transmission and ELISA experiments were positive for CarMV and GFLV, but only for the initial material. No reaction was observed with the other vines.

Transmission tests on woody indicator plants carried out in 1992 have not shown any symptoms yet.

In conclusion, our research revealed that RLD-disease described in the initial vineyard is unrelated with the disease found and studied in the three new vineyards, despite of a strong similarity of the symptoms. In the last case the causal agent needs to be determined.

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## A NEW VIRUS DISEASE, GRAPEVINE BERRY INNER NECROSIS WITH NATURAL SPREAD IN JAPAN

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Berry inner necrosis is the most economically important virus disease of grapevine in Yamanashi, Japan, because it causes severe damage to grapevines and spreads naturally in fields. Mosaic and berry inner necrosis symptoms of the disease were observed in grapevine cv. Campbell Early and Kyoho (2, 4). In 1984, Nishijima *et al.* (3) named tentatively grapevine mosaic after success in graft-transmission of the disease. The disease was renamed grapevine berry inner necrosis in 1992 (6).

**Symptoms:** The affected vines grow less vigorously and sprout late in spring. The diseased vines show inner necrosis in shoots, shortening internodes and various patterns of yellow discoloration on leaves. Berries on affected vines are small, and show discoloration on rind and necrosis in flesh. The fruits often mature late and irregularly, and their flesh becomes harder.

**Causal agent:** The causal virus particle is filamentous and its size is 740 nm long and 12 nm wide (7). The virus did not react with antisera to grapevine virus A, grapevine leafroll virus (type 1) and several potyviruses (9). The virus was back-transmitted to grapevine seedlings using purified virus preparation. The back-transmitted virus induced mosaic on leaves and necrosis in flesh (6, 9).

**Transmission:** The virus can be transmitted by mechanical inoculation from diseased grapevine tissues to *Chenopodium quinoa* or *C. amaranticolor* other than graft-transmission (7). Natural spread was confirmed experimentally by infection of virus free cv. Kyoho plants planted in the fields (5). However, its vector has not been found yet.

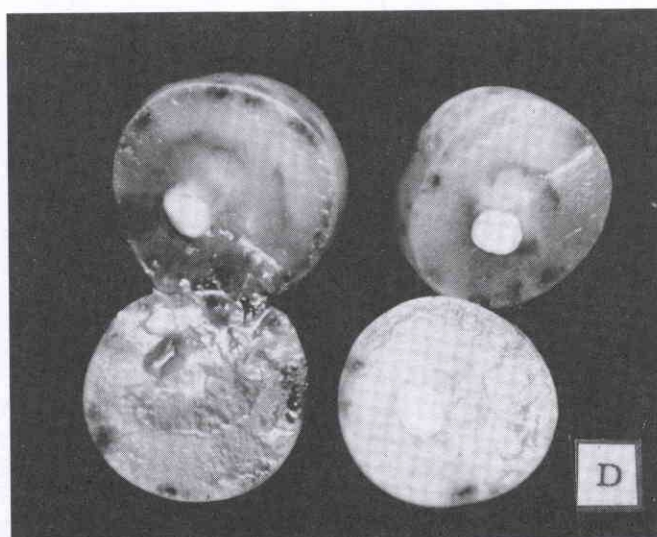
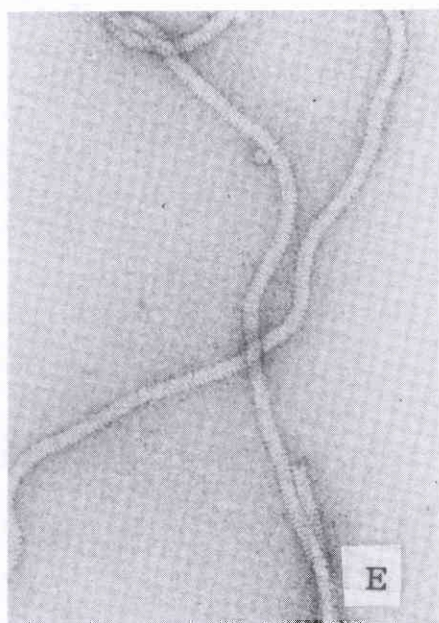
**Varietal susceptibility:** Symptoms expression varies depending on grapevine cultivars. Almost all known Japanese table cultivars cross-made with Campbell Early or its related cultivars, for instance Takao, Kyoho and Pione were susceptible. Some rootstock varieties were also susceptible.

**Detection:** Causal virus can be detected serologically from young leaves of symptomless infected grapevine using ELISA (1, 8). Indexing by grafting on *Vitis* indicators, cv Kyoho and Pione also useful and reliable.

**Control:** In the regions where the disease occurs, replanting of symptomless cultivars is recommended because susceptible cultivars are easily infected with the virus and damaged severely.

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**Figure 1: Grapevine berry inner necrosis on cv Kyoho**

**A:** Necrotic discoloration on rind of berries. **B:** Young shoots with short internodes and zigzag growth with dwarfed leaves. **C:** Various patterns of yellow discoloration on the deformed leaf blade. **D:** Necrosis in flesh. **E:** Causal virus particle.



## ADVANCES IN GRAPEVINE YELLOWS RESEARCH SINCE 1990

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After the outbreak of Flavescence Dorée (FD) between 1955 and 1965 in the South West of France, and the definition of Bois Noir (BN) and Vergilbungskrankheit (VK) in Burgundy and Germany, little attention was given to this type of diseases, except their occurrence in Oltrepo Pavese, Italy and since 1970 in Corsica.

A new interest started at the beginning of the 80<sup>ies</sup> with the spread of grapevine yellows (GY) in Languedoc, Provence (France) and Veneto (Italy). Symptoms of GY, all identical to FD, were then found in Sicily, Emilia Romagna, Piemonte, Toscana, Latium, Apulia, Australia, Israel, the United States and other countries. I proposed at the 9th meeting of the ICVG (1987) and (1) the following classification for GY in order to make clearer the various situations: *Flavescence dorée sensu stricto*, transmitted by *Scaphoideus titanus*; *Mediterranean grapevine yellows* non associated with *S. titanus*; *Bois noir* and *Vergilbungskrankheit* (non transmitted by *S. titanus*); *Subtropical grapevine yellows*; *North American grapevine yellows*. My report underlined also the poor and time consuming methods available at that time for diagnosis and epidemiology. The development of research on GY since 1990 is now characterised by the efforts developed everywhere to access to the new approaches offered by serology and molecular biology. We will first discuss the news in Epidemiology, then the transmission experiments to herbaceous plants and finally the access to Serological and Genomic Tools.

### Epidemiology

No report about GY has been found in new countries since 1990. Apparently the current inventory is not far to be completed over the world. However, the importance and evolution of GY has been investigated in Switzerland (2), Apulia (3), Germany (4), Sicily (5), Greece (6) and Veneto where the average lack of yield in Chardonnay cv. reached 50 % (7).

A better definition of the grapevine yellows diseases has been investigated in several instances by the search of the pathogen agent in grapevine itself, by electron microscopy. Sarachi *et al.* (8) developed the scanning electron microscope investigations and found a good correlation between FD symptoms in grape and MLO-like agglomerates in the phloem tissues. Other scanning studies were carried out with success on the GY occurring in Greece (6). Transmission electron microscope always showed a very low MLO concentration in grapevine tissues (9, 10) except for the samples coming from plants grown in greenhouse where they were found in larger quantities (11). Credi (12) investigated several cultivars for MLO occurrence and found MLO only in cv. Caveccia, a few cells being filled of the pathogen. The necrosed phloem cells, probably previously infected, never contain typical MLO (11, 12).

The spread of the pathogen inside the plant certainly represents a difficult riddle, important for the survey of healthy wood production. The first studies on the evolution of the disease in grapevine tissues already showed an irregular distribution of the FD pathogen and gave an explanation for the recovery and for the localised symptoms in scion cultivars (13). Similarly, graft transmission of FD-like symptoms in Emilia-Romagna/Italy gave relatively low percentages of transmission, ranging from 0 to 15 per cent of the successfully grafted vines (14). The spread of the FD pathogen in the plants since their infection was studied by immunolabelling in both electron and photonic microscopy by Lherminier *et al.* (15). In the case of BN in Burgundy, it appears that the pathogen can remain in symptomless plants (16), and as for VK in Germany that infected cuttings can originate from symptomless wood (4). A study on the transmissibility of FD by symptomless 3309 Couderc rootstock plants showed that the plants, once infected, remain permanently infected. However, there is a very irregular distribution of the infectious cuttings according to the canes and along one cane. Other rootstock varieties seem to behave in the same way (17). This observation will make difficult sampling for FD diagnosis in symptomless rootstocks.

The natural spread of grapevine yellows diseases is another important problem. *Scaphoideus titanus* remains the only one known vector found for a GY. It appears more efficient in France than in Italy where no relationship was detected between the number of *S. titanus* present in the vineyard and the incidence of the GY (7). Search of other leafhoppers as potential vectors were conducted in every region where *S. titanus* is absent or does not account for the epidemiology. In Tuscany, where *S. titanus* was not found, eight most frequently found hopper species were tested for GY transmission but the results were negative (18). It is also the case in Apulia and Sicily where *S. titanus* is not present. Eleven species of leafhopper were found in both regions but they were not tested for GY transmission (3, 5). At the northern border of the *S. titanus* life area in France, it was possible to detect the presence of the leafhopper further north near to the vicinity of Dijon. This fact may probably be related to the warm and dry summers undergone since 1988 in this region which

may provoke fluctuations in the life area (16). *S. titanus* was not found in Germany. Maixner (4) was able to collect 36 species within the canopy of grapevine. Only 6 of them are known to feed on grapevine but 9 species are known as MLO vectors. They were not tested for VK transmission. An interesting way of investigation is the modelisation of the spatial distribution analysis of the infected plants in the vineyards. Credi and Calegari (19) used the van der Plank's doublet analysis technique for the Emilia Romagna GY. Recently Maixner (44) developed the Patchy program for the VK disease in Germany. The results support the hypothesis of a transmission of VK by only occasionally or erroneous ampelophagic vectors. This is in agreement with the observations described for BN in France (20). The random distribution in other vineyards could be an indication of the introduction of the disease by infected planting material).

#### Transmission experiments to herbaceous plants

The transmission of GY to herbaceous plants certainly is an important step for the access to diagnosis tools. The transmission of FD to *Vicia faba* by *S. titanus* (21) always appeared more difficult in Italian conditions than in France. Possibly, a fundamental reason may occur, for ex. a difference in MLO strains or in vector ability. A regular transmission by *S. titanus* of the GY in Piedmont/Italy to *Trifolium repens* (L) is reported by D. Bosco (22). More frequently, transmissions were tried to *Catharantus roseus* (L) either by natural infection or by dodder transmission, because of the facilities offered by Periwinkle to be propagated by grafting, healthy or infected. Healthy seedlings of *C. roseus* placed in a vineyard affected by GY in Emilia Romagna, a region where *S. titanus* is absent, developed symptoms including phyllody and virescence of flowers (Credi cited in 23). The plant is referred as Italian periwinkle virescence (IPVR) in subsequent research. Transmission trials by dodder from Chardonnay stocks affected by GY to young *C. roseus* were started in Udine since 1987. MLO symptoms were obtained on four plants, consisting in virescence, phyllody, vein yellows and witches broom formation. The MLO is easily transmitted from *C. roseus* to *C. roseus* by grafting, but attempts of back transmission to grapevine were unsuccessful (7). The symptoms obtained are referred to as FDU although the original FD of France do not show virescence or phyllody or *C. roseus* (24). A very large trial of dodder transmission from grapevine to *C. roseus* was carried out recently for the GY of Emilia Romagna (25). Four out of 628 (0,6 %) periwinkle seedlings developed symptoms. Two types of symptoms were recorded: either a yellowing of the leaves or a general chlorosis accompanied by virescence or phyllody. In Apulia, Southern Italy, a transmission by dodder was obtained both in the field under screen, and in greenhouse. 9 out of 30 periwinkle plants showed symptoms of small yellow leaves, rosetting and small flowers with colour breaking (3). It is hoped to have by this way a "mediterranean grapevine yellows" on *C. roseus*; it will be referred as FD B for further work. The dodder transmission was also tried in 1991 in Germany under greenhouse for the VK disease. 5 out of 19 plants developed symptoms such as phyllody and virescence (4).

#### Serologic tools

The first polyclonal antibodies for GY were obtained from FD infectious leafhoppers and from FD infected *Vicia faba* and first used for ISEM detection of the pathogen agent (26). The ELISA test and the DOT BLOT (27) were positive on *S. titanus* from FD infected vineyards in France and were used to screen monoclonal antibodies specific for FD (28). The application to immunolabelling *in situ* was developed by Lherminier *et al.*, (29, 30). All these tests were efficient on the infective leafhoppers *S. titanus* or *Euscelidius variegatus* and on infected *V. faba*. The same sera from INRA-Dijon were extensively used to assay *S. titanus* leafhoppers from infected vineyards from Piedmont (22) and from Geneva in the New York state (31) or used in transmission trials in Friuli/Italy (32). All these tests were FD positive and Western Blot patterns were close to those of FD in France. These results may indicate that FD is present in Northern Italy and supports the hypothesis of the North American origin of the FD pathogen (20).

The latter tests were not efficient on FD grapevine tissues. Results were very low or nil for samples coming from the field, low and erratic for samples, from the greenhouse. An important work was carried out since 1990 to adapt the ELISA test to FD infected grapevine tissues. Results concern the best part of the grapevine to sample, the best sampling period and an important adaptation of the extraction medium by addition of detergents (3 % triton X100 or 5 % CHAPS). A 10 times concentration of antigen by molecular filtration was then necessary to obtain positive results with all *Vitis vinifera* scion varieties (33). Up to now the test provided some positive results on symptomless rootstocks grown in the greenhouse, but not from the field. This test was extensively used during the growing season 1992 in collaboration with the "Service de la Protection des Végétaux" in France and with some foreign colleagues, to verify if grapevine leaves from the field in various foci of GY were or not "FD *sensu stricto*". It appeared that no one of the foci of GY in North East of France responded positively to FD (16). This is an information that Bois Noir (BN) is not serologically related to FD. There are "non FD foci" (BN ?) as far South as Ardèche (Rhône valley) where FD was formerly detected in *S. titanus* vectors (see above). Leaf samples with GY symptoms were tested from Sicily (Granata),

Switzerland (Cazelles), South Africa (Shiraz disease, Kriel), Australia (Magarey), New York State (Pearson), Emilia Romagna (Credi), Israel (Tanne) and Friuli/Italy (Osler and Refatti). All were FD negative except for the samples of Friuli/Italy which were very strongly FD positive (34).

A new way to access to the serology of GY was recently developed by Chen. Two monoclonal antibodies were raised in mice immunized with partially purified antigens of FDU-periwinkle, (see above the dodder transmissions). The monoclonals were positive on FDU periwinkle, but serological detection of GY-MLO in diseased grapevines was not reliable because of the extremely low titer of the pathogen and possibly the interference of plant pigments (35). Reciprocal assays were conducted in Chen's and Caudwell's labs: FDU monoclonals did not react positively with FD infected hosts (leafhopper and broadbean from France) and FD specific antibodies did not react on FDU-periwinkle, (36).

Maixner (4) obtained no positive results in ELISA, in immuno-blot or immuno-fluorescence with grape affected by VK or with the *C. roseus* obtained from VK by dodder transmission, using either the anti FD antisera from Dijon or the FDU monoclonal antibodies from T.A. Chen.

FD specific antibodies (INRA-Dijon) were assayed on more than 40 MLO strains and isolates, (including the American Elm yellows). No positive reaction was recorded. In reciprocal assays, specific antibodies to Aster yellows, clover phyllody, tomato big bud, Molière's disease of cherry, peach yellow leafroll (Western X), *Crotalaria* witches broom (Thailand), *Faba bean* phyllody (Sudan) and plum leptonecrosis V<sub>6</sub> (Italy) were tested with FD antigens. No positive reaction was recorded in any of these assays (37).

#### Genomic tools

The 90<sup>ies</sup> are characterised by the quick development of the powerful genomic methods. Their first introduction into the GY problem was the production of FD specific probes from infected broadbean, already presented at the 10th ICVG Congress of VOLOS (38). Since this time, 9 probes specific for FD have been developed. DNA hybridization with these probes demonstrated homologies between FD MLO-DNA and MLO-DNA of American woody plant diseases like Elm yellows, Ash yellows and Western X (39). We have to remember that no serological relation was found between FD and Elm yellows (see above in serological tools). It is time, however, to recall that Elm yellows is transmitted by *Scaphoideus luteolus*, an insect of the same American genus as *S. titanus*, the vector of FD. This would further support the hypothesis of coevolution of MLOs with their insect vector which has probably occurred in America for FD and its vector (20).

Davis *et al.* (23) reported the obtention of IPVR specific cloned DNA fragments produced from the IPVR *C. roseus* obtained in Bologna from a natural infection (see above). At least 6 of the fragments were from chromosomal DNA. Although the results revealed a sharing of nucleotide sequence homologies between IPVR-MLO and MLOs associated with several other diseases including X disease, clover proliferation, Ash yellows, Elm yellows and FDU (see above), IPVR could be distinguished from these and all other MLOs studied.

Chen *et al.* (35) developed DNA probes and primers used in polymerase chain reaction (PCR) from FDU periwinkle (see above). Two recombinant clones which contained inserts of 9,0 and 1,6 Kb were selected. The labelled DNA probes have been used to successfully detect GY-MLO in grapevine samples collected from Italy and the United States. Their oligo nucleotides were designed and synthesized for use as primers in PCR. Using oligos 1 and 2, a 550 bp DNA fragment was amplified from crude DNA extracts of infected periwinkles and grapevines. Employing oligos 1 and 3, a 600 bp DNA fragment was amplified only from infected periwinkles, but not from diseased grapevines. PCR results suggested that related but distinct strains of GY must exist to cause similar yellows symptoms of grapevines.

In an other lab (40), biotinylated cloned DNA probes were employed in dot hybridization and RFLP analysis to compare MLOs from periwinkle associated with FDU, FDB (from *C. roseus* obtained by dodder transmission respectively in North and South Italy, see above) and IPVR (from *C. roseus* naturally infected, see above). Results from dot hybridization revealed that FDU and FDB shared some regions of DNA sequence homology with one another as well as with MLO strains IPVR and Aster yellows 1, but all 4 MLOs were mutually distinct. RFLP patterns of chromosomal DNA from FDB exhibited some similarities with those from IPVR, but were different from those exhibited by DNA from FDU.

More recently, we used in Dijon (41) PCR primers from 16S rDNA conserved regions common to most MLOs according to Ahrens *et al.* (42). A positive result of PCR with such primers gives then a good indication of the occurrence of a MLO in a suspected plant. Furthermore the RFLP patterns after Alu I digestion of the 16S rDNA amplified product gives a profile which indicates specifically to which group the MLO belongs. The method was first adapted to work directly on infected grapevine leaf extracts. It was used during the 1992 growing season to study the samples collected from the field in various regions of France (see above the serological tests). The results were very clear: all the FD foci from southern France gave positive results with specific FD probes, positive PCR response with FD primers and gave a typical and constant "Elm yellow group" Alu I restriction profile after PCR of the 16S fragment. The same results were obtained with samples from Udine (Refatti). On the contrary the "serologically non FD" foci of northeastern France gave for the Bois Noir,



negative results with FD probes and FD PCR primers, but the 16S fragment was MLO positive and the Alu I restriction profile gave a constant "Stolbur profile". The same results with the same profile were obtained in some foci of Ardèche (Rhône valley), in Germany for VK (from Maixner), in Bologna (samples from Credi), in Torino (samples from Arzone), in Sicily (from Granata), in Israel (from Tanne), and with the IPVR periwinkle plants. All these MLO samples were of the Stolbur group and probably belong to the Bois noir disease. An additional 16S Alu I profile was found in some foci of Ardèche which were recalcitrant to the insecticide treatments against *S. titanus*, like the BN foci, but had a more aggressive epidemic spread than BN. This "third 16S profile" was again found in a focus in Champagne region. We can say that BN, described in North Eastern France, seems on the basis of 16S rDNA analysis to be much more widespread than expected, also in Italy and Israel. The relationship between BN and VK was already suspected (20). Three GY are apparently present together in the same French department of Ardèche (16, 41, 43).

In conclusion, one can expect to obtain in the next future a good picture of the various GY, of their number and their distribution over the world. Unfortunately nothing is known about the propagation and the vector of GY other than FD and there is no control method available. The new serological and genomic tools will help to find the potential vectors, and finally to design control methods. An important problem is raised in the fields of production and multiplication of healthy wood material. The current methods adopted in every country were designed for virus diseases with a low spread or no spread. The intrusion of GY renders difficult the production of healthy material anywhere and their multiplication safe from inoculation by flying, unknown vectors.

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## GRAPEVINE MLO TRANSMISSION BY INSECTS

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Since its appearance in France, the disease known as Flavescence dorée was associated with the nearctic cicadellid *Scaphoideus titanus* Ball as a vector (1). Nevertheless, while in France the disease produced great epidemics, spread rapidly and caused heavy damages, in Italy it appeared less virulent and limited to few cultivars. The difference between phytopathologic features observed in France and in Italy, the presence of infected plants in regions where *S. titanus* is not detected (2, 3, 4), the lack of relations between the frequency of *S. titanus* and the number of infected grapevines (5) led us to start researches in order to clarify the role of other leafhoppers in the transmission of MLOs associated with the disease.

For this reason transmission trials between grapevines and herbaceous plants were carried out in northwestern Italy from 1989 to 1992 with *S. titanus*, *Euscelidius variegatus* (Kirschb.), *Anoplotettix fuscovenosus* (Ferr.), *Macrostelus quadripunctulatus* (Kirschb.), *M. sexnotatus* (Fall.) and *Euscelis incisus* (Kirschb.).

### Transmissions from grapevines to grapevines and to herbaceous plants

*S. titanus* adults, that acquired in the field on grapes of cv Chardonnay, both with symptoms and symptomless (the latter having shown symptoms in past years), were transferred onto seedlings and cuttings of cv Chardonnay and Baco 22A and on seedlings of *Vicia faba* L. and *Trifolium repens* L., grown in controlled conditions. Acquisition access periods (AAP) were at least 28 days, inoculation access periods (IAP) were 7-14 days on grapevine, 7 days on broad bean, 3 days on white clover. A different number of *S. titanus* specimens was used in the different trials: 3-8 on Chardonnay seedlings, 10-20 on Chardonnay and Baco 22A cuttings, 3-7 on broad bean, 3 on clover. Leafhopper adults were successively transferred until their death on the above test plants. Grapevines were grown partly in the field, inside insect-proof cages, and partly in a greenhouse; broad beans and clovers were kept in a greenhouse. Three of 193 seedlings and two of 21 cuttings of Chardonnay showed leafroll, interveinal yellowing and necrosis; one of 10 cuttings of Baco 22A showed strong dwarfing, leafroll, yellowing and necrosis spread all over the leaves; none of 127 broad beans showed symptoms; 46 of 95 clovers showed apostasis and/or phyllody of some florets or flower head proliferation.

### Transmissions from clover to herbaceous plants

Nymphs of *S. titanus*, *E. variegatus*, *M. quadripunctulatus*, *M. sexnotatus* and *E. incisus* were isolated for 7 days on *T. repens* plants infected with *S. titanus* as described above; then they were transferred on their host plants to complete a latent period of 28 days; nymphs of *A. fuscovenosus* were isolated on the same clover plants for 28 days of AAP. Adults of the above-mentioned leafhoppers were transferred until their death on the herbaceous test plants for 3 days of IAP. Sixteen of 51 plants of *T. repens* inoculated by *S. titanus* showed the same symptoms as source plants; fifteen of 72 clover plants and none of 20 *Catharanthus roseus* L. inoculated by *E. variegatus* showed symptoms; 28 of 71 clover plants inoculated by *A. fuscovenosus* showed symptoms; one of 76 periwinkle plants and four of 61 *Chrysanthemum carinatum* Schousbce inoculated by *M. quadripunctulatus* showed symptoms; none of 37 periwinkles, none of 29 chrysanthemums inoculated by *M. sexnotatus*, and none of 81 periwinkles inoculated by *E. incisus* showed symptoms. Clover plant symptoms were similar to those described above; periwinkle showed stunting, yellowing, small leaves, virescence and phyllody; chrysanthemum showed apical proliferation without flowering.

### Transmissions from clover to grapevine

Nymphs of *S. titanus*, *E. variegatus*, and *A. fuscovenosus* were isolated on infected plants of *T. repens* as previously described. Adults were transferred until their death on seedlings and cuttings cv Chardonnay for 7 days of IAP. Three of 33 seedlings and none of 21 cuttings inoculated by *S. titanus*, and one of 16 seedlings inoculated by *E. variegatus* showed typical symptoms of grapevine yellows; none of 10 cuttings inoculated by *A. fuscovenosus* showed symptoms.

### Conclusions

Unlike the results obtained in France with FD-infected vines (1, 6), *S. titanus* appeared as a scarcely efficient vector on vine and fully inefficient on broad bean. Among the 3 leafhopper species used in the

transmissions from clover to grapevine, positive results were obtained with *S. titanus*. Although with a low percentage, the transmission from vine to clover and the relative back-transmission were achieved. Among the 3 species that feed on periwinkle, only *M. quadripunctulatus* was able to transmit MLOs from clover to periwinkle and to chrysanthemum. The tested insect species are known as MLO vectors, except *A. fuscovenosus* (7); all species appeared little or not at all efficient, and the transmission did not appear specific. The symptoms shown by periwinkle, i.e. virescence and phyllody, are different from those described for FD *sensu strictu* (8), and are similar to those obtained by other researchers using dodder transmission with infected vines coming from other Italian regions, where *S. titanus* is present (9) or absent (10, 11). These results, particularly the very low efficiency of *S. titanus*, the lack of transmission to *V. faba*, and the symptoms expressed by *C. roseus*, lead us to consider that the grapevine yellows disease spread in northwestern Italy is different from Flavescence dorée.

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# MOLECULAR DETECTION OF MLOs ASSOCIATED WITH GRAPEVINE YELLOWS DISEASE IN PIEMONTE, ITALY

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Grapevine Yellows (GY) is known to be associated with mycoplasma-like organisms (MLO) seemingly spread in the vineyards by leafhoppers (7). A two year study was carried out to check for the presence of MLOs in *Scaphoideus titanus* Ball (1). *S. titanus* was reared in cages in the field and in the laboratory: during June and July 1991 about 300 third and fourth instar nymphs were transferred onto vines in vineyards of cvs Chardonnay, Erbaluce and Nebbiolo, and each plant with nymphs was then isolated in a cage of nylon net. Of the 11 vines of cv. Chardonnay, 4 were symptomless but had shown FD-like symptoms in past years, 4 showed symptoms only on some canes, and 3 showed symptoms on the whole plant. The vines of cvs Erbaluce and Nebbiolo had never exhibited FD-like symptoms. In the laboratory, other nymphs were similarly placed on 3 separately caged cuttings of cv. Chardonnay obtained from plants showing FD-like symptoms on the whole plant. Control insects came from colonies reared on healthy grapevine plants grown from seed. DNA was extracted from samples of leafhoppers from each group and from the controls and submitted to dot hybridization using biotinylated cloned DNA probes (pAY18, pG3, pG30 and pG39) developed in previous works (2, 5). The DNA extracted from 11 of 15 groups of *S. titanus* reared on symptomatic vines showed hybridization signals with probes pAY18 and pG3, pG30 and pG39 at 52 °C. Three of 5 groups of insects reared on symptomless vines (GY-infected in the past) and two groups of insects reared on vines which had never exhibited symptoms were also positive. None of the MLO-DNA probes employed hybridized with the nucleic acid from *S. titanus* reared on GY-healthy plants. The nucleic acid from *S. titanus* bred on GY-insensitive vines (cv. Erbaluce and cv. Nebbiolo) did not hybridize with probes pG3, pG30, and pG39, whereas it did hybridize with probe pAY18. The results clearly indicated the presence of MLOs in *S. titanus* reared on GY-diseased grapevines. This finding was confirmed by the successful transmission of MLO to healthy clover by using similar batches of *S. titanus*. Later, an MLO (GY-T) was transmitted from inoculated clover to periwinkle [*Catharanthus roseus* (L.) G. Don], which subsequently developed symptoms (Alma *et al.*, this book) by using *Macrostelus quadripunctulatus* (Kirschb.). DNA was extracted from healthy periwinkle and clover, as well as from periwinkle and clover inoculated as above and from samples of *M. quadripunctulatus* fed on inoculated and healthy clover. DNAs from Italian periwinkle virescence MLO (IPVR) (2) and MLO strain DNA FD-G (kindly provided by Dr M. Maixner, Germany) were used as positive controls in polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis to characterize GY-T strain at the molecular genetic level.

PCR was carried out for a total of 35 cycles, under previously described conditions (3, 5), in reaction mixtures containing 20 ng template DNA using primer pair G35p/m described elsewhere (3). Sensitivity of amplified DNA detection was increased by high stringency Southern hybridizations with a biotinylated probe (pG39), as previously described (2), to obtain IPVR MLO DNA homologous to DNA fragment G35I. The results revealed amplification of an MLO-specific 1.2 Kb DNA fragment in reaction mixtures containing DNA templates from IPVR, FD-G or GY-T MLOs. In Southern hybridization, a weak signal revealed amplification of the 1.2 Kb MLO-specific DNA fragment in reaction mixtures containing template DNA extracted from *M. quadripunctulatus* that had fed on inoculated clover; no amplification was detected in reaction mixtures containing template DNA from controls.

The same DNA samples were also used as templates in PCR with oligonucleotide designed to prime amplification of 16S rDNA from MLOs (6). An MLO-specific amplification product of 1.2 Kb was observed only in reaction mixtures containing GY-T DNA template or template DNA from a control MLO (IPVR or FD-G). These products were digested with six restriction enzymes (AluI, MseI, RsaI, CfoI, HpaI and EcoRI). The RFLP patterns obtained in polyacrylamide gel electrophoresis indicated that strain GY-T is similar to strains in the type II subcluster described previously (5) in the aster yellows MLO strain cluster and confirmed that IPVR MLO is also affiliated with the aster yellows MLO strain cluster as indicated by other data (3, 4; Davis and Prince, this book; Bertaccini *et al.*, this book). In agreement with data presented elsewhere (Davis and Prince, this book), strain FD-G appeared to resemble an aster yellows MLO cluster strain, type III (5).

The presence of MLOs in *S. titanus* that fed on grapevines, in *M. quadripunctulatus* that fed on inoculated clover, and in periwinkle experimentally inoculated with these leafhoppers, strengthens the conceptual link between MLOs and grapevine yellows disease in Piemonte. However, since several distinctly different MLO groups apparently may infect grapevines (4; Prince *et al.*, in preparation; Davis and Prince, this book), and the symptoms showed by periwinkle differ from those described for flavescente dorée (FD) (Alma, *et al.*, this book), it will be important to detect which MLOs in grapevine are responsible for FD, and which complex of

different MLOs may be involved in GY in Piemonte.

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DETECTION AND CHARACTERIZATION OF MYCOPLASMA-LIKE ORGANISM (MLO) DNA IN NATURALLY INFECTED GRAPEVINE CULTIVARS IN EMILIA-ROMAGNA, ITALY: POLYMERASE CHAIN REACTION AND RESTRICTION ANALYSES.

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Several studies have indicated that grapevine yellows disease is present in several regions of Italy, but identities of MLOs associated with the disease have long remained unknown. Recent data indicates that several diverse genetical groups may infect grapevine in Europe (2, 3, 5, 6; Prince *et al.*, in preparation). At least two of these MLO groups are associated with grapevine yellows in Italy (3, 4, 5, 6). The cloning of DNA fragments from an MLO (strain G), found in a naturally diseased periwinkle plant (*Catharanthus roseus* [L.] G. Don) affected by Italian periwinkle virescence (IPVR) disease, resulted in the development of specific probes for the detection of this MLO (4). A possible relationship between the IPVR MLO and the disease in grapevine has been suggested because the IPVR-diseased plant had been placed as a healthy seedling in an Emilia-Romagna Sangiovese vineyard in which numerous grapevines exhibited symptoms of grapevine yellows, and also because the IPVR MLO was found to be related to a MLO strain (FD-B) associated with grapevine yellows in southern Italy (5, 6). Thus, detection of these MLOs in grapevine tissues was investigated.

Polymerase chain reactions (PCR), using an oligonucleotide primer pair (G35p/m) designed for amplification of IPVR MLO DNA fragment G35I (5). A second primer pair, designed R16F2/R16R2 (F2/R2) (8), was used for amplification of MLO 16S rDNA. In this presentation, data confirming association of MLOs with grapevine yellows are reported and evidence for the presence of aster yellows MLO cluster-related strains in diseased grapevines in Emilia-Romagna region are presented.

Samples of symptomatic and asymptomatic grapevine cultivars Chardonnay, Caveccia, and Sangiovese, were tested for the presence of MLOs by amplification of MLO DNA by PCR. Template DNA from samples of freshly cut leaf midribs was extracted by a method previously described (7). PCR was carried out for a total of 35 cycles, under conditions previously described (9), in reaction mixtures containing 60 ng template DNA from grapevine when using primers G35p/m. When primers F2/R2 were used, 20 ng of template DNA were used in 35 cycles in which the annealing temperature was raised to 52 °C.

When products from reaction mixtures containing primers G35p/m and template DNA from symptomatic yellows diseased grapevine cultivars Chardonnay and Sangiovese were analyzed by gel electrophoresis, a 1.2 Kb amplified DNA fragment was observed, indicating the presence of an MLO in these plants. However, reaction mixtures containing template DNA from symptomatic or asymptomatic Caveccia grapevine yielded amplification of only a nonspecific 600 bp DNA fragment. No DNA amplification was observed in reaction mixtures containing template DNA from asymptomatic Sangiovese and Chardonnay grape vines. Sensitivity of amplified DNA detection was increased by high stringency Southern hybridizations using a biotinylated probe (pG39) containing IPVR MLO DNA homologous to DNA fragment G35I (4). The results revealed amplification of a 1.2 Kb MLO-specific DNA fragment in reaction mixtures containing template from symptomatic Caveccia grapevine, and verified sequence homology of the 1.2 Kb DNA fragments from all three symptomatic grapevine cultivars with DNA of IPVR MLO. No hybridization signal was observed to indicate sequence homology of the probe with the non-specific 600 bp DNA fragment amplified from Caveccia grapevines.

The PCR products of 1.2 Kb obtained from the amplification with the primers F2/R2 from symptomatic Caveccia, Sangiovese and Chardonnay were digested with three restriction enzymes (AluI, MseI and HhaI): the products obtained from the amplification of IPVR-MLO and four MLO isolates in periwinkle, inoculated by dodder (1), were also digested as controls. The polyacrylamide electrophoresis gel of the restriction fragment length polymorphism (RFLP) analysis shows the identity of the patterns obtained with all the enzymes used, providing evidence for a close genetic relationship among MLOs present in grape and MLOs isolated using dodder transmission in periwinkle.

In this study of three naturally infected grapevine cultivars, exhibiting symptoms characteristic of flavescente dorée in Emilia-Romagna, evidence for the presence of MLOs was obtained by PCR using primer pair G35p/m. The result was confirmed by PCR using primer pair F2/R2. Detection of the MLOs with primer pair G35p/m indicated a relationship between IPVR MLO and other aster yellows MLOs. Restriction analyses of amplified 16S rDNA confirmed findings (Prince *et al.*, in preparation) on the four MLO isolates transmitted from grapevine to periwinkle, and illustrated both (a) mutual similarity among the 3 MLOs detected directly in grapevine tissues and (b) differences of these three MLOs from known aster yellows MLO cluster strains.

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# PCR DETECTION OF A MYCOPLASMA-LIKE ORGANISM (MLO) IN FLAVESCENCE DOREE DISEASED GRAPEVINES FROM LOMBARDIA, ITALY.

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Occurrence of grapevine yellows disease in Italy was first reported in the Lombardia region (1). Following this report, grapevine yellows has been observed in several other regions of Italy. Symptoms in affected grapevines were characteristic of the disease known as Flavescence Dorée in France (2), and the leafhopper *Scaphoideus titanus* Ball has been also reported associated with the disease in Northern Italy (3,4): here, therefore, grapevine yellows has been termed Flavescence Dorée (FD). By contrast, in Southern Italy, where *S. titanus* has not been found, the disease is known as Southern Italy grapevine yellows (5). Grapevine yellows in Italy and elsewhere has been attributed to the presence of mycoplasma-like organisms (MLOs); several reports have presented evidence for the presence of MLOs in grapevine. In some cases the association of MLOs with grapevine yellows has been inferred only on the basis of symptomatology in diseased grapevines. Up to now immunological detection has been obtained on infectious leafhopper (6), but not in diseased grapevines. Recently molecular biology approaches supplied diagnostic tools for MLO detection (7); moreover hybridization of cloned DNA from FD-inoculated broadbean with FD-diseased grapevines has been reported (8). In this communication we present the first report of molecular detection of MLOs directly in tissues of grapevine exhibiting typical symptoms of FD in Italy by means of the polymerase chain reaction (PCR) technique.

Aster yellows (AY) MLO strain AY1 (=AY=MAY) was collected in a naturally infected plant of periwinkle (*Catharanthus roseus* (L.) G. Don) in Beltsville, MD (9). Diseased plants were maintained by grafting on a white flowered clone of periwinkle grown in a greenhouse. Samples of grapevine tissue were collected from *Vitis vinifera* L. cultivar Chardonnay. Four different plants exhibiting symptoms typical of infection by FD were sampled from a single vineyard located in the Lombardia region of Italy. Additional samples, numbered 7 and 8, consisted of tissues from leaves of different grapevine plants, upon which field collected *S. titanus* had been permitted to feed for an inoculation access period of 28 days. These numbered samples of grapevine leaves showing typical symptoms of FD were collected three months after *S. titanus* inoculation. Leaves with symptoms (sample n° 22) were also collected from a cutting of a naturally FD infected grapevine. Leaves from healthy periwinkles and healthy seedling grapevines grown in greenhouse were used as controls.

Nucleic acid was extracted from 1 or 2 g of fresh or frozen leaf veins; PCR reactions were conducted according to Schaff *et al* 1992 (10); F2R2 (16S rDNA primers) (11, 12) and G35pm primers (13) have been used in our tests. PCR products were examined by electrophoresis in 1% agarose gels followed by staining in ethidium bromide and visualisation of DNA bands using an UV transilluminator.

In preliminary PCR experiments on all grapevine extracted DNA, no specific amplification products were detectable except for the sample n° 11. Although PCR primer pair F2/R2 has been shown to prime amplification of 16S rDNA from MLOs, the apparent low titer of MLOs and presence of inhibitors in plant tissues necessitated steps to increase sensitivity of detection. In subsequent experiments Southern blot hybridization was conducted after gel electrophoresis of PCR products. 16S rDNA internal probe (758F-1232R) (B.D. Mogen and R.E. Davis, unpublished) was used with digoxigenin labelling and chemiluminescent visualisation (Genius™ Boehringer Mannheim Corporation-Indianapolis). Grapevine sample n° 22 and all field collected grapevine samples showed positive reactions. Moreover samples 7 and 8 (experimentally infected grapevines) and healthy control samples gave negative results in Southern hybridization test.

Thus, in some samples, simple dilution of template DNA preparation allowed detectable sequence amplification, but the levels of amplified DNA were too low in several cases for unequivocal detection by gel electrophoresis alone. Use of an internal probe sequence in hybridization tests has previously been shown to increase the sensitivity of detection at least 100 fold (Prince and Davis, unpublished). Our use of this approach made possible detection of MLO-specific amplified 16S rDNA in dot hybridization as well as Southern blot hybridization of PCR amplified DNA extracted from grapevine tissues. Furthermore, in agreement with the results obtained by Hammond and Podleckis (personal communication) working with viroids and riboprobe, we could detect MLOs in grapevine tissue by using chemiluminescence with digoxigenin-labelled instead of radioactively labelled probes.

Our inability to detect MLOs in symptomatic grapevines experimentally inoculated by *S. titanus* that had fed upon diseased grapevines may reflect low titer of MLOs in those samples; sampling of other tissues from the same plants at different stages of disease might have yielded MLO detection.

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MLO DETECTION BY HYBRIDIZATION AND PCR IN GRAPEVINE STOCKS AFFECTED WITH GRAPEVINE YELLOWS. INVESTIGATION ON SAMPLES FROM VARIOUS AREAS IN DIFFERENT COUNTRIES.

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Flavescence dorée (FD), is a well characterized MLO-induced grapevine yellows (GY) affecting vineyards in Southern France. Its spread results from transmission of FD-MLO from grapevine to grapevine by the leafhopper *Scaphoideus titanus*. Maintenance of FD-MLO in experimental hosts facilitated the construction of DNA probes which allowed the sensitive detection in field-grown *vinifera* cultivars (1). PCR primers specific for this MLO also allowed detection in field-grown symptomless rootstocks and in wild *S. titanus*.

Other grapevine yellows not associated to *Scaphoideus titanus* have been reported in numerous countries of the world (2). The similarities of the symptoms with symptoms of FD, and observations of MLO in symptomatic plants allowed the hypothesis of a MLO etiology.

Samples of diseased grapevine originating from northern and southern vineyards in France were reacted with FD-MLO specific probes and primers. In contrast with samples from southern areas, samples from northern areas did not test FD positive. Their further analysis in a procedure using PCR primers derived from 16S rRNA gene fragment of MLOs, and *AluI* digestion of PCR products (3), demonstrated the occurrence of non-FD MLOs in all the samples which had tested FD negative. Two different RFLP profiles were found, sometimes in close vicinity, and also very close to FD *sensu stricto* infected vineyards (4).

Samples harvested in other countries were analyzed using the same procedures, both with FD specific reactive, and the Ahrens and Seemüller (3) procedure. FD *sensu stricto* was detected in four grapevine varieties harvested near Udine (Friuli, Italy). All other samples originating from Italy (Torino, Bologna, Sicily) and also from Israel were positive for the presence of non-FD MLOs with a constant restriction profile.

This non-exhaustive study suggests MLO diversity in grapevine. It emphasizes the need of specific diagnostic tools suitable to demonstrate the etiology of the diverse GY, to search insect vectors and reservoir plants, and to devise control methods and curative regulations.

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# GRAPEVINE YELLOWS DISEASES: DIVERSE ETIOLOGIES INDICATED BY NEW DNA-BASED METHODS FOR PATHOGEN DETECTION AND IDENTIFICATION-IMPLICATIONS FOR EPIDEMIOLOGY

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Presently, *flavescence dorée* (FD) and similar yellows diseases of grapevine are ascribed to mycoplasma-like organisms (MLOs) (1). In some cases, MLOs have been shown to be associated with disease, whereas in others, the association of MLOs with disease has been inferred on the basis of symptomatology in grapevine. Until relatively recently there has been little direct evidence to indicate whether the various diseases are forms of FD or whether the pathogen(s) involved may be distinct from the FD agent and/or from one another. Our objectives were to develop molecular methods for sensitive detection of MLOs in diseased grapevines and other hosts and to analyze the diversity among MLOs associated with grapevine yellows in different geographic regions. In these studies (4-7, 9-11), methods included dot hybridizations using cloned MLO DNA probes, restriction fragment length polymorphism (RFLP) analysis of total MLO genomic DNA, nucleotide sequencing of randomly cloned MLO DNA fragments and of MLO 16S rRNA gene regions, design of oligoprimers for use in polymerase chain reactions (PCR), PCR using a variety of primers for general or group-specific MLO detection and identification, strain- and subgroup-specific amplification of MLO DNA, and RFLP analysis of amplification products.

Thus far, in independent studies and collaborations with colleagues in other laboratories, we have examined no less than 13 MLO strains associated with grapevine yellows in Italy, France, Germany and the USA. Initially, we examined MLO strains FDU and FDB, originally obtained from naturally infected grapevines in northern and southern Italy, respectively, that had been transmitted experimentally to plants of *Catharanthus roseus* (L) G. Don (periwinkle). These were compared with Italian periwinkle virescence MLO strain G, originally obtained from a naturally infected periwinkle plant that had been placed as a healthy potted seedling in a northern Italy vineyard in which grapevines exhibited symptoms of a FD-like disease. Results from dot hybridizations and from RFLP analysis of chromosomal DNA showed that strain G was more closely related to strain FDB than to strain FDU and indicated that at least two distinct MLOs were associated with grapevine yellows in Italy (4, 7).

Oligonucleotide pairs that specifically primed PCR amplification of DNA from known aster yellows MLO strains, as well as primer pair G35p/m (designed for amplification of DNA from strain G) primed amplification of DNA from strains FDB and G, but not of DNA from strain FDU (5, 9, 11). These results indicated that Italian periwinkle virescence MLO G and southern European grapevine yellows MLO FDB were closely related to MLOs classified in the aster yellows MLO strain cluster but that strain FDU was not (5, 9, 11).

MLO infection in naturally diseased grapevines was detected by use of PCR. Oligonucleotide pair r16F2/r16R2, designed to prime amplification of 16S rDNA from all known MLOs (8), primed amplification of MLO-specific DNA from naturally yellows-diseased grapevine samples from Italy and USA (9-11). When subjected to restriction analyses, amplified DNA from grapevine in Italy gave the same pattern as 16S rDNA from known aster yellows MLO strains, whereas 16S rDNA from diseased grapevine in USA gave the same pattern as that of strains in the X-disease MLO strain cluster (10, 11). Use of additional oligonucleotide primers confirmed that MLOs in grapevine in Italy and USA could be classified with aster yellows and X-disease MLOs, respectively (P. Bianco, J.P. Prince, R.E. Davis, unpublished, 11). Study of insect vectors and plants used in experimental transmission of MLO strain FD70 (= FDF), associated with FD in France (3), indicated that a third MLO group, the elm yellows MLO group, may be represented by MLOs in grapevine (11). This last finding is consistent with possible infection of grapevine by elm yellows MLO (2) and with relatedness of MLO FD70 to elm yellows MLO shown by results from dot hybridizations (3). Thus, it appears that grapevines may be naturally infected by genetically diverse MLOs that can be classified with aster yellows, X-disease, and elm yellows MLOs (11). The breadth of genomic diversity encountered in the grapevine-infecting MLOs was unexpected. Further work will be required to determine the geographic distribution and relative frequencies of grapevine infection by these diverse MLOs, and to establish what etiological roles they may have in *flavescence dorée*/grapevine yellows disease syndromes. Association with grapevine yellows diseases of MLOs classified in three distinct MLO groups points to the need for new studies that could have important implications for epidemiology. For example, vectors known to transmit various strains in these groups may also be important in spread of disease from non-grapevine plant host reservoirs to grapevine, or from grapevine to other economically important plants (11). Sensitive molecular tools now available should make it possible to identify presently unknown insect vectors and alternate host plants of these MLOs. If evidence mounts for a role of all three genetically diverse MLO groups in grapevine diseases, then broader recognition of different diseases caused by distinct pathogens would be warranted.

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## FURTHER STUDIES ON YELLOWS-LIKE DISORDERS IN APULIA

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As a follow up of the studies carried out in Apulia in the framework of a national research program on Flavescence dorée (2) further observations were made in the areas where disease foci had been identified (1) and new viticultural areas of disease in Apulia (southern Italy) were surveyed.

In the search for possible vectors, trap plants (periwinkles and *Aster* sp.) were placed in the vineyards near symptomatic vines, weeds with symptoms like those induced by MLOs were thin sectioned and observed under the electron microscope, and extensive leafhopper collections were made in infected vineyards.

Dodder transmissions were attempted, both in the field and insect-proof screenhouse, from symptomatic vines to periwinkles.

Field surveys led to the identification of new, limited infection foci in the province of Taranto, in vineyards of cv. Malvasia nera, Verdeca and Sangiovese, none of which had been previously found affected by yellows. The symptomatological reactions of vines were typical of yellows disease, consisting of sectorial or extensive yellowing (white-berried cv. Verdeca) or reddening (red-berried cv. Sangiovese and Malvasia nera) of the leaves, rolling of the blades, necrosis of the main veins and parts of the blade, incomplete ripening of the wood and withering of the bunches. A new and alarming disease condition was encountered in vineyards each of cv. Primitivo and Negroamaro in the Salento peninsula, where about one third of the vines showed yellowslike symptoms. These were indeed the most severe outbreaks ever detected in Apulia.

Several plants of the periwinkle and *Aster* trap plants placed in the vineyards, came down with MLO-like symptoms, and several weeds (i.e. *Cichorium* spp., *Sonchus* spp., *Rubus* spp. and *Eryngium* spp.) growing along the edges of the vineyards were also visibly infected.

Electron microscope investigations are still under way and far from being completed. However, preliminary observations showed the abundant presence of MLOs in sieve tubes of symptomatic periwinkles and in an infected *Cichorium* sp. plant.

Leafhopper captures are listed below:

LEAFHOPPER SPECIES	NUMBER OF INDIVIDUALS CAPTURED
<i>Empoasca vitis</i> (Goethe)	81
<i>Edwardsiana rosae</i> (L.)	6
<i>Edwardsiana</i> spp.	2
<i>Ribautiana</i> spp.	2
<i>Zygina rhamni</i> Ferr.	16
<i>Zygina tiliae</i> (Fall.)	14
<i>Fruticoidia bisignata</i> (M. et R.)	10
<i>Fieberiella florii</i> (Stal)	1
<i>Exitianus capicola</i> (Stal)	11
<i>Euscelidius variegatus</i> (Kirschb.)	9
<i>Euscelis lineolatus</i> Brullé	33
<i>Psammotettix alienus</i> (Dahlb.)	37

The absence of *Scaphoideus titanus* Ball in Apulia was confirmed, but at least three known species of MLO vectors were identified, namely *F. florii*, *E. variegatus* and *E. lineolatus*, the latter species having a fairly high incidence.

Dodder transmissions were successful both in the field and screenhouse, with percentages of positive transfer to periwinkle of 25% (5 out of 20 plants) and 48% (10 out of 21 plants), respectively. In all cases diseased periwinkles exhibited yellowing, stunting, undersized leaves, small and virescent flowers.

The present results strongly indicate that yellows are actively spreading in some Apulian vineyards. The natural infection of trap plants and the massive outbreaks newly discovered corroborate this likelihood. Possible sources of inoculum were identified in weeds and possible vectors among leafhopper species captured in infected vineyards. Whether MLOs found in weeds are the same as those present in grapevines and successfully transmitted by dodder to periwinkle was not ascertained. However, the point remains that, with regard to yellows, the situation of Apulian grapevine industry is more serious than previously thought and should be kept under adequate observation.

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## CLONING AND EXPRESSION OF FLAVESCENCE DOREE-MLO MEMBRANE PROTEIN IN LAMBDA ZAP II EXPRESSION VECTOR

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Flavescence dorée-MLO is a leafhopper-transmitted mycoplasma-like organism which can cause serious damage to grapevine. It is maintained in the laboratory in experimental hosts (leafhopper and broadbean) which are infected at high titres.

A genomic library was constructed in the expression vector Lambda ZAP II using *EcoRI*-digested fragments of purified unfractionated DNA which was obtained from MLO particles purified by an affinity column method from highly infected leafhoppers (1). Recombinant phage plaques expressing fusion proteins carrying FD-MLO membrane protein antigenic determinants were identified by screening the phage library with a cocktail of monoclonal antibodies specific for FD-MLO (2, and R. Meignoz, unpublished data).

Three clones were obtained that hybridised specifically to FD-MLO DNA, extracted from both infected leafhoppers and broadbean, in southern blots.

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## FLAVESCENCE DOREE ON ROOTSTOCK VARIETIES: INDEXING RESULTS AND HOT WATER TREATMENTS

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The severity of Flavescence dorée (FD) symptoms on *Vitis vinifera* varieties and the subsequent dramatic yield losses justified thorough study of the behaviour of the various cultivars. On the other hand, the knowledge of vector transmission has allowed an efficient control of the disease in the field. However rootstock varieties may be infected in the mother plant vineyard. The behaviour of rootstock varieties infected by FD is actually very badly understood. Most of them do not show any typical FD symptoms (1) and we don't know if they are or not infected. Moreover we don't know either if they will recover as most of *vinifera* varieties, or if they will remain permanently infected. Finally, it is important to evaluate the chance of FD propagation by rootstock cuttings. The design of a safe method to cure the wood material prepared for multiplication is of great importance. These problems have to be studied on each of the 20 most cultivated rootstock varieties.

Analysis of the carrier behaviour of rootstock varieties towards FD MLO was conducted by monitoring mother plants from vineyards located inside the area which is most exposed to natural infection by FD (2). The fields were controlled against new infections by insecticide treatments since 1985, after the first outbreak of the disease. The observations of the behaviour in the field was completed by an extensive greenhouse indexing of located stocks.

It has been established that the association of a growth delay in spring together with a non ripening of canes in autumn were a reliable sign of the presence of the disease agent. Indexing of these plants confirmed in most cases (60 to 95 percent) the presence of infectivity. However other stocks which were totally symptomless could also happen to index positively.

Differences according to varieties were observed in symptom expression and behaviour. In particular, the number of cuttings that showed graft transmission was very variable, ranging from 6 to 100 percent.

Rootstock varieties 3309 C and Fercal could show both growth delay and bad ripening. They were shown to be permanent carrier of the disease agent. Some infected stocks died. Most of the varieties were able to transmit FD several years after their infection and hence do not recover.

As for other rootstock varieties which show mild or no symptom FD could also be detected by indexing. This showed that symptomless varieties were able to transmit the disease.

An irregular distribution of the infectious cuttings according to the canes and along one cane was demonstrated. The reasons for this remain obscure, but it makes difficult the sampling for the diagnosis tests.

Many plants produced from infected rootstocks could have a healthy appearance and did not show symptoms until several years after planting in the field. Harvesting of wood on such stocks can thus be the origin of new foci of the disease. This risk, which is particularly dangerous for exportation or importation of wood material could be suppressed by a hot water treatment of wood at 50°C during 45 min., according to a formerly published procedure (3). A special device has been designed in the last years for hot water treatment of large quantities of wood. It is actually ready for use at ENTAV France and commercially available.

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## GRAPEVINE YELLOWS IN DIFFERENT AREAS OF THE WORLD: INVESTIGATION BY ELISA USING FLAVESCENCE DORÉE SPECIFIC ANTIBODIES

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Over the last few years grapevine yellows (GY) have been observed in several areas of the world, such as France, North Italy and Sicily, Switzerland, Israel, North America, South Africa and Australia. The disease which induces both severe and mild symptoms on grapevine organs can result in the death of the vine-stock. In every case, the quality and quantity of the grapes produced is affected. One of the most studied GY is flavescence dorée (FD). It is an important disease of grapevine, caused by a mycoplasma-like-organism (MLO) which develops in phloem tissues of infected vines. The MLO is transmitted, according to the persistent mode, in the field by the leafhopper *Scaphoideus titanus* Ball., and is not cultivable *in vitro*. This disease is very epidemic. Diagnosis of FD by symptomatology alone is not possible because of the similarity of the symptoms with those of other yellows diseases and the possible superposition of different yellows in the same area. The recent publication on the use of ELISA to detect FD MLO on grapevine itself (1) has allowed differentiation of FD from the other GY. In order to investigate the epidemiology of FD, grapevine canes or leaves showing GY symptoms were harvested from several areas in the world (France, 138 samples; North Italy and Sicily, 13; Switzerland, 39; Israel, 9; North America, 31; South Africa, 24 and Australia, 26) and tested by ELISA using specific polyclonal and monoclonal antibodies raised against FD (2, 3).

The method used for the preparation of samples has been previously described (1). Briefly, 10 ml buffer F with 5% Chaps were added to 1 g of leaves, and homogenized for 50 seconds in a plastic bag with an universal homogenizer-extractor (Bioreba). Extracts were clarified by centrifugation at 2,900 g for 30 min, and the resulting supernatants were concentrated 10 times by ultrafiltration using a Centriscart Sartorius device with a molecular weight cutoff of 20,000 Daltons.

Enzyme-linked immunosorbent assays (ELISA) were done as previously described (2, 3). ELISA plates were coated with rabbit polyclonal anti FD-broadbean serum (origin INRA-Dijon) in carbonate buffer. Excess buffer was discarded and wells were blocked with casein buffer. The samples, both concentrated or not, were incubated for 1/2 h at room temperature with light shaking, then kept overnight at 4°C; successively, diluted hybridoma supernatants anti-FD (Origin INRA-Dijon: either a hybridoma mixture FD1, 51 and 113 or 12, 17, 18 and 51) in casein buffer and phosphatase alkaline-conjugated goat anti-mouse IgG (H+L) diluted in casein buffer were added. The difference of absorbance at 405nm and 490nm was measured after 2.5 h contact with phosphatase substrate.

FD MLO were specifically detected in various cultivars: Alicante, Baco 22A, Carignan, Chardonnay, Garganeva, Grenache, Perera, Pinot noir, Sauvignon, Ugni blanc from the field and in rootstocks Fercal, 3309 and 1103 growing in the greenhouse and showing mild symptoms (4). In this work, FD was found to be confined to grapevine stocks in two regions of Europe: Southern France and Northern Italy (Friuli). In France, these results have allowed clarification of a very confusing situation concerning FD and Bois noir (BN) (5). All the symptomatic vines of the North East of France reacted negatively, and hence belong probably to BN. Some samples from regions as south as Ardèche reacted similarly. No positive FD reaction was obtained with samples showing GY symptoms from Sicily, Emilia Romagna (Italy), Switzerland (Western Switzerland and Tessin), Israel, U.S.A. (New York state), South Australia or with Shiraz decline disease in the Republic of South Africa. These results suggest that no serological relationship exists between FD-MLO and the other GY. However this study does not exclude that FD could be present in some parts of these areas. This work shows the economic importance of the GY other than FD, the risk of epidemics and the necessity to promote protection, cure and eventual quarantine measures.

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