

STUDIES ON GRAPEVINE YELLOWS (VERGILBUNGSKRANKHEIT) IN GERMANY - DETECTION OF MLOS IN GRAPEVINES AND SEARCH FOR POSSIBLE VECTORS

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A yellows disease of grapevine called 'Vergilbungskrankheit' (VK) is known in German viticulture since the 1930^{ies} (6). Symptoms of VK are very similar to Flavescence dorée (FD) but differences with regard to epidemiology and the range of susceptible cultivars have been reported (3). Whereas VK is present in low incidence in many viticultural regions of Germany it is most important in the valleys of the Mosel- and Middle-Rhine-River where its incidence comes up to more than 40 % in some vineyards. Most of the important cultivars grown in Germany including 'Riesling' and 'Müller-Thurgau' are highly susceptible. Infected vines exhibit symptoms such as discoloration of the leaves, shrivelling of berries and abortion of clusters, black pustules along the internodes, and lack of lignification thus decreasing quality of the yield and vigour of the vines. Infected vines often exhibit symptoms over several successive years. Symptomatic and symptom free shoots usually occur together on infected vines, but cuttings of symptom free wood are able to develop symptoms of VK. Vines with severe symptoms may die within a few years.

Mycoplasmalike organisms (MLOs) have been identified as the causal agents of FD and are suspected pathogens of all yellows diseases of grapevine (2). Since detection of MLOs in grapevines infected by VK by light or electron microscopy failed (7) presumably due to their low titers we performed transmission experiments from grapevine to periwinkle (*Catharanthus roseus*) as an indicator and alternative host. In addition we made use of the highly sensitive polymerase chain reaction (PCR) for experiments to detect MLOs in field grown vines with symptoms of VK.

Scaphoideus titanus, the vector of FD is not present in Germany and no other vectors have been identified. We therefore surveyed the leafhopper fauna of vineyards affected by VK to gain information on possible vector species. Knowledge of the vector of VK is essential for the understanding of its epidemiology and the development of control strategies.

To achieve transmission of MLOs, dodder (*Cuscuta odorata*) bridges were established and maintained for 10 weeks between a nine year old symptomatic 'Riesling' vine kept in the greenhouse and periwinkles. Dodder without contact to the vine was kept on periwinkle as a control. After the dodder had been removed, periwinkles were checked weekly for visible symptoms of MLO infections. Suspicious plants were examined by the DAPI staining technique (8).

Sample preparation and the polymerase chain reaction (PCR) were carried out as described by Ahrens and Seemüller (1). Samples were taken from symptomatic vines growing at different locations of the Mosel-valley, from periwinkle exhibiting symptoms after the transmission experiments, and from periwinkle isolates of different MLOs (see legend to Fig. 1). Grapevine seedlings and healthy periwinkle were used as controls. The primers used have been designed by Ahrens and Seemüller (1) for the amplification of a fragment of the 16S rRNA gene of a wide range of different MLOs. Restriction fragment patterns obtained by *AluI* digestion of the reaction mixtures after 40 cycles were analyzed by polyacrylamide gel electrophoresis.

Leafhopper were collected over two years in two different vineyards affected by VK. Sticky- and pan-traps were placed within the canopy of the grapevine and renewed in weekly intervals throughout the season. Five of 19 periwinkles developed virescence and phyllody as symptoms of MLO infection between 7 to 12 weeks after the end of the experiment. MLOs were detected in the phloem by microscopy after staining with DAPI. Neither symptoms nor MLOs could be observed in the control periwinkles which had not been connected to grapevine. The results of PCR are shown in Fig. 1. Since DNA was amplified also from healthy samples restriction fragment analysis was necessary for the characterisation of the reaction products. The restriction fragment patterns (RFP) of healthy periwinkle, healthy grapevine, and one grapevine sample from the field (EZRE03) were identical and clearly different from all MLO samples. The RFPs of five grapevine samples from the field were identical but differed by the size of one fragment from the periwinkle isolate obtained by transmission from grapevine (PERVK). The latter was comparable to the aster yellows isolate (AV2192). Of 36 leafhopper species collected in the vineyards 15 were present at both locations. Although most species prefer herbaceous plants they were caught within the grapevine canopy. Eight species are known to be vectors of different MLOs.

We were able to detect for the first time MLOs in grapevines showing symptoms of VK. All field collected vines exhibited identical restriction fragment patterns reflecting colonization by the same organism with the exception of one sample (EZRE03) from which no MLO-DNA could be amplified. Equivalent patterns were achieved from isolates of stolbur MLO (1) to which the MLO we detected in grapevine seems to be closely related. It should therefore be different from Flavescence dorée which has been found to be related to Elm

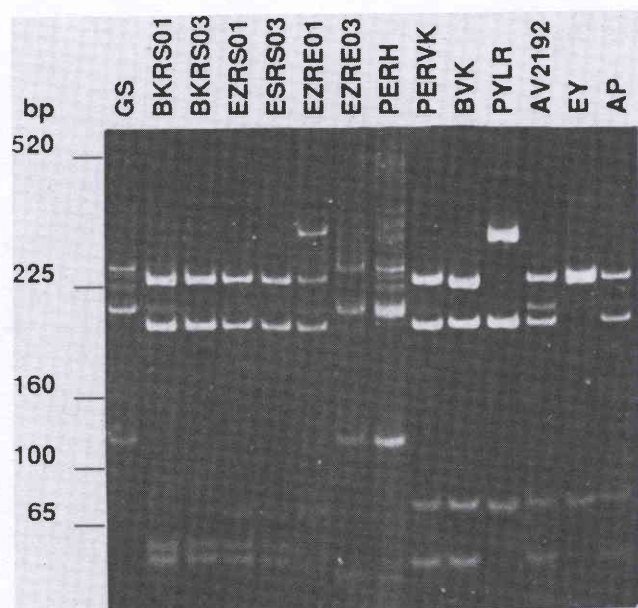


Figure 1. *AluI* digests of fragments of the 16s rRNA gene obtained after PCR amplification and analyzed by polyacrylamide gel electrophoresis. GS: Grapevine seedling; BKRS01, BKRS03, EZRS01, ESRS03, EZRE01, EZRE03: Samples of field grown symptomatic grapevines; PERH: Healthy periwinkle; Periwinkle isolates: PERVK: MLO transmitted from symptomatic grapevine; BVK: MLO transmitted by field collected leafhoppers; PYLR: Peach yellow leaf roll; AV2192: German aster yellows; EY: Elm yellows; AP: Apple proliferation.

Yellows, Ash Yellows and X-disease (4). The periwinkle isolate obtained by dodder transmission differed from the MLO detected in grapevine by the size of one restriction fragment. Its RFP is identical with Aster yellows and other MLOs from herbaceous plants. A double infection of grapevine by two MLOs of different titers could be possible but needs to be confirmed. More grapevine samples of different cultivars and from other viticultural regions of Germany will be tested to assure the association of the detected MLOs with VK infected grapes. No MLO could be detected in the sample prepared from a symptom free shoot of an infected vine (EZRE03). Possibly the pathogen is restricted to the symptomatic tissue. This could account for the observation that symptomatic vines which are cut back to the trunk often stay free from symptoms over several years. However, the distribution of MLOs in infected vines has not yet been studied.

Hyalesthes obsoletus a leafhopper capable to transmit the stolbur MLO (5) was caught regularly although in low numbers in the vineyards affected by VK. Other species which were quite common in the vineyards like *Neolaliturus fenestratus* and *Macrostelus* sp. can carry this pathogen (5). The significance of these leafhoppers for the epidemiology of VK together with common weeds such as *Convolvulus* sp. and *Solanum* sp. which are reservoirs of the stolbur MLO (5) needs to be further investigated.

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EPIDEMIOLOGY OF A YELLOWS DISEASE OF GRAPEVINE IN NORTHERN ITALY

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Grapevine Flavescence dorée was detected and described for the first time in France (3) and the causal agent was experimentally transmitted by the leafhopper *Scaphoideus titanus* Ball. (formerly *S. littoralis* Ball. (7)). *S. titanus* is considered the vector of FD.

In Italy, the first cases of an FD-type disease (based on symptom expression) were reported in Lombardy (1). A severe yellows disease (GYD) occurred in several regions of Italy on different grapevine cultivars in the early eighties (2, 5). Chardonnay appeared to be the most susceptible cultivar. In 1987 heavy damage was detected also on the Perera cultivar and in 1992 also on Garganega, in two peculiar grape areas of the Veneto region. The symptoms of GYD are indistinguishable from the symptoms of FD in France. Since 1983 our Department has followed the disease in the Friuli-Venezia Giulia (F-VG) region and has carried out a series of investigations to clarify some aspects.

At the 10th ICGV meeting we referred on the results obtained during the visual checking carried out for four years on about 15,000 Chardonnay grapevines, in 15 representative vineyards of the F-VG region, to determine the natural progress of the disease on a large scale under field conditions (6). The surveys were continued also during the last 3 years, including the two areas where the GYD was detected on the Perera and Garganega cultivars. The average percentages of Chardonnay grapevines showing GYD symptoms in the 15 vineyards checked during the 6 year period were, respectively, 11, 13, 13, 12, 11 and 10 %; the corresponding increment indices (annual percentage of newly infected plants divided by the percentage of recovered ones) were 2.8, 2.3, 1.0, 0.9, 0.7 and 0.8. The incidence of the disease in the single vineyards varied from 1 to 49%; the increment of the symptomatic plants ranged from 0 to 130 %.

The data obtained from the surveys in the Chardonnay vineyards indicate that the dissemination of the disease gradually decreased in the area. Nevertheless, it continues to spread and is characterized by recoveries and renewed infection. The same is true for the cultivar Perera. The phenomena detected on Garganega are still in an epidemic phase: in 1991 only a few cases were detected but in 1992 the percentage of symptomatic grapevine was in the order of 30%.

In the representative vineyards of the F-VG region the *S. titanus* populations (as well as of other leafhopper species) were monitored. *S. titanus* is present in all the vineyards, though often in limited numbers (one to five individuals for each yellow sticky trap exposed). No relationship has been found between incidence and spread of the disease and the density of the population of *S. titanus* present in the single vineyards.

Transmission trials have been carried out (starting in 1987) using as test vector the leafhopper *S. titanus* captured in the vineyards and fed for 15 to 35 days on affected grapevines. The test plants were Chardonnay, Baco 22A and Perera healthy grapevines as well as *Catharanthus roseus* L. Altogether, about 2,000 potentially infective *S. titanus* individuals were tested. In just one trial conducted in 1990 symptoms of GYD were obtained in one of nine Perera grapevines. The 38 adults of *S. titanus* surviving at the end of the transmission trial were submitted to serological and Western-blot assays by the Station de Recherches on Mycoplasmas of Dijon (4). The ELISA responses recorded for the Udine leafhoppers used in the transmission trials demonstrated the presence of antigens closely related to the French FD 70 isolate: 11 of 38 leafhoppers (29%) scored positively. At the Western-blot assay the antigens obtained with the ELISA - positive leafhoppers from Udine, using rabbit anti-FD 70 antibodies, exhibited a mixed pattern of the two French MLO reference isolates. The results obtained give good evidence of the relatedness of French FD MLO to the agent detected in the *S. titanus* present in F-VG vineyards. The question remains if *S. titanus* can be the main vector of the agent of GYD.

The comparative trials between 100 Chardonnay healthy grapevines planted in the spring 1987 in the open field exposed to natural infections and 100 planted in the same field inside a screenhouse (6) has proved that in the grape area chosen for the trial at least one MLO vector is present. Because in 1987 the first symptoms appeared in early October, the minimum incubation period, if the inoculation is due to insects, does not exceed 5 months. In the first year four grapevines of the exposed ones showed GYD symptoms. During the six years of exposure 30 plants developed symptoms. Some of such grapevines recovered and each year new affected plants could be detected. None of the 100 healthy grapevines planted in the screenhouse exhibited symptoms of GYD during the 6 years of the experiment.

In a parallel trial conducted in the same experimental field, the behaviour of two groups of 3-year old Chardonnay grapevines that had shown GYD symptoms the previous year were compared: one group was transplanted under an insect-proof plastic screen and the other freely exposed to natural inoculations. The aim was to understand the influence of reinfections on the length of the symptomatic period and on transitory recovery. In the year of transplantation (1987) the recovery rate was similar in the exposed and protected

groups of plants (about 65%). The corresponding recovery of the affected plants, pollarded but not transplanted from the original vineyard was 12%. The following years the percentage of symptomatic grapevines was higher in the group of exposed plants (around 55% versus about 25% of the protected ones). The responses of the affected grapevines transplanted to plots in the open field without protection were variable, ranging from no recovery, to temporary or permanent recovery. Some of the results obtained lead to the conclusion that the length of the symptomatic period is variable even when it is not influenced by reinfections. It was also proved that in Chardonnay a symptomatic period exceeding one year is not necessarily the result of successive reinoculation, and that transitory recovery (remission followed by symptom reappearance) is not always due to reinfections.

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SCANNING ELECTRON MICROSCOPY OBSERVATIONS ON FLAVESCENCE DOREE TRANSMISSION BY DODDER

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Using scanning electron microscopy (SEM) during experimental infections of Flavescence dorée carried out from diseased to healthy grapevines, by means of the vector *Scaphoideus titanus* Ball, mycoplasma-like bodies constantly associated to the disease were observed (1-3). Experimental transmission trials of the disease from grape to grape through *Cuscuta campestris* were carried out in order to verify the role of dodder as a vector and to confirm the relationships between the disease and the MLO related bodies, observed by SEM.

SEM investigations on phloem tissues of *Vitis vinifera* cv. Chardonnay and *C. campestris* were carried out during all the stages of the experimental transmission of the disease, to verify the passage of the MLO bodies through dodder and their colonization of inoculated plants.

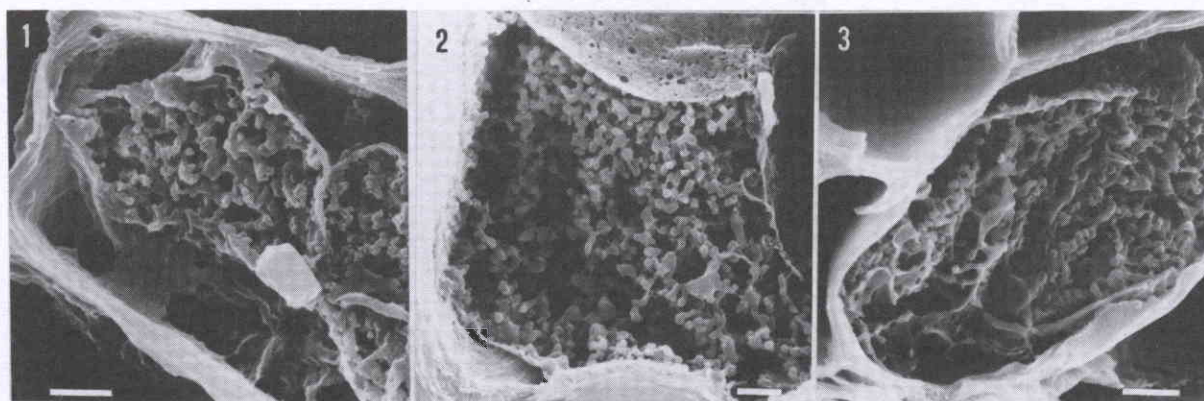
Source of inoculum was a one year old cutting obtained from a heavily diseased grapevine. This plant has been grown in pot and kept in greenhouse, and showed the typical symptoms of Flavescence dorée as leaf yellowing, rolling and poor lignification of the shoots.

Colonization of the diseased grapevine was obtained with dodder filaments originated from seed and grown on a healthy *Catharantus roseus* plant. The parasite plant was left to grow on grapevine for at least 3 weeks. Afterwards dodder filaments were connected to a healthy grapevine cv Chardonnay in order to transmit the disease. The dodder bridges were cut after 6 weeks and the parasite was completely removed two weeks later. Control trials were always prepared.

Samples for SEM observations were periodically collected from all the different plants and prepared with the freeze-drying technique as previously described (4)

Circular to oblong bodies, sized between 0.4 to 0.9 μm , often coated with an amorphous layer were observed in the phloem tissue of the grapevine used as inoculum source (Fig. 1). Their morphological characteristics corresponded to those previously described (1-4) confirming further their MLO nature, strictly related to the disease.

After 3 weeks, particles showing the same size and shape were detected in the dodder phloem cells (Fig. 2). Only 2-3 colonized phloem cells were found in each cryofracture of the dodder filaments. This poor diffusion of the pathogen inside the parasite plant reflects the similar colonization that may be found in grapevine phloem. The dodder phloem didn't show remarkable structural or morphological changes, as usually occurs in grapevine tissues.



Figures 1 - 3: MLO bodies in phloem tissues. Fig. 1: diseased grapevine used as inoculum source. Fig. 2: dodder filaments used in transmission tests. Fig. 3: grapevine parasitized for 8 weeks by infectious dodder. The markers correspond to 2 μm .

As previously observed in sieve tubes of grapevine affected by *Flavescence dorée*, the phloem cells containing the MLO bodies were entirely filled by the microorganism. Frequently the MLO mass was surrounded by the cell membrane which appeared completely detached from the wall as a consequence of the freeze-drying technique.

Two weeks after the connection with the dodder filaments the MLO bodies were observed in the receiving grapevine (Fig. 3). Few sieve tubes of the leaf petiole appeared completely filled by MLOs, showing the same morphological characteristics described in diseased grapes and in other symptomatic plants (1-5). The phloem of this grapevine showed an abnormal proliferation of sieve tubes, typical of the phloem of *Flavescence dorée* infected plants. The symptoms of the disease appeared 3 months after the observation of the MLO bodies in the phloem cells. These observations confirm similar results previously obtained during transmission trials, from grape to grape, by the vector *S. titanus* (2).

In the phloem tissues of control grapevine and dodder plants, MLO bodies were never observed.

Previous research (6-7) reported the use of dodder to transmit the *Flavescence dorée* agent from grapevine to herbaceous hosts, however the percentage of success was low. This study shows that dodder can transmit *Flavescence dorée* MLOs from grapevine to grapevine and confirms that SEM is a useful technique for early detection of this disease.

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TWO PROCEDURES FOR IMMUNOPURIFICATION OF FLAVESCENCE DORÉE MYCOPLASMA-LIKE ORGANISM (FD-MLO), AND EVIDENCE OF THE PATHOGENICITY OF PURIFIED MLO

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To date, MLO (Mycoplasma-like organisms) remain non-culturable organisms and are difficult to extract in good conditions of purity and conservation from infected hosts (plants or leafhopper vectors).

Two immunological purification methods have been devised, which yield FD-MLO antigens, one-step procedures. The previously described FD-specific monoclonal antibody, 51B5 (1), is used as a ligand on two synthetic supports, in low pressure columns. In both cases, the active sites of IgGs are oriented towards the outside, since the antibodies are bound via the Fc region of the molecules. The specificity of the monoclonal antibody ensures the binding of the FD-MLOs only.

In the first procedure, the ready to use Bioprobe Chromatography System (protein A AvidGelF and buffers), which permits a very good binding of the Fc region of IgGs, was tested for the purification of the FD-MLO. However, the elution of the immunocomplex in acidic conditions did not yield the mollicutes in a good state of preservation. In the second procedure (2), the monoclonal antibody 51 B5 was covalently bound on the chromatographic matrix of Hydrazide AvidGel (Bioprobe) via oxidized sugar moieties of their Fc part. Elution was obtained with 0.1 M glycine buffer pH 11.5.

In both procedures, successive fractions from the eluted MLOs have been analysed by SDS-PAGE. Western blot profiles of the SDS-PAGE gels profiles show that 2 major bands and some minor ones are revealed using anti FD-MLO rabbit polyclonal antibodies (fig 1). Healthy host rabbit polyclonal antibodies reveal no bands (2).



Fig. 1. Western blot of SDS-PAGE protein profiles of FD-MLO incubated with rabbit anti FD-MLO polyclonal antibodies. Lane 1, Immunocomplex purified on the protein A AvidGelF. Lane 2, FD-MLO purified on the Hydrazide-51 B5 column.

An infectivity test was conducted to examine if the pathogenicity of purified MLO is preserved: MLOs purified by the second procedure were injected into healthy leafhoppers (artificial acquisition). The injected leafhoppers were then allowed to feed on healthy *Vicia faba*. After 23 days of incubation, these plants showed symptoms of Flavescence dorée. This indicated that the purified MLOs may retain their pathogenicity. These results are in agreement with the already obtained data (2), and indicate that the MLOs were obtained by immunoaffinity chromatography in good conditions of purity and preservation.

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GRAPEVINE FANLEAF VIRUS IN CANARY ISLANDS AS A MODEL FOR MEDITERRANEAN REGION.

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Grapevine was introduced in Canary Island in the middle of the XV century, not only from other Spanish regions but also from Portugal, France and Italy, leading to the large number of about 100 catalogued varieties. The quality of *Malvasía* wine, made of the grape of the same name, a variety originating from Creta Island, must be pointed out. It is also noteworthy to remember that most of the grapevines grown in America originate from Canary Islands. Vineyards cover 14.535 ha and spread over the whole Archipelago, except Fuerteventura island where only few and small vineyards exist (Fig.1). Generally, vines are cultivated without watering, in a very special way, on volcanic ashes that sometimes reach 2 m of depth. They are not grafted, as phylloxera did not reach the islands. For this reason and because of the scarce vine cane exchange it was thought that no virus diseases would be there (7). Several foci of Grapevine Fanleaf Virus (GFLV) infected grapevines were detected in two localities of Tenerife in 1991, as well as the vector nematode, *Xiphinema index*, in La Gomera and Tenerife and *X. italiae* in vineyards, orchards of fruit trees and uncultivated areas of Gran Canaria, El Hierro, La Palma and Tenerife (5). The presence of *X. index* had been reported for the first time for Tenerife in 1963, without any specification on host plant or locality (3). Thereafter, the presence of virus vector nematodes was studied in the islands (1). The distribution of GFLV and the vector nematode in relation with the geographical situation and environmental characteristics of Canary Islands was most suitable for an epidemiological study and lead us to carry out a thorough survey of the occurrence of GFLV in relation with the presence of its nematode vector not only in vineyards but also in orchards of fig tree, the nematode's best host plant, in the whole Archipelago. The results could be of great interest for disease control in Mediterranean countries.

Materials and methods: A total of 509 samples of vine plants from the different islands were tested for GFLV during 1991, 1992 and 1993: 53 from Lanzarote, 50 from Fuerteventura, 24 from Gran Canaria, 291 from Tenerife, 18 from La Gomera, 40 from La Palma and 33 from El Hierro. Previously, 766 soil samples from the rhizosphere of cultivated plants and naturally formed soils from Gran Canaria, Tenerife, La Gomera, La Palma and El Hierro were analyzed. During 1991 to 1993, soil samples were taken in every island, especially in vineyards and fig tree orchards, in order to detect *X.index*. The presence of virus was determined by ELISA with Tris-HCl extraction buffer. Nematodes were extracted from soil by Flegg's method (5).

Results and discussion: GFLV is widespread in all the islands except Lanzarote and La Gomera, where a more thorough survey must be done. Fig. 1 shows the islands in which virus was found. GFLV appeared in 18 % of the samples studied from a nursery in Fuerteventura, in three samples of cv. Negramoll at Breña Baja and Puntallana (La Palma) and in one sample of cv. Negramoll at Frontera in El Hierro. All the infected material was introduced in those Islands from the locality of Tacoronte in Tenerife. On the other hand, 22 out of 24 samples taken in vineyards that were more than 100 years old at Monte Lentiscal (Gran Canaria) 22 were found infected with GFLV. In Tenerife, of 56 plants found infected, only four originated from old vineyards, two at Tegueste, one at Los Silos and possibly one at San Miguel. The remaining infections were found in vines recently introduced, most of them as plants from Tacoronte and in two cases as plants from abroad, introduced by the farmers. 36 infected plants were found in the northern part of the island, five foci at Tacoronte, where in a nursery 5 out of 7 samples were GFLV infected. The other foci were localized at La Guancha Baja, Icod and Garachico. 20 plants from the southern part of the island were GFLV positive: three foci localized at San Miguel and one at Arico, where 15% of the infected plants were found (6 positive from 40 tested plants were introduced from Tacoronte through Icod). In an experimental farm at San Miguel 72 plants had died and 10 from the 39 remaining vines were infected. The infected plants belong to the varieties Bermejuela, Breval, Listan blanco, Listan negro, Málaga, Malvasía, Negramoll, Pedro Ximenez, Torrontés and Verdello.

Xiphinema index is widespread in the whole Archipelago, with highest populations in orchards of fig trees, especially in places with a high soil moisture content. Indeed, water is a main growth factor of *X. index* populations, as found in the central region of Peninsular Spain (2). On the other hand, *X. italiae* was also found in the occidental islands, El Hierro, La Gomera, La Palma and Tenerife (6). Although it has been accepted that the latter is not a virus vector (4), both species appeared in several points associated to GFLV, and tests carried out in our laboratory have proven their capability of virus transmission. The transmission efficiency of *X. italiae* might be lower than that of *X. index* (8) and possibly depends on environmental factors or local virus strains. *X.index* was found at Haria (Lanzarote), Tetir (Fuerteventura), Monte Lentiscal (Gran Canaria), Arafo, Garachico, Granadilla, Güimar, Los Silos, San Juan de la Rambla, Tacoronte, Teganana, Tegueste and Valle Guerra (Tenerife), Barranco de Santiago (La Gomera), Garaffa, Tijarafe and Mazo (La Palma) and at Frontera (El Hierro). *X. italiae* occurred at Agüimes and Telde (Gran Canaria); Arafo, Garachico, Granadilla, Icod de los

Vinos, Los Silos, San Juan de la Rambla, Tacoronte and Taganana (Tenerife); San Sebastian (La Gomera); Breña Baja and El Paso (La Palma) and Erese, Frontera, Isora and at La Dehesa (El Hierro).

The most important problem is the dissemination of infected vine plants into new vine growing areas. It is therefore important to control the plants foreseen for new vineyards as well as to advise the farmers on the consequences of the introduction of non certified plants. The biggest foci are localized in zones with the highest moisture content of the soil, since moisture increases the development of *X.index* populations, especially where watering systems are established. It must be taken into account that the vector nematode is spread over all the islands, which means that the virus infectivity could be maintained for several years in the presence of vines or even some months without the host plant. The special agronomic characteristics of grape crops in Canary Islands, especially on substrata of the volcanic ashes of Lanzarote, La Palma and El Hierro, together with the traditional methods of grapevine selection, have prevented the dispersal of the disease through the vector, in spite of the presence of localized foci that have been increasing because of the introduction of infected plants and changes in the traditional agronomic techniques, especially those that increase soil moisture.

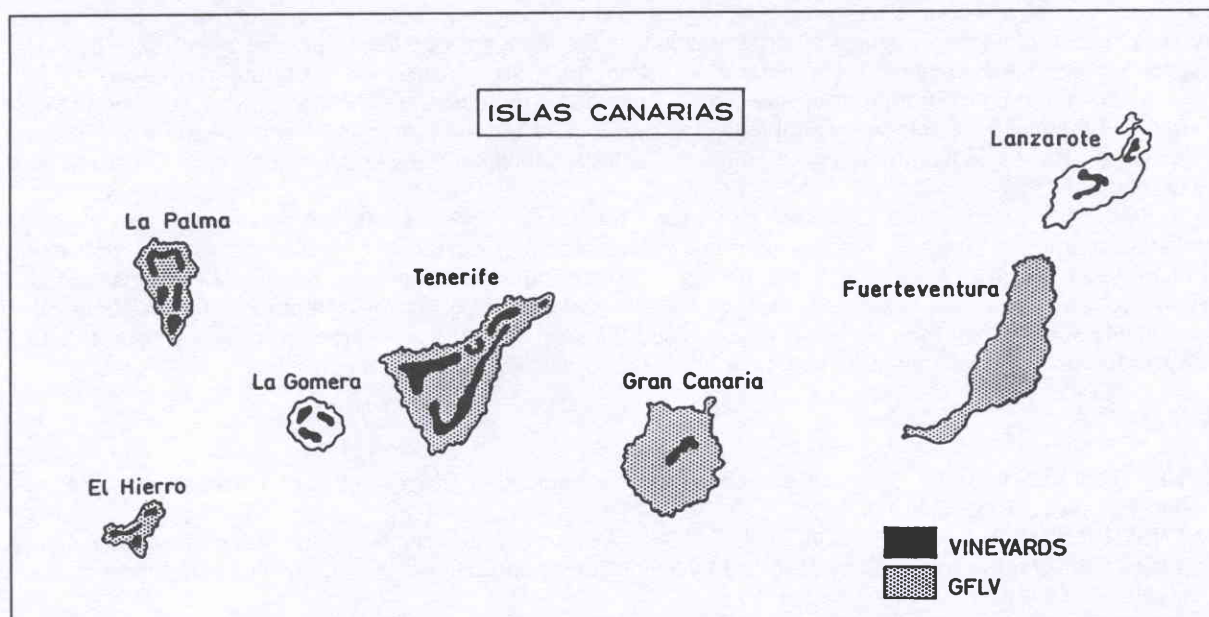


Figure 1. Distribution of vineyards and GFLV in Canary Islands

Acknowledgments: Authors are indebted to Dr. J.M. Rey, Miss A. Gala, Mr.C. Martínez and J.García Vaquero and all colleagues from the Laboratory of Virology for their cooperation. We are also grateful to the Comisión de Investigación Científica y Técnica (CICYT) for financial support to projects PB 89-0034 and NAT 91-0763.

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NATURAL SPREAD OF GRAPEVINE LEAFROLL DISEASE IN A VINEYARD OF NORTHERN ITALY

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It is generally accepted that the frequent occurrence of grapevine leafroll disease in vineyards is mainly due to the use of infected propagation material. However, a few cases of natural spread were reported (1, 2) and two species of mealybugs (*Planococcus ficus* and *Pseudococcus longispinus*) were shown to be capable of transmitting closteroviruses associated with the disease (3, 4). In this paper we report a case of natural spread of grapevine leafroll in a vineyard of Northern Italy where other insect species and not those cited were found.

The vineyard where observations were made is located in the selection Center of Riccagioia (Oltrepo Pavese) and was planted in 1976 by using buds of a virus-free *Vitis vinifera* clone (Pinot noir clone 5V-17) grafted on cuttings of a virus-free rootstock (Kober 5BB clone MI-K-3) in order to produce virus-free material for nurseries. From then on, the sanitary condition of the vineyard has been checked every year by visual inspections and bio-serological tests without detecting any virus infection for more than ten years.

In late summer 1989 four grapevine plants, located in a restricted area of the vineyard, started to show leafroll symptoms. Two more vines showed symptoms in 1990, two more in 1991 and one more in 1992. All those vines resulted to be infected with grapevine leafroll associated virus III (GLRaV-III) in ELISA serological tests made in 1992.

From 1991, inspections have been conducted in order to check the possible presence of mealybugs or similar insects in the vineyard. Mealybugs (fam. *Pseudococcidae*) have never been found; on the other hand, both in 1991 and 1992, several scale insects (fam. *Coccidae*) belonging to the genera *Eulecanium* and *Pulvinaria* were found on canes and shoots of some vines showing leafroll symptoms and on some other surrounding vines. Therefore, recently, transmission tests of leafroll disease from grapevine to grapevine by using scale insects of the cited genera have been set up. Observations are in progress.

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OCCURRENCE AND NATURAL SPREAD OF GRAPEVINE LEAFROLL-ASSOCIATED CLOSTEROVIRUSES IN CYPRUS

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Results of indexing tests carried out since 1982 show that leafroll is an extremely widespread disease of grapevine in Cyprus, affecting virtually all traditional and introduced cultivars at an average incidence of about 45% and 80%, respectively (2). Since many aspects of the etiology and epidemiology of this disease are still uncertain the present study was undertaken in order to: (a) determine the occurrence, prevalence and distribution of different grapevine leafroll-associated closterovirus (GLRaV) types in Cyprus, (b) investigate the relationship between these viruses and the leafroll disease, and (c) investigate the possibility and means of natural spread of GLRaV type III, which appears to be the most widespread among grapevine closteroviruses in Cyprus.

The presence or absence of leafroll disease was established by biological indexing on four standard indicators, namely the vinifera cultivars Cabernet Franc and Mission and the hybrid cultivars Baco-22A and LN-33. The indexing procedure involved graft-inoculation (chip budding) of potted indicator rootings in the greenhouse and transplanting of inoculated plants in an outdoor indexing plot where they were regularly examined for symptom development over a period of 3-4 years (1,2).

The closteroviruses concerned were serologically detected using the double antibody sandwich method of the enzyme-linked immunosorbent assay (DAS-ELISA), as described elsewhere (1,2). Tests were conducted with antisera to GLRaV type I from Bioreba, Switzerland, type II from D. Gonsalves, Cornell University (CA-5 isolate), type III from either D. Gonsalves (NY-1 isolate) or Bioreba, and type IV from D. Gonsalves (CA-4 isolate). No tests were conducted with GLRaV type V. Grapevine tissues subjected to ELISA testing were mature basal leaves or petioles, which were collected during autumn and macerated in extraction buffer, usually at a dilution of 1:10. Alternatively, ELISA was applied to cortical scrapings from mature canes which were collected in winter and processed as above. Tests were performed in ELISA plates coated with 1 or 2 µg of γ-globulin per ml of coating buffer. The conjugate was used at a dilution of 1:500 or 1:1,000 in enzyme buffer and the substrate at a concentration of 1 mg of p-nitrophenyl phosphate per ml of substrate buffer. The reaction was assessed by visual observation or measurement of absorbance at 405 nm.

The results of a series of tests carried out during 1988-92 (Table 1) show that all four GLRaV types investigated are present in Cyprus. Type III appears to be the most widespread as it was detected in 286 out of 928 vine specimens tested (overall incidence 31%). By contrast, levels of infection determined for types I and IV were much lower, i.e. about 12% and 10%, respectively. Type II was also detected in 8 out of 15 specimens tested but these figures are too small to enable a reliable quantitative estimate of its incidence.

Table 1. Serological detection of different types of grapevine leafroll-associated closterovirus (GLRaV) among vines indexing positive or negative for leafroll disease.

GLRaV type	ELISA reaction	Indexing		Total
		LR+	LR-	
I	+	32	2	34
	-	112	142	254
		144	144	288
II	+	8	0	8
	-	7	0	7
		15	0	15
III	+	208	78	286
	-	285	357	642
		493	435	928
IV	+	2	32	34
	-	163	127	290
		165	159	324

Despite its low incidence, type I appeared to be consistently associated with leafroll, as it was almost entirely detected in vines indexing positive for this disease (Table 1). By contrast, type IV was almost exclusively detected in vines indexing negative for leafroll, whereas type III was detected in both leafroll-positive and leafroll-negative vines, at an average incidence of about 42% and 18%, respectively. The latter figure poses some questions as to the actual role of GLRaV-III in the etiology of leafroll disease in Cyprus. Finally, the role of GLRaV-II remains uncertain due to the very small number of specimens tested.

The widespread incidence of GLRaV-III among vines grown in Cyprus prompted a more detailed investigation into the possibility of existence and means of natural spread of this virus. The following data suggest that GLRaV-III is transmitted naturally in Cyprus by means other than infected propagating material:

a) In two grapevine plots known to be infected by the virus there was a definite increase of virus incidence from 1991 to 1992, i.e. from 44% to 51% at Akhelia and from 41% to 68% at Zyghi.

b) Low levels of infection were detected in 1992 among clean grapevine stocks, which had been introduced from California in 1985 and maintained since at the Plant Quarantine Station, Kornos. The infection was less than 1% and concerned only 4 out of 55 grapevine cultivars. Such an infection could have either been introduced with the propagating material from California or occurred locally through natural virus spread. Further observations at Zyghi, however, where progeny plants of the same cultivars were planted in 1989, proved the existence of natural virus spread, as the infection here increased to 19% and included 15 of the 55 cultivars.

c) Low levels of GLRaV-III infection (1.7%) were likewise detected among locally selected clean stocks of traditional cultivars grown in commercial vineyards at various sites of Cyprus. When such stocks, however, were clonally propagated and transferred to a greenhouse in Nicosia infection by GLRaV-III increased to 57% within 2-5 years.

In the greenhouse mentioned above there was heavy mealybug infestation suggesting a possible involvement of this insect in the natural spread of GLRaV-III (3,4). Therefore, during the second half of 1992 a series of laboratory transmission tests were initiated in order to verify mealybug transmission under controlled conditions. Data already obtained from one of these tests (initiated on July 27, 1992) show that positive virus transmission was obtained in 3 out of 15 mealybug-inoculated plants.

The above findings on natural spread and mealybug transmission of GLRaV-III, provided they will be further substantiated by additional tests already in progress, may explain the extremely widespread distribution of leafroll disease, and of GLRaV-III in particular, among both local and imported vine cultivars grown in Cyprus. Since the island is still Phylloxera-free and vinifera grape cultivars are grown self-rooted, dissemination of the disease cannot be attributed to American rootstocks which, being symptomless carriers of leafroll-associated viruses, are often considered as the major means of leafroll disease spread in other viticultural areas of the world.

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SPREAD OF GRAPEVINE LEAFROLL AND ITS ASSOCIATED VIRUS IN NEW ZEALAND VINEYARDS

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Grapevine leafroll is not a new disease for the New Zealand grape and wine industry. This disease, although never killing vines, can reduce vine productivity by about 30%, delay maturity, and reduce colour in red varieties. Leafroll infected vines are generally associated with fruit of inferior quality compared to fruit from vines free of the disease.

Until recently, leafroll was thought to be spread only by infected propagation material - cuttings, scions and/or rootstock wood. However, in many New Zealand vineyards spread has occurred between vines. This is a great concern to the New Zealand industry because they do not have a method to control spread.

This paper describes the first studies undertaken to characterise spread of leafroll in four vineyards in the North Island of New Zealand.

Vineyard assessment

Visual symptoms of leafroll were used to assess four vineyards. Often the assessments of the different vineyards and at the different years were made by different people.

VINEYARD ONE: A Cabernet Sauvignon clonal trial in an Auckland vineyard. Eighteen clones were planted as grafted vines in 1985 and randomly distributed throughout the area as single vine replicates. Since 1988 the vines have been annually assessed for visual symptoms of leafroll. Adjacent vineyards had a long history of leafroll infection.

VINEYARD TWO: A commercial vineyard of Cabernet Sauvignon in Hawke's Bay planted in 1979. In the autumns of 1991, 1992 and 1993 the vines were visually assessed for leafroll.

VINEYARD THREE: A commercial vineyard of Pinot noir in the Auckland area planted in 1988. In autumns of 1991 the vines were visually assessed for leafroll. The vines with leafroll were removed during the winter of the same year. The vineyard was assessed again in 1992 and 1993.

VINEYARD FOUR: This is a commercial Merlot vineyard in the Auckland area. Over two years a portion of a single row has been monitored for leafroll symptoms and samples analyzed by ELISA. ELISA samples were either leaf tissue collected during the autumn (between veraison and harvest) 1992 or vascular tissue from beneath the bark of spurs collected in the spring 1992 (bloom in the 1992/93 season). Antibodies for GLRaV I and III were obtained from BIOREBA AG and the procedures were those outlined by the supplier.

Results and Discussion

The pattern of leafroll spread within VINEYARD ONE, the Cabernet Sauvignon clonal trial in Auckland, over five years from 1988 to 1992 showed that the spread was generally along the rows, although some across the row spread also occurred.

The initial infection in this vineyard could be traced to two clones. The random planting pattern resulted in a random distribution of leafroll throughout the trial.

At the first three vineyards the number of vines infected with leafroll doubled each year (Fig. 1). This graph was first established with the visual assessments from VINEYARD ONE and then the assessments from other two vineyards overlaid.

VINEYARD TWO is six years older than VINEYARD ONE but the level of infection is about two third less. This could have resulted from three situations. There may have been less vines infected with leafroll at planting or leafroll was introduced later in VINEYARD TWO compared to VINEYARD ONE. Alternatively, the vector for spread arrived later after planting in VINEYARD TWO compared to VINEYARD ONE. It appeared that the level of infection in VINEYARD THREE was more advanced for its age compared to VINEYARD ONE. Note, at year two both VINEYARD ONE and THREE had similar levels of leafroll but VINEYARD THREE was two years younger than VINEYARD ONE. VINEYARD THREE may have a greater number of infected vines at planting or the vector was present earlier than in VINEYARD ONE.

At VINEYARD FOUR spread was confirmed by ELISA. Vines adjacent to infected vines that tested negative in late 1991/92 season tested positive at bloom in the next season.

Leafroll did not spread great distances within the monitored vineyards between years. Often the spread was only to vines adjacent to infected vines. This suggests that the vector for spread does not have the ability to travel large distances throughout the vineyard or between vineyards.

The vector for spread in New Zealand is unknown. Mealybug have spread the disease in Sicily (1) and

Israel (2) and can be present in high numbers in New Zealand vineyards. Mealybugs do not travel large distances so would match the pattern of spread observed. Future research will determine if mealybugs are vectors for leafroll in New Zealand.

Conclusions

Grapevine leafroll has spread within New Zealand vineyards at a rate that doubled the number of infected vines from one year to the next. The vector for spread in New Zealand is unknown. If the vector is an insect, it is not mobile because the spread is restricted to vines adjacent to infected vines. Mealybug spread leafroll in other countries, so they are likely to be also involved New Zealand.

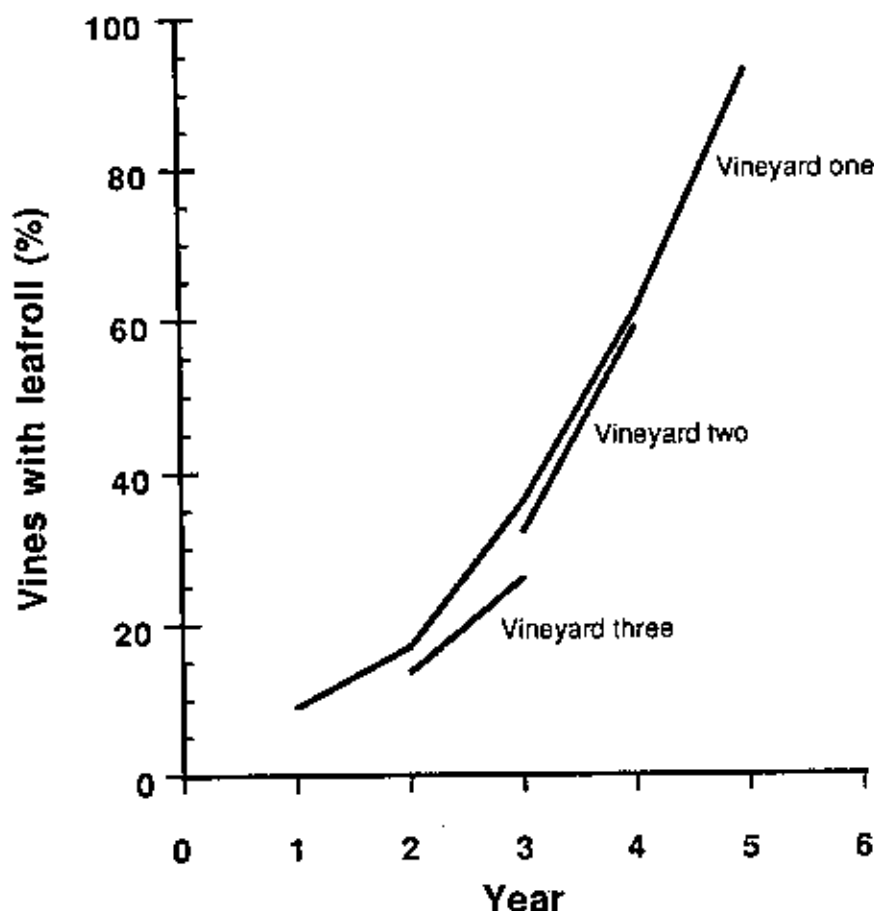


Figure 1. Spread of leafroll in three North Island vineyards. VINEYARD ONE - 3 (Year 1) to 7 (Year 5) years old; VINEYARD TWO - Cabernet Sauvignon in Hawke's Bay, 12 (Year 3) to 13 (Year 4) years old; and VINEYARD THREE - Pinot noir in Auckland, 2 (Year 2) to 3 (Year 3) years old. Leafroll infection based on visual symptoms.

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VIRUSES AND VIRUS DISEASES OF THE GRAPEVINE IN ALBANIA

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The Albanian viticultural districts of Shkoder, Durres, Lezha, Tirana, Elbasan, Lushnje and Vlora were surveyed in 1988, and the following virus diseases were observed: fanleaf, leafroll, rugose wood, enations and fleck (1).

Whereas symptoms like those induced by distorting strains of grapevine fanleaf virus (GFLV) were widespread, though with a variable incidence, infections by chromogenic strains of the same virus were rare. Yellow mosaic with a patchy distribution was observed near Tirana and at Shkoder, in a cv. Afuzali vineyard, whose soil hosted *Xiphinema index*. Leafroll and rugose wood were ubiquitous and frequent in native cultivars. Enations occurred in a few vines of cv. Alphonse Lavallée near Durres, which were also heavily affected by rugose wood. Fleck was observed in a few volunteer *Vitis rupestris* vines at Elbasan, but not in comparable vines in other parts of the country, nor in mother vine plots in nurseries.

Samples consisting mostly of mature canes were collected in 1991-92 and brought to Bari for examination. Sap transmission tests did not reveal viruses other than GFLV, an isolate of which was investigated in detail (2). Serological tests were performed on bark scraping extracts with commercial or locally produced ELISA kits to the following viruses: GFLV, grapevine fleck (GFkV), grapevine virus A (GVA), grapevine leafroll-associated virus I (GLRaV I), and grapevine leafroll-associated virus III (GLRaV III).

A total of 473 samples from 16 native cultivars and 81 samples from two imported European grape varieties (Afuzali and Merlot), and two American rootstocks (Kober 5BB and 140 Ru) were tested. The following average infection rates were detected:

1. Native cultivars: GFLV, 5.5%; GFkV, 15%; GVA, 59%; GLRaV I, 10%; GLRaV III, 50%.
2. Imported cultivars: GFLV, 40%; GFkV, 30%; GVA, 53%; GLRaV I, 7%; GLRaV III, 56%.
3. American rootstocks: GFLV, 8%; GFkV, 4%; GVA, 25%; GLRaV I, 0%; GLRaV III, 17%.

Although no yellow speckle or vein banding symptoms were observed in the course of the surveys, viroids were proven to occur in Albanian vines (3).

The data relative to the presence and distribution of viruses are in fairly good agreement with estimates on the incidence of the diseases they cause, as derived from visual observations. Although there is no information on the possible presence of virus-like diseases such as vein mosaic and vein necrosis because the results of the graft transmission tests are not yet available, there is little doubt that the sanitary conditions of the Albanian viticultural industry are comparable to those of the surrounding European and Mediterranean countries.

The high level of occurrence of rugose wood and leafroll in native varieties, corroborated by a comparably high level of detection of GVA and GLRaV III can be taken as an indication that these diseases (and their putative agents) have been with Albanian vines for long time.

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FREQUENT OCCURRENCE OF GLRaV-I AND GLRaV-III IN LEAFROLL AFFECTED GRAPEVINES IN LOMBARDY (NORTHERN ITALY).

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In previous studies carried out on 23 clones belonging to four different cultivars of *Vitis vinifera* (Barbera, Croatina, Merlot and Cortese), grown in Oltrepo pavese (Southern Lombardy), it was found, by DAS-ELISA, that all grapevine clones with leafroll symptoms (17 on 23) were infected mainly with GLRaV-III or GLRaV-I or both. The use of IEM on the same samples showed the occurrence in very few cases of GLRaV-II and GVA (1).

In this paper we report the results about further studies, on the same clones, in order to verify the occurrence of GLRaV-IV and GLRaV-V. Furthermore, we carried out a wider serological investigation to ascertain the possible presence of GLRaV-I and GLRaV-III in leafroll affected grapevines grown in a different viticultural area (Valtellina, Northern Lombardy) using standard DAS-ELISA.

Referring to the further studies on the 23 clones of Oltrepo pavese, partially purified extracts obtained from 10 g of leaves by the method of Gugerli *et al.* (2) were tested with antisera to GLRaV-IV (supplied by Dr. Gonsalves, Geneva, U.S.A) and to GLRaV-V (supplied by Dr. Walter, Colmar, France) for identification of viruses through decoration method (4). This IEM application detected the presence of GLRaV-V only in one sample of cv Barbera already infected with GLRaV-III, while GLRaV-IV was never detected. These and previous results are summarized in Tab. 1.

The wider investigation regarded n. 136 grapevines sources with leafroll symptoms belonging to cv Chiavennasca found in 10 different vineyards of the Valtellina valley. Samples of basal leaves were harvested in late September and kept frozen at -20°C until the use.

All samples were tested as crude extract obtained by grinding 2 g of leaves with liquid nitrogen and the powder obtained was thawed in extraction buffer with a 1:3 ratio (w/v). Subsequently the samples that produced negative reactions were partially purified by the method of Gugerli *et al.* (2) with 2 g of leaves and tested again to obtain more accurate results.

DAS-ELISA tests on the 136 crude extract samples gave the following results: GLRaV-I was present in 36 samples; GLRaV-III in 50. In 14 of these the infection was mixed thus the total number of infected grapevines was 72.

The 64 samples that had not reacted in the first test, after partial purification showed the following results: 48 reacted with GLRaV-I, 5 with GLRaV-III, 3 with both closteroviruses and 14 did not react. All these results are shown in Tab. 2.

The results of these studies show that GLRaV-III is the most frequent closterovirus found in leafroll diseased grapevines of Oltrepo pavese; while the frequent occurrence of GLRaV-I in Valtellina is in agreement with the results obtained in some viticultural areas of France (5) and Germany (3).

Tab. 1. - Results of DAS-ELISA and IEM decoration tests on leafroll-infected grapevine clones with leafroll symptoms (Oltrepo pavese).

CV	CLONES	DAS-ELISA		IEM decoration test			
		GLRaV-I	GLRaV-III	GLRaV-II	GLRaV-IV	GLRaV-V	GVA
BARBERA	6	3	6	0	0	1	1
MERLOT	5	1	5	1	0	0	0
CROATINA	5	2	4	1	0	0	0
CORTESE	1	1	0	0	0	0	0
TOTAL	17	7	15	2	0	1	0

Tab. 2. Results of DAS-ELISA on crude extracts and partially purified grapevine samples of Valtellina.

	SAMPLES	POSITIVE	GLRaV-I	GLRaV-III	Mixed inf.
Crude extract	136	72	36	50	14
Partially purified samp.	64	50	48	5	3
TOTAL	200	122	84	55	17

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INVESTIGATIONS ON THE OCCURRENCE OF THE NEMATODE GENERA *XIPHINEMA*, *LONGIDORUS* AND *PARALONGIDORUS* IN VINEYARDS OF BADEN-WÜRTTEMBERG (GERMANY)

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A survey on the occurrence of the nematode the species *Xiphinema*, *Longidorus* and *Paralongidorus* was carried out in vineyards of Baden-Württemberg (South-West Germany). These investigations allowed us to estimate the share of potential vectors and the relation between species range and site conditions.

In 49 % of the sites sampled we found six species of *Xiphinema* randomly spread over the entire study area. For each species a distinct centre of distribution could be established.

X. index Thorne & Allen 1950, vector of the grapevine fanleaf virus (GFV), was found in 6 % of the sites studied. It occurred in various soil types, but clearly preferred soils of keuperous and shell-lime origins. *X. index* was found exclusively in traditional vineyards where no large scale terracing had been carried out. With a few exceptions the population density was very low, generally between 1-5 adults, in a few cases 5-10 adults per 200 ml soil. On 22 of the 45 sites a close relation between the occurrence of *X. index* and fanleaf diseased grapevines was observed. As tested by ELISA, these vines were infected with grapevine fanleaf virus (GFV).

X. diversicaudatum (Micoletzki, 1927) Thorne 1936 is considered to be the vector of Arabis mosaic virus (ArMV) in grapevines. The species was also found in 6 % of the study sites. We observed a population centre of this vector (48 % of all findings) at the western slopes of the central Black Forest. The analysis of grapevines from vineyards with *X. diversicaudatum* in most cases yielded an infection with Arabis mosaic virus (ArMV). The majority of the sample sites was located on granite-, gneiss- and keuper-based soils with pH-values below 7. The species was not only found on sites planted with grapevines but also in soils of embankments and orchards (i.e. cherries, peaches, strawberries).

X. vuittenezi Luc, Lima, Weischer and Flegg 1964 was found in 30 % of the entire study sites. In loess, loess-lime and volcanic soils *X. vuittenezi* reached the highest population densities with up to 150 adults per 200 ml soil.

X. pachtaicum (syn. *mediterraneum* Martelli & Lamberti, 1967) Kirjanova 1951 was found in 8 % of the sample sites. The species was observed in all soil types with the exception of granitic and volcanic soils. Frequently the species was found together with *X. vuittenezi*.

X. coxi Tarjan 1964 was isolated once in an embankment next to a vineyard.

X. rivesi Dalmasso 1969 was found once together with *X. vuittenezi* and *X. pachtaicum* in a vineyard on loess. This is only the second proof of the species in Germany.

Nematodes of the genus *Longidorus* were found in 18 % of the study sites and until now six species could be identified. In just a few cases identification up to the species was impossible and they were placed either in the *Elongatus*- or the *Vineacola*- group.

L. macrosoma Hooper 1961 communicates the raspberry ringspot virus (RRV) to grapevines. The species was spread over almost the entire study site but exclusively on medium to heavy clay soils. In connection with *L. macrosoma* a hearth of RRV-infected grapevines was observed only once.

L. profundorum Hooper 1961 had a similar distribution as *L. macrosoma*. The species also prefers heavy soils and was occasionally found together with *L. macrosoma*. Amongst all species of *Longidorus* this one was found most often (on 9 % of the sites studied) but could not be linked to grapevines showing symptoms of grapevine degeneration.

L. caespiticola Hooper 1961 was present in seven vineyards, of which five were on medium-weight gneiss soils.

L. elongatus (de Man 1876) Thorne & Swanger 1936 communicates tomato blackring spot virus (ToBRV) to grapevines. It occurred on four sites with light soils but could not be linked to ToBRV.

L. attenuatus Hooper 1961, also a vector of ToBRV in grapevines, was only observed in two vineyards on loess. The vines showed no signs of ToBRV.

L. vineacola Sturham & Weischer 1964 was found only once on a granitic soil in central Baden.

Paralongidorus maximus (Bütschli, 1874) Siddiqi 1964 is the only species of the nematode genus *Paralongidorus* found in Germany. It is suspected to communicate RRV to grapevines. The species was evidenced in eight sites on predominantly light soils. In one case this species was found together with *L. macrosoma* in an RRV-diseased vineyard.

PRELIMINARY RESULTS ON THE OCCURRENCE OF GRAPEVINE LEAFROLL ASSOCIATED VIRUSES (GLRaV's) IN HUNGARY

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Grapevine leafroll (LR) is a widespread disease causing severe damage all over the world. Over the last decade thorough research was made which led to the isolation and study of closteroviruses, the preparation of antiserum and to the development of ELISA (1). LR symptoms were already described in Hungary in 1895 (2), but the occurrence of the disease was proved with graft transmission experiments only in 1969 (3). The subject of this paper is the investigation of the occurrence of viruses causing grapevine LR in the Hungarian vine regions. The survey covering 20 regions started in 1991 as project No. 1015 supported by the National Committee for Technical Development (OMFB, Hungary).

Detection of grapevine leafroll associated viruses (GLRaV's) was made by DAS-ELISA using antibodies prepared by GUGERLI as well as commercial antisera to GLRaV I, II, III, IV and GVA manufactured by BIOREBA and SANOFI. Since 1992 detection of GVA was also made with antiserum kindly provided by Dr. P. MONETTE. Leaves and petioles collected in September as well as cane shavings without bark were tested. ELISA was first carried out on plants with LR symptoms collected earlier (1978-86) in different regions of the country and which were then maintained in a virus collection and simultaneously indexed on Pinot noir indicator. The survey included diseased plants of those varieties which were typical for a given vine region. Results obtained by ELISA in 1991 allowed to select the plants which were indexed. Three donor plants per LR infection type (single or mixed infection) were used for chip grafting onto the following woody indicators: Pinot noir, Cabernet franc, Carignan, Alicante Bouschet, Grenache and Merlot.

Results

Two out of 7 plants with LR symptoms collected between 1978 to 1986 gave negative results when indexed on Pinot noir, but GLRaV's I and GLRaV's II+IV were detected by ELISA in one and the other, respectively. Four plants, which were positive on the indicators, were infected by the following viruses, as determined by ELISA: GLRaV I alone, GLRaV's I+II+III+IV and GLRaV's II+III+IV. However, in one plant which induced severe LR symptoms on the Pinot noir indicator, none of the GLRaV's was detected by ELISA.

In comparative experiments closteroviruses were detected in leaf samples from 34 out of 60 plants and in cane samples from 52 out of 81 plants showing LR symptoms in September 1991. In 1992, viruses were detected in leaf samples of only 34 out of 92 diseased vines compared to cane samples of 44 out of 72 diseased plants. In both years, higher ELISA readings were recorded in the cane samples.

The surveys in 1991 and 1992 comprising tests on 173 vines with LR symptoms, from 32 varieties or clones originating from 11 vine regions of Hungary, showed that closteroviruses could not be detected by ELISA in five varieties: Ezerjő, Sauvignon, Juhfark, Nemes Olaszrizling and Szürkebarát. Single infections were observed on five varieties whereas mixed infections of two or more closteroviruses occurred in various combinations on the other varieties. GLRaV III was most frequently detected, e.g. in 72 out of 173 plants, followed by GLRaV I in 45 plants. GLRaV's II and IV as well as GVA were detected with lower but nearly identical frequencies: 28, 23 and 27 respectively (Table 1). Evaluation of ELISA reactions for GLRaV II and IV, using the kits from SANOFI, needed long incubation periods of the substrate.

Indexing in the field of the serologically selected grapevines were started on 6 indicator varieties and showed promising results after one year. However, the observation of the indicators needs be continued.

Conclusions

- Samples obtained from canes by shavings were more appropriate for virus detection by ELISA than leaf samples.
- The detectability of ELISA requires further improvement.
- GLRaV's I, II, III, and IV as well as GVA occur in grapevines grown in Hungary.
- GLRaV III was most frequently detected followed by GLRaV I, whereas GLRaV's II and IV as well as GVA were observed rarely.
- Pinot noir was not sufficient as an unique LR indicator in Hungary.
- Other and new indicators are required to improve reliability of LR indexing under Hungarian climatic conditions.

LR diseased plants, which failed to react in the serological or biological tests, will be further investigated, especially by electron microscopy. The production of new antisera will be investigated.

Table 1. Occurrence of closteroviruses in Hungarian grapevine varieties, found with ELISA in leaf and cane samples in 1991 and 1992.

Variety	No of tested plants	Detection of GLRaV's I to IV and GVA by ELISA					
		Negative	I	II	III	IV	GVA
Ahuz A5	2	0	1	1	1	1	0
Alicante Bouchet	10	1	1	3	6	2	1
Bánfi rizling	3	1	1	0	0	0	1
Cabernet franc	13	4	5	3	4	0	1
Cabernet Sauvignon	3	0	2	1	0	0	0
Chardonnay	2	0	2	1	0	0	2
Coglód szilve	1	0	0	1	0	0	1
Chasselas bl.	8	1	2	2	4	1	2
Chasselas r.	1	0	0	0	1	1	0
Ezerjórfő	3	0	3	0	0	0	0
Ezerjó	2	2	0	0	0	0	0
Hárslevelő	9	6	3	1	2	1	0
Izráki mérfőhár	6	3	0	0	3	0	0
Juhfark	3	3	0	0	0	0	0
Karmin	4	1	2	1	2	0	0
Kékfrankos	18	12	7	1	4	0	2
Isáryka	10	7	1	1	1	1	1
Merlot	16	0	0	2	15	3	2
Múzeum Lunai	2	0	0	0	2	2	2
Nemes Olaszrizling	3	3	0	0	0	0	0
Nozteri rizling	2	0	0	1	1	0	2
Olaszrizling	8	2	2	0	3	1	0
Operto	8	3	2	1	3	1	4
Pinot noir	7	0	6	2	5	4	3
Pirce tramini	3	1	2	0	0	0	0
Pirce veltelini	6	0	3	3	3	3	1
Rubintoe	3	1	0	0	2	0	0
Sauvignon	4	4	0	0	0	0	0
Sémillon	4	1	1	2	0	0	1
Székelybárát	1	1	0	0	0	0	0
Zöld veltelini	3	0	0	0	3	2	1
Zweigelt	4	0	0	0	4	0	0

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SPATIAL PATTERN ANALYSIS FOR EPIDEMIOLOGICAL STUDIES ON GRAPEVINE DISEASES

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For a number of grapevine diseases epidemiological problems need still to be investigated. Modes of spread, existence of vectors, and sources of infections are important components for the understanding of disease progress. The detection and analysis of spatial patterns of diseased grapevines is an approach to acquire information about those components of disease epidemiology.

Different techniques are available to study the distribution of diseased plants. In many cases mapping allows the detection of specific patterns or disease gradients. However, spatial patterns are not always such evident and often information on the significance of detected patterns or a quantification for the comparison of different plots or times of assessments are required. Out of the different statistical techniques commonly used in plant disease epidemiology (1) the calculation of dispersion parameters is of special advantage. Vineyards are usually planted in a defined structure of rows and columns, which makes data collection easy. The variation within the host plant population is low since planting material of the same cultivar and age is used. The distribution of infected plants is therefore mainly a result of the properties of the disease under investigation and its interrelations with the environmental factors influencing its epidemiology. Indices of dispersion like Lloyd's index of patchiness (LIP) or Morisita's index of dispersion (I_d) indicate regular-, random-, or aggregated distributions by values less, equal, and greater than one. The significance of their deviation from random distribution can be calculated.

The procedure described here requires mapping of the entire area to be analyzed and recording of the position of symptomatic or infected vines in a data file. The plot is then divided into a grid of subunits of variable size and LIP and I_d are calculated for all possible sizes of subunits. If significant aggregations are detected, the average focus size can be determined by the size of subunits which lead to maximum dispersion parameters or by another parameter (index of clump size) which is calculated from I_d. Disease incidence is calculated for individual rows and columns and subjected to regression analysis to detect disease gradients within the plots.

The application of the above mentioned techniques and the use of a computer program written for the analysis of dispersion parameters, frequency data and disease gradients (2) is demonstrated for 'Vergilbungskrankheit' (VK) a yellows disease of grapevine in Germany which is presumed to be caused by mycoplasma-like organisms. Its epidemiology is not yet well understood. Modes of transmission, the existence of vectors and alternative hosts and the effects of culturing practice on this disease are open questions. Vineyards of the Mosel- and Middle-Rhine valleys were mapped for the location of symptomatic vines. Severity of symptoms for individual vines was not recorded.

Data collected from 1990-1992 in 12 vineyards of different cultivars, age and disease incidence varying between 4 % and 44 % have been used for the analysis. 7,361 of 38,865 grapevines (18.9 %) exhibited symptoms of VK. With two exceptions, a four year old 'Riesling' and a '12 year old 'Scheurebe' vineyard, LIP and I_d were significantly greater than one for some sizes of subunits. This is an indication for the existence of foci of VK in the vineyards. The average size of these foci as determined by the size of subunit which led to a maximum of LIP varied between 8 and 45 vines. Disease gradients were detected in some of the analyzed vineyards. They were most evident in plots adjacent to non cultivated fields and bushy areas. Incidence was usually negatively correlated to the distance from such areas.

The presented data show that non-random distribution of symptomatic vines is common with VK. The existence of aggregations indicates that the disease is transmitted in the field from grape to grape or from alternative hosts to grape. This finding is supported by the detection of disease gradients in vineyards adjacent to fallow fields or bushy areas which could be sources of vectors, pathogens or both. Many leafhoppers species occurring in vineyards exhibit corresponding gradients of abundance (unpublished data).

The technique and program presented allow the valuation of statements about the spatial distribution of diseased vines and the comparison of different experimental plots by standardized parameters. It was demonstrated with grapevine yellows but its use is not restricted to this disease. It may be of value also for studies on other infectious diseases for which epidemiology is still a problem.

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VIRUS DISEASES OF GRAPEVINE IN SOUTHEASTERN ANATOLIAN REGION IN TURKEY

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Anatolia is the origin of *Vitis vinifera*. Therefore vineyard farming is an important traditional agricultural sector in Turkey. Turkey is the 8th biggest grape production country in the world. 30% of total vineyard in Turkey is planted in southeastern Anatolian region and more than 150 local ancient *Vitis vinifera* varieties grow in this area.

In the last 2 decades the traditional grape production has been losing importance and economy due to the introduction of new varieties and the infestation by *Phylloxera*. In most places farmers are not aware of viruses and their transmission. Viruses are disseminated by propagation material as well as by pruning, harvesting and grafting tools (grafting is not so common in this region)

The reason of the wide spread of viruses in this region (table 1) is related to the traditional plantation system (4). Between 1988 and 1991 surveys have been made by indexing (5,6) with *Vitis* indicators and ELISA (2,3).

Table 1. The differences of grape production and grape occupied lands in the southeastern Anatolian region between 1990 and 1988 (1)

Name of location	Total land (ha)		Difference (%)	Production (t)		Difference (%)
	1990	1988		1990	1988	
GAZIANTEP	51 707	50 150	-4	271 138	270 850	-1
SANLIURFA	18 984	11 844	-38	69 070	57 140	-18
DIYABAKIR	22 500	21 500	-5	117 650	116 170	-1
MARDIN	26 526	24 437	-5	167 842	152 522	-10
ADYAMAN	18 135	11 858	-27	100 825	77 858	-23
SIRT	7 583	7 086	-7	42 395	21 831	-51
Total	142 436	126 975	-11	758 818	686 471	-10

LN 33, Baco 22 A, LOT, Mission, 5-BB and R-110 varieties were used as *Vitis* indicators and *Chenopodium quinoa* and *Nicotiana benthamiana* as mechanically inoculated herbaceous indicators (5,6).

For ELISA antisera were diluted 1/2000 for grapevine fanleaf (GFLV) and arabis mosaic virus (AMV) and 1/1000 for grapevine leafroll associated viruses (GLRaV's). Antigens were diluted 1/10 and 1/5 respectively (2, 3).

GFLV, GLRaV type I and GLRaV type III, arabis mosaic (AMV) and grapevine fleck (GFV) viruses are present in the region. Among these viruses GFLV is the prevalent virus and causes important yield losses on local varieties in ancient vineyards. Many of the plants were found to be infected by more than one virus.

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RESULTS REGARDING THE IDENTIFICATION OF CLOSTEROVIRUSES ASSOCIATED WITH THE LEAFROLL DISEASE ON SOME GRAPEVINE VARIETIES GROWN IN ROMANIA

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Over the last years progress has been made regarding the etiology of grapevine leafroll disease. Various types of virus particles were found in grapevine plants with leafroll symptoms (3). Recently, closterovirus-like particles of a length of 1800 to 2300 nm were shown to be constantly associated with the grapevine leafroll disease. These particles were of distinct serotypes and were tentatively called grapevine leafroll associated viruses: GLRaV I, GLRaV II and GLRaV III (1, 4, 5). As leafroll symptoms are relatively frequent in some red varieties grown in Romania a study was initiated to determine the presence of grapevine leafroll associated viruses in the most important red and white varieties and rootstocks grown in different parts of the country. The first results of serological investigations are presented in this work.

During the period of 17th September to 2nd October 1991 serological tests were performed on grapevine plants with and without leafroll symptoms, belonging to three red varieties grown near Bucharest in experimental plots planted with vines of standard category. During the period of 13th to 15th October 1991, tests were done on plants of white varieties without leafroll symptoms which were grown at the Experimental Station Blaj (Transylvania). They belonged to the visually selected clones Feteasca alba 29, Feteasca regala 21, Muscat Ottonel 55/53, Neugurger 31-10, Pinot gris 34, Riesling Italian 3 and Sauvignon 9 grafted on rootstocks of standard category and to the varieties Ezerfürü, Feteasca alba and Furmint from a commercial plot planted with standard vines.

All tests were done by DAS-ELISA, using for the coating and the alkaline phosphatase labelled conjugate antibodies to GLRaV-I, GLRaV-II and to GLRaV-III. Tests were carried out as described in the literature (2). Extracts were obtained from basal leaves which were homogenized in plastic bags (1). Optical density (E_{405}) was measured with a SUMAL automatic analyzer (Combinat Carl Zeiss Jena).

As shown by the data presented in table 1, tests using antisera to GLRaV-I and GLRaV-II were essentially negative for all except one of the 36 Cabernet Sauvignon and Merlot plants without symptoms and all the 34 Cabernet Sauvignon, Merlot and Cinsaut plants with symptoms, whereas when tested with antibodies to GLRaV-III these plants without symptoms gave clearly negative and all the plants with typical leafroll symptoms gave intensely positive reactions. Therefore GLRaV-III appeared to be strictly associated with the leafroll disease in these red varieties.

The visually selected clones belonging to the white varieties proved to have a low infection rate of GLRaV's I, II and III. Thus, by testing 10 plants of these selected clones negative results were obtained in all the plants belonging to Feteasca alba 29 and Riesling Italian 3. In Feteasca regala 21 two plants with GLRaV-III were found ($E_{405} = 0.476$ and 0.400), in Pinot gris 34 two plants with GLRaV-II ($E_{405} = 0.369$ and 0.364), in Sauvignon 9 one plant with GLRaV-I ($E_{405} = 0.866$) and one with GLRaV-III ($E_{405} = 0.460$) and in Muscat Ottonel one plant with GLRaV-III ($E_{405} = 0.481$). Similarly, by testing 10 plants from the white varieties grown on commercial plots planted with standard vines, one plant with GLRaV-I was found in each of the varieties Ezerfürü and Furmint ($E_{405} = 0.528$ and 0.708 respectively), one plant with GLRaV-III in Neuburger ($E_{405} = 1.325$) and one plant with GLRaV-I and GLRaV-III in Feteasca alba ($E_{405} = 0.155$ and 0.806 respectively). Comparing these data with those from table 1, we observe that extinction values obtained from positively reacting plants of white varieties are lower than the values corresponding to plants of red varieties with typical leafroll symptoms.

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Table 1. DAS-ELISA (E_{405} readings) of grapevine plants with and without leafroll symptoms using antisera to three grapevine leafroll associated viruses (GLRaV-I, GLRaV-II, GLRaV-III)

No of plant	Plants without leafroll			Plants with leafroll		
	GLRaV I	GLRaV II	GLRaV III	GLRaV I	GLRaV II	GLRaV III
Variety Cabernet Sauvignon						
1	0.016	0.033	0.041	0.029	0.024	1.892
2	0.020	0.029	0.084	0.030	0.028	1.545
3	0.024	0.025	0.110	0.037	0.032	1.334
4	0.029	0.036	0.049	0.046	0.039	1.254
5	0.032	0.023	0.057	0.050	0.027	1.301
6	0.043	0.027	0.053	0.040	0.027	1.480
7	0.033	0.035	0.061	0.046	0.032	1.355
8	0.037	0.038	0.047	0.041	0.033	1.902
9	0.043	0.026	0.123	0.024	0.024	1.490
10	0.064	0.035	0.218	0.038	0.033	1.751
11	0.034	0.035	0.260	0.122	0.030	0.905
Variety Merlot						
1	0.123	0.113	0.141	0.170	0.019	1.128
2	0.124	0.130	0.211	0.035	0.019	1.330
3	0.129	0.156	0.144	0.049	0.026	1.023
4	0.131	0.118	0.141	0.068	0.039	1.524
5	0.126	0.124	0.142	0.180	0.026	1.745
6	0.126	0.118	0.152	0.182	0.027	1.762
7	0.121	0.119	0.145	0.107	0.026	1.048
8	0.127	0.123	0.141	0.079	0.022	1.309
9	0.130	0.119	0.140	0.054	0.025	1.329
10	0.127	0.124	0.142	0.054	0.029	1.517
11	0.123	0.120	0.149	0.045	0.028	1.162
12	0.531	0.121	0.143	0.040	0.087	1.454
13	0.126	0.124	0.141	0.046	0.088	1.575
14	0.124	0.120	0.140	0.002	0.023	1.409
15	0.123	0.126	0.140	0.004	0.021	1.765
16	0.125	0.127	0.140	0.006	0.022	1.479
17	0.121	0.125	0.147	0.054	0.025	1.730
18	0.124	0.124	0.143	0.005	0.031	1.507
19	0.128	0.124	0.152	0.011	0.028	1.624
20	0.126	0.122	0.149	0.013	0.027	1.542
21	0.125	0.120	0.151	0.014	0.029	1.672
22	0.124	0.116	0.150	0.005	0.023	1.407
23	0.127	0.124	0.148	-	-	1.730
24	0.125	0.123	0.152	-	-	1.429
25	0.126	0.119	0.149	-	-	1.504
Variety Cinsaut						
1	-	-	-	0.208	0.263	1.652

PRESENCE OF GRAPEVINE LEAFROLL IN NORTH WEST OF SPAIN

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From 1990 to 1992 a survey on vineyards in Galicia was carried out in order to estimate the sanitary status concerning the most important virus diseases of the grapevine (1). This was done by following the sampling methodology suggested by Barnett (2). Virus detection was done by ELISA by means of commercial polyclonal antisera against 4 serotypes of grapevine leafroll associated viruses (GLRaV's): GLRaV I, II, III, and V (3,4) and GFLV. Results from this survey indicated that GLRaV III was the main problem, with about 46% of the vines and almost 80% of the vineyards infected (Figure 1). Autochthonous plant material from some areas was revealed to be heavily infected and it seems difficult to find healthy vines for new plantations. In the last 15 years many new plantations were established using mainly the white cultivar "Albarino" and these new vineyards, now in their first years of production, are the most infected. Leaf symptoms are evident on this white variety, with rolling and yellowing of the leaf blades. The relationship between symptoms at the end of the summer and the results obtained by ELISA is good for the most frequent serotype, GLRaV III. Serotype I was found in few very old, isolated, non-grafted vines.

This ubiquitous presence of leafroll in vineyards, most of them less than 10 years old, raises the question of its possible incidence on yield, vine growth and must quality. The weather conditions (fresh and wet) and varieties in Galicia (North West) are quite different from those in other places of Spain (5) and in other Mediterranean countries. Possible damage by leafroll is probably more important here and especially affects the content of must sugar.

A study was initiated for the white cultivar Albarino, which is predominant in the coastal area (Rias Baixas) and produces high quality wines. Two vineyards established in 1979 and 1989 were examined. The 13 year old vineyard with about 35% GLRaV III infected vines showed the problem of low sugar and high acid content during the last years. 15 GLRaV III infected and 15 virus-free vines were studied. The 3 year old vineyard is an experimental plot. In this case, 22 GLRaV III infected and 22 GLRaV III-free vines were analysed. Data on vine development and production (pruning wood weight, perimeter, fruit weight, number of bunches) and quality of the must ($^{\circ}$ Brix, titratable acid, pH) were measured for all vines.

Preliminary results recorded in 1992 indicate a clear effect of the leafroll virus infection in the 13 year old vineyard. All production and growth parameters were negatively affected with a 30% decrease in fruit weight/vine and significantly ($p < 0.005$) lower number of bunches/vine (164 on healthy and 121 on GLRaV-III infected vines). The perimeter and weight of pruning were also significantly lower in the infected vines. Concerning the must quality, the sugar content was 1,1 $^{\circ}$ Brix lower in the infected vines ($p < 0.005$) than in the healthy plants but no differences were found in both pH and titratable acid. The 3 year old vineyard appeared not to be affected by the virus, both in the vine growth and yield. Only the sugar content was lower (about 0.8 $^{\circ}$ brix) in the infected vines.

The results of the survey confirm the presence of leafroll virus in this viticultural area. Serotype III was dominantly detected, as in other Mediterranean countries. It was the first time that serotype I was found in Spain, but only in a few, more than one hundred years old, vines. The study on the leafroll incidence on wine quality and quantity is only preliminary and the data need to be confirmed in the next years. The results for the 13 year old vineyard agree nevertheless with those described in the literature for other grapevine varieties in various locations (6, 7). The absence of differences found in the young vineyard could indicate that during the first years the effect of the virus is not very important and infected vines grow and develop in the same way as the healthy ones. Damage could occur only after some years. Considering the high percentage of leafroll infected vineyards, serious problems might be encountered over the next years.

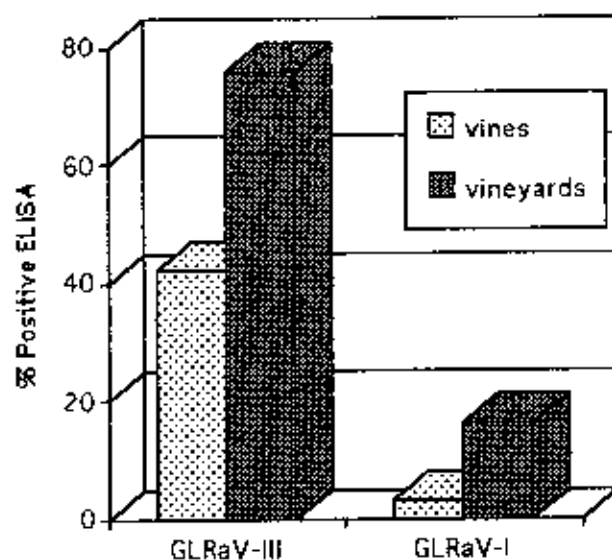


Figure 1. Percentage of vines and vineyards positive for GLRaV infections (serotypes I and III).

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ADVANCES IN GRAPEVINE VIRUS DISEASE DIAGNOSIS SINCE 1990.

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Detection and diagnosis of virus and virus-like diseases of grapevine are essential for the control of these diseases. The importance of a reliable diagnosis is presently increasing because of four main reasons.

(a) Up to now, there are only two efficient ways of controlling in some extent the spread and thus the detrimental effects of the viroses, namely sanitary selection and chemical control of vectors (nematodes, leafhoppers). Sanitary selection is based on reliable and easy-to-use methods for the detection of pathogens.

In the countries where mother blocks of grapevines were planted 20 to 40 years ago, recontamination by some viruses, as for example Nepoviruses, has been demonstrated to occur. This underlines the requirement for a constant and efficient survey of the sanitary status of the base and certified vines at the different steps of their multiplication.

(b) The use of chemicals for the control of vectors of viruses or virus-like diseases - such as nematodes, leafhoppers or mealybugs - is becoming more and more regulated or has even been forbidden. As grapevines resistant to the pathogens or to their vectors are not yet available, routine and early detection of contaminations will become more and more essential.

In parallel, evaluation of the risks of contamination by quantifying the infectious potential of nematode - or of insect - populations will be helpful to avoid too rapid recontamination of base or certified vines.

(c) Due to economic factors and political changes, the trade with grapevine plant material is increasing. In addition the quality of this plant material - including its sanitary status - is a factor of increasing importance on a competitive market.

(d) Finally, diagnosis is an essential tool for research in the field of etiology, epidemiology and virus characterization (e. g. the grapevine closteroviruses).

All these reasons are strong encouragements to all of those who are involved in the development of detection and diagnosis.

Advances in grapevine virus and virus-like disease diagnosis since the last ICVG Meeting in Volos can be noticed in three main fields : 1. - technical improvements and new applications; 2. - new techniques or reagents; 3. - guidelines and regulations.

1. Technical improvements and new applications

1. 1. Serological techniques

The use of F(ab')₂ antibody fragment for the detection of GFLV in grapevine sap makes it possible to retain the benefits of indirect - ELISA (high sensitivity and low background), while avoiding the necessity for two sources of antiserum (1).

A study was carried out in California (2) to compare the efficiency of serological detection of 3 isolates of GFLV and an isolate of TomRSV. Whereas ELISA readings of extracts from TomRSV-infected vines were relatively constant over the season regardless of the type of sample examined (shoot tips, mature leaves, cortical scrapings), variations were observed in the detection of GFLV. These differences did not depend on the virus isolate nor on the donor cv., but were mostly due to seasonal variation in virus titre. Shoot tip and mature leaf values were highest in May, but decreased rapidly to reach a minimum in September.

At Colmar, the detection of ArMV and GFLV was not always possible on *Vitis vinifera* in leaf samples collected during the hottest period in summer. Detection is possible during the entire vegetative period when leaf samples are collected from rootstock varieties.

In a study of the vertical distribution and the infectious potential of *X. index* in fields affected by GFLV in Champagne, the detection of GFLV was possible by ELISA with samples of 2 nematodes (3). This method might adequately establish the infectious potential of a nematode population.

The possibility of developing monoclonal antibodies (MAbs) with a broad spectrum reactivity that could be used for quarantine purposes as detection assays for nepoviruses has been investigated (4). Cross-reacting and heterospecific antibodies have been produced with ArMV virions used as immunogen: one hybridoma line reacted with ArMV and GFLV, one with ArMV and RRV; five lines did not react at all with ArMV but reacted with a different nepovirus (RRV, TBRV or CLRV). The lack of extended sequence homology between the coat

protein genes of nepoviruses confirms that a MAb capable of detecting all nepoviruses cannot be readily produced.

Using MAbs, the detection of the MLOs is possible in leaves of *Vitis vinifera* cvs infected by Flavescence dorée but only during the season when symptoms are strong (5). The use of detergent (4% Triton X 100 or 5% CHAPS) is essential for ELISA. Results have not been obtained with the symptomless rootstock varieties. Daire *et al.* (6) showed that ELISA can distinguish between different yellows: the symptoms occasionally present in the north-eastern part of France are due to a MLO probably different from the MLO of flavescence dorée.

Antibodies to GLRaV being highly specific, Hu *et al.* showed that for general diagnosis ELISA can be simplified by using a blend of antibodies (7). In this way, the cost and labour of screening samples can be reduced by 65%. ISEM was reliable in detecting GLRaV: the use of antibodies as a blend considerably reduces the time for analysis if the objective is to simply detect GLRaV particles. However ISEM requires an electron microscope and antisera to different GLRaV types. Considering the advantages and disadvantages of 3 assays (ELISA, ds RNA analysis and ISEM), the authors recommend ELISA to be done with multiple antisera to test many samples. Samples for which ELISA results are inconclusive should be retested with ISEM and/or ds RNA (7).

Immunogold labelling techniques have been used for localization and identification of closteroviruses on grapevine thin sections. Faoro *et al.* (8) pointed out that cytopathic effects should be used as a valuable aid in the diagnosis of the closterovirus type when the appropriate antiserum is not available. P-protein structures can be recommended for screening the presence of closteroviruses. Vesiculating mitochondria and enclaves of virus particles seem to be associated with GLRaV III infection, whereas masses of filaments occupying the cell lumen are always present in plants infected with GVA.

1. 2. Detection of nucleic acids

Hu *et al.* (7) showed that analysis of ds RNA for GLRaV is not as sensitive as ELISA and is more time consuming, though it has the advantage of being a nonspecific assay.

Rezaian *et al.* (9) described a procedure involving cellulose chromatography and gel electrophoresis for reproducible detection of ds RNAs from leafroll-infected grapevine stem cortex tissues and leaves. Ds RNA detection will be suited for detection of diseases such as leafroll for which specific virus detection methods are not likely to be applicable because more than a single virus may be involved in the disease.

The method has also been used for assessing the success of eliminating leafroll by tissue culture techniques (9), though the simultaneous presence of ds RNAs from other viruses infecting the samples may be confusing (10). Ds RNA detection may also be complicated by "contaminations" such as ds RNA species associated with the powdery mildew fungus (11).

Two cloned cDNA probes to genomic RNA of grapevine closterovirus A (GVA) were utilized successfully for the detection of GVA in infected *Nicotiana benthamiana* and grapevines in spot- or northern blot-hybridization. However virus detection was difficult and unreliable from woody tissue (12). cDNA clones and a riboprobe transcribed from a cDNA were successfully used as probes for detecting GLRaV III sequences in grapevine extracts from leaves and petioles, or cortical tissues (13).

cDNA probes have also been used to compare the viroids derived from grapevines (14).

Hybridization analyses using random probes or specific probes resulted in the definition of four major groups of grapevine viroids, in agreement with the previous general clustering of viroids based upon their recovery from specific hosts.

2. New techniques or reagents

Daire *et al.* (15) obtained DNA probes from flavescence-dorée (FD) diseased broadbean which hybridize with the total DNA from FD-infected leafhoppers or broadbeans and not with the DNA from healthy hosts. A dot blot assay was developed with the main leaf veins from field-grown grapevines: all samples with FD symptoms strongly reacted with the FD probe whereas no signal was recorded with samples from healthy grapevines. In addition RFLP patterns observed in southern-blot analysis of MLO isolates from grapevines are consistent with serology and indicate variability in isolates of grapevine MLOs (6, 15, 16).

Detection of ArMV using the polymerase chain reaction (17) makes a diagnosis possible by hybridization without the disadvantages of radioactively-labelled probes. Specific sequences of ArMV RNA present in total nucleic acid extracts of infected *Vitis vinifera* or *Chenopodium quinoa* were initially reverse-transcribed into a complementary DNA, then amplified by PCR using specific oligonucleotide primers. Different primer combinations distinguished between an ArMV infection and an infection with GFLV or RRV.

Routine diagnosis based on PCR will need a simplified protocol (such as a dot-blot assay). Primers have to be carefully chosen in order to avoid false positive responses or to simultaneously detect different viruses

sharing common sequences.

A PCR based method using viroid-specific oligonucleotide primers offers a greater degree of specificity compared to probe hybridization: it allowed Rezaian *et al.* (18) to propose a uniform nomenclature of grapevine viroids found in different geographical regions.

Reverse transcription-polymerase chain reaction (RT-PCR) was successfully applied to detect GVA RNA in nucleic acid extracts of infected grapevines (19). The detection limit of this technique was estimated to be 200 fold higher than by molecular hybridization or by ELISA. Until now the major shortcoming of RT-PCR application is the requirement for nucleic acid purification because of the presence of inhibitors in extracts of woody plants.

Polyclonal antibodies have been raised to Grapevine Fleck Virus (GFkV) and used for etiology and diagnosis (20, 21). Leaves and cortical scrapings can both be used as antigen sources for ELISA. Under the climatic conditions in Colmar, a dramatic decrease of ELISA readings during the hottest period brings the response very close to the background level.

Monoclonal antibodies to GVA gave efficient and reproducible results for identification of GVA in field-grown grapevines (22).

Biological indexing remains a valuable and, in some cases, irreplaceable method for the diagnosis of virus-like diseases of grapevine. Grafting onto indexing varieties has the advantage of low specificity allowing the simultaneous detection of a broad spectrum of virus isolates or even of different viroses on the same indicator. We determined, in our lab, controlled conditions for a reliable detection of stem pitting and stem grooving by greengrafting (23). Diagnosis of stem grooving on Kober 5BB is possible within 8 months by observing only external symptoms: darkening and grooving on the bark just below the grafting point. Stem pitting can be diagnosed 12 months after grafting onto *V. rupestris* or 8 months after grafting onto 161-49C by observing histological modifications on transverse sections of the indicator.

3. Guidelines and regulations

Guidelines for the safe movement of grapevine germplasm were developed at a meeting in Athens (11-13 sept 1990) organized by FAO/IBPGR in collaboration with ICVG (24). Technical recommendations are given for collecting and movement of grapevine seed, cuttings, *in vitro* cultures and pollen. Therapy and indexing strategy is described that will help to ensure phytosanitary safety when germplasm is moved internationally.

For commercial exchange of grapevine plant material, quarantine measures exist in different countries. The theme of the 73rd General Assembly of the OIV was "How to facilitate international trade for products of the vine" including exchange of plant propagation material.

The main grape-growing countries of EEC are on the way to harmonize their certification schemes. An international Group of Experts composed of ICVG members was established. This Group issued a booklet summing up the state of the art in the certification in EEC countries (25). A concerted action funded by EEC has started recently which aims at proposing internationally recognized protocols for the detection and diagnosis of viruses and virus-like diseases (26).

G.P. Martelli published an extensive handbook for detection and diagnosis of graft-transmissible diseases of grapevine, edited by the FAO (27). Detection of major virus and virus-like diseases of grapevines was also reviewed by R. Bovey and G.P. Martelli (28).

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DETECTION OF GRAPEVINE CLOSTEROVIRUSES ASSOCIATED WITH LEAFROLL BY ELISA TEST IN VITIS ROOTSTOCKS

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Immunoassays such as ELISA are commonly applied to detect nepo- and closteroviruses of grapevine by testing leaf and wood samples of the *Vitis vinifera* varieties. ELISA did however not always reliably detect closteroviruses on grapevine rootstocks (1, 2). This report shows results on the detection of these viruses on rootstocks by ELISA and biological indexing.

ELISA was carried out with antigens both extracted from leafstalks partly with leafy texture, picked up towards the end of summer, and from wood collected during winter. We used the following commercial ELISA kits: Grapevine Fanleaf Virus (GFLV), Arabis Mosaic Virus (ArMV), Grapevine Leafroll associated Virus (GLRaV) I, II, III and V.

147 rootstock cultivars were analysed by testing two plants per variety for the presence of nepo- and closteroviruses. The detection of GFLV, ArMV, GLRaV I and GLRaV III in wood samples is reported in table 1. The antigens extracted from leaves with leafstalk reacted only occasionally with the antibodies to the nepoviruses and hardly ever with those specific of closteroviruses.

Table 1. Detection of nepoviruses and closteroviruses by ELISA in woody samples of rootstocks

Cultivars	GFLV ^{*)}	ArMV ^{*)}	GLRaV I ^{*)}	GLRaV II ^{**)}	GLRaV III ^{*)}	GLRaV V ^{**)}
tested	147	147	147	130	147	130
healthy	129	143	135	126	141	124
infected	18	4	4	0	6	0
dubious	0	0	8	4	0	6

*) ELISA Kit from Bioreba; **) ELISA Kit from Sanofi

GFLV was detected most frequently (12.2%), followed by GLRaV III (4%) and then by GLRaV I and ArMV (2.7%). The absorbance values (A_{405}) were particularly high for GFLV, ArMV and GLRaV III, while the readings for the GLRaV I, GLRaV II and GLRaV V were dubious. The American rootstocks which reacted positively in ELISA for the following closteroviruses were:

- GLRaV I: *V. riparia* cvs. Seribner and Martin;
V. riparia x *V. rupestris* cvs. 3309 C and 3310.
- GLRaV III: *V. berl.* x *V. rup.* cvs. 57 Richter, 1043 Paulsen and 301 A;
V. rip. x *V. rup.* cvs. 16.108 Prosperi, 16.109 Prosperi;
V. berl. x *V. rip.* x *V. rup.* cv. 11.72 Prosperi.

The reliability of the immuno-enzymatic testing was evaluated by comparing ELISA with biological tests made by indexing on the following indicator plants: *V. rupestris* St. George for infectious degeneration (nepoviruses) and *V. vinifera* cv. Cabernet Sauvignon for leafroll (closteroviruses). 45 American rootstocks from an ampelographic collection and 6 cultivars, graft-inoculated with leafroll diseased Chardonnay, were tested.

The detection of GFLV by ELISA proved to be reliable, even better than the biological indexing carried out on St. George. Furthermore ELISA detected closteroviruses in almost all the samples from vines which proved to be infected by leafroll as shown by indexing on Cabernet Sauvignon. Only *V. rupestris* du Lot did not respond, while samples of Kober 5BB and 161-49 yielded unreliable results, often in the form of a low infected/healthy absorbance ratio (3-5).

It could be suggested that the failure of detection of closteroviruses by ELISA in wood and leaf samples of rootstocks might be due to the buffer used to extract the antigens, i.e. Tris/HCl pH 8.2, which would create unsuitable conditions with some varieties. Another cause might be the presence of some proteins, that stick to the closteroviruses particles preventing them from multiplying and moving into the leaf or wood, as in the

case of young leaves and canes of *V. vinifera*.

Diagnosis of grapevine rootstocks by ELISA is therefore reliable for diseases caused by nepoviruses. In the case of leafroll associated closteroviruses, ELISA produces variable results, but the method is sufficiently valid as a preliminary screening, especially if applied on mature cane samples.

Table 2. Comparison of ELISA on wood samples and woody indexing for the detection of virus diseases in rootstocks

Cultivars	Grapevine fanleaf virus		Grapevine leafroll associated viruses		
	ELISA	INDEXING St. George	ELISA		INDEXING Cabernet S.
			GLRV I	GLRV III	
<i>V. rip.</i> Seribner	+++	+	+	-	+++
<i>V. rip.</i> Baron Pierres	+++	++	-	-	-
<i>V. rip.</i> Sombre n.2	+++	+	-	-	-
<i>V. rip.</i> Martin	-	-	+	-	++
<i>V. rip.</i> x <i>V. rup.</i> 3309 C	-	-	+	-	+
<i>V. berl.</i> x <i>V. rup.</i> 57 Richter	-	-	-	++	+++
<i>V. berl.</i> x <i>V. rup.</i> 140 Ruggeri	-	-	-	++	+++
<i>V. berl.</i> x <i>V. rup.</i> 17-37	-	-	-	++	+++
<i>V. berl.</i> x <i>V. rup.</i> 110 Richter	-	-	-	++	++
<i>V. berl.</i> x <i>V. rip.</i> 161-49	-	-	-	+/-	++
<i>V. berl.</i> x <i>V. rip.</i> Teleki 5C	-	-	-	++	+++
<i>V. berl.</i> x <i>V. rip.</i> Kober 5BB	-	-	-	+/-	++
<i>V. rupestris</i> Du Lot	-	-	-	-	++
(Coudere 1613 x <i>V. berl.</i>) LN33	-	-	-	++	+++

- : negative replies or symptoms;
 +/- : dubious replies;
 +, ++, +++ : positive replies (presence of infection shows up differently).

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MONOCLONAL ANTIBODIES TO GRAPEVINE FLECK VIRUS

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The identification of grapevine fleck virus (GFkV) as the agent of the homonymous disease (1), and the possibility of using ELISA as an alternative to indexing on *Vitis rupestris* for routine detection of fleck infected vines, has prompted studies for improving serological diagnosis, in whose framework, monoclonal antibodies to GFkV were produced.

Virus sources were three grapevine donors of different geographical origin: Italy (MT48), Malta (M8) and Bulgaria (BU40). Virus isolates were purified as described (3) from roots of greenhouse-grown rootlings forced in sand.

Six week-old BALB/c female mice were immunized with two injections of GFkV-MT48 given two weeks apart from one another, followed 8 months later by a booster injection of GFkV-M8 and GFkBBU40. Splenocytes were fused with mouse myeloma cells line NS/O. Hybridoma cells secreting GFkV-specific antibodies, identified by indirect DAS-ELISA, were cloned and subcloned by the limiting dilution method and cultured as described (2).

Two hybridoma cell lines denoted FK.89 and FK.117 were found to produce antibodies that reacted specifically with GFkV, but not with healthy plant sap extracts. Both cell lines were stable and continued to produce large quantities of GFkV-specific antibodies (direct DAS-ELISA titres between 1:8,000 and 1:16,000) after three successive cycles of freezing and thawing in liquid nitrogen. Monoclonal antibodies (MAbs) were of the IgG 1 isotype and were probably originated by cryptotopes for they did not induce detectable decoration of virus particles. MAbs were readily produced in RPMI 1640 medium with the addition of 2mM L-glutamine and 10% fetal bovine serum. Both cell lines induced in mice the formation of peritoneal tumours secreting antibodies.

For GFkV detection in grapevines several antigen sources (leaf or root extracts, cortical scraping from mature canes) and ELISA protocols were tested. MAbs were always used as revealing antibodies whereas trapping antibodies were either MAbs or IgGs from a polyclonal antiserum to GFkV, utilized directly or after pre-coating plates with protein A.

Protein A coating proved unnecessary, if not detrimental, because of the frequent development of non specific reactions. By contrast, both MAbs could be used equally well for coating plates (trapping) and for antigen detection. Reactions were virus-specific with both MAbs, although MAbFK.117 yielded consistently stronger responses than MAbFK.89.

Bark scrapings were the best antigen source, for they afforded reproducible results over time, without appreciable loss of titre.

Thus, large amounts of high-titre monoclonal antibodies to GFkV can now be produced in mice ascitic fluids. These antibodies allow detection of GFkV in infected vines in a reliable and specific manner. Their availability overcomes the problems linked with the necessity of repeated production of polyclonal antisera to a virus whose extraction in purified form from infected sources is not deprived of difficulties.

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ELISA FOR DETECTION OF ArMV AND GFLV IN GRAPEVINE: SCHEDULE OF 3 YEARS OF CONTROL TESTS

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In 1990 we started a systematic program of detection of ArMV and GFLV on all basic (about 100 ha), and on some mother vines of certified grapevine material in order to estimate the extend of new contaminations after 20 years of planting in the field.

Samples were collected by agents of ONIVINS during spring (from May to June) and autumn (from September to October). They collected one young leaf per plant and pooled 20 to 25 leaves (i.e. 20 to 25 plants) in a plastic bag. They also established a map of the sample origins. The samples received a code reference to remain anonymous and they were quickly sent to the laboratory at E.N.T.A.V.

As soon as the samples arrived in the laboratory they were weighted and pounded to avoid injuries on the leaves. The extracts were immediately tested or kept frozen for one or two weeks according to the testing capacities of the laboratory.

During 3 years we realized 22 850 tests. Basic material should be virus free. Positively reacting combined samples were therefore reexamined by testing plant by plant. These tests revealed either some plants, or sometimes only a single plant with a positive reaction. In rare situations, parts of blocks were contaminated. We found 6 percent of positive tests in all experiments. This high percentage probably results from the plant by plant retesting of all the positive samples. For a juridical procedure, the grower can require a re-examination of the positive plants by an other laboratory to confirm the first result.

For stock mother vines of basic material which gave positive reactions, ONIVINS services can order to destroy the plot. For scion mother vines the basic material is eliminated from the control system and plants can only produce grapes.

In spite of the care at the moment of planting this work confirms the risk of new contaminations although the reasons remain obscure.

In the future the basic material could be periodically retested. The tests will be extended to the mother vines of certified material but with a simpler method. On the other hand E.N.T.A.V. establishes agreements of sanitary monitoring with premultipliers.

EXPERIENCES WITH THE DETECTION OF CLOSTEROVIRUSES OF THE GRAPEVINE WITH A GREEN-GRAFTING METHOD

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In German vineyards viral diseases of the grapevine caused by Closteroviruses play just as an important role as those caused by NEPO-viruses. Grapevines with the symptoms of the grapevine leafroll disease are quite frequent, whereas the corky bark disease has not been observed yet. However, many grapevines exhibit broad symptoms like growth depression and leaf discoloration undoubtedly suspicious of a latent corky bark disease. For this reason a safe and clear proof of closteroviruses causing diseases in grapevines is needed. Green grafting is an available method that allows to detect viral diseases of the grapevine within a very short time period.

1. Materials and methods

The virus-infected material and the indicator varieties came from a collection in Freiburg or Colmar, whereas the healthy, virus-tested control plants descended from an *in vitro* culture of the varieties "Blue Burgundy".

Green grafting was carried out according to the method of WALTER (INRA Colmar). The graftings were cultivated for 14 days at 28 °C in a nebulizer to develop a callus and roots. After the healing of the grafting site the plants were cultivated in the green house at ideal conditions.

2. Results

2.1. Leafroll disease

Plants infected with GLRaV type 1 and type 3 (proof by ELISA) were used to detect the leafroll disease. Green shoots of the varieties "Blue Burgundy" (Pinot Noir) and "Gamay" were grafted with GLRaV infected and healthy green shoots. For comparison buds of GLRaV positive and healthy plants were grafted on the indicators according to the chip-budding method.

Within a year the chip-budding method revealed that graftings with infected material yielded the typical symptoms of leafroll disease. A positive correlation was established between symptoms and a positive ELISA reaction. At the same time green graftings exhibited no significant symptoms of leafroll disease. Even the reddening and leafrolling of the GLRaV positive shoots in some variants seemed to be strongly temperature dependent. We observed, that with a change of temperature in the greenhouse these symptoms disappeared and the plants once again looked healthy. Combinations with healthy shoots showed occasionally symptoms that possibly resulted from an insufficient adhesion of the graftings.

2.2 Corky bark disease

To detect corky bark disease shoots of corky bark infected plants were grafted on shoots of the indicator variety LN 33. The same material was used in comparative studies applying the chip-budding technique.

Infected specimens of the green graftings all showed typical primary symptoms of corky bark disease after 8 weeks. The comparable "chip-budding plants" in the field took 18 weeks, or until the end of August, to exhibit first symptoms. Additionally we observed that in the green graftings as well as in the outdoor plants with old shoots an initial swelling of the shoot was followed by the tearing of the bark, reddening of the leaves and finally caused the death of the plants.

3. Conclusion

If temperature and moisture requirements for callus formation and rooting are met exactly, the green grafting method according WALTER yields a sufficient number of united grafts. Green grafting is a very safe method to detect corky bark disease.

From our experiences symptoms are clearly visible after eight weeks at average temperatures of 25 °C. Thus the method can be used in routine testings to successfully select for healthy propagation material.

Until present our results don't give clear evidence of GLRaV type 1 and 2. Therefore the conventional chip-budding method with old shoots and ELISA to detect leafroll disease is still safer.

DETECTION OF THREE GRAPEVINE CLOSTEROLIKE VIRUSES BY NON RADIOACTIVE MOLECULAR PROBES

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The use of molecular probes for sensitive detection and identification of plant viruses is becoming increasingly popular, and represents more and more a complement, or an alternative, to serological diagnosis. Molecular detection techniques, however, largely utilize radioactive probes, whose use requires properly trained personnel, provisions for a safe manipulation and disposal, and, in any case, is not deprived of hazards. Most, if not all, of these impairments can be overcome by the development of cold probes, whose successful hybridization with target nucleic acid is revealed by reactions other than those depending on radioactivity.

As reported in the present paper, cold probes were produced to three major grapevine closterovirus, namely grapevine virus A (GVA), grapevine virus B (GVB) and grapevine leafroll associated virus III (GLRaV III), and were successfully applied to their detection in host tissue extracts.

The probes were cRNAs transcribed from cDNA templates to viral genomic RNA, excised from previously described plasmids and subcloned into the transcription vector pGEM4z. The plasmids were: pGA240a for GVA (1); pGV85 for GVB (2); pGLRaV23ds for GLRaV III (3). A commercial kit (Boehringer, Mannheim) was used both for labelling probes and for the chemiluminescent detection of hybridization products. Labelled riboprobes were generated by SP6 or T7 transcription in the presence of digoxigenin-UTP, and chemiluminescent detection was performed following the manufacturer's instructions.

Total nucleic acids (TNA) were obtained from grapevine tissues (cortical scrapings or leaf petioles) as described (4) but after phenolchloroform extraction, the aqueous phase was clarified in the presence of cellulose CF-11 and 35% ethanol. TNAs were then eluted directly in eppendorf tubes, ethanol precipitated, and spotted onto nitrocellulose membranes (Hybond N+, Amersham).

All three digoxigenin-labelled riboprobes specifically detected homologous viral sequences with clearly visible hybridization signals in TNA extracts from as little as 250 mg of tissue. However, as ascertained by comparative trials, signals from CF-11 cellulose-treated TNA preparations were stronger and cleaner, because of the lower background. Chemiluminescent detection was in complete agreement with the results of other tests by which the presence of GVA and GLRaV III (ELISA) and GVB (sap inoculation) had been ascertained in the samples examined.

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SENSITIVE IMMUNOCAPTURE AND MULTIPLEX REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION FOR THE DETECTION OF GRAPEVINE LEAFROLL ASSOCIATED VIRUS III AND GRAPEVINE VIRUS B.

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Leafroll and corky bark are economically important diseases of grapevine which cause significant yield losses worldwide. Grapevine leafroll associated virus III (GLRaV III) and grapevine virus B (GVB), are highly associated with leafroll and corky bark diseases, respectively. Improving the detection of these two viruses in infected grapevines by polymerase chain reaction (PCR) technology, may help elucidating the etiology of the diseases they are associated with. Recently, we have cloned GLRaV III and GVB cDNAs, transcribed from purified and denatured dsRNA of GLRaV III and RNA of purified GVB virions, respectively (2, 3), and partially sequenced clones pGLRaV9ds and pGVB5. DNA primers specific for GLRaV III and GVB were constructed based on the nucleotide sequence of each of the above clones. DNA primers were utilized for cDNA synthesis and PCR amplification of a 340 bp and a 450 bp DNA fragments from extracts of GLRaV III- and GVB-infected grapevine tissues, respectively.

One hundred mg of infected grapevine tissue (leaf midrib and petiole or bark scrapings from dormant mature canes) were ground in a mortar containing 1 ml citrate buffer (50 mM sodium citrate, pH 8.3, 20 mM DIECA; 2% PVP 40K). Crude extracts of each sample were centrifuged at 8,000 rpm for 10 min, 200 µl of the clarified extract were placed in an ELISA microplate well that had been coated with GLRaV III antiserum (diluted 1:100 in PBS) for 2 hrs, and the microplate was incubated at 4 °C overnight (4). Each well was washed thoroughly with PBS-Tween buffer. Aliquots of 25 µl of transfer buffer (10 mM Tris-HCl, pH 8; 1% Triton X 100) were added to each well and the microplate was used directly for GLRaV III cDNA synthesis.

Alternatively, when the immunocapture procedure was omitted, crude grapevine extracts of each sample were serially diluted in 1% Triton X 100, each dilution was incubated at 65 °C for 5 min, and 5 µl aliquots of each dilution were used for viral cDNA synthesis.

For viral cDNA synthesis, each grapevine extract was added to a mixture containing 1 µg of a 22-mer primer complementary to a segment of GLRaV III genomes (immunocapture PCR assay), or 2 µg of two 22-mer primers complementary to segments of both viral genomes (multiplex PCR assay) (1). The mixture was boiled for 5 min to denature viral RNA, chilled on ice for 2 min, then incubated at room temperature for 1 hr to allow primer annealing to the RNA template. The annealing reaction mixture was mixed with 20 µl of cDNA reaction mixture containing 1 µl (200 units) cloned Moloney murine leukemia virus reverse transcriptase and incubated at 42 °C for 1.5 hr. Aliquots of reverse transcriptase reaction mixture were transferred to tubes, each containing PCR buffer. The buffer contained 5 units of AmpliTaq™ DNA polymerase and 200 pM of each the complementary and homologous primers to a segment of GLRaV III genome (immunocapture PCR assay), or 200 pM of each primer complementary, or homologous, to segments of both viral genomes (multiplex PCR assay). PCR amplification was for 30 cycles (denaturation at 94 °C for 30 sec, annealing at 62 °C for 30 sec, and extension at 72 °C for 45 sec with a final primer extension cycle of 7 min). Amplified PCR products were analyzed by electrophoresis through 6% polyacrylamide slab gels and by Southern hybridization using ³²P-labelled GLRaV III cRNA and GVB cRNA probes.

The size of amplified GLRaV III DNA and GVB DNA was, as expected, 340 bp and 450 bp, respectively. Each DNA fragment hybridized specifically with the homologous viral cRNA probe. No DNA fragments or hybridization signals were obtained with extracts from uninfected tissues. The 340 bp GLRaV III DNA was obtained regardless of whether immunocapture or multiplex RT-PCR were utilized. The best results of multiplex RT-PCR were obtained when GLRaV III and GVB were from dormant canes or leaves, respectively, and also when crude grapevine sap extracts were diluted 1:50 to 1:100 before cDNA synthesis. Our results also revealed that multiplex RT-PCR is easier and faster than immunocapture RT-PCR for the detection of GLRaV III from infected grapevine tissue and it allows the sensitive detection of GVB, for which a high titre antiserum is not yet available.

Acknowledgements

This study was conducted while the senior author was at the National Germplasm Laboratory, ARS, USDA, Beltsville MD, USA, on a NATO fellowship.

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DETECTION OF GRAPEVINE VIRUS A IN SINGLE MEALYBUGS BY IMMUNOCAPTURE - REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION

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The mealybug *Pseudococcus longispinus* is a vector of the closterovirus grapevine virus A (GVA) and B (GVB), both of which are thought to be involved in the etiology of diseases of the rugose wood complex. Sensitive detection of these viruses in insect vectors may prove valuable in epidemiological studies, for investigating virus-vector-host interactions, and for controlling rugose wood disease. Recently reverse transcription-polymerase chain reaction (RT-PCR) was utilized for detection of GVA in infected grapevine tissues, and proved more sensitive than other detection methods (1). Based on these findings, RT-PCR coupled with immunocapture (2) has now been used to amplify a segment of GVA genome from viruliferous insects.

Adult mealybugs were maintained in a greenhouse on GVA-infected grapevine plants of cv. Perricone for at least 20 days prior to processing. Non viruliferous mealybugs were maintained on potato plants. Extracts from one or more mealybugs were incubated overnight with polyclonal or monoclonal antibodies to GVA. The antibody-bound virus particles were then treated with 1% Triton X 100 at 65 °C for 5 min to release viral RNA. For controls, total nucleic acids were isolated directly from insect extracts, i.e. omitting the immunocapture step. Reverse transcription of GVA RNA and amplification of synthesized GVA cDNA were performed using DNA primers that specifically amplify a 430 bp GVA cDNA fragment. DNA primers were constructed based on the nucleotide sequence of GVA clone pGA240. GVA cDNA was amplified in a programmable DNA thermal cycler for 40 cycles: template denaturation at 94 °C for 1 min, primer annealing at 55 °C for 2 min, and DNA synthesis at 72 °C for 3 min. A final 7 min elongation step at 72 °C was also performed. Amplified products were analyzed by electrophoresis in 6% polyacrylamide slab gels followed by silver staining to visualize separated products.

The size of GVA amplified cDNA fragments from viruliferous mealybugs was 430 bp, as expected. These fragments hybridized specifically with RNA transcripts of ³²P labelled, cloned GVA cDNA. The 430 bp fragment, however, was absent from samples from non viruliferous mealybugs, for no hybridization signals were obtained when the probe was tested on these samples.

Comparison of RT-PCR with immunocapture and simple RT-PCR indicated that the former was easier to perform, and that the expected 430 bp GVA cDNA fragment was the only amplified product from viruliferous mealybug extracts. To our knowledge, GVA is the first plant virus definitively detected in its vector by immunocapture RT-PCR.

ACKNOWLEDGEMENTS

This study was conducted while the senior author was at the National Germplasm Laboratory, ARS, USDA, Beltsville MD, USA, on a NATO fellowship.

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USE OF IEM FOR THE DETECTION OF THE VIRUSES OF THE GRAPEVINE LEAFROLL COMPLEX IN SOUTH AFRICA

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In an attempt to eradicate the grapevine leafroll disease from South African vineyards, new clonal material and selections from existing vineyards undergo virus elimination by either heat therapy and meristem tip culture or by somatic embryogenesis (1). Effectiveness of the virus elimination is tested using commercially available GLRaV type I and III ELISA systems. Continued elucidation of the leafroll complex however now necessitates testing for at least GLRaV type I, II, III, IV, V and GVA (3,4,6,7,8). As the wine and table grape industries are primarily interested in whether any viruses occur after elimination, and not necessarily in which viruses occur, a cocktail ELISA, detecting all the viruses would be ideal. Developing such an ELISA requires antisera to each virus individually, which can then be blended in proportions for the optimal detection of each virus. Single virus sources of each type are not yet available locally. The aim of this study was to obtain *Vitis* samples with single virus infections, for use in the development of ELISA systems to each.

Numerous samples with known multiple virus infections have been detected in South Africa, and antisera to these have been useful in the detection of the leafroll associated viruses in immuno electron microscopy (IEM) (2).

For the purposes of this study, antiserum was made to a *Vitis vinifera* cv. Black Spanish source known to contain at least a spherical virus, GLRaV type I, II, III, GVA and an unidentified closterovirus. The antiserum was used in an IEM technique. The specificity of the IEM was tested by trapping the homologous virus mixture with the complex antisera and then decorating the virus particles with specific antisera received from a number of colleagues worldwide (Gonsalves; type IV, Gugerli; type I and III, and GVA; Martelli, GVA, and grapevine fleck; Monette, closterovirus isolated from corky bark-affected vines (CB) (5); Walter, type I and V; Zimmerman, type II). The vine contained, and had elicited antibodies, to at least grapevine fleck virus, GVA, GLRaV type I, II, III, IV, V, and CB. This is the first positive identification of GLRaV type IV, V and CB in South Africa. The IEM system, lacking the decorating step, could therefore be used to serve the needs of the industry in the non-specific detection of these viruses in the interim, until a cocktail ELISA could be developed. Sensitivity of detection of each virus by IEM could not be assessed until single virus sources were available.

To obtain single virus sources, 244 vine accessions, having indexed positive for either leafroll, corky bark, stem grooving or grapevine fleck were collected from the mother block of the quarantine station at Stellenbosch. Plants were tested for GLRaV type III infection by a locally developed ELISA. Of these, 118 tested positive. GLRaV type III infected plants were removed from further tests as a single virus source was already available to this virus.

The remaining 126 plants were further tested, sequentially, for GLRaV type I; type II or III or grapevine fleck (mixed antiserum, produced locally); II; III; V; IV and GVA, using the described IEM. Accessions with viral mixtures (containing decorated and undecorated particles) were discarded after each test. This was necessary due to the small amount of specific antisera available for tests.

Most samples tested had multiple infections. However, one sample had a single infection of grapevine GLRaV type I. In three samples all particles were decorated in tests for CB and type II, suggesting that the two viruses are serologically closely related. Four samples were negative for all viruses tested but nevertheless contained closterovirus(es) particles. In the type I test, closterovirus-like particles in 47 samples were decorated with a different density compared to samples interpreted as type I positive, suggesting the presence of a virus related to type I, or the presence of contaminating antibodies in the antiserum. These particles were in apparent single infections in 21 samples, which lacked any closteroviruses in further tests suggesting that the virus is labile under the conditions used. In five samples only spherical virus particles were detected. Of the 57 samples tested for GLRaV type V, only one contained this virus, unfortunately in a multiple infection. More samples will have to be tested to obtain single virus sources of type IV and V, and further work will have to be performed on the potentially undescribed virus(es) detected here.

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DETECTION OF GRAPEVINE CLOSTEROVIRUS ASSOCIATED WITH LEAFROLL AND CORKY BARK *IN VITRO* USING F(ab')₂ ELISA

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An enzyme-linked immunosorbent assay using F(ab')₂ fragments of virus-specific antibodies (F(ab')₂ - ELISA) was used to detect grapevine leafroll associated virus (GLRaV) and grape corky bark associated virus (GCBaV) in *in vitro* cultures of infected *Vitis vinifera* cv. Cabernet Sauvignon. Infected vines included GLRaV isolates 101 and 109 (type III), 102 (type II), 106 (type IV) and GCBaV isolate 100, each of which tested ELISA positive with the homologous antisera.

Green cuttings from infected and healthy vines were collected in late summer of 1991 and 2 cm long nodes were cultured in tissue culture media. The basic nutrient solution (BSN) contained 1/2 strength (final concentration) inorganic salts of Murashige and Skoog(2) with 0.1 mg/l IAA, 0.05 mg/l nicotinic acid, 0.01 mg/l thiamine, 0.05 mg/l pyridoxine, 10.0 mg/l myo-inositol, 0.20 mg/l glycine, and 20 g/l sucrose. The callus promoting medium contained 0.1 mg/l of NAA and 0.01 mg/l of BAP instead of IAA. The pH of the media was adjusted with 0.1M NaOH to 5.7 +/- 0.2. After shoots had grown from the nodes to approximately 5 cm high (35-60 days) the first assay was done. Sufficient explants were cultured to provide samples for destructive ELISA assays and propagation of subsequent tissue culture sub-cultures. Propagation of a first sub-culture was done by removing leaves from stem pieces and dividing the stems in two node pieces. The two node pieces were divided into two different categories: bottom and top. The bottom constituted the first two nodes above the root system, and the top constituted any other set of two nodes above the bottom ones. Node pieces (bottom and top) were cultured in the same media. When new shoots were about 5 cm high (105-135 days after subculturing) the first sub-culture plants that resulted from the top parts were used for the second assay. Additional explants which had been cultured in the first sub-culture were used for propagation of the second sub-culture. After shoots had grown for 75-95 days after subculturing, the second sub-culture plants resulting from the bottom-bottom combination were used for the third assay. A fourth assay was done for a set of second sub-culture plants that were 180 days old (second-aged sub-culture). Callus tissue from all different isolates were also tested. Samples weighting 50, 100, or 200 mg were collected, depending on the amount of available tissue. Polyclonal antibodies against the three different types of GLRaV and the GCBaV were produced in rabbits (4, 5). They were used in an F(ab')₂ - ELISA procedure described previously (3). Cross absorption of the purified IgG with healthy grape tissue was done before the IgG/Protein-A conjugate incubation step for leafroll type IV (isolate LR106), corky bark (isolate CB100) and in the first assay (1) and for all the isolates in first and second sub-culture assays. Tissue was harvested and analyzed separately for a) petioles and leaves, b) stem including the shoot tip, c) original field node, and d) roots either from field node (first culture) or from the 2 node pieces (first and second sub-culture) of the explants. The number of young plants from one node field explant and from two subsequent sub-cultures that were tested depending on survival rate varied from 21 to 32 plants. Nonparametric analyses were performed in this experiment to analyze the difference between infected and healthy tissue.

Results are shown in Figure 1. First culture plants had the highest titer of the virus particles for GLRaV 101, 102, and 109 in the original node while the virus titer for GCBaV 100 and GLRaV 106 was highest in stem+tip samples. Second sub-culture for GLRaV 102, GLRaV 106, and GCBaV 100 had the highest virus concentration in both leaf+petiole and stem+tip samples while for third sub-culture the highest titer was found in stem+tip samples for GLRaV 102 and 106, and in stem+tip samples for GCBaV 100. Third sub-culture (over 180 days old) had the highest titer in stem+tip for GLRaV 101, GLRaV 106 and the highest titer in leaf+petiole samples for GLRaV 102 and GCBaV 100. Roots samples had consistently the lowest titer. The viruses were detected in callus cultures from only two of the five infected vines. This information should be useful to both grapevine virus elimination programs and to researchers using *in vitro* systems to study the biology of GLRaV and GCBaV.

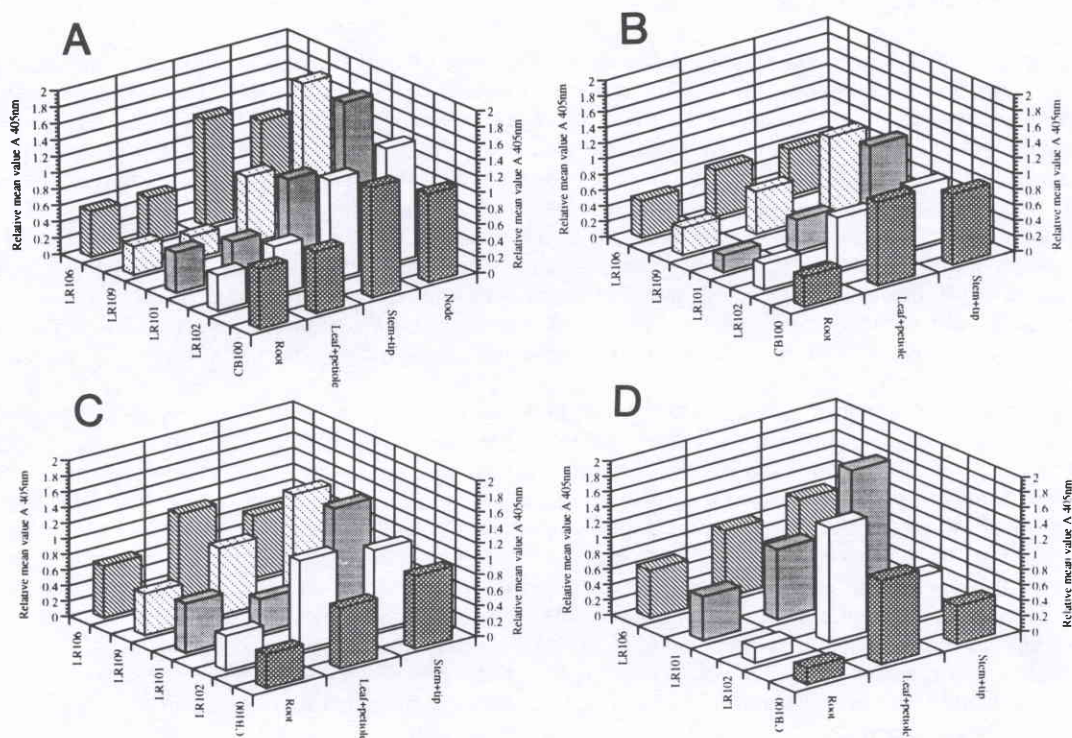


Fig.1 - Results from indirect F(ab')₂ ELISA *in vitro* representing the means of the Friedman test for leafroll isolates 106 (type IV), 109 (type III), 101 (type III), 102 (type II), and corky bark isolate 100 (X-axis); relative mean value (Z-axis); and node (only first tissue culture), stem + tip, leaf + petiole, and root (Y-axis). First tissue culture (A), first sub-culture (B), second sub-culture (C), and aged second sub-culture (D). 'Healthy' values have already been subtracted for all the mean values.

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RAPID DIAGNOSIS OF GRAPEVINE CORKY-BARK AND LEAFROLL DISEASES BY *IN VITRO* MICROGRAFTING

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Grapevine leafroll and corky-bark diseases are most likely of viral etiology (3, 6). Both may cause severe economic damage, reducing yield and quality in certain cases or remaining latent in others. Disease symptoms are better pronounced on *Vitis vinifera* which serve as indicator plants for these diseases. The use of serological methods (ELISA) to detect virus particles is not satisfactory, especially because none of the associated viruses has been proven to be the causative agent. Diagnosis is therefore still based on indexing by graft transmission, a procedure which takes 2-3 years to completion (8).

Diagnosis is important especially in latent infections and symptomless carriers. The preparation of virus-free propagative material and quarantine procedures depend on reliable diagnosis, which, due to its length, has become the bottleneck in releasing new and improved varieties and rootstocks.

Rejuvenation and incompatibility studies and the use micrografting has been reported for virus elimination procedures in various crops (1, 2, 4, 5, 7,). In this paper we report the use of micrografting for rapid indexing in grapevine.

Explants of grapevines, diagnosed by conventional indexing to be infected with either leafroll or corky-bark, were grown aseptically *in-vitro* in MS-based medium, at 25°C and 16 hours light. Explants of grapevine indicator rootstocks, such as LN-33, Mission, Cabernet-franc, were cultivated *in-vitro* under the same conditions. Shoots from explants to be tested, were allowed to grow to 2-3 cm, when they were excised to a wedge-shape scion and kept in moisture. In parallel, shoots of grapevines to be served as rootstocks were topped at 2 cm above the root system. The scions were grafted into a slopping cut of the rootstocks, and held in place with a sterile aluminum foil wrapped around the "graft". Grafting was performed aseptically. The grafted exemplars were kept in MS medium with NAA, in test tubes or Magenta boxes under the above-described conditions. In some cases plants were transferred to Jiffy pots.

Corky-bark symptoms (swelling of the nodes, longitudinal cracks and downrolling of the leaves) appeared on LN-33 eight to twelve weeks from grafting (Fig. 1). Leafroll symptoms (downrolling of leaves and stunting) appeared on Mission and Cabernet-franc three months after grafting (Fig. 2).

Micrografting can be used for rapid indexing, releasing the bottleneck resulting from the currently used conventional indexing practice.

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Figure 1: Corky-bark symptoms on micrografted LN-33

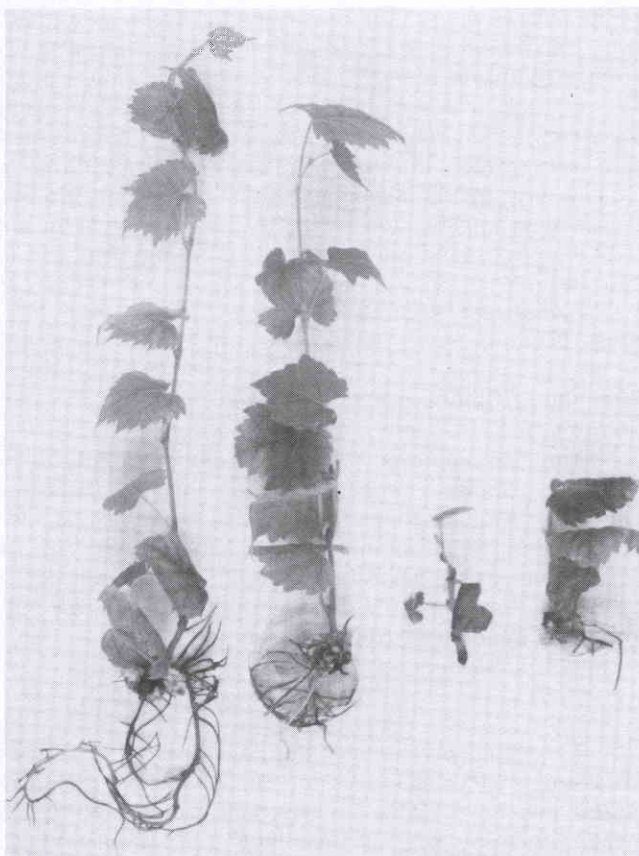


Figure 2: Leafroll symptoms on micrografted Cabernet-franc. Two left plants: grafted with healthy scions. Two right plants: grafted with leafroll-diseased scions.

USE AND LIMITS OF ELISA FOR ROUTINE DETECTION OF ArMV AND GFLV IN GRAPEVINES AND IN *XIPHINEMA* INDEX

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Control of grapevine diseases induced by Nepoviruses is based on sanitary selection and soil disinfection with nematicides. Chemicals are not always efficient as nematicides and can become dangerous pollutants.

Thus reliable techniques such as ELISA are essential not only for the initial selection of non infected clones but also for the routine control of mother blocks in the propagation of selected clones.

Evaluation of the infectious potential of a soil may help to use the chemical nematicides in a more rational way.

We have checked some essential factors for the routine ELISA detection of ArMV and GFLV; in addition, we studied the detection of GFLV in *Xiphinema index* and we established simultaneously the vertical distribution of the nematode in a given pedological profile.

Essential factors affecting detection of ArMV and GFLV by ELISA in grapevines

In previous reports (1, 2, 3) it was demonstrated that up to 50 leaf samples of grapevine can be mixed in a single test to detect one sample infected by ArMV or GFLV, but the reliability of the assay is highly dependent on the tissues that are sampled and on the time of the year at which the samples are harvested.

We confirmed that, in the climatic conditions of Colmar, the detection of ArMV and GFLV is not always possible during the hottest period of the year on *Vitis vinifera* (Cabernet Sauvignon, Chardonnay, Colombard, Gros Vert), whereas it is reliable all over the year on rootstock varieties (41 B, 5 BB, 99 R, *V. rupestris*).

The sensitivity of ELISA in leaves and wood shavings from Chardonnay and Kober 5BB grown in the field in Colmar was estimated in May. The test is much more sensitive on leaves (positive with dilutions down to 10^{-5}) compared to wood shavings (positive only down to 10^{-1} - 10^{-3}).

Leaves were harvested in June, July, September and October from the upper, middle and lower parts respectively of GFLV - infected Cabernet Franc grown at Le Grau du Roi: OD readings in ELISA were always higher for the upper leaves but detection was possible in all three parts of the plant at all periods.

In another assay we tested *V. vinifera* plantlets multiplied in pots and grown in a greenhouse at Le Grau du Roi. When leaf samples were taken from GFLV plants at regular time intervals it was shown that the detection of the virus was no longer reliable from the end of July onwards. When 5 leaves from an infected plant were mixed with 15 leaves from a healthy control, the detection was possible only until the end of June.

Quality and optimal conservation of reagents are essential factors for routine ELISA. We confirmed that the use of an amplification system by biotinylation of the antibodies considerably increases the ELISA response.

Keeping suspensions of reagents from a diagnosis kit for 6 months at 4°C significantly lowers the OD readings.

Bad conservation of leaf samples from a GFLV - infected grapevine (rotten or desiccated leaves) makes it impossible to detect the virus.

Detection of GFLV in *Xiphinema index*

The distribution of *Xiphinema index* in three fields infected with GFLV in the Champagne vineyard was studied in samples from each horizon of the pedological profile (4). The highest nematode populations were detected in a loam horizon (55 - 70 cm) in one field and in calcareous chalk parent rock (> 90 cm) in the two other fields. The GFLV infection potential of *X. index* from the first field was determined using ELISA on samples increasing in size from 2 to 256 individuals: detection was positive in samples of 2, 4 and 8 *X. index* individuals. However, the threshold number for GFLV detection was 10-15 adult nematodes in similar tests with other geographical populations.

Discussion

The results presented in this paper clearly show that care has to be taken when ELISA is used for routine analysis of grapevines for the detection of nepoviruses: leaf samples should not be harvested during the hottest periods and have to be processed as rapidly as possible. Reagents cannot be kept for long times after suspending them: keeping them lyophilised is more advisable. Thus, care has to be taken especially in large

scale analysis for sanitary selection and in routine controls, not to use reagents which are too old or conserved in inconvenient conditions.

In practical terms, evaluation of the infection potential of a soil based on detection of the virus in nematodes may enable the viticulturists to limit the length of the interval between uprooting and replanting grapevines to that strictly required for eliminating the virus in the nematode population.

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DETECTION OF GRAPEVINE FANLEAF VIRUS (GFLV) IN VINEYARDS ALONG THE WHOLE YEAR AND IN ITS VECTOR NEMATODE *Xiphinema index*

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Grapevine fanleaf virus (GFLV), transmitted by the nematode *Xiphinema index*, is known to be widespread in Spain, but the infection rate seems to be much lower than previously estimated on the basis of visual diagnosis, mainly because neither the presence nor absence of nepovirus-associated symptoms is sufficient to assess the status of infection (1, 3). ELISA allows the detection of small amounts of virus (6, 7), both in plant tissue and in vector nematodes but detection depends on the season (2, 7, 8) and varies from one year to the other (4). Furthermore, in a study carried out in Central Spain, intended to determine the importance of GFLV in vineyards, some differences were observed in virus concentration in leaves of different ages. Consequently, we carried out work in order to analyze the virus concentration occurring in different vine tissues along the whole year.

The study was carried out in a GFLV infected vineyard at the experimental station "La Higuera" in Santa Olalla, Toledo, Central Spain, on samples from the field and also from infected plants grown in the glass house. Samples from some infested sites in La Mancha were also studied. Virus concentration was analyzed in leaves, tip shoots, shoots, vine canes, tendrils, roots, buds, apical meristem, primordia, bunches of grapes, isolated grapes, grapes skin and seeds every five weeks, when available, by DAS-ELISA (5). Leaves, vine canes and roots of different age and locations and leaves and vine canes from 8 different levels of the plant were tested twice a year. Vector nematodes were also tested in batches of one, five, ten and twenty specimens, using, in the later case, infected grapes as positive and virus-free nematodes as negative controls.

Results show that virus concentrations in leaves in spring or early summer are higher than those in other plant tissues. Even leaves developed during the seasons with the highest temperatures gave rather high absorbance values, but with increasing leaf age absorbance values decreased, although remaining positive until the first frost, when the tissues were practically destroyed and the virus no longer detectable, results which all confirm the assertions of some authors (2, 3, 8) on the possibility of virus detection in relation with the seasons. Figure 1 represents absorbance values observed with different plant and fruit tissues along the whole year. The highest virus concentration was in young leaves, seeds, young roots and grape juice, in spite of the fact that this virus is not transmissible by the seed. Absorbance values of vine cane samples were very constant along the whole year; even those from samples kept at 4°C for one year remained clearly positive. Secondary root samples also gave constant positive reactions along the whole year with higher readings from the samples of the youngest rootlets (white ones). Absorbance values from bunch and bud samples were higher than from any other tissue. Virus was also detected, with rather high absorbance values, in fruit, grape skin, pulp, seed and stalk. No differences were observed in virus concentration in roots, leaves, or vine canes from different positions on the same plant.

Samples of young leaves of the same age taken in February from different positions of the same vine cane or from different stems of the same plant showed no differences in virus concentration. However in August, cane samples from eight different levels of the stem gave rather constant absorbance values while those of leaf samples increased from the cane base to the top. Tests carried out in spring with the same plant tissues gave intermediate values with leaf samples and nearly constant with cane samples. In autumn absorbance values from leaf samples decreased according to the season. It must be pointed out that one year old leaves from vines grown in the glasshouse gave positive ELISA readings, although much lower than those of young leaves of the same plant. Finally, virus could also be detected in the nematode samples with a minimum of five specimens per sample. Absorbance values of samples with only two specimens were lower than those of the negative control, even after several hours of substrate incubation. However, with five or more nematodes per sample the readings were clearly positive after one hour and with twenty nematodes per sample the absorbance values were close to those recorded with leaf samples.

It can be concluded that GFLV can be detected in grapevine plants along the whole year. Detection is possible in any tissue, but young leaves, along the season, contain higher virus concentrations and are easier to extract. Virus can be detected in vine canes or roots at any season. This is of interest for virus tests on propagation material in sanitary selection programs and custom control. The possibility of detecting the virus in samples of small numbers of nematodes is also useful for advisory services in charge of new plantations.

Acknowledgements: Authors are indebted to Prof. Bello and Drs. Almendros and Rey for their suggestions, and to Alicia Gala and C. Martinez for their cooperation. We are also grateful to the Comisión de Investigación Científica y Técnica (CICYT) for financial support to project PB 89-0034.

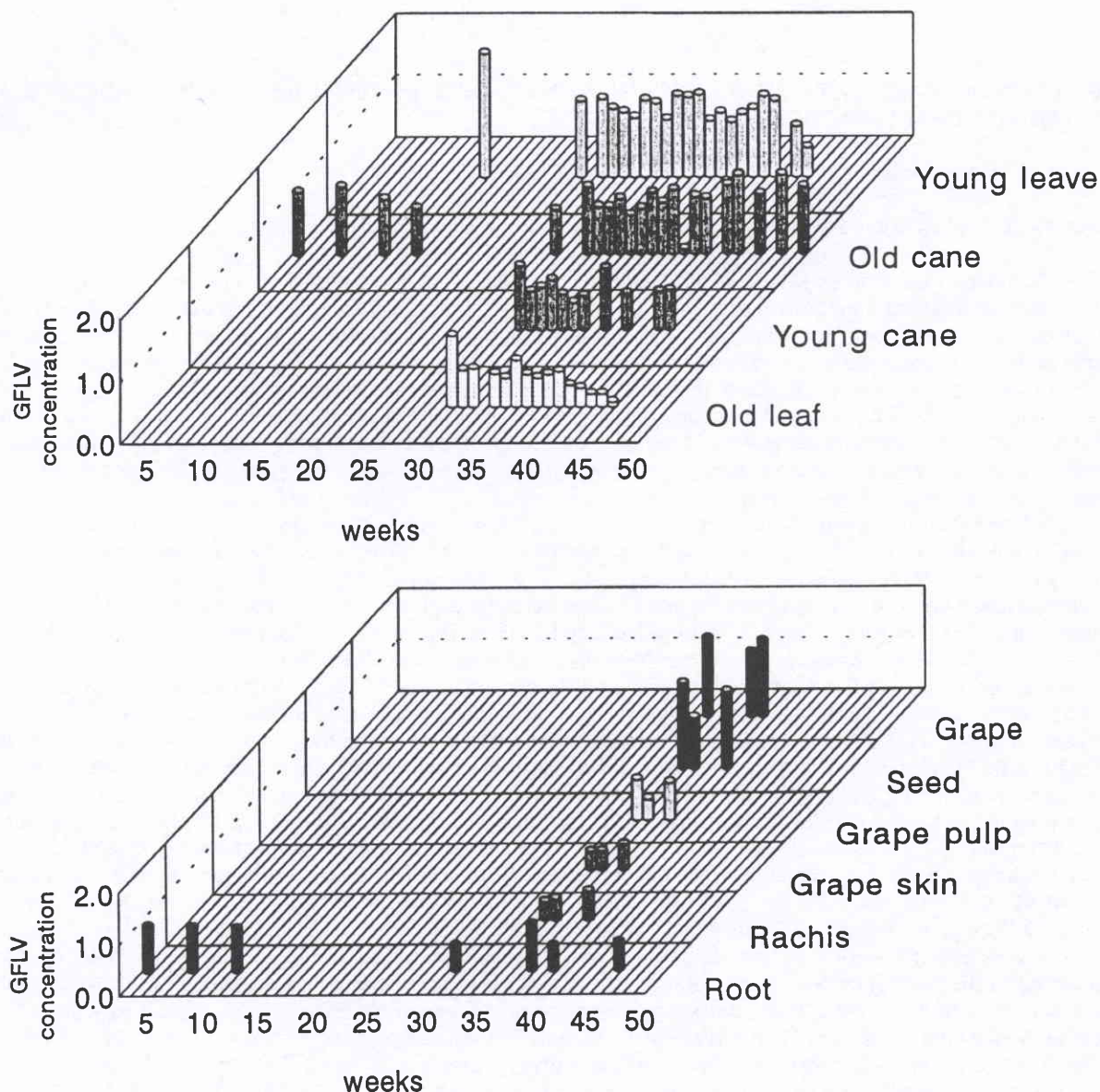


Figure. 1.- Virus concentration in the different plant tissues throughout the vegetation cycle

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DETECTION OF GRAPEVINE FANLEAF VIRUS (GFLV) FROM WOODY MATERIAL BY USING IMMUNOCAPTURE POLYMERASE CHAIN REACTION

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Grapevine fanleaf virus (GFLV) is a Nepovirus known to infect only grapevine in nature, causing severe damage in all Austrian vine-growing regions. Up to now, 840 grapevine samples have been tested by DAS-ELISA and 86 were found to be infected by GFLV. Although DAS-ELISA seems sufficiently reliable for large scale testing, efforts were made to establish the polymerase chain reaction (PCR) for diagnostic purposes in order to increase sensitivity. However though PCR could be applied for diagnosis in herbaceous plants and leaves, virus could not be detected in woody material so far, probably because the reverse transcription of the viral RNA to cDNA is inhibited by tannins. To resolve this problem, a sensitive immunocapture polymerase chain reaction (IC/PCR) assay (1) has been adapted to detect grapevine fanleaf virus from naturally infected grapevine away from the period of vegetation.

Polypropylen microcentrifuge tubes (0,5 ml) were coated with 120 μ l of polyclonal anti-GFLV-serum (Bioreba) diluted 1:500 in coating buffer pH 9,6 and incubated 4h at room temperature. Grapevine shoots were freshly harvested from field-grown GFLV-infected and virus-free plants. After removing the bark, the shoots were scratched and the thus obtained wood shavings homogenized (0,25 g) in 5 ml Tris-HCl (0,5 M pH 8,2) buffer containing 2 % PVP, 1 % PEG, 0,14 M NaCl and 0,05 % Tween 20. Aliquots of 100 μ l of plant extract were applied to the precoated and washed tubes and incubated at 4°C over night.

The tubes were washed five times with washing buffer and twice with PBS, then loaded with 10 μ l of a 1% Triton X-100 solution containing RNase inhibitor (10 U) in order to denaturate proteins by vortexing and incubation at 60°C for 15 minutes. To disrupt the secondary structure of the thus released RNA, samples were treated with 10 mM methyl mercuric hydroxide for 10 min at RT. Following neutralization of the methyl mercuric hydroxide by addition of 20 mM β -mercaptoethanol (10' at RT), the reverse transcription step was performed in the same tubes loaded with a mixture containing 50 mM Tris-HCl buffer pH 8,3 at 42°C, 50 mM KCl, 7,5 mM MgCl₂, 1 mM dNTPs, 1 μ M 3'primer, 10 U RNase inhibitor (Boehringer Mannheim) and 22 U of AMV reverse transcriptase (Boehringer Mannheim) in a final volume of 20 μ l. The reaction mixture was then held at 42°C for 45 - 60 min. For the PCR reaction, 10 mM Tris-HCl buffer pH 8,3, 50 mM KCl, 1,5 mM MgCl₂, 0,01% (w/v) gelatin, 0,25 mM dNTPs, 0,25 μ M of each primer and 2,5 U of Taq DNA polymerase (Cetus) were added to a final volume of 100 μ l/tube. The reaction mixes were overlaid with mineral oil and subjected to 35 thermal cycles of denaturation at 92°C for 60 sec, primer annealing at 54°C for 60 sec and DNA synthesis at 72°C for 60 sec. Both oligonucleotide primers used for reverse transcription as well as for PCR were selected from homologous regions of the two GFLV sequences GFLV strain fanleaf (2) and GFLV strain F13 (3). The sequences of sense and antisense primer are 5' ATGTGGAAGAGGACGGAAGT 3' (corresponding to position 815 - 834 of the capsid protein gene) and 5' CCTAGACTGGAAAACCTGGTTC 3' (position 1496 - 1516) respectively, resulting in a 700 bp product. The IC/PCR amplification products were analysed by electrophoresis in 1% agarose gel and visualised by ethidium bromide staining: Amplified 700 bp fragments were obtained from GFLV-infected woody material, whereas no fragment was amplified from healthy controls.

Following this first result, DAS-ELISA and IC/PCR were compared as to their sensitivity. For this purpose, extract of wood shavings from infected grapevine was stepwise diluted 1:20, 1:80, 1:320, 1:1280, 1:5120 and 1:20.480 in healthy extract, which was also used as negative control. In the following, with the same samples DAS-ELISA and IC/PCR were simultaneously performed as described above. Whereas the absolute ELISA detection limit layed between 1:320 and 1:1280, it was possible to detect viral RNA until dilution 1:20.480, when a second PCR-reaction was carried out as formerly described with 5 μ l of IC/PCR-product as template.

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DEVELOPMENT OF THE IMMUNOCAPTURE - REVERSE TRANSCRIPTION - PCR PROCEDURE FOR DETECTION OF GVA IN GRAPEVINE TISSUES.

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For grapevine viral diseases, progresses are made to complement or to replace indexing with serological and biochemical detection methods. However, in the case of the grapevine closteroviruses, serological assays such as ELISA are not always reliable enough mainly because of the small amount of virus in the plant tissues. To solve this problem, we have used the immunocapture - reverse transcription - PCR procedure (IC - RT - PCR), as described by Wetzel (1).

IC - RT - PCR combines a serological procedure, the capture of viral particles on a solid support with an antiserum raised against the virus, and molecular biology techniques: reverse transcription of the viral RNA and PCR amplification of cDNA. This procedure was used with success for the detection of grapevine virus A (GVA) in grapevine and *Nicotiana benthamiana* tissues.

Crude sap. either of grapevine or of *Nicotiana benthamiana*, was loaded on an ELISA plate previously coated with polyclonal antibodies raised against GVA. Trapped particles were decapsided with a detergent (Triton), and the released RNA denatured by methyl mercure hydroxyde. Synthesis of cDNA was initiated with random hexanucleotides or a GVA specific primer (2). A fragment of the resulting cDNA was then amplified by PCR using a couple of primers.

This procedure allowed us to detect GVA on very young leaves (two weeks after start of vegetation) of infected grapevines (figure 1), whereas no positive result could be obtained by ELISA on the same plant material.

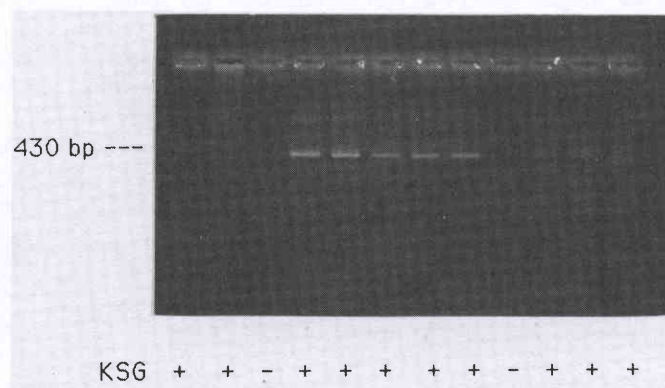


Figure 1 : GVA - specific PCR products obtained from plants affected by Kober stem grooving (KSG).

In addition, we have confirmed by this way the correlation between the Kober stem grooving disease (KSG) and the presence of GVA in a survey of 60 plants. This technique will also allow us to follow the transmission of GVA at early stages in heterografting experiments from infected *Nicotiana benthamiana* to Kober 5BB.

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DETECTION OF GRAPEVINE FANLEAF VIRUS BY ELISA AND ELECTRON MICROSCOPY: COMPARISON BETWEEN DIFFERENT SOURCES AND ORGANS

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Direct double antibody sandwich ELISA (DAS-ELISA) (1) and electron microscopy with indirect immuno-gold labelling (2) were applied to investigate the presence and localization of fanleaf virus (GFLV) in grapevine.

We report a comparative analysis of the virus content and infectivity in *in vitro* shoot tip cultures from frozen tissue (-20 °C) harvested during the vegetation period of last year and extracts from various organs collected in April of this year from greenhouse-grown grapevines affected by fanleaf.

The plant extracts obtained from grapevine leaves, petioles, young cuttings, tendrils, wood shavings and rootlets showed that young cuttings were as good virus sources as upper leaves, the best sources of material for routine diagnosis (3) by ELISA.

For virological studies by DAS-ELISA and electron microscopy the use of tissue samples from *in vitro* shoot tip cultures revealed to be better virus sources than extracts from infected vines grown in the field.

Extracts from grapevines with mosaic leaf symptoms gave positive reactions, but lower absorbance readings than extracts from fanleaf type leaves.

The ELISA readings recorded with leaf samples collected in the field and stored at -20 °C were very low as well as with samples from *in vitro* shoot tip cultures that were also stored at -20 °C for more than 5 months.

The indirect immuno-gold labelling of grapevine fanleaf virus in crude extracts gave best images with samples from bark scrapings, probably due to a lower content of disturbing compounds.

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INDEXING BY GREEN-GRAFTING TECHNIQUE

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The sanitary selection is based on the efficiency of the methods of virus detection. ELISA is used for the diseases whose pathogen agent is identified. For other virus-like diseases the diagnosis uses normally indexing by grafting of woody cuttings of candidate vines on indicator varieties of the different virus-diseases. Recording of typical symptoms needs observations in indexing nurseries during 3 years.

The green-grafting method was used because it presents some advantages over other grafting techniques (1).

Woody cuttings of the candidate vines to be indexed were forced in a mixture of 1/2 sand + 1/2 perlite. The woody cuttings of the indicator varieties were cultivated on rockwool cubes with a nutrient solution.

The herbaceous cuttings of the candidate vines were grafted as six replicates per indicator. Green-grafting was performed with the GCEV machine (2). The two parts of the assembly were held together with a small clothes pin, pricked into moist rockwool cubes in a mini plastic greenhouse with saturated humidity which was maintained in a climatic chamber at 26° C.

Maintenance of these conditions was very important to obtain a good callogenesis and rhizogenesis. The plastic cover was progressively removed. After one month the young plants were transferred to the greenhouse for acclimatization. Normally we obtained over 80 percent of successful graftings. At this step it was possible to observe the first Corky Bark symptoms on LN33. For other diseases the plants were transferred into an other climatic chamber. A range of temperature and day and night conditions promoted the optimal expression of the symptoms on the leaves of the indicators. The detection of clear symptoms could be observed 3 to 7 months after grafting according to the viral diseases.

The high level of success of grafting and the speedy transmission of virus-diseases on indicators are considered as two determinant improvements for the sanitary selection of virus-free grapevines. Furthermore this indexing under greenhouse conditions, without seasonal climatic variations, allows virus testing all along the year.

In fact, the green-grafting technique revealed to be a valuable new tool for routine screening for viruses. This technique will soon reach an official status.

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THE DEVELOPMENT OF A NUCLEIC ACID-BASED PROBE FOR THE RAPID DETECTION OF GRAPEVINE CORKY BARK DISEASE

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Major research efforts to characterize the causal agent(s) associated with grapevine corky bark disease (CB) have revealed that corky bark may be more complex than originally believed. Attempts to develop a rapid detection method for CB include the production of a polyclonal antiserum to an isolate of CB that was originally described in California, designated CB 100 (1). ELISA results have shown a variation in the specificity of the CB 100 antisera to different isolates of corky bark. This indicates that there may be more than one causal agent associated with the symptoms of corky bark disease. In order to elucidate the cause of grapevine corky bark disease, as well as to provide a more effective tool that will allow the detection of serologically diverse CB isolates, we have utilized molecular techniques to develop a nucleic acid-based probe for CB. In addition, we have conducted several experiments to further characterize the CB disease complex, including insect transmission tests with the mealybug, *Pseudococcus longispinus*, and the screening of various CB isolates using the CB 100 antiserum.

The production of nucleic acid probes was accomplished using dsRNA extracted from CB-infected tissue as the template for cDNA synthesis. CB dsRNA was extracted from grape tissue as follows: grape phloem tissue was powdered in liquid nitrogen and the nucleic acids were extracted from the powdered tissue with phenol and chloroform. The dsRNA was isolated by chromatography on CF-11 (Whatman) columns (2). Concentrations of the dsRNA were estimated from ethidium-stained bands after polyacrylamide gel electrophoresis. Denaturation of CB dsRNA was accomplished with methyl mercury hydroxide (3). Single stranded DNA complementary to CB RNA was synthesized using random primers with MMLV reverse transcriptase followed by second strand synthesis of the cDNA using DNA polymerase I. The resulting ds-cDNA was d(C)-tailed and annealed to d(G)-tailed pUC9 plasmids (4). Recombinant plasmids were then introduced into DH5 α cells (Bentheseda Research Laboratories) by transformation and plated on LB-ampicillin plates containing X-gal. Ampicillin-sensitive, X-gal-bacterial colonies (white pigmented) were selected and screened by colony hybridization using ³²P-labelled cDNA probe synthesized with CB dsRNA template. Once the CB clones are identified, the specificity of the probes will be tested by nucleic acid hybridization to screen dsRNA samples from grapevines infected with serologically distinct CB isolates. Characterization of the clones will also be attempted, including Northern blot analysis to determine the relationship of the clones to the various dsRNA molecules associated with CB.

Insect transmission tests were carried out to determine if the mealybug *Pseudococcus longispinus* is capable of acquiring and transmitting CB from grapevine to grapevine and from grapevine to herbaceous hosts. Results from the transmission tests enabled us to further characterize the virus-vector relations of CB disease.

Non-viruliferous colonies of the mealybug *Pseudococcus longispinus* were established on rooted cuttings of *V. vinifera* cv. Semillon originating from CB 100-infected vines located in the Davis grapevine virus collection, and maintained in individual insect cages to prevent contamination. After an acquisition period of four weeks, the highly infective mealybugs were transferred onto virus-free, rooted LN-33 single node cuttings, tomato seedlings, and *Vinca* seedlings, and maintained in a growth chamber. After an inoculation period of three weeks, the mealybugs were eliminated and the plants were moved to an insect-free green house.

Two months after infestation with the viruliferous mealybugs, many of the LN-33 indicator plants showed reddening, scorching, and downward rolling of the leaves, accompanied by moderate to severe stunting for some plants. The symptoms observed are suggestive for CB infection. The tomato, *Vinca* and control plants showed no symptoms. These observations were corroborated by ELISA using the CB 100 antiserum. All of the LN-33 indicator plants infested with the viruliferous mealybugs reacted positively with the CB antiserum, while the tomato, *Vinca* and control plants reacted negatively. Results from these transmission tests provide the first evidence that the mealybug *Pseudococcus longispinus* is capable of transmitting CB from grapevine to grapevine. Although attempts to transmit CB to the herbaceous hosts, tomato and *Vinca* were unsuccessful, attempts to inoculate new hosts will continue since successful transmission of CB to herbaceous hosts may increase the efficiency of dsRNA isolation and virion purification procedures.

The screening of different CB isolates using antiserum to the CB100 isolate was carried out to investigate the specificity of the CB 100 antiserum among different isolates of corky bark. The F(ab)₂ type ELISA method was used and include a pre-absorption step with healthy grape tissue to reduce high background levels. Results showed strong positive reactions of the CB 100 antiserum to CB 100-infected grape tissue. However, mixed reactions resulted from the ELISA tests when different CB isolates were screened, with only five of the nineteen CB isolates reacting positively to the CB 100- antiserum. Evidence from these tests thus supports the

idea that different CB isolates may be serologically distinct, and further demonstrates the need for a more effective detection method that can identify all isolates of CB.

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USE OF DOUBLE STRANDED RNA FOR DETECTION OF VIRUS DISEASES OF GRAPEVINE

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Ds-RNA associated with virus diseases of plants may be used as hybridization probes for the detection of the causal viruses (1,2). The method of hybridization with ds-RNA for the detection of virus diseases of grapevine was not used so far.

Ds-RNA was purified by column-chromatography on CF-11 cellulose (3). The nature of purified RNA was established by treatment by Ribonuclease A in 2 x SSC and 0.1 x SSC buffers. The radioactive labelling of ds-RNA was done by the method of chemical modification of cytidine residues using the alkaline solution of I-125. Based on this technique, we obtained probes for testing the non-sap transmissible viruses associated to grapevine vein mosaic (GVMAV), grapevine stem pitting (GSPaV) and grapevine leafroll (GLRaV).

The probes were used for hybridization with the following samples: tissue of healthy and diseased grapevine with symptoms of stem pitting, vein mosaic, leafroll and fanleaf (Fig.1). The results of hybridization with the three probes did not differ from each other. All three probes did not hybridize with extracts from the leaves of healthy plants. The positive reactions were obtained with leaves from grapevines affected by different virus diseases. So, the GVMAV probe hybridized with grapevine affected by GLRaV, GVMAV and grapevine fanleaf virus (GFLV). The GLRaV probe hybridized with GVMAV, GSPaV and GFLV. We interpreted this result by the presence of extensive complementary sequences in the virus genomes. Besides, it may be explained by complex virus infections. Such complex infections were often found by Tanne *et al.* (4).

For the investigation of possible reasons of such low specificity, we hybridized the GVMAV probe with different samples of diseased and healthy grapevine and we also used the cross hybridization of GLRaV probe with pure preparations of some other viruses of grapevine. The results suggest that the low specificity of hybridization was not due to the presence of grapevine RNA and DNA in the leaf extracts. This conclusion is based on the following results: 1) the absence of hybridization with extracts from healthy grapevine and 2) the absence of hybridization with pure DNA from grapevine.

According to the investigations of Azzam *et al.* (5), ds-RNA may be found in grapevine affected by some fungus diseases. For this reason we carried out the hybridization of the GVMAV probe and extracts of grapevine affected by downy mildew and powdery mildew. The results of hybridization showed that low specificity was not associated with fungal diseases. Extracts from the leaves affected by downy mildew gave only a weak signal which was close to background reaction.

The absence of hybridization with extracts from the leaves of stramonium affected by potato virus X suggested that the nonspecific character of hybridization with GVMAV- and GLRaV-probes had a certain limit.

The GLRaV-probe hybridized with pure ds-RNA from grapevine affected by GLRaV, GSPaV and GFLV. This is further evidence that the hybridization occurred with RNA associated with the grapevine viruses. We used the GVMAV-probe to test grapevine rootstocks. About 10% of all samples gave positive reactions. Diseased plants were found in four varieties of grapevine, but the most infected were the varieties Kober 5BB and S04. As we expected, in most cases all the bushes of one rootstock clone were infected by the viruses.

Our results give evidence that this method may be used for the detection of virus diseased grapevine clones. However, the low specificity of the probes does not allow to identify the virus.

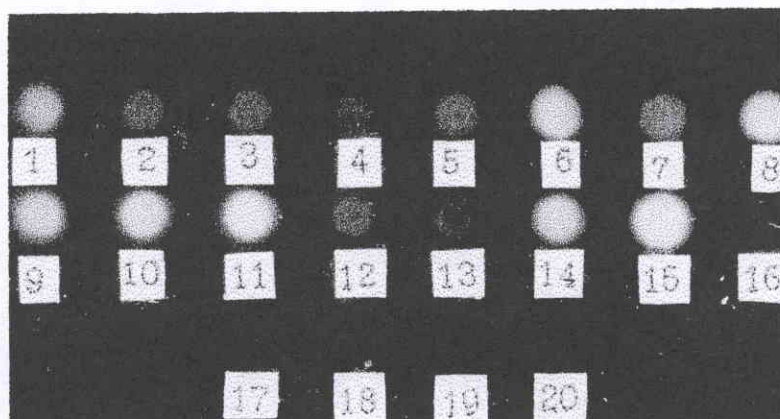


Figure 1

Dot-hybridization with ds-RNA probe associated with GVMaV: samples 1, 2, 3, 9, 10 and 11 = GVMaV; samples 4, 12, 13, 16, 17, 18, 19 and 20 = healthy grapevine; sample 5 = GVMaV and GSPaV; samples 6, 14 = GLRaV; samples 7 and 8 = GSPaV; sample 15 = GFLV.

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IMMUNOCAPTURE POLYMERASE CHAIN REACTION (IC/PCR) IN THE DIAGNOSIS OF GRAPEVINE FANLEAF VIRUS (GFLV) IN GRAPEVINE FIELD SAMPLES.

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Polymerase Chain Reaction (PCR) was shown to be a very sensitive technique for the diagnosis of viral infections. Protocols for its use in the detection of several plant viruses have been described but, apart from some work with plum pox virus (1), very little information is available about the use of this technique in field surveys of viruses in woody hosts.

In the case of grapevine nepoviruses a method was described (2) that enabled the detection of Arabis Mosaic (ArMV) or Grapevine Fanleaf Virus (GFLV) RNAs in preparations of nucleic acids obtained from *C. quinoa* and from grapevine. Due to the laborious manipulations involved, this method is not suitable for large scale screenings and, according to the authors, its performance was not satisfactory in the case of grapevine.

An improved method of IC/PCR that avoids nucleic acid extractions was recently described (3) for the detection of GFLV and other viruses and sub-viral pathogens. In this work we report the assessment of this technique as a diagnostic tool for the detection of GFLV in infected field grapevines.

Material and Methods

Grapevines from a vineyard heavily infected with GFLV and also infested with *Xiphinema index*, located in Algarve (southern Portugal) and several isolates from different locations and maintained under greenhouse at Estação Agronómica Nacional were used.

ELISA was performed by the direct double antibody sandwich technique. IgG for coating the plates and for the phosphatase conjugate was obtained from an antiserum against an isolate (U9) of GFLV belonging to the collection of Estação Agronómica Nacional.

The IC/PCR protocol was basically, with minor alterations, the same as presented by Nolasco *et al.* (3). Microtiter plates were coated with IgG, the plant tissue extracts to be tested were added and the plates incubated and washed as for the ELISA technique. A mixture containing the reagents for the cDNA synthesis was then added to each well. No additional step designed for virion disruption was needed. After reverse transcription, the mixture containing the cDNA was transferred to a tube containing the remaining reagents for amplification. The thermal cycling consisted of five cycles of: 92°C for 30 s, 52 °C for 30 s and 72°C for 30 s followed by thirty cycles in which the denaturation time was reduced to 5 s. The process ended with a final cycle with the elongation time of 5 minutes. Usually one tenth of the amplified product was analysed by electrophoresis and viewed under UV light after ethidium bromide staining.

The design of the primers (3) was based on the sequence (4) of an isolate from the collection maintained at Davis, California. The primer pair chosen permits the amplification of a segment of 568 nucleotides at the beginning (nt 2040) of the coat protein cistron. Each of the primers differed at one position relatively to the sequence (5) of the GFLV F13 isolate. The 5' (upstream) primer sequence was: 5'-CCGTGAGAGGATTGGCTGGTA-3' and the 3' (downstream) primer was: 5'-ATGGGAGGGCAAGTGAGAAAT-3'.

Results

Amplified fragments of the expected size were usually obtained from infected material. No amplification was obtained from uninfected plants (Fig. 1). In a few cases, minor variations in the size of the amplified fragment or the occurrence of extra bands were noticed.

The sensitivity of IC/PCR method for the detection of GFLV was roughly determined through a series of dilutions of infected grapevine tissue extracts. The endpoint dilution at which amplification was no more detectable was found to go beyond 1/800000. In a parallel experiment with ELISA the infected samples were not detectable at dilutions greater than 1/3200, in our conditions.

A comparison of the results obtained by ELISA and IC/PCR analysis of 109 samples was performed. From a total of 22 samples scored as ELISA negative 6 were IC/PCR positive, a direct consequence of much higher sensitivity of IC/PCR. This technique can thus be an important tool to complement ELISA data in viral diagnosis, especially when false ELISA negatives could have deleterious consequences as in quarantine, certification of plant material or nursery activities.

On the other hand, from 85 samples scored as ELISA positive, 5 were found IC/PCR negative. In a few cases the electrophoresis band corresponding to the amplified product was so faint that it could only be

detected after silver nitrate staining. A possible explanation could be that the nucleotide sequence of some of the samples could lack sufficient homology with the primers in order to secure an efficient amplification. The existence of a significant diversity in the nucleotide sequence of the amplified zone is suggested by the minor size variations already mentioned and is confirmed by the results presented in a companion paper in this meeting. For diagnostic work there is therefore the need to look for a more conserved sequence to design the primers.

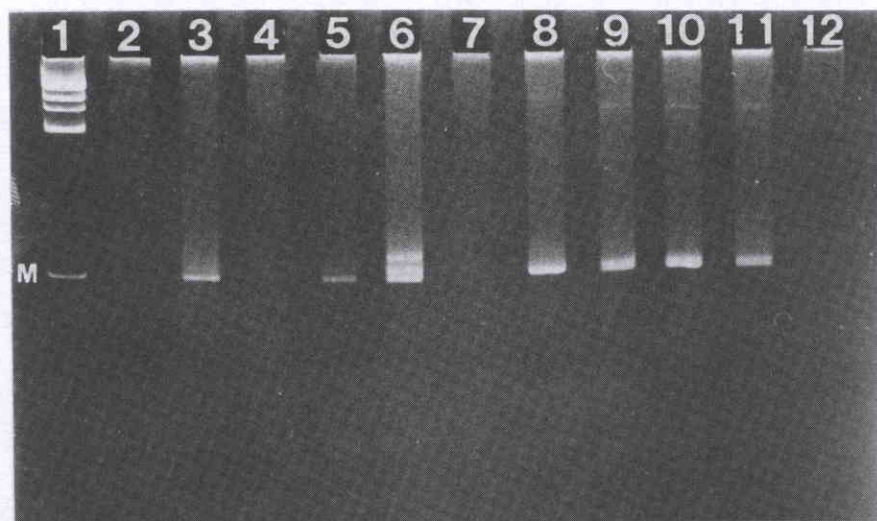


Fig. 1. Electrophoretic analysis of grapevine samples after IC/PCR (1/10 of the amplified product). Lanes 3, 5, and 6-11 were scored as ELISA positive for GFLV. Lanes 2, 4, 7 and 12 were ELISA negative for GFLV. Lane 1, Lambda DNA Hind III digest used as marker (M = 564 nt). Electrophoresis was in a 6.5% polyacrilamide gel. Staining with ethidium bromide.

Compared to other so far described PCR based procedures for diagnostic purposes, the present methodology is highly simplified and does not involve more work in the preparation of the samples than those required for ELISA, thus enabling its applicability to large scale surveys. Furthermore it proved to be an excellent preparative technique for molecular epidemiological and genomic studies.

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PROBLEMS OF ELISA DIAGNOSIS OF GRAPEVINE LEAFROLL ASSOCIATED VIRUSES

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From 654 clones analyzed by ELISA, to verify their sanitary status in relation to leafroll associated viruses (GLRaV's), the following results were obtained (table 1):

Table 1: Clones of grapevine analysed by ELISA for the presence of GLRaV's

	Number of clones			
ELISA	GLRaV I	GLRaV II	GLRaV III	GLRaV IV
negative	654	654	630	654
positive	0	0	24	0

Out of these 654 clones, 192 were indexed in the field using as an indicator the cv. 'Cabernet Sauvignon'. Fifteen clones induced symptoms of GLRaV. After analysis by ELISA 10 out of these 15 clones positive to Type III whereas 5 were inconclusive.

Conclusions:

1. In our region only GLRaV III is present.
2. Results obtained by ELISA may be influenced by:
 - the cultivar
 - the phenologic state of the plant at sampling time
 - the type of material used (bark, leaf, etc.)
3. Detection of the four GLRaV's by ELISA should be made in at least two or three different geographic environments, because it seems that the climatic variables prevent the manifestation of types I, II and IV.

MONOCLONAL AND POLYCLONAL ANTIBODIES FOR THE DETECTION OF GRAPEVINE FLECK ASSOCIATED VIRUS

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Antiserum against an Italian isolate of grapevine phloem-limited isometric virus (GPLIV) allowed Boscia *et al.* (1) (1991) to monitor the virus during indexing, transmission and sanitation experiments and subsequently to demonstrate that GPLIV is associated with grapevine fleck disease. Isometrical particles that were serologically related to GPLIV (as demonstrated with an Italian antiserum, kindly provided by G.P. Martelli) were also consistently detected by electron microscopy in fleck infected grapevines in Switzerland (5). We have now prepared improved antisera and monoclonal antibodies to a Swiss isolate of GPLIV and have further corroborated its association with grapevine fleck disease.

Virus purification: Virus particles were extracted from leaves of infected *V. rupestris* St. George according to (4), with minor modifications. The isopycnic centrifugation on a Nycodenz gradient was omitted when virus particles were only partially purified. More recently, we used the purification method described by Boulila (1990) (2). Its enzymatic maceration of the leaf tissue allowed us to extract significantly more particles than the method mentioned first.

Immunization and production of monoclonal antibodies: White New Zealand rabbits and Balb/c mice were injected up to five times with purified virus preparations, each from 80 g of leaf tissue. Out of five rabbits only rabbit K3 gave sufficiently specific antisera for reliable use in ELISA. Antisera from the other animals could only be used to immuno-precipitate and to decorate virus particles for observation by electron microscopy. Eleven mice were immunized for the production of hybridoma. The fusion and sub-cloning protocols were as described by Gugerli and Fries (1983) (3) except that X63/O myeloma cells were used. Standard protocols were used for antibody processing and ELISA. GPLIV occurred to be poorly immunogenic, since we obtained only few hybridoma that produced virus-specific antibodies. However, we produced a significantly higher proportion of virus-specific hybridoma, when we induced tolerance to healthy plant antigens in neonatal mice as described by Hsu *et al.* (1990) (6). In this case, mice were pre-immunized between one to eight days after birth by injection of extracts from healthy plants and then further immunized with virus preparations at the age of five to seven weeks. From all fusion experiments, we retained four hybridoma and characterized their antibodies: **MCA-13, MCA-17, MCA-28 and MCA-47.**

We found DAS-ELISA most appropriate for large-scale testing of grapevine. Performance of ELISA was best when polyclonal immunoglobulin from rabbit K3 (**PCA-K3**) was used for virus trapping and alkaline phosphatase labelled **MCA-47** as a second antibody. The performance was further improved with a modified extraction buffer: 0.4 M Tris/HCl, 0.14 M NaCl, 2 % PVP K25, 1 % PEG 6000, 0.05 % Tween 20 and 0.02 % Na₂S₂O₃ pH 7. Virus detection worked equally well with leaf samples of symptom bearing *V. rupestris* St. George and of symptomless, but infected, grapevine cvs Chasselas, Gamay Rouge de la Loire, LN33, Muscat and Räuschling.

Electron microscopy of immuno-precipitated virus particles (IPEM) was useful to analyze small numbers of grapevine samples. 10 µl of clarified, partially purified virus preparations from leaf extracts (as mentioned above) were mixed with 10 µl of immunoglobulin (1 mg/ml) and 80 µl of 0.018 M citrate-phosphate buffer pH 7 and incubated on a shaker for 30 min at room temperature. The immuno-precipitate was sedimented by centrifugation at 10'000 g for 5 min, collected in 10 µl of dist. water, negatively stained with phosphotungstic acid and observed with the electron microscope. IPEM of GPLIV particles performed well with either purified polyclonal immunoglobulin (**PCA-K3**) or monoclonal antibodies **MCA-13, MCA-17 and MCA-47** (Fig. 1).

SDS-polyacrylamide gel electrophoresis (12 % w/v) of partially purified leaf extracts combined with an electrophoretic blot onto nitro-cellulose membranes, followed by immuno-staining of the protein bands (Western analysis), was first used to determine the molecular weight of the viral coat protein and subsequently to detect the virus. **PCA-K3** recognized a major band of protein in extracts of GPLIV infected vines, which migrated in the gel closely ahead of the 31000 dalton molecular weight marker protein (carbonic anhydrase from Bio-Rad) (Fig. 2). The band represented therefore most likely the viral coat protein. Its molecular weight was about 30800 daltons, which is close to published data (2). The technique allowed us to detect GPLIV in several clones of local grapevines. The selected **MCA's** mentioned above did not work in Western analysis since they might not recognize the depolymerized and denatured protein, possibly due to their specificity for the coat protein in its native structure.

In order to estimate the correlation between biological indexing and serological analysis, we examined by ELISA 28 fleck indicator plants *V. rupestris* St. George from the 1991 indexing program. Five out of these 28 plants showed fleck symptoms, entirely concordant with the five positive serological reactions. We further analyzed by electron microscopy plants issued from diseased *V. rupestris* St. George submitted to heat therapy.

We selected randomly four healthy and four diseased plants and found, in partially purified extracts, no virus particles in the former but a significant number of particles in the latter.

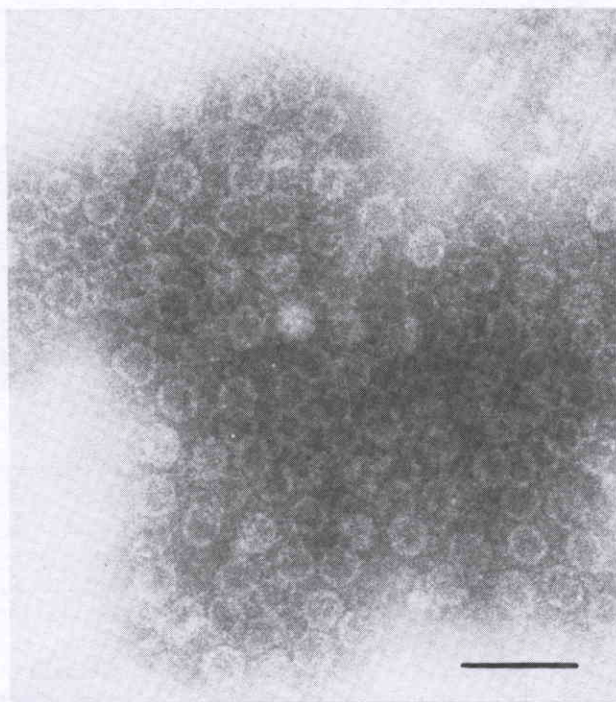


Figure 1. IPEM: GPLIV particles immuno-precipitated with MCA-47 (bar = 100 nm).

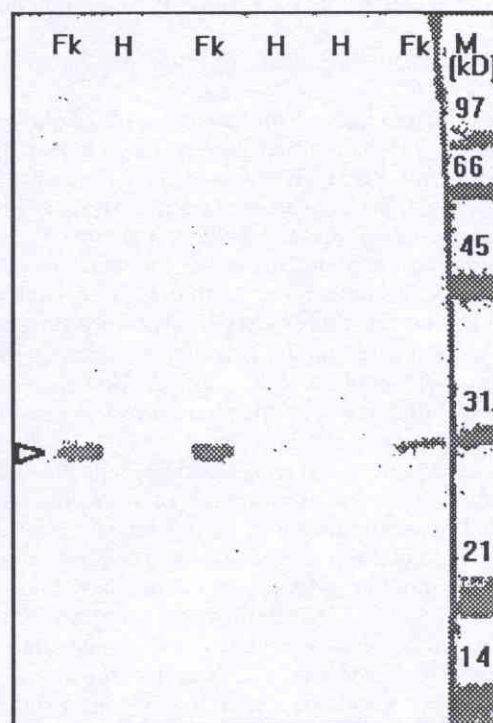


Figure 2. Western analysis of GPLIV in partially purified extracts of fleck infected (Fk) and fleck-free (H) Gamay Rouge de la Loire, Räuschling and Chasselas vines. Coat protein identified with phosphatase labelled PCA-K3.

Conclusions: Our data further confirm that GPLIV is associated with fleck disease of grapevine and justify the renaming of GPLIV to grapevine fleck associated virus **GFkaV**. The difficult preparation of specific antisera, due to the poor yield of GFkaV immunogen from grapevine tissue, can be circumvented by the production of monoclonal antibodies, especially by inducing tolerance to antigens from healthy plants in neonatal mice. ELISA, IPEM and Western analysis can favourably replace the traditional field or greenhouse indexing techniques.

Acknowledgments: We thank N. Dubuis, S. Bonnard and J.-J. Brugger for excellent technical assistance. This work was supported by the COST 88 action from the Federal Office of Science and Education.

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THE TUNISIAN PROGRAMME FOR CLONAL AND SANITARY SELECTION OF GRAPEVINE

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The Tunisian grapevine industry is severely affected by a number of virus and virus-like diseases that are widely distributed in the major viticultural areas of the country (2,4,5). In the last few years, an increasing attention was paid to these virological problems, some of which were investigated in detail (1,3,7) making it clear that provisions of some sort for the sanitary improvement of grapevines were desirable. This realization, and the launching of the UNDP/FAO Regional Project RAB/88/025 for the "Control of Virus and Virus-like Diseases of Fruit Crops", whose headquarters are in Tunis, prompted the establishment in 1991 of an ad hoc "Committee for the Sanitation of Tunisian Viticulture". This Committee, which is composed of viticulturists and virologists from the Institut de la Recherche Agronomique de Tunisie (INRAT) and representatives of professional organizations, elaborated in 1992 a "National Programme for Selection and Production of Healthy Grapevine Material".

The main objectives of this programme are:

(a) Pomological and sanitary selection of wine and table grape varieties: conceived as an emergency short cut that aims at distributing to the growers propagating material of improved quality identified through a preliminary pomological (1 year of observation) and serological screening of local germplasm. It also represents the first step of the more complex and thorough selection process mentioned below. This programme was first applied in 1992 to Alicante Grenache, a valuable variety which is disappearing from Tunisian vineyards. Vines selected in the field because of desirable pomological characters were serologically tested for the absence of grapevine fanleaf virus (GFLV), grapevine leafroll-associated viruses I (GLRaV I) and III (GLRaV III).

(b) Clonal and sanitary selection: a long term activity for the production of sanitarily improved clones of the major varieties and rootstocks grown in the country, through a procedure that follows the main lines of comparable programmes now being implemented in the EEC (6). Clonal and sanitary selection of cv. Alicante Grenache is currently under way.

(c) Establishment of a repository of healthy imported material: Many of the grapevine cultivars grown in Tunisia are of European origin, and several of them were introduced in relatively recent times. Thus, a way for a quick renewal of mother vine stands is the introduction of certified stocks from abroad, to substitute for those currently utilized in the country, which are infected or have an undetermined sanitary status. Grapevine imports of 1992 were strictly of certified category, were tested for the presence of viruses and planted in soils with no grapevine history. In 1993, only material of basic category will be imported which will be retained in part by INRAT and checked for virus presence, before being multiplied by authorized nurseries.

(d) Establishment of mother vine stands of American rootstocks selected in Tunisia: Clonal and sanitary selection of 110R, 140Ru and 1103P was initiated in mother vine plots established some 20 years ago with imported material that has never been submitted to virological controls. Visually selected vines were tested for the presence of GFLV, GLRaV I and GLRaV III, and those that proved free from these viruses, were multiplied and planted in soils where grapevines have never been grown.

(e) Sanitation of native germplasm: Native germplasm from the central and southern areas of the country is being collected and submitted to sanitation treatments through meristem tip culture. In 1992, two accessions denoted Oasis 14 and Oasis 31 were regenerated from meristematic explants and underwent preliminary sanitary controls.

The results of the 1992 activity of the National Programme for Selection and Production of Healthy Grapevine Material was critically reviewed and approved by the ad hoc Committee, which recommended its further implementation. The financial support of professional organizations was also secured. It is hoped that the grapevine programme may serve as a model for other fruit crops.

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THE USE OF *IN VITRO* SOMATIC EMBRYOGENESIS TO ELIMINATE PHLOEM LIMITED VIRUS AND NEPOVIRUSES FROM GRAPEVINES

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The effectiveness of *in vitro* somatic embryogenesis in eliminating phloem limited viruses from grapevines has been demonstrated (3). Detailed studies proved this procedure to be ineffective in the elimination of grapevine fanleaf virus (GFLV) from infected material when performed under normal culture conditions (25 °C) (4). Heat therapy of infected vines followed by *in vitro* shoot apex cultures has been used successfully to eliminate fanleaf virus from grapevines (2, 5, 7). This paper reports on the utilization of somatic embryogenesis in combination with heat therapy as a single procedure to eliminate both phloem limited virus and nepoviruses from infected material.

Dormant canes from field grown vines showing severe symptoms of leafroll, fanleaf and yellow mosaic, respectively, were collected. The virus status of source material was confirmed by subjecting samples to both immunosorbent electron microscopy (ISEM) with decoration (6) and enzyme-linked immunosorbent assay (ELISA) (1). The canes were stored in sealed plastic bags at 3 °C. Large quantities of elongating shoots were procured by forcing the buds at 26 °C with the basal parts of canes immersed in water. Flower development on shoots bearing inflorescences was promoted by the removal of all vegetative tissues. Anthers and ovaries used as explants were aseptically excised from the flower buds. Explants were cultured at 35 °C for 60 days on the basal medium (BM) of Nitsch & Nitsch (8) supplemented with specific growth regulators. Pro-embryogenic masses (PEMS) that developed under these conditions out of nodular callus (produced by explants) were transferred to BM (hormone-free) and grown at 25 °C. Mature embryos (containing cotyledons and roots) obtained at the lower temperature regime (25 °C) were germinated and somatic plantlets acclimatized and transferred to soil.

At normal culture conditions (25 °C) somatic embryogenesis as a means to eliminate GFLV proved to be ineffective. In detailed studies on the ontogeny of grapevine somatic embryos, no vascular tissue in nodular callus as well as no vascular connections between any two neighbouring embryoids or between embryoids and the parent tissue could be observed. This explains the elimination of phloem limited viruses but indicates that fanleaf virus particles (not restricted to vascular tissue and present in very young meristematic tissues) are translocated to callus and to subsequently regenerated embryoids. Somatic embryogenesis performed under heat therapy conditions (35 °C for 60 days) resulted in the elimination of GFLV and the yellow mosaic serotype of GFLV. PEM formation at 35 °C was enhanced substantially and abundant somatic embryos was produced. Following cotyledon expansion and root formation germination of embryos was achieved without special modifications i.e. cold treatments and/or growth regulators. Indexing results by means of ISEM and ELISA showed that regenerated somatic embryos and plantlets were free of the nepoviruses present in the source material.

Anthers and ovary derived somatic embryos gave rise to vigorous growing plants displaying the normal characteristics of the grapevine. After three years no somaclonal variation or virus symptoms can be detected. Vines established in the field by aerial grafting are still without any aberrations and produce fruit of exceptional quality.

It has been proved by this research that somatic embryogenesis could be successfully carried out at temperatures high enough to eliminate GFLV and the yellow mosaic serotype of fanleaf. This technique could be advantageously applied for the elimination of GFLV as well as leafroll-associated viruses from grapevines utilizing only one procedure. It is further postulated that this technique could also be effective in eliminating other nepoviruses normally eliminated by heat therapy. This will then offer the added advantage of producing grapevine material for reinfection with single or other combinations of virus isolates for research purposes.

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PRELIMINARY RESULTS OF CROSS-PROTECTION EXPERIMENTS AGAINST GRAPEVINE FANLEAF VIRUS (GFLV) IN THE VINEYARDS.

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Cross-protection has been already used to control virus diseases of perennial crops such as *Citrus* and Papaya.

Nematode-borne viruses can induce severe losses on grapevine. In some vineyards soil disinfection using chemical nematicides remain inefficient due to compacity and depth of the soil. In addition, the use of nematicides becomes more and more regulated or even forbidden due to their detrimental effects on the soil inhabiting fauna and on dripping water. Crossprotection could be an alternative for the control of nepoviruses infecting grapevines.

We started cross-protection experiments in the region of Burgundy in vineyards heavily infested by GFLV, where it was until now impossible for the viticulturists to eradicate the fanleaf disease: even after repeated soil disinfection with nematicides, in "hot spots", vines become infected rapidly after replanting and in some cases rapidly decline never producing any bunch.

In previous experiments we characterized hypovirulent isolates of GFLV and ArMV (Arabis Mosaic Virus) (1). We further demonstrated that cross-protection can be induced on *Chenopodium quinoa* by some of these hypovirulent isolates (2). We checked the protective effects of these isolates on grapevine by evaluating the multiplication rate of a challenge hypervirulent strain after transmission by nematodes in controlled conditions in the greenhouse (3).

Material and methods

To carry out experiments in natural conditions in the vineyards, we checked for the presence of GFLV or ArMV in leaf samples and *Xiphinema* in soil samples from various vineyards in the region of Burgundy. In these vineyards, fanleaf appears as "hot spots" of declining vines, in most cases chromogenic, in other cases with typical short internodes. In these "hot spots" some plants were missing or severely stunted.

Seven vineyards were selected, four of Pinot Noir and three of Chardonnay. In the "hot spots", groups of 5 plants each were chosen for replanting. A few months after uprooting of the remaining plants in these groups, the experimental vines were planted. Each group of 5 is composed of a healthy control and 4 vines of the same clone infected by ArMV (isolates A1 or A2) or GFLV (isolates G1 or G2).

The vines were produced by grafting non-infected Pinot Noir or Chardonnay onto Kober 5BB non-infected (control) or previously inoculated with ArMV (A1 or A2) or GFLV (G1 or G2) by heterografting with *C. quinoa*.

Each group (5 vines side by side) was planted on the row, with a total of 20 groups in each vineyard. The vines were regularly observed for vigour and symptoms. Those infected with protecting ArMV isolates and the healthy controls were also checked for the presence of GFLV (challenge isolate in the vineyards) by ELISA. For the plants previously infected with protecting GFLV isolates, it is not possible to distinguish by serology between these isolates and the challenge GFLV isolate.

Results

- Due to frost or mechanical injuries 5 to 9 plants out of 100 in each of 6 vineyards did not develop. In the seventh vineyard 38 out of 100 plants died because of lack of care by the viticulturist.

- Both for Chardonnay and Pinot Noir the healthy controls grew the most rapidly after planting. Especially in the second year after planting the vigour of the plants infected by ArMV A1 was comparable to that of the healthy controls. The presence of GFLV G1 or G2 induced a growth delay. ArMV A2 slightly reduced the vigour of Chardonnay in comparison to the healthy control, whereas it induced more severe stunting on Pinot Noir suggesting that it could not be considered as hypovirulent on this cultivar.

- Until now typical symptoms induced by the chromogenic isolate present in the vineyards only occasionally appeared on the control plants.

- ELISA with polyclonal GFLV antiserum on leaf samples collected 12-24 months after planting revealed that infection of the control plants had occurred in 4 of the 7 fields in rates varying from 21% to 47% with a total of 20 infected plants out of 67 (table). In the 3 remaining fields natural infection had not yet occurred.

Discussion

The results presented here clearly demonstrate that in the natural conditions of GFLV-infected vineyards, the hypovirulent ArMV A1 isolate induces a delay in the infection process by GFLV. Of course, it is too early to conclude that a cross-protection effect occurs: this can only be evaluated by comparing the harvest from infected and possibly cross-protected plants.

If the natural infection is only delayed, it is possibly sufficient from an economical point of view if it allows a minimal level of production. This is particularly true in the conditions of our experiments because of the severe limitation of the production in these vineyards of Burgundy and because until now the vines planted to replace the declined vines in the "hot spots" of fanleaf areas produced nearly nothing.

We report here results obtained with only one of the four isolates we test as possible protecting isolates: the second ArMV isolate shows too severe symptoms on Pinot Noir and the evaluation of the effects of the GFLV isolates will be possible only at production because these isolates can not be distinguished from the challenge isolate by ELISA.

Other hypovirulent isolates will be tested, in the same and in other vineyards.

Table 1 - Infection by GFLV in 7 fields in Burgundy of previously healthy controls and plants previously infected by ArMV A1. ELISA was performed 12 to 24 months after planting.

Field	previously healthy	previously ArMV A1
A	4/19*	0/20
B	0/15	0/17
C	0/10	0/20
Ma	3/10	0/7
Mo	9/19	0/25
N	4/19	0/18
V	0/15	0/4
	20/67	0/70

*) number of ELISA positive plants/number of tested plants

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CERTIFICATION SCHEME FOR PRODUCTION OF VIRUS-FREE GRAPE PROPAGATION MATERIAL AND ITS RESULTS IN HUNGARY

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Use of virus-free propagation material is an important factor to improve quality and quantity of grape production. In this publication we describe the screening system used in Hungary during production of virus-free propagation material and results.

Regular virological screening of grape varieties started in 1972 (3) and fifteen viruses have been identified until 1993 (4). The present system of screening (visual selection, indexing, ELISA) has been established using methods with continuous improvement according to recommendations of international organizations (1,2).

Certification scheme

The 1st year selection of basic material is performed by visual observation of grapevine stocks, twice during the vegetation period, and by ELISA. Since 1985 ELISA has been routinely applied for the detection of 7 viruses/strains: grapevine fanleaf (GFV), GFV yellow mosaic, GFV vein banding, arabis mosaic, grapevine chrome mosaic, tomato black ring and alfalfa mosaic virus. Since spring 1993 raspberry ringspot and strawberry latent ringspot viruses have also been serologically screened.

In autumn, canes of symptomless and ELISA negative plants are collected for further investigations. In the spring of the second year overwintered canes are checked by ELISA and mechanical transmission onto herbaceous indicator plants: *Chenopodium quinoa*, *C. amaranticolor*, *Cucumis sativus* "Delicates", *Gomphrena globosa*, *Nicotiana clevelandi*, *N. tabacum* "Samsun", *N. glutinosa*, *Phaseolus vulgaris* Beautiful. Woody indexing is also carried out on 8 indicator species in the field (Table 1). (In the present system FS4 and Chardonnay are regularly used, but they will be omitted or only occasionally used in the future. Symptoms are registered in June and in September.

Table 1: Woody indicators and detectable viruses in the Hungarian certification scheme

Indicator	
<i>Vitis rupestris</i> St. George	Fanleaf and strains, Fleck, Arabis mosaic, Rupestris stem pitting
Siegfriedrebe (FS4 201-39)	Fanleaf and strains
<i>Vitis vinifera</i> cv. Pinot noir (and other red berries cultivars)	Leafroll, Chrome mosaic, Tomato black ring, Arabis mosaic, Alfalfa mosaic, Line pattern
<i>Vitis vinifera</i> cv. Chardonnay	Fanleaf and strains, Alfalfa mosaic, Tomato black ring
<i>Vitis riparia</i> Gloire	Vein mosaic, Bulgarian latent, Line pattern
<i>Vitis berlandieri</i> x <i>Vitis riparia</i> Kobber 5BB	Kobber stem grooving,
LN33 (Couderc 1613 x <i>Vitis berlandieri</i>)	Corky bark, Enations, LN33 stem grooving
<i>Vitis rupestris</i> x <i>Vitis berlandieri</i> 110 R	Vein necrosis

In the third year symptoms are registered twice in the nursery. Grape clones that react negatively on all the indicator species are considered as virus-free. If there are varieties from which it is not possible to select healthy plants, cuttings are rooted and treated by heat, or adapted to *in vitro* culture for the production of virus-free progenies. After treatment, they are re-tested for viruses and trueness to type.

In autumn of the third year 30 plants per treated, virus-free variety are transferred as basic plants into a special mother block for maintenance and for further propagation. Plants derived from plants grown in mother blocks are used to establish nuclear stock. Plants of nuclear stock (superelite) produce propagation material (elite) which will be planted out in propagation stock. The progeny of elite plants originating from the propagation stock is source material for nursery propagation. Propagation material derived from mother vines established in nurseries is delivered to the growers as certified material.

During these steps of propagation, visual observation and random tests by ELISA are done to monitor

the virus status of the plants. The propagation is performed under strict official control by the nationwide plant health organization. Trueness to type is also monitored by the inspectors of the Institute for Variety Control.

Results

Mother blocks of virus-free scion varieties have been established on 2 ha and those of rootstock varieties on 0,5 ha including the following number of varieties : *Vitis vinifera*: 47, interspecific: 6, rootstock: 10, other varieties/variety candidates and clones: 33 (Table 2). There are 28 ha of virus-free nuclear stock from 51 European scion varieties and 31 ha from 10 rootstock varieties/hybrids. Virus-free propagation stock of scion varieties has been planted on 56 ha and of rootstocks on 22 ha.

Table 2: Virus-free grape varieties in Hungary until 1993

Grape varieties for red vine:	
Blauburger /2-III-4/ Cabernet s. E.153 /22-VIII-1/ Fűszeres kadarka /16-I-1/ Karmin /5-4/ Medina/ 69-VI-3/*	Pinot noir P.1 /8/ Tf. Kékfrankos /2-IV-3/ Tf. Kék Oportó /10-XX-4/ Zweigelt /11-IV-4/
Grape varieties for white wine	
Badacsony 10 /2-50/ Bianca /66-I-3/* Cserszegi fűszeres /2659/* Ezerfürtű /11-9/ Furmint T. 85 /2.5.8./** Furmint T. 92 /1.2.3./** Gyöngyrizling /40/* Hárslevelű P. 41 /8.9.10./** Hárslevelű T. 311 /1.3.4./** Izsáki /2-230/ Jubileum 75 /10-23/ Karát /6-20/ Kerner S. /5-XI-4/ Királyleányka 21 /2-I-2/ Korai piros veltelini /9 -VIII-4/ Kövidinka K-8 /31-40/ Nektár /71/ ** Olasz rizling B-20 /7.9./**	Ottonel musk. D-90 /16-II-3/ Rajnai rizling B. 7 /1-VIII-6/ Rajnai rizling Gm. 239 /3-20/ Rajnai rizling Ni. 391 /18-X-4/ Rajnai rizling T. 68 /4-VII-4/ Rizlingszilváni /10-7/ Rizlingszilváni D-100 /8-III-4/ Sauvignon /10-2/ Szürkebarát B-10 /8/* Szürkebarát 34 /1-VIII-1/ Zala gyöngye /1.3.4.5./*, ** Zefir /5- V- 1/ Zengő /10-XI-7/ Zöld szilváni Fr. 7 /4-XIX-3/ Zöld veltelini Kj.3 /19-X-2/ Zöld velt. Kr. 100 /15-II-1/ Zöld velt. La. 10/83 /14-I-6/ Zöld velt. M-25 /15-IX-7/
Table grape varieties	
Boglárka /5-8/ Cegléd szépe K. 73 /4-VII-2/ Chasselas bl.: Fr. 38-95 /46-V-2/ Chasselas r. Fr. 38-28 /15-VII-2/	Favorit /2-3/ Pölöskei musk. /63-VII-2/* R. 58 Teréz /4-177/* Néró /426-15/ *
Rootstock varieties	
Berl.x Rip. T 5C /E-20/ Berl.x Rip. T 5C Gm-6 /64-I-4/ Berl.x Rip. T 5C Gm-10 /74-III-2/ Berl.x Rip. T 5C Wed /103-III-3/ Berl.x Rip. TK 5BB /Fv.A.3.V.21./	Berl. x Rip. TK 5BB Cr.2 /18-IV-3/ Berl. x Rip. K 125AA /147-11/ Berl. x Rip. SO4 /133-16/ Chasselas x Berl. 41 B /2-9/ Fercal /25-1/

Number of other virus-free varieties and clones: 33 Note: * = interspecific varieties; ** = heat-treated varieties

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SEARCH FOR RESISTANCE GENES TO GRAPEVINE VIRUSES IN VITIS SPECIES

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Several hundreds of genotypes belonging to the different classes of the *Vitaceae* family have been collected to search for natural resistance to GFLV, ArMV and GLRaVs. Most of them were *V. vinifera* cultivars.

The grapevines have been first subjected to sanitary controls by ELISA, to eliminate the plants already infected by these viruses. For the remaining plants, the screening consisted of an inoculation by green cutting-grafting, followed by recurring ELISA to detect the virus in the inoculated plant. The time required for the transmission to occur was variable, from a few months to almost one year, but not ascribed to a certain resistance of the variety tested.

As yet, our survey did reveal one likely source of resistance to GFLV: a wild Iranian *V. vinifera* accession, in accord with the results of Walker and Meredith (1990)(2). Indeed, virus transmission has not been obtained in spite of a succession of graft-inoculations. Now, this resistance has to be checked by inoculating with several strains of the virus and by using natural transmission with viruliferous nematodes. If it is confirmed, crosses with susceptible rootstocks will be engaged to study its inheritance, with the aim of creating a resistant rootstock.

In the case of ArMV, lack of virus transmission has been observed in a great number of varieties. It seems unlikely that so many grapevines are resistant. The graft-inoculations are now being done again with a *V. vinifera* cultivar as the inoculating genotype, instead of the Kober 5BB.

Concerning GLRaVs, results are more difficult to interpret because of the weak sensitiveness of the available antisera.

Based on these preliminary results, it can be expected, according to Walker *et al* (1985), that resistance sources should mainly be found in wild Middle Eastern *vinifera* cultivars.

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A MODIFIED METHOD FOR *IN VITRO* THERMOTHERAPY AND MERISTEM CULTURE FOR PRODUCTION OF VIRUS-FREE GRAPEVINE PLANT MATERIAL.

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Elimination of grapevine leafroll has been achieved by *in vitro* regeneration of fragmented (2) or unfragmented shoot apices (1,3,4,7,8), combined with or without thermotherapy of whole potted plants.

Thermotherapy of *in vitro* plantlets is more advantageous than the use of potted plants because the procedure requires less space and uses no surface sterilizers, which may be detrimental to the culture efficiency of the explants. However, survival of vitroplants under prolonged thermotherapy is quite difficult.

In this communication, we report the use of one-node unrooted green shoot explants for thermotherapy followed by *in vitro* culture of the axillary bud for the production of virus-free plants.

More specifically, green shoots from greenhouse or *in vitro* grown *Vitis vinifera* L. cv. Italia were used. The presence of grapevine leafroll associated closteroviruses (GLRaV's) I and III was determined by ELISA by means of commercial antisera from Bioreba Ltd. One-node segments were surface sterilized, positioned in Roubelakis solidified culture medium (6) and kept in a tissue culture room at 26 ± 1 °C, 16/8 h photoperiod and total energy of $55 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent lamps. After 2 weeks, the explants were transferred into a heat chamber (Forma Sci.) kept at 37 ± 0.1 °C with a photoperiodism and light intensity as mentioned before. After a 70 d heat treatment period, $57 \pm 7\%$ of the explants survived. No apparent rhizogenesis and bud sprouting were visible. Thereafter, the axillary bud from each green shoot explant was aseptically excised and cultured on Murashige and Skoog medium (5) supplemented with $8.9 \mu\text{M}$ 6-benzylaminopurine. After approx. 3-4 weeks all the buds had developed shoots and were transferred to Roubelakis medium for rhizogenesis. The rooted plantlets were then transplanted, hardened and moved to the glasshouse. The elimination of the virus was checked by ELISA and will be further evaluated in the current season.

The described method can be successfully used for *in vitro* heat treatment of grapevine. Since the axillary buds consist of meristematic tissues with no active growth during heat treatment, the movement of virus to the growing points of the bud is not favoured and therefore virus elimination can be achieved. Also, regeneration of the new plants by this method is significantly faster compared to apical meristem culture and no callogenesis interferes, which may result in somaclonal variation.

Acknowledgments

This work was supported by the EEC ECLAIR AGRE-CT91-0060 Project.

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EXPRESSION OF SEVERAL MODIFIED GRAPEVINE FANLEAF NEPOVIRUS COAT PROTEIN GENES IN TRANSGENIC TOBACCO PLANTS.

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Introduction

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

One modern approach is the introduction of resistance genes into existing grapevine varieties by gene engineering. Over the past decade, several strategies have been used by many different groups to engineer virus resistant plants, including antisense RNA strategy, modulator satellite RNA and coat protein mediated resistance. The last strategy has been reported to be the most successful way to confer resistance against virus infection. Therefore the goal of this work is to use the coat-protein strategy to induce resistance to nepovirus in *Vitis spp.* As a first step, several chimeric coat protein genes have been constructed and their ability of conferring resistance to GFLV will be tested in a model system such as tobacco.

Materials and methods

GFLV genome is composed of two single-stranded positive sense RNAs. Translation of the two RNAs gives two polyproteins. RNA-1 encodes the RNA polymerase and the protease which cleaves the two polyproteins into functional units, whereas RNA-2 encodes a 66 kD protein of unknown function and the coat protein (1).

- Virus isolation: GFLV isolate 837 (RAC, Nyon) was propagated in and purified from *Chenopodium quinoa* according to (2). Genomic RNAs were isolated from virus particles and cDNA was synthesized.
- cDNA synthesis: RNA from GFLV was used as a template for cDNA synthesis with an oligo(dT)₁₇ primer/adaptor (ADT17). After synthesis of the first strand by reverse transcription, the resulting cDNA was directly used in various polymerase chain reactions (PCR).
- PCR: The CP gene was modified at the 5' and 3' ends through mismatched primers in PCR, in order to facilitate the cloning of the gene, and to provide it with a methionine initiation codon. Three primers were used, each containing a number of non-complementary bases. Conditions designed to maximize the fidelity of the PCR reaction were used. 45 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min were carried out.
- Cloning and sequencing: Cloning of the PCR products was done with standard procedures. The recombinant clone (pGFLV-1) was characterized by restriction analysis, before being completely sequenced.
- Construction of functional chimeric genes: Two different promoter regions were added upstream of the CP coding region. First, the CP gene present in pGFLV-1 was directly inserted in the binary vector pBI121.1. The resulting plasmid (pCCP-4) contained the CP gene under the control of the CaMV 35S promoter and the nopaline synthase terminator. Second, the promoter for the soybean translation elongation factor 1a (tefS1) was added in front of the CP gene in the pGFLV-1 plasmid. Then a fragment containing the tefS1-CP sequence was inserted in the binary vector pBI101.3. The resulting plasmid (pSCP-4) contained the CP gene under the control of the tefS1 promoter and the NOS terminator.
- Plant transformation: The plasmids pCCP-4 and pSCP-4 were transferred into *A. tumefaciens* LBA4404 by triparental mating. Using standard methods, leaf discs of *Nicotiana tabacum*, *N. clevelandii* and *N. benthamiana* were transformed with *A. tumefaciens* containing the plasmids pCCP-4 or pSCP-4, and putative transformants selected by their resistance to kanamycin (100mg/l).

[illegible]

Nucleic acid and protein sequences: The nucleotide sequence of the 1.5 kb PCR amplified clone was determined. Comparison of the coat protein sequence of isolate 837 (RAC Nyón) with that of isolates F13 (2) and "Davis" (3) revealed 90% identity with both isolates. Comparison of the deduced amino acid sequences of the three GFLV isolates showed a very high degree of homology (figure 1) :

- The high degree of homology found between the three capsid proteins is in good correlation with the lack of immunological heterogeneity reported for the virus.

Expression of the modified genes in transgenic plants: The level of expression of the modified CP genes in transgenic *Nicotiana tabacum*, *N. clelandii* and *N. bentamiana* plants will be determined by Northern and Western blots hybridization analysis. The ability of the modified CP genes of conferring resistance to GFLV infection will be tested in *N. clelandii* and *N. bentamiana* transgenic plants.

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STUDIES ON COAT PROTEIN MEDIATED CROSS PROTECTION OF NEPOVIRUSES

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Arabis Mosaic Nepovirus (ArMV) and Grapevine Fanleaf Nepovirus (GFLV) are closely related viruses that cause a severe disease in grapevine. The coat protein (CP) genes of the two nepoviruses, (both isolates from grapevine), have been cloned and sequenced, and were introduced into herbaceous host plants. Several constructs, containing different translational enhancer sequences, CP sense and antisense sequences, were made and transgenic plants were screened for the expression of the inserted viral genes. The main goal of our work is to get ArMV and GFLV tolerant or resistant plants by expressing viral sequences in transgenic plants.

Materials and Methods

Cloning and sequencing of the ArMV and GFLV were carried out as described elsewhere (Steinkellner *et al.*, 1989, 1990, 1992, (3, 4, 5) and unpublished data). Plasmid pROK-AMV, a binary plasmid that contains the entire ArMV coat protein and the omega leader sequence, was obtained from J.I. Cooper (Bertioli *et al.*, 1991) (2).

Transgenic plants were made by leaf disc transformation (Horsch *et al.*, 1985) (6) using *Agrobacterium tumefaciens* LBA4404. Bacteria, containing a binary plasmid, were cultivated overnight in LB/Kanamycin medium until OD₆₀₀ reached 0,6. Then 20 µm Acetocyringon was added as inductor. The culture was then diluted 1/50 in 10% glucose to achieve optimal conditions for transformation. Leaf discs were prepared from sterile cultures (Horsch *et al.*, 1985) (1) and placed onto regeneration medium. Transformants were selected on kanamycin sulphate.

Expression of the viral coat protein of transgenic plants were tested by double antibody sandwich ELISA.

Results

pROK-AMV was used for *Agrobacterium tumefaciens* mediated transformation of *Nicotiana tabacum* cv. White Burley. Several regenerated plants were tested by ELISA for coat protein expression. The expression level varied from 0,01-0,1% of total plant proteins. Although coat protein could be detected in all parts of the plants, a tendency of its accumulation in older leaves was observed.

Our preliminary results concerning the influence of different leader sequences (e.g. TEV- or omega-leader) do not give significant differences in the expression level of the coat protein. Several plants were challenged with purified ArMV particles. Different degrees of virus tolerance were observed. It is not yet clear whether the degree of resistance depends on the expression level of the coat protein or if other mechanisms are involved like it has been reported for other virus families (Beachy *et al.* 1990) (1). Experiments concerning this topic are under investigation.

Different plasmid constructs containing the GFLV CP gene, its antisense sequence and truncated CP sequences were transferred into *Nicotiana tabacum* cv. White Burley as described in Materials and Methods. The expression of these constructions are under investigation.

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LUSH GROWTH COMBINED WITH CONTINUED GREEN CUTTING PROPAGATION - AN EFFECTIVE MEANS OF ELIMINATING VIRUSES FROM GRAPEVINE SHOOT TIPS.

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I. The speculative part of the science

The high incidence of virus and virus-like diseases and of phloem- or xylem-limited procaryotes in commercially grown grapevines makes the use of sanitary procedures highly desirable, if not compulsory. Regardless of the procedure used, testing of the treated material for the assessment of its health status must follow. The techniques that must be used for detection and identification of infectious diseases inside the treated material are: a) indexing on *Vitis* indicators, b) inoculation to herbaceous hosts and c) ELISA testing. Generally, the term virus-free is used with the meaning that the material concerned has been found free of specified viruses or virus-like diseases after indexing, serological tests or other suitable tests. If anybody has omitted only one of these recognised and recommendable tests, it may be impossible to speak about "healthy" plants. This, however, is the speculative part of the questions put forward for solution !

II. A very old means of obtaining clean tissue

The major sanitary procedures are in-vitro techniques. Some explants, such as woody tissues, may contain pathogens within the tissue, in which case surface sterilisation will not be effective. Other means of obtaining clean tissue must be used in this case, the easiest being to use lush growth followed by the propagation of shoot tips grown very rapidly. Lush growth or abundant growth or luxuriant growth may be compared with a sudden start up of game-birds. Such conditions have been the basis for obtaining virus-free plants in other virus host systems (3).

III. Some experiences with regard to shoot tips.

1. In the use of grapevine in-vitro shoot tip culture for detection of fanleaf virus (GFLV) by ELISA, 27 of 29 shoot tip cultures, grown at 23°C +/- 2°C, tested by ELISA were positive for GFLV (4). The variation in the A-405 values obtained with these cultures may reflect differences in the virus content of the shoot tips used to initiate the cultures, as GFLV is not homogeneously distributed throughout a grapevine (2). Similarly, the low values obtained with the remaining two cultures (0.088 and 0.089 A-405 units) may be due to an absence of virus in the shoot tips used to initiate those cultures. Alternatively, the two cultures yielding negative results may have contained too few virus particles to permit detection even by ELISA.

2. Shoot tips of GFLV-, ArMV-, or RRV- infected vines, taken after forcing mother plants at 30°C, mist propagating the explants (6-10 cm long) near 30°C and establishing them in greenhouse containers after rooting, did not show any symptom of infection for 7 years and were negative by ELISA (5). The positive therapeutic effect of grapevine propagation under moderately high temperatures (30°C) may reside in the consistency of the temperature conditions under which mother plants were grown. It is unfortunate that in order to be effective in grapevines, the moderately high temperature must be kept constant around the clock. This prevents the possibility of a natural sanitation of nepovirus-infected grapevines taking place even in countries with a hot climate.

3. In experiments with the aim of elimination by heat treatment and meristem culture of four serotypes of closteroviruses associated with leafroll in grapevines (*Vitis vinifera* L. cv. Black Seedless) it was found that thermotherapy treatment at 38°C over a period of 150 days, using the three most distal buds (3 cm in all) for propagation from the treated shoots, was the most effective method for eliminating the four serotypes at a level of 100%. The vegetative tissues of the middle section of the shoots treated for 150 days at 38°C was not free of virions. Therefore, the removal of buds or meristems from this section after a real thermotherapy is not an appropriate method for obtaining grapevine plants free of this type of virus (1).

4. The first trials to eliminate the closterovirus Grapevine leafroll-associated virus III (GLRaV III) by a single step of grapevine propagation under moderately high temperature, failed. This means that some of the green shoot tips, 6-10 cm long after growing rapidly at 30°C and rooting, have been found still infected (see 2). However, when the shoot tips of the rooted plantlets had been used for a second propagation step under the same conditions, no virus was detectable for two years. Shoot tips smaller than 3 cm were rooted *in vitro*. Bigger green plants were rooted *ex vitro* in buckets covered with plastic film and filled with artificially aerated water.

IV. Some unresolved problems

At optimum growing temperatures, we must look for a mechanism different from thermotherapy to explain virus freedom in grapevine green shoot tips. Is it entirely a matter of growth rates at 30°C, or is it a matter of virus immobilization involving virus invasiveness which is mostly reduced within a second propagation step at 30°C ?

V. The most important question

It is recognized that the major viruses of grapevines belong morphologically to the polyhedral group (nepoviruses) and to the filamental group (closteroviruses). The question is, whether not only three plus one (see III. 2. and III. 4.) but all members of the named groups - more than twenty viruses - do not invade grapevine shoot tips after lush growth combined with continued green cutting propagation. If the answer is "yes", - and why shouldn't it be ? - a lot of problems connected with clonal selection and international trade with grapevine planting material may become simpler and cheaper.

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GRAFT INCOMPATIBILITY BETWEEN GRAPEVINE CLONES: TECHNICAL APPROACH

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In France, for many years, anomalies have been seen by nurserymen and grape growers when they graft some *vinifera* clones onto certain rootstocks clones. They speak of "graft incompatibility". The symptoms are: late sprouting, slow growth, weakening of the vines, year after year, and finally death. These symptoms are sometimes accompanied by yellowing or reddening of the leaves. Defective grafts between clones are periodically reported.

Previous research (1,2,3) has established that unknown pathogens are the cause of some graft incompatibilities.

We have made a study of incompatibilities where different varietal clones of *Vitis vinifera* were grafted onto clones of the rootstock 3309 C. We have studied too, the graft-incompatibilities between Syrah clone N°101 on SO4 clone N°5 (3), Pinot Entav N°95 on SO4 clone N°15 and Jaoumet on 57 Richter (4).

We have examined different techniques in order to discover a screening method for early detection of the clonal incompatibilities: wood grafting, green grafting and *in vitro* micrografting. Histochemical and histo-enzymatical analysis were made on the site of grafting in micrografts.

Field observations in the nursery on woody grafts have expressed some symptoms associated with the graft incompatibility.

Some unions of the green grafts in the glasshouse have given quick exteriorisation of viral symptoms.

The *in vitro* micrografts have shown, in some unions, the incompatibility symptoms within the first month after starting the culture on artificial medium.

We conclude that the organisation of the vascular connection, the starch reserves and proteins repartition, as well as the evolution of the peroxydasic activities are the important events for the appreciation of the incompatibility level.

Our research is continued and we expect to obtain an early screening method for graft incompatibility between clones of *Vitis vinifera* varieties and clones of rootstocks.

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OPTIMIZATION OF *IN VITRO* MICROGRAFTING: ITALIAN EXPERIENCE

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In vitro micrografting is a well known technique routinely used for eliminating viruses and viroids from many fruit tree species, including citrus. Its use in grapevine sanitation programs is still not well established due mainly to the fact that the micrografted apex does not grow easily.

In this paper some modifications, applied to optimize the different steps of *in vitro* micrografting, are reported.

1. Production of virus-free seedlings

Grapevine seeds of cv. Mission or Vialla are sterilized and let to grow on solid medium or in Grodan substrate containing liquid medium. To eliminate any environmental and mechanical stress, each seed is germinated in a polyallomer tube (25 x 90 mm) contained in a bigger glass tube (30 x 200 mm). Murashige and Skoog (1962) medium, modified by Galzy (1964) and supplemented with 20 g/l sucrose, is used either liquid or solidified with 6.5 g/l Phytogel Agar. Stratified seeds are maintained in the dark for about 20 days at 30 °C and then transferred in a growing chamber at 24 °C supplying 2000 lux for 18 hours. 90-95 % of the seeds germinate on both substrates. The tissues of the seedlings reveal to be more suitable to be micrografted than the ones obtained in the traditional way (1).

2. Tip isolation

No marked difficulties are met in this step of micrografting. Our experience confirms that the best results are achieved when apexes are excised from shoots grown *in vitro*. In this conditions, in fact, the isolation of the apex is easier and quicker.

3. Micrografting

This step is performed without causing any stress to the rootstock as it is micrografted without being extracted from the medium. Also the place where to put the apex is modified. In fact, it is placed on a lateral cut of the rootstock stem that will be excised after two weeks.

4. Acclimation and growth of micrografted plantlets

After 40-50 days, the plantlets are ready to be transferred into glasshouse. Those grown on solid medium are transferred, under mist, in perlite and, one month later, they develop a suitable rooting system and are ready to be transplanted in pots. The plantlets grown on Grodan perform better as they do not undergo stress transplant. The growth is assured hydroponically by supplying liquid nutrient containing Hydrokani AOⁿ at 4.5‰.

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La Confrérie des Vignerons et les Fêtes des Vignerons

Ancienne corporation dont l'origine se perd dans la nuit des temps, la Confrérie des Vignerons de Vevey a pour but unique d'encourager la bonne culture de la vigne. Ses experts - des vignerons très qualifiés - visitent trois fois l'an les vignes placées sous la surveillance de la Confrérie et attribuent des notes aux ouvriers chargés de la culture. Tous les trois ans, une généreuse distribution de primes et de médailles vient récompenser les meilleurs ouvriers de la vigne. Cette cérémonie solennelle se termine par un grand banquet réunissant les vignerons, les propriétaires des vignes et les autorités du pays.

Les Fêtes des Vignerons, manifestations artistiques et populaires d'envergure internationale, s'inscrivent dans le droit fil de ces cérémonies d'hommage aux vignerons. Quatre à cinq fois par siècle, elles réunissent sur des estrades construites tout exprès sur la vaste Place du Marché de Vevey des spectateurs venus de tous les horizons pour assister au couronnement des vignerons méritants, puis à la célébration des saisons, dansée et chantée par plus de 4000 artistes et figurants. Chaque fête donne l'occasion de créer un spectacle totalement renouvelé, avec des textes, des musiques, des décors et des costumes originaux. La dernière fête, en 1977, a enthousiasmé 200'000 spectateurs et les cortèges - près de 5 km de parade en ville de Vevey - ont mobilisé et enchanté les foules.

La Confrérie des Vignerons de Vevey remercie les membres de l'ICVG de leur engagement constant en faveur de la vigne et souhaite les voir nombreux à Vevey pour célébrer la prochaine

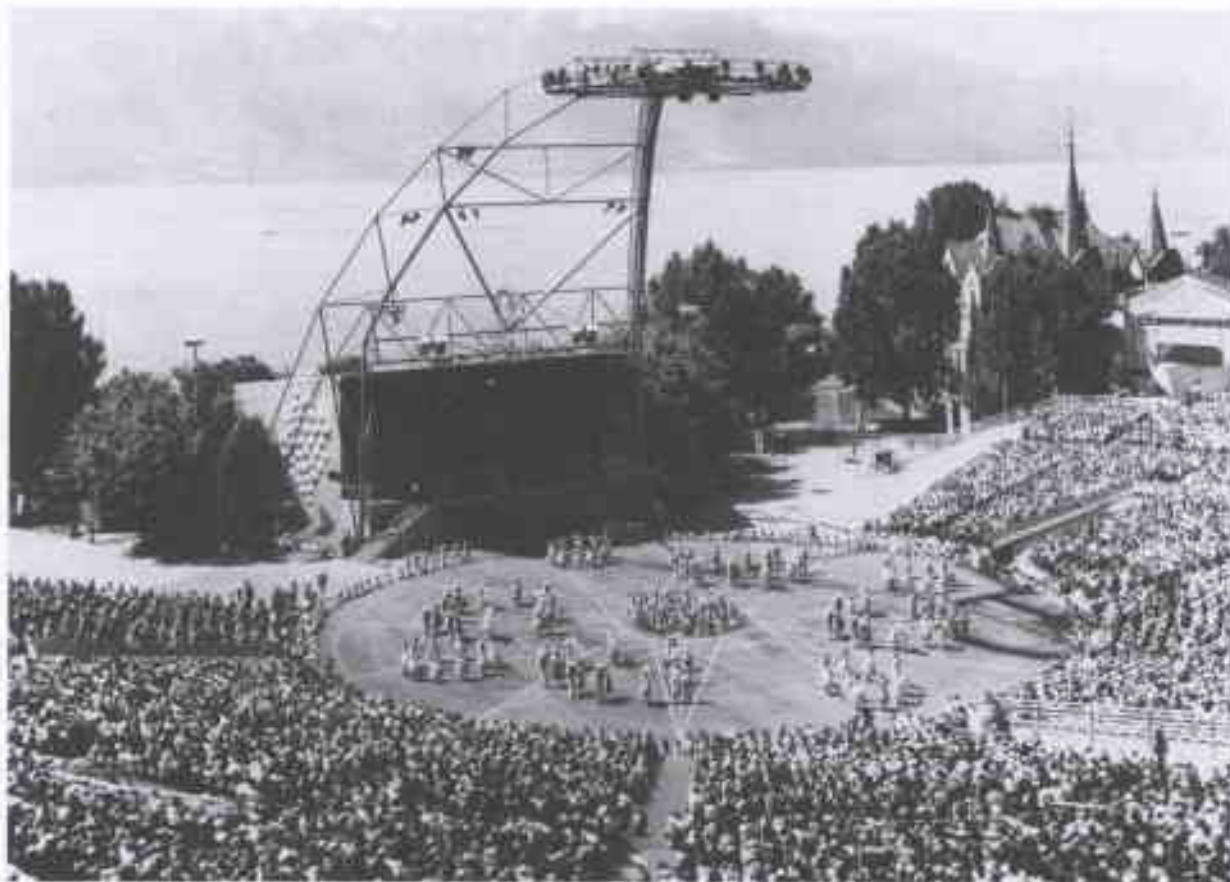
Fête des vignerons au début d'août 1999.

The "Confrérie des Vignerons" or Wine-growers' brotherhood of Vevey is an old corporation whose origin is lost in the mists of time. Its aim is to encourage careful cultivation of the grapevines. Its experts, who are highly experienced wine-growers, visit three times a year the vineyards that are under the supervision of the Confrérie, and attribute marks to the workers who are in charge of the cultivation. Every third year, the best workers are generously rewarded with prizes and medals. This solemn ceremony is followed by a large banquet where the vineyard workers, the owners of the vineyards and the country's authorities have an opportunity to meet.

The "Fêtes des Vignerons" are artistic and popular festivities of international fame which complete the tribute paid to the wine-growers. Four to five times each century, the festival brings together thousands of spectators from Switzerland and all around the world on a vast stage built up on the Market Place of Vevey. They attend the crowning of the most deserving vineyard workers and the celebration of the seasons. More than 4000 artists and supporting cast enliven the celebration with dances and songs. Each festival is a unique opportunity to create an entirely new spectacle with original text, music, scenery and costumes. The last "Fête des Vignerons", in 1977, fired with enthusiasm more than 200 000 spectators and the cortege, a three-mile long parade in the town of Vevey, attracted and delighted crowds.

The "Confrérie des Vignerons" of Vevey expresses its gratitude to all members of ICSVG for their constant devotion to improving the health of grapevines and wishes to meet many of them at Vevey for the celebration of the next

Fête des Vignerons at the beginning of August 1999.



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Appendix 1

THE INVESTIGATION OF MOLECULAR WEIGHT OF COAT PROTEIN OF GRAPEVINE FANLEAF VIRUS USING POLYNOMIAL REGRESSION

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Hydrolysis of viruses for the determination of molecular weights (mol. wt.) of their coat protein and nucleic acid represent one of the most important phases in the process of virus identification. So far several methods have been used for the determination of these molecular weights with variable accuracy (chromatography, logarithmic method, polynomial regression).

This paper reports the determination of the molecular weight of the coat protein of grapevine fanleaf virus (GFLV) by comparing its migration in gels with those of marker proteins with known molecular weights.

The results were computed with the method of polynomial regression of the first to the fourth degree. The first degree equation resulted in a coat protein mol. wt. of 67,14 kd with a coefficient of correlation (r) of 0.934, the 2nd degree polynomial equation in a mol. wt. of 63,615 kd with $r = 0,9906$ and the 3rd degree equation in a molecular weight of 58,7 kd with $r = 0,9979$. A value of $r = 0,9999$ and a mol. wt. of 56,85 kd were obtained with the 4th degree polynomial equation.

Logarithmic computing was used as a reference. A mol. wt. of 62,52 kd was calculated (standard deviation: $s = 95\%$). The estimated molecular weights found in this study were between the limits of molecular weights of the coat protein of GFLV reported by other authors.

Appendix 2

GRAPEVINE FANLEAF VIRUS - ULTRASTRUCTURAL INVESTIGATIONS

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During some years the ultrastructural aspect of the fanleaf virus was investigated. *Rupestris* du Lot grapevines showing symptoms of fanleaf, *Riparia Rupestris* 101-14 grapevines with yellow mosaic and cv. Matrasa exhibiting symptoms of vein banding contained membrane-associated 30nm spherical viral-like particles in the cytoplasm, vacuoles and in the cell wall plasmodesmata. Similar results have been obtained by Corbett and Podleckis (1985) with infected 'Colombard' and 'Chenel' grapevines. Only in the shoot apical tissue and in the cells of young leaves of infected grapevines it was possible to identify the presence of virus particles.

Further ultrastructural research of 'Matrasa' grapevine with vein banding symptoms showed crystalloid type inclusions consisting of icosahedral subunits each composed of 6 spherical virus-like particles (fig. 1). Later, different configurations of such crystalloid structure were found for two other strains of fanleaf. Some sections permit to determine the approximate distances between the centres of the adjacent and diametrically opposed spherical particles: 32 and 143 nm respectively.

Probably it would be mistaken to consider the crystalline structure as a new form of intracellular inclusions. They are often found in close contact with the well-known tubular structures (fig. 1) and in some electron micrographs we observed the appearance of the crystalloid subunits as the inner structure of the tubular inclusion (fig. 2).

Thus we can assume that the crystalline structure is only one of the aspects of the tubular intracellular formations.

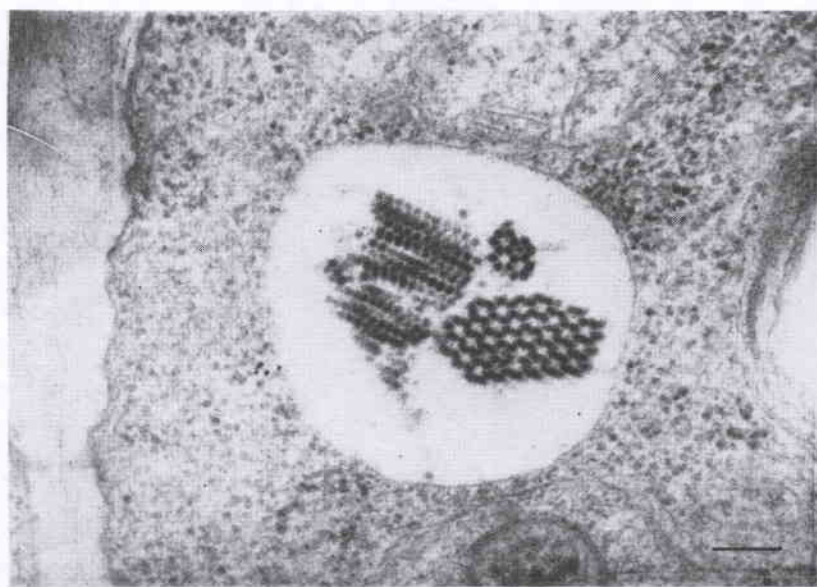


Figure 1. The presence of the tubular and crystalloid structure in the vacuole of the grapevine meristematic cell (c. Matrasa). Magn. bar 200nm

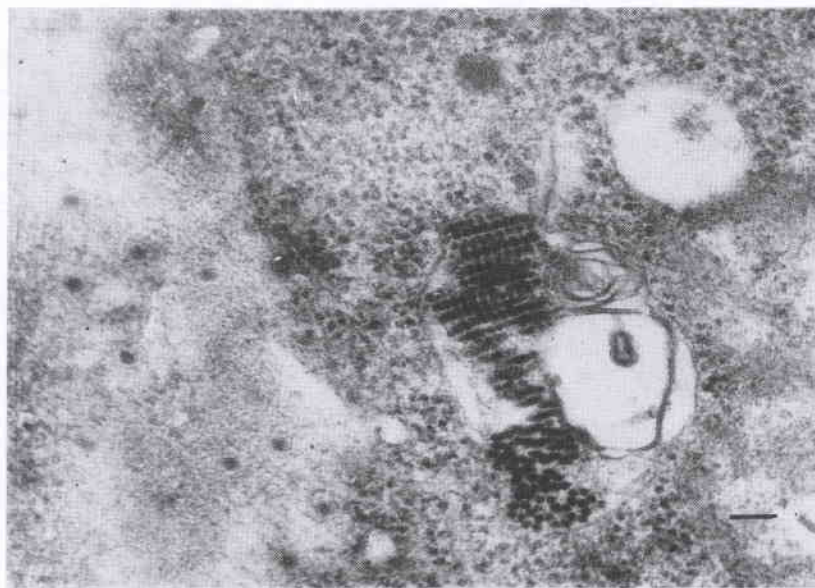


Figure 2. The appearance of the crystalloid subunits as an inner structure of the tubular inclusion (*Riparia Rupestris* 101-14) Magn. bar 200nm

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TRANSFER AND EXPRESSION OF THE COAT PROTEIN GENE OF GRAPEVINE FANLEAF VIRUS IN GRAPEVINE

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The use of grapevines resistant to Nepoviruses could be an advantageous alternative to soil disinfection with nematicides which are not always efficient enough and constitute dangerous pollutants.

One possible way to obtain resistant vines is to transfer into their genome the coat protein gene of the virus.

We have transferred the coat protein gene of Grapevine Fanleaf Virus (GFLV) into *Vitis Berlandieri* x *V. rupestris* 110 Richter, using *Agrobacterium tumefaciens* as a vector.

Anthers were cultured at 20°C, in the dark, on Murashige and Skoog (MS, 1962) medium containing the vitamins of Morel and Martin (1987), 36,7 mg/l Fe-EDTA, 30 g/l sucrose, 8 g/l bacto-agar, pH 5,8 ; three auxins have been compared (2,4 D at 1 mg/l ; NAA or NOA at 0,2 mg/l) in combination with one cytokinin (BAP at 0,25 mg/l).

Agrobacterium tumefaciens construct was GV 3101 [pCP1660]. This strain contains a disarmed Ti plasmid and a binary vector containing between its T-DNA borders a GUS-intron gene, the NPT-II gene and the CP gene of GFLV.

For the transformation, fragmented embryogenic calli were immersed for 5 to 75 min in a bacterial suspension and cocultured for 2 days on MS 1962 medium with 1 mg/l NOA and 0.25 mg/l BAP.

The embryos were they transferred onto MS 1962 medium containing 1 mg/l NOA, 0,25 mg/l BAP, 25 or 50 mg/l kanamycine and cefotaxime at a concentration stepwise decreased from 500 to 100 mg/l during the weekly transfers.

The rate of transformation of the embryos was checked by GUS- and NPT II -tests.

We have obtained several rooted plantlets which show positive reactions in GUS test, NPT II test and ELISA using a GFLV polyclonal antiserum.

These transformed 110 R plants expressing the GFLV coat protein gene are now under further investigation for their possible resistance to GFLV infection.