IMPROVED PROCEDURE FOR THE ISOLATION OF DOUBLE STRANDED RNA FROM VIRUS-INFECTED GRAPEVINE

Carstens, R.

Agricultural Research Council - Fruit, Vine and Wine Research Institute, Private Bag X5026, Stellenbosch, 7599, South Africa
E-mail: roleen@nietvoor.agric.za

Introduction

The presence of double stranded RNA (dsRNA) in virus-infected tissue has been well documented by Morris and Dodds (4), Dodds and Bar-Joseph (2). The analysis of dsRNA proved to be useful for determining the virus-free status of propagation material after sanitation treatments. Isolated directly from grapevine, dsRNA is also a good source of viral RNA for synthesizing and cloning cDNA from non-mechanically-transmitted closteroviruses (3). Isolation and purification of dsRNA from grapevine tissue, however, is problematic. Relatively large amounts of tissue are needed and the procedure is time-consuming. The high concentration of polyphenols in grapevine tissue can also cause problems in the isolation of dsRNA.

The objective of this study was to find a simplified procedure for the isolation of dsRNA from virus-infected grapevine that will yield enough dsRNA to produce detectable bands on a gel. The recovery of dsRNA was investigated during various phases of growth.

Materials And Methods

The method used by Bar-Joseph et. al.(1) was adapted and used for the isolation of dsRNA from phloem shavings of *Vitis vinifera* cv. Crouchen infected with viruses and harvested every 3 weeks from the 10th of August up to the 2nd of November. This involved the grinding of phloem shavings (8 g) to a powder in liquid nitrogen; recovery of dsRNA using 6% p-aminosalicylic acid (PASA); purification of the dsRNA on cellulose columns and two cycles of precipitation with ethanol and sodium acetate. Following this, nucleic acids were electrophoretically fractionated on 5% polyacrylamide gels. Gels were stained with 5µg/ml ethidium bromide and the bands were visualised by ultra-violet fluorescence at 302nm.

Results And Discussion

High quality dsRNA was obtained using this simple and relatively inexpensive technique. Phloem shavings from dormant shoots (just before pruning) gave the highest yield of dsRNA. If dsRNA isolations must be done earlier in the season, the amount of tissue used should be increased to produce detectable bands.

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INVESTIGATIONS ON THE AETIOLOGY OF CORKY BARK: ROLE OF GVB

Chabbouh N[1]., Marrakchi M.[2] and Bel Falah Z[1].

Institut National de la Recherche Agronomique de Tunisie[1], Rue Hedi, Karray 2049, Ariana, Tunisia
Faculté des Sciences de Tunis[2], Rue Hedi, Karray 2049, Ariana, Tunisia

The assessment of virological problems in Tunisian vineyard has been carried out in the framework of a Regional Project for the control of virus and virus-like diseases of fruit crop and the National Program for selection and production of healthy grapevine materiel (1). The present survey brings back results of investigations, on the main grapevine varieties grown in Tunisia, for detection of corky bark, an economically important disease.

Material And Methods

The study of corky bark disease in wine and table varieties has been carried out using biological tests based on the assessment of symptoms in the field. All candidate clones expressing visual symptoms are biologically indexed on LN33 that provides a specific reaction of corky bark (2). Three indexing techniques are used (traditional indexing with omega grafting, green grafting and in vitro micrografting). Individual clones indexed positive by grafting on LN33 are checked serologically (ELISA) for the presence of GVB associated with corky bark disease (3). ELISA tests use polyclonal and monoclonal antibodies (provided by Dr D.Boscia). The immuno-capture reverse transcription- polymerase chain reaction (IC-RT-PCR), proved to be a more sensitive method, is used to confirm serological results for detection of GVB. PCR was used as described by Minafra and Hadidi (4), using amplification of 450pb GVB fragment with specific primers (kindly provided by Dr. A. Minafra). These primers present the following sequences:
GVBh: 5'ATCAGCAAACACGCTTGAACCG3 '
GVBc: 5'GTGCTAAGAACGTCTTCACAGC3 '

Results

Based on field observations, the presence of corky bark is confirmed in Tunisia: 386 clones out of 4148 prospected exhibited corky bark symptoms.

The transmission experiments, from clones expressing field symptoms, through indexing on LN33 specific indicator plants, permitted to identify the presence of swelling and cracking of the basal internodes, rolling and reddening symptoms of indicator leaves. 77 clones out of 386 show specific response; however, these indexed positive clones for corky bark give irregular response, probably due to uneven distribution of viruses in the plants. Two types of reactions were observed on LN33 in the same repetition and by three techniques of indexing used:

- specific reaction
- unachieved reaction characterized by the presence of rolling, total reddening symptoms on indicator leaves and absence of swollen and cracked internode symptoms.

TAS-ELISA test achieved on the 77 indexed strictly positive clones, showed the presence of GVB in mixed infection on 49 samples; the 28 remaining positive clones didn't reveal the presence of GVB by serological test.

The IC-RT-PCR, used for a more appreciable detection of the GVB, confirmed ELISA results in most cases(5)

In conclusion

The results of this survey show the presence of the disease in the Tunisian vineyard as well as Grapevine Virus B (GVB) on different prospected varieties. Although the incidence of GVB is high, in indexed positive grapevine clones for Corky bark, specific symptoms on LN33 may be induced in the absence of GVB.

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DETECTION OF GRAPEVINE VIRUSES BY SEROLOGICAL METHODS IN SOUTH-EAST OF TURKEY

Çigsar I.[1] Yilmaz M.A.[2]

[1] Plant Protection Research Inst., 21100, Diyarbakir, Turkey, icigsar@hotmail.com
[2] Plant Protection Department, University of Çukurova, Adana, Turkey, yilmazma@mail.cu.edu.tr

Grapevine is widely cultivated since ancient times in Anatolia which is origin of *Vitis vinifera*. It is important crop in South-East Anatolia with the surface of about 85 Ha which covers 15% of total vineyards of Turkey. More than 150 varieties (1) are widely grown in this region which are mostly table grapes presented by native (Kabarcik, Agin, Kirmizi, Köhnü, Manzuri, Kirfok, Kerküs, Harmani, Gildini, Zeyti, Çilores, Res, Vanki, Hasani, Cevizeni, Hacikiran, Sam, Razaki, Müsküle) and foreign cultivars (Alfons, Perle de Csaba, Muscat de Vignes, Cardinal), a few wine varieties (Öküzgözü, Bogazkere, Kalecik karasi) and raisin (Besni). Productivity of grapevine is insufficient in this region that average yield is 3300 kg/Ha (2). As well as lack of water and cultural practices, the sanitary status of vineyards plays important role at this result in this region. Sanitary status of viticulture in South-East of Turkey is not clear since necessary studies are still not done.

This limited survey was done in the autumn 1997 and 1998, and mature canes were randomly collected from vineyards in Diyarbakir, Sanliurfa, Mardin, Siirt, Adiyaman, Elazig and Malatya provinces. Collected samples were analyzed for the following viruses by DAS-ELISA; GVA, GLRaV-1, -3, and -7, GFkV, GFLV, ArMV, and TAS-ELISA; GLRaV-2. Mechanical transmission to the herbaceous hosts were made 20% of total collected samples (3).

Total 171 samples were tested and 78 samples (45,6% of total samples) were found to be infested by one to five viruses. GLRaV-1 is dominant pathogen at the vineyards in this region, and it was followed by GVA, ArMV, GFLV and others. This is the first report for GVA and GLRaV-7 from South-Eastern Anatolian region. GLRaV-2 could not detected at the tested samples by the serological assay. GVA was transmitted to *N.benthamiana*.

Table 1. Incidence of eight different viruses in South-Eastern Anatolian vineyards:

Virus	Infected samples (Total 171 samples were tested)	% Infection
GLRaV-1	44	26,2
GVA	25	14,9
ArMV	19	11,3
GFLV	17	10,2
GFkV	13	7,7
GLRaV-3	10	6,0
GLRaV-7	5	3,0
GLRaV-2	-	-

According to these results, leafroll and rugoose wood complex (Kober stem grooving) are most widespread viruses in South-East Anatolian region, on the contrary of preceding reports (1, 4). The reason of this situation maybe, after phlloxera invasion at 1960s has been brought uncontrolled infested production materials by these viruses from west of Turkey to this region. Foreign cultivars are not common in the region, but native virus diseases, like GFLV and ArMV are rare than leafroll and rugoose wood complex.

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THE N-TERMINL REGION OF A TUNISIAN GFLV-COAT PROTEIN SEQUENCE: CLONING, SEQUENCE ANALYSIS, AND EXPRESSION IN *E.coli.*

Fattouch, S[1]. Achèche, H[2]. M'Hirsi, S[1]. Chabbouh, N[3]. Mellouli, L[4]. Marrakchi, M[2]. and Marzouki, N[1].

Institut National des Sciences Appliquées et de Technologie, Tunis. E-mail : Mn.Marzouki@insat.rnu.tn.
[2] Faculté des Sciences de Tunis. [3] Institut National de Recherches Agronomiques de Tunis. [4] CBS Sfax. Tunisie.

Introduction

Grapevine fanleaf virus (GFLV) is a plant virus which belongs to the genus Nepovirus of the family Comoviridae. GFLV is widely distributed in most vineyards and causes serious economic damage. Important areas of Northern Tunisia are contaminated by the virus and its corresponding vector *Xiphinema index* (1). Detection of grapevine viruses is needed in sanitary improvement programs in which the purpose is the exclusive propagation of plant virus-free material. We used powerful optimized techniques such as IC-RT-PCR (2) and RNA oligoprobe capture RT-PCR for GFLV detection in infected Tunisian grapevine plants. Therefore, we studied the virus polymorphism in our grapevine cultures. We identified two GFLV strains with a predominant one (GFLV-Tun2) using PCR-RFLP analysis. In this work we report the cloning of a 5' coat protein coding region, its sequence analysis and expression in *E.coli*.

Material and methods

Grapevine from a vineyard heavily infected with the identified predominant GFLV strain (GFLV-Tun2), located in Rafraf (North Tunisia). Immunocapture and RNA oligoprobe capture RT-PCR protocols are used to amplify DNA. The design of the primers was based on the sequence of the GFLV-F13 French isolate (3). The primer pair chosen permits the amplification of a segment of 605 nucleotides at the beginning (nt 20412) of the coat protein cistron. The 5' (upstream) primer sequence was 5' -GTG AGA GGA TTA GCT GGT AGA GG - 3'; And the 3' (downstream) primer was 5' - AGC ACT CCT AAG GGC CGT GAC C -3'. The 605pb amplified fragment from the GFLV-Tun2 strain doesn't carry a methionine initiation codon, we have cloned this sequence in the beta galactosidase coding sequence of the pBluescript SK+. The recombinant plasmid pGFLV-T2 was characterized by restriction analysis and completely sequenced. The fusion protein expression of the pGFLV-T2 was performed in a BL 21 *E.coli* strain with standard procedures.

Results

Nucleic acid and protein sequences: The nucleotide sequence of the 605pb PCR amplified clone was determined. Comparisons of this Tunisian isolate (GFLV-Tun2) sequence with that of F13 isolate (3) revealed 94% identity. Comparison of the deduced amino acid sequence of the two GFLV isolates showed a very high degree of homology (97%). This high degree of homology is previously described for this virus (4).

Expression of the fusion protein in *E.coli*: The expression of the recombinant protein in the BL21 *E.coli* strain was determined by dot-immunoblot and ELISA tests. The identification of the expressed peptide was confirmed with Western blot hybridization technique. The fusion protein was specifically recognized with GFLV specific antibodies (SANOFI) and showed the expected molecular weight.

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SANITARY SELECTION OF THE GRAPEVINE IN CYPRUS (pasted into oral/posters)

Ioannou, N.

Plant Pathology and Biotechnology Section, Agricultural Research institute, Nicosia, Cyprus.
Email: ioannou@arinet.ari.gov.cy

Grapevines comprise the third most important crop for Cyprus. During the 1980s a research project was initiated by the Agricultural Research Institute with main objective to provide the Cypriot grower with healthy propagating material of the various grapevine varieties grown in Cyprus. The first phase of the project was centered on the identification of the most important virus and virus-like pathogens of grapevines grown in the different viticultural areas of the country. Virus detection and identification was based on symptomatology, the reaction of woody indicators following graft-inoculation, the reaction of herbaceous indicators after mechanical transmission, and the use of serology, in particular the enzyme-linked immunosorbent assay (ELISA). The most important grapevine diseases identified were the infectious degeneration complex (fanleaf, yellow masaic), the leafroll complex (induced primarily by grapevine leafroll associated virus 3/GLRaV-3), *Rupestris* stem pitting, corky bark, fleck, vein necrosis and yellow speckle (1). Of these, leafroll disease was the most widespread, with average incidence of about 80% in introduced varieties and 45% in local/traditional ones (2, 3).

In order to resolve the severe virus problem on introduced varieties, an introduction program of basic (where possible) or certified material from reliable foreign sources, such as the Foundation Plant Material Service of the University of California, Davis was implemented. About 60 varieties were introduced through this program and subjected to a quarantine period of 4 years, during which the material was multiplied while being re-indexed for major virus and virus-like diseases. Material shown to be free of these pathogens was used to establish basic plantations under the responsibility of the Agricultural Research Institute, as well as mother plantations for production of healthy propagating material under the responsibility of the Department of Agriculture.

The elimination of virus and virus-like pathogens from local and other traditional varieties is being pursued through a sanitary selection program implemented since 1987. Selection of healthy clones is based both on phytotechnological characteristics such as trueness-to-type, plant vigour, productivity and grape quality, and on the results of visual, biological, and serological phytosanitary controls (5). The scheme adopted comprises the following phases: 1) The pre-selection phase of about 1 year duration during which the sanitary status of candidate clones is assessed visually and serologically with ELISA. 2) The main selection phase of 3-4 years duration during which the test material is subjected to full bio-indexing on a prescribed set of indicators, while being multiplied in a clonal propagation repository. At the end of this phase, virus infected clones are either rejected or go through the third phase. 3) Sanitization of infected clones through a combined program of tissue culture and thermotherapy, followed by complete re-indexing. The duration of this phase is 4-5 years. 4) Virus-free clones selected with the procedures described above are used to establish basic and mother plantations for the production of healthy propagating material.

So far, 286 clones representing 15 traditional varieties have been processed through the sanitary selection program outlined above. Of these, 61 clones were rejected during the pre-selection phase while 195 were found infected by one or more viruses during the main selection phase (bio-indexing). Thus, only 30 clones (less than 10%), representing 10 of the 15 varieties under sanitary evaluation, were found free of major virus and virus-like diseases and were finally selected. These 10 varieties, available now in a virus-free state, are: Mavro, Aspro or Xynisteri, Malaga, Levcada, Ophthalmo, Maratheftico, Moschato, Promara, Spourtico and Morocanella. The five varieties which proved to be totally infected will be subjected to thermotherapy and tissue culture (phase 3) for virus elimination.

During the early stages of the sanitary selection program both the basic and the mother plantations were maintained outdoors. However, it was soon noticed that clean material, either introduced from abroad or selected locally, became severely infected by GLRaV-3, transmitted by mealybugs (3, 4). In order to protect the material from the mealybug/leafroll complex, since 1997 the basic material has been transferred into insect-proof screenhouses while the mother plantations are being moved to an isolated area with relatively low mealybug activity.

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REGENERATION AND GENETIC TRANSFORMATION OF TUNISIAN GRAPEVINES USING PROTOPLASTS AND SOMATIC EMBRYOS (pasted into oral/poster)

Mliki A., B. Bouamama, R. Jardak, G. Reustle and A. Ghorbel

Institut National de Recherche Scientifique et Technique, Laboratoire d'Adaptation et d'Amélioration des Plantes, B.P 95, 2050 Hammam-lif, Tunisia
e-mail: ahmed.mliki@inrst.rnrt.tn

Tunisia possess a considerable phytogenetic patrimony of autochtonous grapevine varieties. These varieties, mostly for table grapes, are well adapted to the local edaphic conditions and represent an excellent reserve of genetic resources. The major part of these grapevines is located in the south of the country which is arid and desertic. These varieties are very appreciated by consumers and represent a vital resources for a non-negligible part of the nomads living in the desert. The plantations represent 18,6 of the national viticulture sector. Therefore, they have to be considered as a priority in the conserving and breeding programs.

Recent prospecting on the hole Tunisian territory revealed that these varieties are suffering from viral diseases, mainly fanleaf, leafroll, and Corky bark. Furthermore, these local varieties are being neglected by growers who are attracted by introduced varieties which seem to be more profitable.

Taking into consideration this situation, we collected the existing autochtonous varieties for a rehabilitation program. Today we are in possession of 61 varieties which are preserved at the National Institute of Scientific Research and Technology, as a germplasm repository and as a vitrocollection. In order to better manage this germplasm, molecular markers are being used for the characterisation and identification of these varieties.

Recently, we started a research program on the regeneration and genetic transformation of these varieties through protoplasts and somatic embryos. The short term aim is to establish reliable systems of regeneration and genetic transformation, the long term aim is to produce transgenic grapevines resistant to viral diseases. In this work, we present, for the first time, the use of protoplasts and somatic embryos to transform and regenerate Tunisian grapevines.

1. PEG mediated transformation of leaf protoplasts

Protoplasts were isolated from leaves of *in vitro* cultivated grapes. The donor plants, from varieties Sakasly and Muscat, derived from meristematic culture and were maintained on hormone free MS medium. Optimum yields were obtained from leaves of 3 to 5 week old vitroplants, digested for 13 hours under 25 rpm agitation with an enzymatic mixture containing 0.25% cellulase of *Aspergillus niger*, 0.25% cellulase of *Penicillium funiculosum*, 0.5% cellulysin of *Trichoderma viride*, and 0.2% macerozyme R-10 of *Rhizopus* sp. Cell division was obtained after 7 days in protoplasts immobilised in sodium alginate layers, at a density of $0.5.10^6$ cell.ml^{-1}, and cultivated in modified CPW-13 medium containing 4 mg. l^{-1} of NOA and 0.88 mg. l^{-1} of TDZ.

This kind of protoplasts have been used to perform genetic transformation using the Polyethylene glycol (PEG, type 3.350) technique. The DNA used for the transformation is that of the plasmid pBI426 carrying the neomycin phosphotransferase II (NPTII) and □**-glucuronidase (GUS) as selection and reporter genes. After incubation withe PEG, the protoplats were diluted** and cultivated as suspension or as sodium alginate layers. 48 hours later, transient expression of GUS gene was positively detected histochemically and fluorimetrically, in the protoplast suspensions. One week later, the histochemical GUS test carried on sodium alginate layer cultures revealed transformed protoplasts which entered cell division. These results showed clearly the success of the PEG in the transformation of grape protoplasts and offers new tools in the genetic transformation of this crop.

2 Coupling of biolistic and *Agrobacterium tumefaciens* in the transformation of somatic embryos from immature seeds

Immature seeds were used to establish somatic embryogenesis and regenerate twelve autochtonous grapes. The grape berries were harvested at the right stage of their development, disinfected and the seeds were extracted, severed into two meioties and cultivated in media based on MS and NN-69 salts supplemented with different hormonal combinations. Two weeks after their incubation in the dark, callogenesis started to develop all around the explants from the most of the varieties. This happened in all of the media independently of the hormonal nature and combination. However, not all of the calli were embryogenic. Indeed, somatic embryogenesis was preferentially inducted in calli obtained on NN-69 medium containing 2, 4-D or NAA as auxins and BAP or Kin as cytokinins. We didn't proceed to any renewing or changing in the composition of the medium and the hole embryogenesis process took place in the same medium. The embryogenic calli and the mature somatic embryos were used for genetic transformation using *Agrobacterium tumefaciens* strains harbouring a pBIN-19 plasmid that carry NPTII and GUS genes. In order to induce injuries, the explants, were bombarded with 10 µm diameter tungsten microprojectiles using a gene gun or biolistic. Few days later, positive transformants, from both explants were obtained as revealed by histochemical GUS tests. The results showed that all of the twelve varieties were sensitive to the transformation with *Agrobacterium tumefacien*. The response of the same variety was different from one *Agrobacterium* strain to another since the pGV2260 strain was more efficient than the LBA4404 and pMP90 strains. Results of transformation of embryogenic calli were better than those of mature embryos. Bombardment of mature embryos provoke their oxidation and necrosis. Therefore, the coupling of biolistic to Agrobacterium transformation represents a very promising transformation technique in grapes.

For the genetic transformation, the next step will be the use of viral genes in the aim to produce viral resistant genotypes. Protoplasts will represent a powerfull system in the analysis of the viral resistance in the transgenic grapevines. Though, it would be possible to infect transgenic protoplasts and follow the viral replication in cultures of these cells. Our experience in indexing using *in vitro* and green grafting will also be very helpfull in this purpose.

NEW INDEXING STRATEGY OF VIRUS AND VIRUS-LIKE DISEASES OF GRAPEVINE

F. Ben Abdallah, A; Fnayou, A. Mliki and A. Ghorbel

INRST, BP 95, 2050 Hammam-Lif, Tunisia
e-mail: ahmed.mliki@inrst.rnrt.tn

Most of grapevine severe diseases are caused by viruses that have been clearly identified such as GFLV (grapevine fanleaf virus) and GLaRV (grapevine leaf roll associated virus), etc., whereas virus-like diseases are caused by agents of unknown etiology, as a case of point vein necrosis and vein mosaic.

The agents of the first diseases group can be detected by immunological technics such as ELISA, whereas for the detection of the second group agents, grafting onto indexing varities (indicator vines), has to be used. Although this method is nowdays used for the detection of virus, however, it requires sophisticated materiel (highly regulated green houses). Therefore, the present work is an attempt to device an *in vitro* indexing technique proved to be efficient for virus and virus like-disease symptom expression in a very short period. This method is based on the adaptation of the usual indexing by green grafting technique (Walter et *al*. 1990) to the *in vitro* culture conditions.

Three Tunisian grapevine varieties were tested: Akhel Meguergueb, Khamri and Asli. Fragments of the vine to be tested were grafted onto different indicator varieties, each has expressed typical virus disease symptoms:

- Degeneration on *Vitis rupestris*
- Leafroll on Cabernet Sauvignon
- Vein mosaic on *Vitis riparia* Gloire
- Vein necrosis on 110 R

Our results have shown that with *in vitro* indexing, symptoms appeared within 30 to 45 days whereas with green grafting technic, symptoms do not appear before 2 to 3 months.

VARIETAL CONFORMITY OF TUNISIAN GRAPEVINES OBTAINED *IN VITRO*

F. Ben Abdallah, A. Fnayou, A. Mliki and A. Ghorbel
INRST, BP 95-2050 Hammam-Lif- Tunise

Prospections effectuated in tunisian vineyards have shown that the phytosanitary state of tunisian vineyards is degraded as anywhere else in the mediterranean region. Virus and virus like-diseases symptoms were observed everywhere. In view of such a situation, meristematic culture and apex micrografting were adopted in our laboratory to cleanse all prospected local vines. After a succeeded acclimatization, serological tests were performed periodically to control plant sanitary state, mainly for detection of GFLV and GLRaV. A soiless collection of 21 virus free local vines, growing on Rockwool substrate, is then established.

Our regular observations showed that juvenil characters appearing during the first weeks of acclimatization have disapeared progressively, while adult characters (apex and stem erected structure, presence of tendrils, phyllotaxy ½ etc.) were preserved whatever was the variety. In addition, these regenerated plants have shown a considerable active growth.

Some cautions such as reduction of subculture number, use of hormone free culture medium during growth phase, were proved to be efficient in ensuring normal fertility and maintainig genetic stability of rgenerated varieties. In some cases where the huvenil characters presisted, application of long pruning during the second year can induce the reversibility of the pheneomenon.

The relative success of this procedure allows us to extend this technique for cleansing and regenerating all the local cultivars threatened by extinction.

POTENTIAL VECTORS OF GRAPEVINE YELLOWS IN ISRAEL (pasted into oral/poster)

Tanne, E., 1 Melamed, S.,2 Koznetsova, L.,2 Davidovich, M.,1Weintraub, P.,1. Klein, M.1

1 Institute of Plant protection, ARO, Volcani Center, Bet Dagan, 50250, Israel.
2 Faculty of Agriculture, Rehovot 76100, Israel

Grapevine yellows diseases have been spreading in Israel in the last decade. They were found in all geographical regions, infecting most of the wine varieties grown in the country. They are causing substantial economical damage in susceptible cultivars such as Chardonnay, and brought about the cessation of new planting in the Golan Heights, and substantial reduction of planting in other regions. Phytoplasmas has been found to be the causal agents of this disease in many countries as well as in Israel (1). An extended survey has been carried out over several years, including all grape-growing regions and most of the wine cultivars. It revealed that three types of phytoplasma: AY, Stol and WX are associated with the disease. Phytoplasmas are transmitted by phloem feeding insects, mainly leaf- and plant- hoppers, and to a lesser extent by psyllids. Some phytoplasmas, such as AY, are vectored by a number of leafhopper species, in other cases a specific insect vectors only type of phytoplasma.

Major factors determining the spread and progress of plant diseases are inoculum density and the presence of a vector. In recent years, yellows diseases have been detected in Israel in different crops and weeds, and their phytoplasmas have been typed and found to be identical to the types infecting grapevines. The density of inoculum is therefore increasing dramatically.

This paper reports the first two steps of an epidemiological study (collecting and identifying insects carrying phytoplasma and detection of this organism in the saliva of these insects), aiming to predict disease progress, the risk of new infections, and attempts to develop control methods.

Materials And Methods

Insect sampling. Insects were collected using yellow sticky-traps or D-vac suction traps. Trapping was performed at two sites in the Golan Heights, and in the coastal-centre of Israel. Insects were collected from grapevines and weeds growing in and around these locations, and were identified.

DNA extraction from leaf/planthoppers. DNA was extracted according to the procedure described by Maixner et al (2) from insects removed from sticky-traps and stored in 70% ethanol or from insects trapped from grapevine and weeds by D- vac.

DNA extraction from TE-sucrose. TE-sucrose solution was collected after an inoculation period of 48-72 hours and DNA was extracted from it according to Zhang et al (4).

PCR and RFLP analysis. Insect and saliva DNA was amplified and RFLP-typed as cited by Tanne et al., (3).

Inoculativity assays. Five different insect species collected by D-vac from grapevines and weeds were fed on a TE-sucrose solution through a parafilm membrane according to the method described by Zhang et al (4). DNA was extracted from the sucrose solution, and PCR-tested for phytoplasma.

Results And Discussion

Throughout the survey of leafhopper and planthopper populations in the country's various regions several species have been trapped and identified. The most abundant ones were four leafhoppers, Neoaliturus spp., Macrosteles quadrapunctulatus, Circulifer spp.Orosius albicintus and one planthopper Hyalesthes obsoletus. Molecular analysis confirmed that all five species were carrying phytoplasma. Samples from this two-year survey are summarised in table 1

The seasonal trapping of H. obsoletus indicates that this planthopper has two generations per year. Neoaliturus spp. was collected from early spring till late in the year on various plants in different regions. O. orientalis and M. quadripunctulatus were trapped mainly from weeds, using D-vac throughout the summer months, as well as Circulifer spp. that were trapped on sticky traps.

Inoculativity tests were carried out with: Neoaliturus, O. orientalis, M. sexnotatus, Circulifer spp. and Anaceratagalia laevis (trapped in large numbers in 1999 in the Golan Heights) The PCR analysis of DNA extracted from the saliva, indicated the presence of phytoplasma in all five insects tested. Nested PCR and RFLP analysis detected AY in specimens of all five species. In addition, WX was detected in some Neoaliturus and STOL in A. laevis.

A pre-condition for the transmission of phytoplasmas is their circulation in the vector and their presence in the saliva. We have detected phytoplasma in the insect bodies and have demonstrated their presence in the saliva of five different leafhoppers, indicating that phytoplasma interacts with the insect in a circulative manner. We therefore suggest that these leafhoppers can be considered inoculative.

Transmission tests are currently being carried out to establish the vectorship of these insects.

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AN ASSOCIATION OF ROOTSTOCK STEM LESIONS IN VITIS SPECIES AND DIFFERENT GRAFT-TRANSMISSIBLE AGENTS (pasted into oral/poster)

J. K. Uyemoto[1], A. Rowhani[2], and D. Luvisi[3].

[1]USDA-ARS, [2]Department of Plant Pathology, and [3]Cooperative Extension, University of California, Davis 95616; [3]Bakersfield 93307, USA.

Introduction

In 1992, 15 table grape-rootstock trials were established in Kern County, California. Collectively, the trials involved 5 cultivars grafted onto 9 rootstocks plus own-rooted scions with each plot comprised of 5 complete replications per scion-rootstock combination. Various horticulture measurements were made. Three sites were planted with Redglobe. A fourth site planted with a different scion was later top-worked with Redglobe; thereby creating grapevines with an interstem. A year or so after vineyard establishment, all four plots with Redglobe scions, with and without interstems, on 5BB, 3309C, 1103P, and 5C roots had severely declined or died. Because involvement of a graft-transmissible agent (GTA) was suspected, a study was initiated in 1996 to determine the etiology of the Redglobe decline problem. Some preliminary findings are presented.

Materials And Methods

Fifty-grafted plants of Cabernet Sauvignon each on 9 different rootstocks (Ramsey, Harmony, Freedom, 039-16, 5C, 5BB, 3309C, 101-14Mtg, and 1103P) were established in individual rows during spring 1996. In the summer 1997, known sources of grapevine leaf roll associated viruses (GLRaVs 1, 2, 3, 4, 5, and an undescribed GLRaV), and corky bark, and two Redglobe sources (from UCD Foundation Plant Materials Service and a commercial nursery) were collected. These were chip-budded (2 per plant) onto 3 plants per scion-rootstock combination. Several non-grafted grapevines per rootstock served as healthy controls. Chip-bud success was determined ca. 30 days later and the grapevines read for canopy symptoms a year after.

Results

Among a total of 239 graft-inoculated grapevines, 16 inoculum chip-buds on 8 plants had not survived 30 days. On all others, at least one bud chip was sound and all inoculum sources had one to three indicator scion-rootstock representatives in the trial. Also, all GLRaV and corky bark source produced leaf roll symptoms on one or more Cabernet Sauvignon scions per rootstock during 1998. Canopy symptoms of a solid red leaf coloration were observed also on at least one grapevine grafted with either Redglobe sources and involved the rootstocks: 1103P, 5BB, 5C, and 3309C i.e. the same ones affected in the Kern County trials. Interestingly, the GLRaV 1 source also induced similar red foliaged scions on the rootstock 3309C, and two others, Freedom and 101-14Mtg.

In spring 1999, several of the symptomatic grapevines grafted with Redglobe or GLRaV 1 did not break dormancy. For example, among Redglobe-infected grapevines, only 2 of 4 symptomatic 5BB; 2 of 3 on 3309C; zero of 2 on 5C; and zero of 1 on 1103P survived and resumed spring growth. With GLRaV 1, 3 symptomatic grapevines on rootstock 101-14Mtg survived also. However, 3 of 3 symptomatic grapevines per rootstocks Freedom and 3309C had not.

During summer 1999, leaf petioles were assayed by RT-PCR using primers designed to detect grapevine virus B, GLRaV 2, Rupestris stem pitting virus (RSPaV) strains 1, 2, & 3, and the Redglobe closterovirus (for latter, see Rowhani et al. in these proceedings). In the fall 1999, all surviving, symptomatic grapevines grafted with Redglobe or GLRaV 1 were sacrificed and trunk stems inspected.

RT-PCR assays. With the occasional exception, symptomatic and asymptomatic grapevines receiving Redglobe inocula tested positive by RT-PCR with the Redglobe closterovirus primers. Among the Redglobe-rootstock stem lesioned (RSL; see below) grapevines, two 3309C and one 5BB collections were positive. Also, three 101-14Mtg grapevines with RSL (grafted with GLRaV 1) were negative with all 4 primers. Overall, and irrespective of inoculum sources, a few grapevines were positive for RSPaV. The appropriate homologous reactants and healthy controls were included in all of the RT-PCR assays and reacted as expected.

Wood markings. By November 1999, RSL of varing severities (length and area) were developed and the more extensive necrosis created on the rootstock 3309C with Redglobe inoculum. Moderate- to smaller-sized lesions was observed also on 5BB grafted with Redglobe, and 101-14Mtg with GLRaV 1. In the previous November (1998), one symptomatic Redglobe grafted 3309C grapevine was sacrificed. Also, in 1999, the rootstock of another Cabernet Sauvignon on 1103P grapevine (regrafted in 1998 with Redglobe and displaying red foliage in 1999) was removed and inspected. The stems of both rootstocks, with year-old infections, appeared normal. In addition, we examined the rootstocks: 1103P, Harmony, Ramsey, 5BB, 5C, and 039-16 grafted with the GLRaV 1 source and 101-14Mtg grapevines grafted with GLRaVs 2, 3, 4, 5 the undescribed GLRaV, corky bark, and two Redglobe and several non-grafted grapevines. All of these grapevines were asymptomatic and stem surfaces were smooth.

Discussion.

Based on these results, the rapid decline and death of grapevines was associated with two GTAs causing RSL. The stem lesions required more than a year to fully develop. One GTA was present in Redglobe and a different one in the GLRaV

1source. In the Redglobe source, we have isolated a new closterovirus and developed specific RT-PCR primers for it. The primers amplified only homologous (Redglobe) virus. No further reactions using Redglobe virus primers were noted when RT-PCR assays included preparations of all virus sources employed in our inoculation trial. The Redglobe closterovirus has an estimated coat protein of 22 kDa and share 74% homology with GLRaV 2 (Rowhani et al., these proceedings). At this time, we do not believe GLRaV 1 causes RSL, but that this symptom was more likely due to an as yet unconfirmed GTA.

In the literature, grapevine graft incompatibilities have been reported by Greif et al. (1) for GLRaV 2 on Kober 5BB. Likewise, Monis and Bestwick (2) found by western blot assays an association of a 24 kDa protein and rootstock-scion incompatibility symptoms expressed on rootstocks: 5C, 3309C, SO4, and *Vitis riperia* Glorie. In the current trial, our source of GLRaV 2 did not incite graft incompatibility symptoms on nine different rootstocks, including 5BB, suggesting the existence of a rather wide variation among GLRaV 2 isolates or that some GLRaV 2 sources may be co-infected with a RSL-like GTA.

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TOWARDS DIRECT INOCULATION OF GFLV INTO GRAPEVINE

Valat[1] L., Mode[1] F., Mauro[2] M.C., Burrus[1] M., Pinck[3] L.

[1]Laboratoire de Biologie et Physiologie Végétales, Université de Reims Champagne-Ardenne, BP 1039, F-51687 REIMS Cedex 2, [2]Moët & Chandon, Laboratoire de Recherche, BP 140, F-51333 EPERNAY Cedex, [3]IBMP du CNRS, Département de Virologie, 12 rue du Général Zimmer, F-67084 STRASBOURG.
e-mail : monique.burrus@univ-reims.fr

Introduction

The " court-noué " disease, one of the most widespread and damaging diseases of grapevine, is caused by two nepoviruses, GFLV (*Grapevine fanleaf virus*) and ArMV (*Arabis mosaïc virus*). In nature, inoculation of these viruses solely occurs through *Xiphinema* feeding on grapevine roots; mechanical inoculation is therefore not feasible (1, 2). This characteristic renders studies of grapevine resistance to GFLV very difficult, time-consuming and hardly reproducible as plants have to be challenged in nematodes-infected vineyards or greenhouses. So far, *in vitro* cultures of *Xiphinema* have been described but they remain difficult to master. We have developed two different methods of direct GFLV inoculation, namely protoplast electroporation and particle bombardment.

Materials And Methods

Grapevine was *in vitro*-propagated by one-node cuttings or embryogenic cell suspension. *Chenopodium quinoa* was grown from seeds in the greenhouse (3).

GFLV (isolates F13 and GH) and ArMV were routinely propagated on *Chenopodium quinoa*. Virus and RNA extractions and purifications have been described previously (4).

Protoplast isolation was done from leaves or embryogenic cell suspension. Electroporation was done with 10ng RNA or 2µg GFLV under different electrical settings. Replication was detected by Western blotting using anti-P38.

For bombardment, the pVT-GUS plasmid was used. It was propagated in *E. coli* HB 101-p35S, purified by the Qiafilter plasmid maxi kit method (Qiagen).

Leaves were bombarded once with 0.5mg gold particles coated with 1µg DNA or with 1µg virus. The Helios Gene Gun (Biorad) as used at 2.04MPa. Two leaves were bombarded by plant.

Results

It is of great importance to study virus multiplication in grapevine at two different levels: the cell level with electroporation of protoplasts; the whole plant level with bombardment of *in vitro* or greenhouse grown plants. Indeed, GFLV spread *in planta* is the result of cellular events, cell-to-cell movement and long distance migration.

Electroporation

Electroporation of mesophyll protoplasts led to significant GFLV replication detected as soon as 24 hours after treatment. Optimum was observed between 48 and 72 hours. GFLV replication was observed using either RNA or viral particles. Inoculation was not specific of a given GFLV strain as replication of both GFLV-GH and GFLV-F13 was observed. Replication of ArMV was also possible in grapevine protoplasts.

Finally, inoculation was not specific of the protoplast type: GFLV replication was observed in mesophyll or embryogenic cell suspension-derived protoplasts.

Bombardment

Preliminary experiments of grapevine leaf bombardment with plasmidic DNA were conducted in order to determine best shooting conditions: 0.6µm-gold particles, 2.04 MPa shooting pressure. Although quality of cartridges varied among preparations, less than 2% of them gave no GUS expression at all.

Introduction of GFLV into grapevine through biolistics requires an efficient coating of gold particles with RNA or virus particles, while keeping their infectious potential. *C. quinoa* which is a good indicator of GFLV infection was the best candidate to test cartridges.

Shooting conditions were 1.36MPa and 0.6 µm gold particles. GFLV replication was observed in *C. quinoa* after 6 days with RNA or at day 10 with GFLV particles: clearly visible chlorotic lesions appeared on newly formed leaves. All these conditions adapted to grapevine will be presented.

These new methods offer other possibilities to understand virus multiplication and spreading in grapevine, to screen transgenic plants or to test hybrids plants for their resistance to the virus.

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ANALYSIS OF THE VARIABILITY IN THE MOVEMENT PROTEIN GENE AMONG GRAPEVINE FANLEAF AND ARABIS MOSAIC NEPOVIRUS ISOLATES (pasted into oral/poster)

Wetzel T., Meunier L., Jaeger U., Krczal G and Reustle G

Zentrum Gruene Gentechnik, Staatliche Lehr- und Forschungsanstalt, Breitenweg 71, 67435 Neustadt/Weinstrasse, Germany
Email: twetzel.slfa-nw@agrarinfo.rpl.de or thierrywetzel@hotmail.com

Grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV) are the causative agents, together with other nepoviruses, of the widespread fanleaf disease in grapevine. They also represent one of the main virus problems in vineyards in Rheinland Palatinate, which is the biggest wine producing area in Germany. In order to develop a PCR-based assay for the detection of the GFLV/ArMV in grapevine, a survey of the variability of these viruses was implemented, to enable the design of primers susceptible to amplify a broad range of GFLV and/or ArMV isolates. This analysis is also of importance for the choice of viral sequences to be used in the establishement of transgenic varieties/rootstocks against GFLV/ArMV, if a broad range resistance is desired. The movement protein gene, described as being conserved among nepoviruses, was chosen for this study.

GFLV and ArMV isolates were collected from different infected parcels in South-West Germany but also in other wine growing countries. Total RNA was extracted from infected leaf material using the Plant RNeasy kit, (Qiagen), and/or from phloem scrapings from infected wooden material using the newly commercially available RNA extraction kit from D-Genos. A fragment of approximately 1.3 kb, encompassing the full movement protein gene, was amplified from GFLV or ArMV RNA by RT/PCR using degenerate and/or specific primers. The PCR products were cloned and sequenced. Pairwise sequence comparisons and multiple alignments were performed using the Maximum Matching and Multiple Sequence programs respectively from the DNAsis programme package (Hitachi).

Preliminary sequence comparisons between the different GFLV isolates sequenced so far revealed homologies of at least 97% at the amino acid level. However, the variability observed at the nucleotide sequence level ranges between 3 and 12%. For ArMV, homology levels of 93% and 85% were found at the amino acid level and at the nucleotidic level respectively between the movement protein gene of the local isolate of ArMV and the corresponding sequences of other ArMV isolates found in the databases. The homologies found in the movement protein genes between the Rheinland Palatinate isolates of GFLV and ArMV were of 83% at the amino acid level, and 73% at the nucleotidic level.

Additional isolates of both GFLV and ArMV have been collected. The amplification by RT/PCR of their movement protein gene, and the cloning and sequencing of the resulting PCR products is currently underway. The results of these investigations, and their signification in terms of design of a PCR-based detection test and/or establishment of transgenic resistant grapevines, will be presented and discussed.

TRANSMISSION OF GRAPEVINE FANLEAF VIRUS BY XIPHINEMA INDEX TO DIFFERENT NEWLY BREEDED ROOTSTOCKS IN GREENHOUSE AND FIELD TRIALS (pasted into oral/poster)

Ipach, U.[1], Kling, L.[1] and Rüdel, M.[2]

[1]Staatliche Lehr- und Forschungsanstalt für Landwirtschaft, Weinbau und Gartenbau, Fachbereich Phytomedizin, Neustadt/W., Germany;
[2]Neustadt/W., Germany
uipach.slfa-nw@agrarinfo.rpl.de

Introduction

Grapevine fanleaf virus (GFLV) transmitted by its specific vector Xiphinema index is one of the major viruses affecting grapevine world-wide causing important damages (1). Since 1988 there exists no possibility in Germany to combat virus transmitting nematodes by nematicides. Therefore investigations are forced to find alternative combating methods. One method would be the use of nematode- and virus-resistant rootstocks respectively. The phylloxera breeding programmes have shown that hypersensitive reactions in root cells were responsible for the resistant reaction against phylloxera (2). By continued selection the variety 'Börner' was developed from a crossing of Vitis riparia 183 G and Vitis cinerea Arnold (3) and is now registered for planting in different countries in Europe, USA and Canada. It would be possible that 'Börner' and other cinerea-crossings can prevent the transmission of GFLV by nematode as well. In trials with pot-plants Sopp examined the host plant ability of 'Börner' against X. index and showed that it was not such a good host compared with usually used rootstocks. The susceptibility of Börner against GFLV by nematode transmission could not yet definitely cleared up (4).

In the following 'Börner' and some other new created rootstocks (cinerea-crossings) were tested for their ability to reduce X. index populations and their possible GFLV-resistance. Tests were first conducted with pot-plant experiments followed by examining the results in field tests.

Material And Methods

The "double plant test-method" was chosen for greenhouse virus-transmission tests (5 replicates, 40 adult Xiphinema index/pot, 10 cm in diameter per pot, loess-loam/sand/gravel = 1:3:5) using rooted cuttings of V. vinifera 'Gewürztraminer' diseased with GFLV as virus donor. Rooted cuttings of the following healthy varieties were used as test plants: Kober 5BB (V. berlandieri x V. riparia, control), Börner (V. riparia x V. cinerea), A9 (Rici, V. riparia x V. cinerea), C3 (Cina, 125AA x V. cinerea) and 5 other V. cinerea-crossings named only by working-numbers A8, A17, B4, C5 and C7. Four months after planting the number of nematodes was determined and the test grapevines were replanted in vegetable mould. For three years the shoot tips of the test plants were serologically tested by DAS-ELISA for reinfection with GFLV.

In a GFLV-infected vineyard with X. index as vector a part of the diseased plants was rooted out and replanted with the following healthy combinations: V. vinifera 'Scheurebe' as scion grafted on 125 AA (V. berlandieri x V. riparia, control, 38 plants), Börner (44 plants), A9 (Rici, 35 plants) and C3 (Cina, 37 plants) as rootstocks. Every year all of these newly planted grapevines were tested serologically (DAS-ELISA) for reinfection with GFLV.

Results And Discussion

Although 'Börner' had reduced propagation rates of X. index compared to the control 'Kober 5BB' (5) the pot-plant trials showed that the transmission of GFLV by X. index was possible in principle to V. cinerea-crossings. One year after beginning the test the first GFLV-infected plants could be found in Kober 5BB and A17. After the second year in all rootstock groups some plants were tested positive for GFLV except A9. Three year after inoculation no rootstock group was completely healthy.

These results could be confirmed by the field trial. Two years after replanting the infection rate of all combinations reached at least 55 % whereas the control plants (Scheurebe/125 AA) were infected 100 %. In the third year at least 80 % of the vines of each rootstock group tested were infected with GFLV.

The results obtained show that in particular 'Börner', but also the other V. cinerea-crossings tested, are not suitable to be used as alternative combating method to solve the GFLV-X. index-problem in vineyards.

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UIGV AND GLRaV-1 INTERACTIONS IN MIXED INFECTION OF GRAPEVINE (pasted into oral/poster)

Faoro F.[1], Scattini G.[1], Sant S.[1] and Gugerli P.[2]

[1] CNR, Centro Miglioramento Sanitario Colture Agrarie, Milano, ltaly. [2] Federal Agricultural Station of Changins, Nyon, Switzerland

Introduction

An unidentified isometric grapevine virus, provisionally named UIGV, was first isolated from Chasselas vines (1). UIGV is a phloem-limited virus, that: i) does not cause apparent external symptoms in the infected vines, including *Vitis rupestris*; ii) is found very frequently in mixed infections with other grapevine viruses, mainly closteroviruses ; iii) induces peculiar cytophatic effects (double-membraned vesicles in plastids) in the infected parenchyma phloem cells (2).

Field observations carried out in the past years on grapevine clones doubly infected by UIGV and one of the different leafroll-associated closteroviruses (GLRaV-1 to 7) suggested that the former may interfere with symptom expression of the latter. In particular, it seemed that the presence of UIGV could often account for a reduction in symptom severity. To throw some light on the interaction between UIGV and leafroll associated closteroviruses we have investigated two Barbera accessions, one doubly infected by UIGV + GLRaV-1 (MIB7) and the other by GLRaV-1 only (MI2B).

Materials And Methods

MIB7 and MI2B accessions were free from other leafroll associated closteroviruses (GLRaV-2 to 7), GFKV and the most frequent nepoviruses as assessed by ELISA and indexing. GLRaV-1 infecting MIB7, though serologically indistinguishable from GLRaV-1 infecting MI2B, had been previously regarded as a hypovirulent virus strain due to the lack of symptoms showed by all the MIB7 plants over a 5-year observation period, in contrast with the typical leafroll symptoms visible on MI2B vines. The two clones were green-grafted on rooted cuttings of different cultivars (Barbera, Cabernet, Pinot Noire) and symptom expression, virus concentration and ultrastructural alterations were monitored over two growing seasons and compared with those observed in the mother plants. Ultrastructural alterations were also compared with the cytopathic effects detected in a Chasselas clone infected by UIGV only.

Results And Discussion

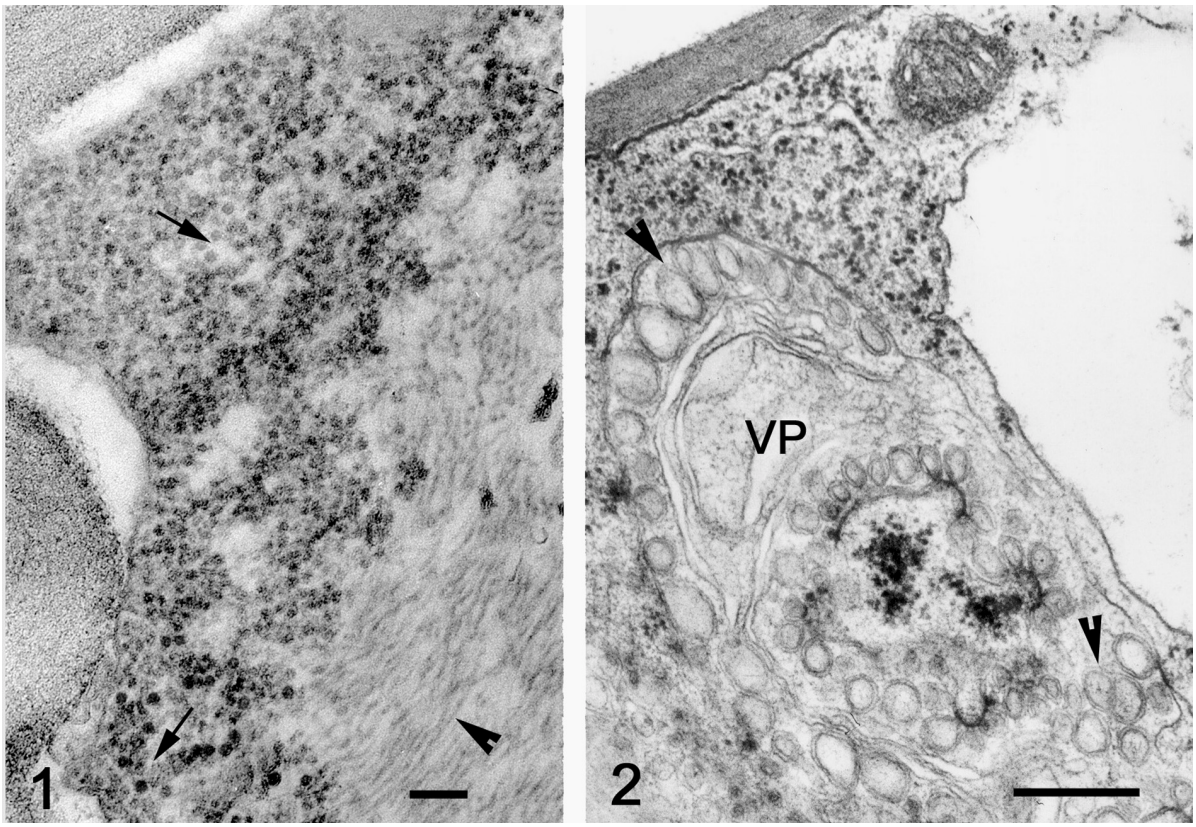
Surprisingly enough, all the infected indicator vines grafted with MIB7 showed during the second growing season the typical leafroll symptoms at the same extent as those grafted with MI2B, thus demonstrating that the GLRaV-1 infecting MIB7 is not an hypovirulent strain, at least in the tested cultivars. Therefore, the absence of symptoms in MIB7 accession could be really due to the presence of UIGV. ELISA tests showed that GLRaV-1 concentration in MIB7 mother plants was usually twice lower than in MI2B vines. On the contrary, in all the inoculated plants, either grafted with MIB7 or MI2B, GLRaV-1 concentration was similar after the second growing season (see table 1). As regards UIGV, ELISA detected it only in the MIB7 mother plants, but not in the grafted ones, probably because of its very low concentration and/or the low antiserum titer. In fact, electron microscopic observations proved that some UIGV particles were present in MIB7 grafted plants.

Ultrastructural observations carried out on MIB7 mother plants revealed the presence of both closterovirus particles and isometric virus-like particles (~20 nm in diameter) in the phloem cells (Fig.1), besides some vesiculating plastids (Fig.2), very similar to those observed in UIGV infected vines. However, in very rare occasions the vesiculating mitochondria typical of GLRaV-1 infection (3) were present. Instead, in the Cabernet and Pinot Noire plants grafted with MIB7, closterovirus particles and vesiculating mitochondria were easily detected, while isometric-like particles were very rare and no vesiculating plastids were visible. The cytopathology of either the MI2B mother plants and the plants grafted with this accession were very similar and consisted of vesiculating mitochondria and bundles of closterovirus particles in different arrangements and amount.

The above results indicate that UIGV can interfere with symptom expression of grapevine leafroll disease when UIGV infection has been well established (possibly since a long time before) and the virus titer has risen to a discrete concentration in the host plant. Instead, in the plants infected contemporaneously by UIGV and GLRaV-1, that is the case of our grafted vines, the closterovirus replication is probably more efficient than the UIGV one, possibly due to the high ratio mitochondria/plastids (replication sites of GLRaV-1 and UIGV, respectively) present in the parenchyma phloem cells. It will then be interesting in the following years to monitor symptom expression and virus titer of the plants grafted with MIB7 to verify if UIGV can rise up to a concentration suitable for interfering with leafroll symptoms.

Table 1- Results of ELISA tests after two growing seasons (o.d. : + = 0.3÷0.69; ++ = 0.7÷1.2; +++= >1.2)

	MIB7	MI2B	Barbera/ MIB7	Barbera/ MI2B	Cabernet/ MIB7	Cabernet/ MI2B	Pinot Noire/ MIB7	Pinot Noire/ MI2B
GLRaV-1	+	++	++	+	+++	+++	++	++
UIGV	+	—	—	—	—	—	—	—



Figs. 1 and 2. Thin sections of a leaf midrib from a MIB7 Barbera plant double-infected with UIGV and GLRaV-1; bars=100nm.
Fig.1. Aggregates of UIGV (arrows) and GLRaV-1 (arrowheads) particles filling the whole sieve tube.
Fig.2. A vesiculating plastid (VP) in a parenchyma phloem cell; note the double-membraned vesicles typical of UIGV infection (arrowheads).

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ISOLATION AND PARTIAL CHARACTERIZATION OF TWO NEW VIRUSES FROM GRAPEVINE

Adib Rowhani, Y.P. Zhang, D.A. Golino, and J.K. Uyemoto[1], [1]USDA-ARS, Department of Plant Pathology, University of California, Davis, CA 95616, USA

Introduction

In the course of cDNA cloning of dsRNA species from grapevine, two new grapevine viruses have been cloned and partially sequenced. The first of these viruses was found in Redglobe, a popular table grape variety developed in California which is now being grown throughout the world. The other virus was found co-infecting vines used for a study on grapevine rupestris stem pitting disease.

In 1996, we received a report that Redglobe scions grafted on certain rootstocks dies a year or two after planting in the vineyards. Because the propagating material originated from our foundation vineyard at FPMS, UC Davis, the Redglobe source was re-indexed across a panel of grape and herbaceous virus indicator hosts. Following up to two years incubation, these bioassays detected only the rupestris stem pitting disease. Concomitantly, another trial using indicator plants of Cabernet Sauvignon scions established on 9 different rootstocks were graft-inoculated with the FPMS source of Redglobe. After 15 months incubation, a decline of infected indicator scions occurred on 4 rootstocks (see Uyemoto et al., these proceedings). In the laboratory, a dsRNA species was purified from source Redglobe similar in size known for closteroviruses, which has been cloned, partially sequenced, and some details are presented.

In a separate study and during characterization of grapevine rupestris stem pitting associated virus (GRSPaV) (Zhang et al., 1998), a second virus, with properties described for members of the marafivirus group, was discovered from the same cDNA library. Data for this new virus to be presented also.

Materials And Methods

A dsRNA species was extracted from a Redglobe vine (Valverde et al., 1990) and a cDNA library was obtained using random priming as described by Zhang and Rowhani (1999). Selected clones were sequenced and a pair of PCR primers was made from HSP70 region and used for PCR detection of the associated virus. The sequence of PCR primers were: RGHSP227V 5' GCG ACT CCA GCA ACT TTA GTG A 3' and RG777C 5' GTC TAA CGA AAG ATC GGG TTC TAA G 3'. The sequence was compared with other viruses in the GenBank.

The same procedure was used also in cloning and sequencing the new marafivirus in grapevine. Selected clones sequenced. The sequence was compared to other GeneBank viral sequences. PCR primers were designed and used to test a number of grapevine collections.

Results And Discussion

A dsRNA species of about 15kb in length was isolated from the Redglobe vine. The sequence information showed that the isolated virus had 74% similarity to grapevine leafroll associated virus 2 (GLRaV-2). The PCR primers consistently detected the associated virus in Redglobe vines or other vines to which Redglobe had been grafted. Samples from Redglobe vines showed a weak reaction in Western blot and ELISA using antiserum obtained from Sanofi for GLRaV-2. However, repeated testing of Redglobe vines on leafroll indicator host, Cabernet Franc, in the field did not produce any symptom typical of leafroll infection. Considering the fact that this new virus has about 74% sequence homology (from the segments which have been sequenced up to date) and weak cross reaction with GLRaV-2 antibody, it may be one of the variants of leafroll disease complex in grapevine which is latent in the indicator host Cabernet Franc. In addition, this virus is economically important in commercial vineyards because certain rootstocks are sensitive to this virus. Data are not available on the impact of this virus on yield and plant performance.

The genome of the virus isolated from grapevine co-infected with GRSPaV had a high GC content (about 75%) similar to oat blue dwarf virus (OBDV), a member of marafivirus group and its nucleotide sequence shared 55.7% similarity. To check the association of this virus with rupestris stem pitting (RSP) disease, 17vines which tested positive for RSP on field indicator *Vitis rupestris* cv. St. George, were tested by PCR using specific primers designed for the new virus. In this PCR test only 3 vines tested positive indicating that this virus is not associated with RSP. The same pair of PCR primers was used to test 39 vines which were positive for grapevine fleck virus on St. George indicator and all tested negative. Additionally, the 3 vines which tested positive for the virus tested negative on St. George indicator host for fleck disease. These data support the concept that the isolated virus is not associated with grapevine fleck disease and its etiology is unknown.

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GRAPEVINE RUPESTRIS STEM PITTING ASSOCIATED VIRSUS: POPULATION DIVERSITY, TITER IN THE HOST AND POSSIBLE TRANSMISSION VECTOR

Adib Rowhani[1], Y.P. Zhang[1], J. Chin[1], A. Minafra[2], D.A. Golino[1], and J.K. Uyemoto[3], [3] USDA-ARS, [1]Department of Plant Pathology, University of California, Davis, CA 95616, USA, and [2]Dipartimento di Protezione delle Piante, Universita di Bari, Bari, Italy

Introduction

Grapevine rupestris stem pitting (RSP) is a graft-transmissible disease which causes slow decline in the growth of grapevine (Goheen, 1988). RSP is detected by indexing on *Vitis rupestris* cv. St. George. Usually small pits form on the woody cylinder immediately below the inoculum chip bud, but occasionally pits and grooves occur elsewhere on the woody cylinder. Rupestris stem pitting associated virus (RSPaV), in the Foveavirus genus (Martelli and Jelkmann, 1998) was associated with the disease. The entire genome of this virus was sequenced and consisted of 8,725 nucleotides excluding a poly A tail (Zhang et al., 1998; Meng et al., 1988). A study was initiated to investigate: 1) variability among the coat protein gene sequences of different RSPaV isolates, 2) seasonal variation of virus titer in grapevine, and 3) possible natural transmission of the virus by pollen.

Materials And Methods

Seventeen different isolates of RSPaV were included in sequence comparison of the coat protein gene. A pair of primers, RSP52, 5' TGA AGG CTT TAG GGG TTA G 3' and RSP 53, 5' CTT AAC CCA GCC TTG AAA TG 3' which flank the coat protein region, were designed from previously published sequence and used in RT-PCR assay. All PCR products were cloned into TA cloning vector and sequenced. The sequences were compared using the PileUp Program of GCG (Wisconsin Genetic Computer Group, Madison, WI) with a gap weight of 3 and a gap length weight of 1. Monitoring virus titers in the field in different season were studied with 4 RSP-infected and 2 healthy vines. Two samples per plant (shoot tips and basal leaves) were collected at two-week intervals (May through October) and tested by RT-PCR using dilution end point of extract to determine virus titer. This experiment continued through the year. In addition, pollen samples were collected from 71 vines and tested by RT-PCR for RSPaV. The pollen grains were washed and prepared as described by Digiaro and Savino (1992). After the final wash, the pollen grains were crushed mechanically in microfuge tubes in presence of carborandum and prepared for RT-PCR.

Results

Phylogenetic analysis of 17 isolates of RSPaV showed that these isolates separated into three major groups (denoted strains 1, 2, and 3). The nucleotide sequence alignment showed that the members in different group share as little as 79% similarity. PCR primers were designed from this comparison to amplify specifically virus members in a group and universally all 17 isolates in a single reaction. Irrespective of tissue source virus titers were similar during the sample period. Overall, virus titers increased in August and continued to October. In pollen assays, RSPaV was detected in 19 collections. In a preliminary test, 10 seed samples (20 seeds per extract, seeds harvested from positive grapevines) had tested negative by RT-PCR for RSPaV.

Discussion

Variations among the sequences of the coat protein gene of different isolates of GRSPaV were found. The differences in sequence homology of these 17 isolates was great enough to put them into three distinct groups or strains with as high as 79% homology between them. Using strain-specific primers and testing number of GRSPaV-infected vines with different degrees of symptom severity (observed on the woody cylinder of the indicator host St. George), we were not able to associate any of these strains or combination of strains to symptom expression on the indicator host. Therefore, it seems that symptom severity (or mildness) may cause by the association of a different, unidentified virus, or the initial titer of virus in the candidate vine used for indexing. Titer changes of GRSPaV in grape grown in the field were monitored from May to October. In this experiment no significant changes in virus titer was observed during this period. Additionally, virus titer was quite similar in both young and old basal leaves, which suggested that any type of tissue could be used in PCR for virus detection. Virus was detected in pollen grains even after thorough washing in 1% SDS indicating that the virus may be carried internally by the pollen. We do not have any other evidences to show in which parts of the pollen the virus is located. Our PCR test revealed that few of the grapevine seedlings tested were positive for the virus, but it is not clear yet that whether the virus transmitted through the ovary or pollen to these seedlings. The test results from seeds collected from infected vines were not conclusive.

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SIMPLIFIED SAMPLE PREPARATION METHOD AND ONE-TUBE RT-PCR FOR GRAPEVINE VIRUSES

Adib Rowhani[1], L. Biardi[1], R. Johnson[2], P. Saldarelli[3], Y.P. Zhang[1], J. Chin[1], and M. Green[2]

[1]Dept. of Plant Pathology, University of California, Davis; [2]Center for Plant Health, Canadian Food Inspection Agency, Sidney, British Columbia; [3]Dipartimento di Protezione delle Piante, Universitn degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italy.

Introduction

Diagnostic methods employing the polymerase chain reaction (PCR) provide the most sensitive means currently available for detecting viruses in woody plants. However, sample preparation for PCR testing has proven to be difficult, due to plant compounds that inhibit various enzymes in the reaction. Several methods were developed to overcome this limitation. Immunocapture PCR which utilizes virus-specific antibodies (Nolasco et al., 1993, Rowhani et al., 1995) which is fast and relatively inexpensive, however, the virus initially has to be purified for production of antibodies. Total nucleic acid extraction can be used as an alternative method of sample preparation, but this method is cumbersome, and is not practical when analysis of several hundred of samples is required. Finally, commercially available sample purification kits can be used (MacKenzie et al., 1997), but such kits are very costly. The sample preparation method we developed and report on in this paper is simple, fast, and inexpensive and requires minimal sample manipulation. In addition, samples processed by this method can be stored at -20C for several months. To further simplify the process of sample analysis, we have developed a one-tube RT-PCR method. In this method, all the necessary reagents for reverse transcription and PCR amplification are added to the RNA template. Once the reaction is complete, the sample can be loaded onto an agarose gel for analysis.

Materials And Methods

The extraction protocol was a modification of La Notte *et al*. (1997) and consisted of the following steps: grape leaf petioles or cambial scrapings were macerated in extraction buffer (1.59g/L Na_2CO_3, 2.93g/L $NaHCO_3$, pH 9.6, 2% PVP-40, 0.2% bovine serum albumin, and 0.05% Tween 20) at a 1:10 to 1:50 dilution. Two microliters of this crude extract were added to 25 µl of GES buffer (0.1M glycine, pH 9.0, 50mM NaCl, 1mM EDTA) containing 0.5% Triton X-100 and 1% ß-mercaptoethanol. The mixture was then denatured at 94C for ten minutes and placed on ice. Two microliters of the GES mixture were then added to 23 µl of PCR cocktail containing 12.71 µl sterile distilled water, 2.5µl 10X PCR buffer (500mM KCl, 100mM Tris-Cl, pH 9.0), 0.7µl 50mM $MgCl_2$, 2.5µl 10X sucrose-cresol (20% (w/v) sucrose, 1mM cresol red), 0.5µl 10mM dNTP mixture, 1.24µl 10mM DTT, 1.25µl each sense and anti-sense primers (10µM), 0.25µl *Taq* DNA polymerase (5U/µl, Promega), and 0.05µl AMV reverse transcriptase (1.08U/µl, Gibco-BRL). The samples were subjected to RT-PCR in GeneAmp 9600 thermal cycler (Perkin Elmer) as follows: reverse transcription reaction consisting of 30 minutes at 54C followed by 35 cycles of 94C for 30 seconds, 54C for 45 seconds, and 72C for 60 seconds. A final extension at 72C for 7 minutes was followed by storage at 4C until removal from the thermal cycler. PCR products were resolved on a 1.4% agarose gel and stained in ethidium bromide solution.

Results And Discussion

We have developed a simple extraction method for the preparation of samples for RT-PCR and combined it with a simple one-tube RT-PCR protocol that allowed for fast processing of samples. We have employed this method for the detection of grapevine viruses: grapevine leafroll-associated virus (GLRaV)1, GLRaV 2, GLRaV 3, GLRaV 4, GLRaV 5, grapevine virus A (GVA), GVB, GVD, grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine fanleaf virus (GFLV), grapevine fleck virus (GFkV), and arabis mosaic virus (ArMV).

We have compared leaf, petiole, and cambial scraping samples using the GES extraction method. Cambial scrapings gave consistent results, however petiole and leaf tissue samples sometimes gave false negative results, attributed to higher concentration of inhibitory compounds found in leaf tissue. We have also compared various dilutions of the samples. Our findings indicated that although a 1:10 dilution (w/v) of sample in extraction buffer gave consistent results using cambium, petiole and leaf samples needed to be diluted at a 1:20 or 1:50 to eliminate false negatives due to inhibitors. Use of one-tube RT-PCR was evaluated for all of the above-mentioned viruses. After optimization of reaction conditions, all virus-specific primers amplified a product of the correct molecular weight. In addition, utilization of AMV-reverse transcriptase allowed use of higher temperature in the synthesis of cDNA, therefore, templates with secondary structures and high GC content were denatured and could be amplified. Specifically, GFkV could not be detected by PCR using the conventional reverse transcription conditions of 42C due to its high GC content.

The simplified sample preparation method reported here combined with the one-tube RT-PCR method allowed for very simple, fast, and cost-effective analysis of a large number of samples. In addition, use of an indicator dye in the RT-PCR cocktail allows the PCR products to be loaded directly onto a gel after the reaction is complete. The simplicity of the method eliminated chances of error and contamination.

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RFLP ANALYSIS INDICATES THAT THE GENOME OF GRAPEVINE FANLEAF VIRUSR IS COMPLEX

P. Naraghi-Arani[1], Adib Rowhani[1], and M.A. Walker[2], [1]Department of Plant Pathology, and [2] Department of Viticulture and Enology, University of California, Davis, CA 95616

Fanleaf degeneration is caused by grapevine fanleaf nepovirus (GFLV), and was named for the distinctive fan-shaped leaves of infected vines. Vein-banding and yellow mosaic are also associated with this disease. GFLV isolates associated with these three symptoms have been designated GFLV 100, 101 and 102 respectively (2). These and other isolates are serologically and physiochemically indistinguishable from one-another (1, 3).

The objective of this study was to determine the genetic variation among GFLV isolates. Reverse transcriptase polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) were used to examine variation in coat protein region (CP) of nine GFLV isolates. A 1,500bp fragment of GFLV was amplified from the 3' end of RNA2 by immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR). This specific fragment was recovered from nine California GFLV isolates, four from the UC Davis Clonal Virus Collection (2), and one from Italy. Sequence data from Genbank showed that AvaII should cleave the PCR fragment into 6 diagnostic pieces. However, AvaII digestion of the PCR products generated many fragments, of varying intensities, with patterns distinct to each viral isolate. The data suggest that GFLV infections are composed of a complex mixture of closely related genomes (quasispecies).

To further elaborate the quasispecies nature of the GFLV genome, virus from each isolate was passaged in *Chenopodium quinoa* and *Niccotiana occidentalis* and the resulting virions examined with the above-described system. The resulting RFLPs suggest rapid evolution in the type and titer of quasispecies members after passage in alternate hosts. Quasispecies may be composed of members with different host optimizations that are selected by their adaptation to varying hosts. Unique RFLP patterns seem to modifications as a result of several passages in particular host plants.

Samples were taken from shoot tips and young leaves for IC-RT-PCR analysis. Plant material was ground in IC-PCR buffer and extract loaded onto microtiter plates pre-coated with anti-GFLV IgG using a modification of the method used by Brandt et. al. (5). After an overnight incubation, plates were washed, and viral RNA released using TritonX-100 and heat. The RNA was then subjected to RT-PCR and a 1,500bp DNA fragment was generated from all isolates. After digestion with AvaII, the RFLP banding patterns were complex and suggested that infection by a single bipartite RNA genome was unlikely.

Several lines of evidence support this conclusion. The relative intensities of the bands comprising each RFLP were quite different one from the other indicating non-identical DNA molecules. Secondly, when the band lengths of the isolates were summed none equaled 1,500bp, and ranged from a few to many times 1,500bp. Thirdly, after passage into *Chenopodium quinoa* the RFLP pattern changed markedly for each isolate, indicating adaptation of quasispecies members to their new host environment. Finally, the cloning of 1,500bp fragments into bacteria and RFLP analysis of specific clones produced patterns that would normally be expected from a single DNA species.

GFLV appears to consist of quasispecies capable of co-infecting grape and alternate hosts, resulting in the multiple RFLP bands detected in this study. GFLV infection appears to consist of a set of closely related, co-infecting genomes.

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THE USE OF PCR FOR THE DETECTION OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3.

Van der Merwe, M.*, Kasdorf, G. G. F. & Pietersen, G.

Virology Unit, ARC-Plant Protection Research Institute, Roodeplaat, Pretoria, South Africa, 0001.
*E-mail: nipbmvm@plant1.agric.za

Introduction

Grapevine leafroll is one of the most widespread viral diseases of grapevine. Seven serologically distinct closteroviruses have been associated with grapevine leafroll disease and have been designated GLRaV-1 to –7. GLRaV-3, together with GLRaV-1, has most often been associated with leafroll disease and observations suggest that leafroll disease does not spread readily within infected vineyards except when GLRaV-3 is present, emphasizing the economic importance of GLRaV-3 (2). To date five species of mealybugs and one scale insect have been identified as vectors of GLRaV-3.

Use of virus-free propagation material in vineyards is the primary control measure. Sensitive assays are required to ascertain the virus status of treated plant material because of low viral titer in infected grapevine, uneven distribution throughout the plant, high concentration of phenolic compounds (which interfere with assays) and varying virus concentrations throughout the year. The advantage of PCR is its high specificity and the theoretical sensitivity to detect a single target molecule in a complex mixture (3).

We compared the sensitivity of immunocapture-reverse transcription-nested PCR (IC-RT-nested PCR), using primers designed by Ling et al (2) to ELISA and ISEM. Various extraction buffers were compared and various applications investigated such as the length of time that a macerated sample can be stored in extraction buffers and still test positive for the virus.

Materials And Methods:

Winter-wood cuttings of a specific vine (93/1053), cultivar Black Spanish, were used to compare the sensitivity of ISEM, ELISA and IC-RT-nested PCR. Bark scrapings were cut into small pieces, mixed and divided into three equal parts. Each pool was macerated in liquid nitrogen and suspended in one of the buffers given below, each of which is preferred for one of the three assays.

Exponential dilution series from 1:5 to 1:10 240 (plant material: extraction buffer) (w:v) were prepared in each of the three buffers and each technique was done in its preferred buffer. ISEM was done in all three buffers and the nested PCR was done using buffers A and B. ELISA tests were done with antiserum produced by Goszczynski et al (1).

Table 1: Extraction buffers used in assays

IC-RT-nested PCR (A)	ISEM (B)	Grapevine ELISA (C)
0.02M PBS pH7.4	0.1M PO_4 buffer pH7	0.1M Tris-HCl pH7.6
+0.5ml Tween 20	+2% PVP	+4% PVP-P (insoluble)
+2% PVP		+0.2% ß-mercapto-ethanol
+0.2% ovalbumin		+2% Triton X-100

As some of the results of the first experiment were inconclusive, a second experiment was done. Here the sensitivity of the three assays were compared using macerated bark shavings of the vine (93/1096), cultivar Margaretha resuspended in buffer C only. Dilutions of 1:50 to 1:12 000 (w:v) were made and tested in each of the three assays.

In a third experiment designed to compare the influence of buffers A and C on the sensitivity of ELISA and IC-RT-nested PCR, macerated bark shavings of the vine (93/0939), cultivar LN33 was used. Exponential dilution series in each of the two buffers were made ranging from 1:3 to 1:24 576 (w:v) and tested in both assays.

Black Spanish (93/1053) and Margaretha (93/1096) were macerated in liquid nitrogen, resuspended in extraction buffer and stored at 4°C. These samples were tested periodically over a period of months with IC-RT-nested PCR to determine after what period the virus can still be detected.

IC-RT-nested PCR was used in vector studies to detect GLRaV-3 in *Planococcus ficus* and *Acia lineatifrons*. Insects were tested in groups of ten, five as well as single insects.

The GLRaV-3 IC-RT-nested PCR was used in our laboratory for the indexing of propagation material and rootstocks from nurseries and independent producers that had tested negative in ISEM.

Results And Discussion

In the first experiment virus was still detected at the highest dilution (1:10 240) in both ISEM and ELISA when using buffer C. When using buffers A and B the PCR was respectively 4 and 16 times more sensitive than ISEM. No conclusions could be made concerning PCR and ELISA as no limit of detection was reached in the ELISA test.

When comparing the three assays using buffer C it was shown that the PCR is the most sensitive of the three assays, detecting the virus at dilutions of 1:10 750 with ISEM detecting the virus at 1:10 500 and ELISA at 1:2 000.

When comparing the two buffers, PCR detected the virus at 1:768 in buffer A while ELISA detected the virus at 1:48 (PCR was 16 times more sensitive). In buffer C PCR detected the virus at a dilution of 1:6 144 while ELISA detected it up to a dilution of 1:192 (PCR was 32 times more sensitive). It was shown that buffer C improved detection of GLRaV-3 in PCR and ELISA compared to buffer A. The use of buffer C increased detection in ELISA 4-fold and in PCR-8 fold, making this the preferred buffer for Grapevine in both ELISA and PCR testing.

Black Spanish (93/1053) macerated in buffer A as well as in buffer C still tested positive after 3 months in both buffers. Margaretha (96/1096) macerated in buffer C was also still positive after 3 months. This stability allows the possibility of material being prepared at one location and being tested elsewhere within a three-month period. This .allows for reduction in labor and materials if general large-scale screening is done with ELISA and then verified later with PCR.

Insect vector studies were assisted by using the PCR to test for the presence of the virus in known and potential vectors. Because of the small size of these insects the PCR was the preferred method for virus detection. Also the sensitivity of the IC-RT-nested PCR procedure, shown to be capable of virus detection in single insects (where ELISA results were negative), has potential value for epidemiological studies as it is capable of ascertaining whether a vector does acquire the virus from the host.

Rootstock and propagation material, which had tested negative in ISEM, was tested with the nested PCR and revealed some positive results. Virus infected material was prevented from being propagated which otherwise would have been propagated.

Each of these three assays tested has advantages and disadvantages and they can be used to complement each other. IC-RT-nested PCR is the most sensitive assay, but is time consuming and impractical when testing large numbers of samples. This can be overcome by applying the colorimetric-PCR assay (3).

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IMPROVED DETECTION OF FLAVESCENCE DOREE AND RELATED PHYTOPLASMA IN THE ELM YELLOWS GROUP IN DIFFICULT MATERIAL, WITH SPECIFIC PCR PRIMERS THAT AMPLIFY A VARIABLE NON RIBOSOMAL DNA FRAGMENT

Clair D.[1], Frelet A.[1], Aubert G.[2], Collin E.[3] and Boudon-Padieu E.[2]

[1] Equipe de Recherches sur les Phytoplasmes, INRA Dijon, France
[2] Génétique et Amélioration des Plantes, INRA-Dijon, France
[3] Ressources Génétiques et Plants Forestiers, CEMAGREF, Nogent-sur Vergisson, France

Detection of phytoplasmas in grapevine and woody hosts is sometimes tedious in spite of the high sensitivity of molecular-based methods, mainly because of their low and irregular titre. The reliability of detection also depends on the period of sampling. Hence, the literature most often reports of characterisation of phytoplasma associated to affected plants with conspicuous symptoms; very few reports are given of detection of phytoplasma in non symptomatic hosts or in dormant material. There is a need for routine diagnosis of Grapevine Yellows (GY) on dormant material that could be included in sanitary certification scheme of grapevine, especially for Flavescence dorée (FD) which is a dangerous quarantine phytoplasma. Such protocols should include both reliable sampling procedures and reliable diagnosis in plants with very low titre of the pathogenic agents.

In former studies, specific non-ribosomal DNA of FD phytoplasma had been randomly cloned and several fragments had been selected for use as specific probes in DNA-DNA hybridisation (1). Later on, two of these fragments, i.e., FD2 (1.7 kb) and FD9 (1.3 kb), were partially sequenced at both ends and oligonucleotides were selected that could be used as primers for amplification in PCR of FD2 and FD9 fragments, respectively (2, 3). Primers FD2 f/r were specific for PCR amplification of FD2 fragment in FD isolates, and they did not detect periwinkle-maintained AEY or ULW phytoplasmas, isolated from American and European elms respectively (2). Primers FD9f/r were demonstrated to specifically amplify all known phytoplasma in the Elm yellows group (also referred to as 16S rV) in periwinkle reference strains and in natural hosts such as grapevine and elm (3, 4, 5, 6). Furthermore, RFLP of the FD9 fragments amplified in reference strains and in natural hosts allowed to differentiate at least 9 subgroups, among which two FD *sensu stricto* isolates from France (namely FD70 and FD88) (4) also present in Italy, grapevine isolates from Italy and Germany not associated to *S. titanus* transmission (4, 5), AEY and ULW strains and HD1, a phytoplasma associated to hemp dogbane (*Apocynum cannabinum*) in USA (6). In addition, a survey of FD epidemics in four regions of Veneto (Italy) showed a geographical distribution of the two FD *sensu stricto* grapevine isolates, referred to as 16SrV-C and 16SrV-D by the authors (5) and similar to FD70 and FD88, respectively.

Amplification and restriction analyses of the FD9 fragment are thus most valuable tools both for specific detection and for epidemiological studies of phytoplasmas related to FD in grapevine, in other natural hosts and in known vectors or potential vectors (2, 5, 7). However FD9 primers have not being widely used in routine diagnosis because they lacked sensitivity and failed to give a positive response in a number of difficult samples of grapevine which were nevertheless affected with FD. Instead, a nested-PCR procedure with two pairs of primers for rDNA amplification followed with RFLP analyses (8) is currently used by the Plant Protection Laboratory in charge of GY survey in France. Even though, positive detection is not regularly obtained with symptomatic material.

Full length sequences of the FD9 fragment in FD70 and AEY reference strains and in three German grapevine isolates were recently obtained and aligned (W. Reinert and M. Maixner, personal communication). We have used the latter data to delineate and construct new primers that could improve the detection of EY-group phytoplasma in natural hosts. The new primers were designed according to several prerequisite conditions: they should be located close to the position of the initial FD9f/r primers (renamed f1 and r1), in order to retain as much information as possible in RFLP analyses; they should contain about 20 nucleotides with a “high” G+C content and G and C be located near to the ends of primers, both conditions to provide a good stability at the annealing step of amplification; finally their respective locations should allow their use in combinations for nested-PCR assays for still higher sensitivity. According to the latter requirements, two alternative forward primers, namely FD9f2 and FD9F3 and one reverse primer, FD9r2, were designed.

The use of either pair FD9f2/r1 or FD9f3/r2 in one-step PCR assays showed a higher sensitivity of detection than the former pair FD9f1/r1 on conventional samples such as leaf tissues, main veins and petioles of symptomatic grapevine and elm. Their use in nested assays provided an excellent signal, especially from phloem chips or whole tissues of dormant elm and grapevine collected in winter, taken either on canes, or on rootlets and medium-size roots of the plants. The restricition patterns of either FD9f2/r1 or FD9f3/r2 products of several EY-group strains and isolates were shown to be as informative as the restriciton patterns of the initial FD9 product, since only very low molecular weight restriction fragments were lost.

The new primers will help in development of routine diagnosis towards sanitary certification protocols and in quick assessment of strain variability in the EY-group phytoplasmas, especially of grapevine origin. It will also be valuable to investigate the possible products of the phytoplasma gene to which the FD9 fragment belongs, as reports on its variability seem to be related to serological differences (9, 10) and to epidemic traits (5, 10). In particular, the relationship to transmission characters by vectors will be investigated.

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OCCURRENCE AND SYMPTOM EXPRESSION OF BOIS NOIR IN BURGUNDY OVER A 15 YEARS PERIOD

Larrue J., Caudwell A. and Boudon-Padieu E.

Equipe de Recherches sur les Phytoplasmes, INRA Dijon, France

Bois noir (BN) and Vergilbungskrankheit (VK) were described almost 40 years ago (1, 2) because of their epidemiological importance and of their similarity of symptoms with Flavescence dorée (FD). They were actually known in these viticultural areas since the 40's at least and they were reported to have progressed very rapidly in the middle of the decade (3). Since the development of phytoplasma detection and characterisation methods they have been shown to be associated with similar etiological agents (i.e., a stolbur phytoplasma) (4, 5) and similar diseases associated to stolbur phytoplasma were identified in a number of European vineyards (6, 7, 8, 9). Their occurrence can be related to the wide geographical distribution of the main vector of stolbur phytoplasma, *Hyalesthes obsoletus* (10, 11).

The significance of BN/VK propagation through grapevine multiplication material has been examined (3, 12) and appeared to be very limited. However, epidemiological surveys of VK and BN report of very different situations regarding the importance of the disease. In some cases nowadays it is regarded as an endemic disease with a low incidence (13) and little economic consequences on harvest. Other situations nevertheless report of very high sanitary and economic importance (14) and are very similar to the situation in Burgundy in the past, when as many as 90% of the stocks were affected (3). It is believed that some factors are still unknown, such as the presence of alternative vectors (Maixner, personal communication) or varietal differences in sensitivity, tolerance or possible recovery.

Several vineyard plots affected with BN in Burgundy were noted every year over a 15-year period. All the stocks were noted each year with notes ranging from 1 (one cane affected) to 5 (whole stock affected). Data show that symptoms developed regularly on the same stocks and that some stocks were never affected though they were in-between deeply affected stocks. Symptoms on individual stocks would sometimes disappear for a few years, then appear again with variable severity.

The study presents an analysis of the different situations and evolution in time, with regard to the age of the plants and origin of the planting material. Tolerance and temporary recovery can be assumed from the data. The importance of these phenomenon in the propagation of the disease through planting material will be discussed.

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PCR DETECTION OF PHYTOPLASMA-DNA IN ARTIFICIAL FEEDING MEDIUM OF PHYTOPLASMA INFECTED LEAFHOPPER : A METHOD TO SCREEN FOR NATURAL VECTORS OF PHYTOPLASMA DISEASES.

Tanne E.[1], Clair D.[2], and Boudon-Padieu E.[2]

[1] Department of Virology, ARO, The Volcani Center, Bet Dagan, Israel
[2] Equipe de Recherches sur les Phytoplasmes, INRA Dijon, France

Grapevine yellows (GY) associated to phytoplasma are known worldwide. However, only the main vectors of Flavescence dorée (FD), Bois noir (BN) and Vergilbungskrankheit (VK) are known sofar. Alternative or secondary vectors for the latter diseases and vectors of other GY have not been identified yet. This is also the case for vectors of numerous phytoplasma diseases of other crops.

Sensitive techniques (ELISA, DNA hybridisation and PCR) are now available to detect phytoplasma in individual naturally or experimentally infected insects (1, 2, 3, 4, 5). These procedures are being used in epidemiological studies for searching potential vectors of phytoplasma diseases or surveying wild populations of a vector species (2, 3, 4, 6). However it is known that phytoplasma transmission by insect vectors is an active process with several key-events (7), some of which depend on specific recognition between phytoplasma and host cells (8). After feeding acquisition, multiplication and movement of phytoplasma in the gut and the haemocell, the penetration of salivary glands is a prerequisite to transmission (1, 8, 9). Consequently, detection of phytoplasma in the body of wild insect specimen is not enough to demonstrate transmission ability. On the other hand, transmission trials require large quantities of insects and of phytoplasma susceptible plants on which the insects could feed and survive (3, 4, 6). We present here an alternative method which could be used at a large scale to check if insects are infective, by feeding them on an artificial medium and then checking the medium for the presence of phytoplasma (10).

Euscelidius variegatus leafhoppers were reared in healthy colonies or in colonies fed-infected with FD or Clover phyllody (Phy) phytoplasmas. Leafhoppers in each colony were hatched simultaneously and were assumed to be all of the same age. Leafhoppers in infected rearings were fed-infected during a limited period (7 days) and the delay from acquisition was dated (11). Batches of 20 leafhoppers taken from one colony were individually fed for 3 days on 200 µl of TE-sucrose medium (10 mM Tris, 1 mM EDTA, 0.5% sucrose) covered with stretched Parafilm. They were then transferred individually for feeding transmission to healthy broadbean (*Vicia faba*) seedlings (1). The 200 µl TE-sucrose samples fed by infected leafhoppers or control healthy leafhoppers as well as control non-fed samples were submitted to a 15,000 *g*, 20 mn centrifugation. DNA was extracted from the individual pellets according to Daire et al. (12) and assayed for phytoplasma DNA by PCR amplification using universal (13) or specific primer pairs (12). Alternatively, 2 µl of feeding medium were added to the PCR mixture for "direct" amplification. After a one-week feeding transmission period on broadbean, individual leafhoppers were collected and PCR assayed for the presence of phytoplasma in their whole body.

A first experiment was conducted with FD or Phy-infected leafhoppers known to be in the inoculative period (1, 8, 9). Positive results were obtained as well from directly assayed 2µl aliquots of infected leafhopper-fed TE-sucrose samples as from DNA extracted from whole 200 µl samples. No amplification was obtained from control healthy leahopper-fed samples or from control non-fed samples. PCR on the whole body of leafhoppers from the infected rearings tested positive.

A second experiment was set up to correlate detection in fed-infected TE-sucrose samples to stages of inoculativity build-up in the insect body. Twenty male *E. variegatus*, known to be more regularly infected than females (1), were randomly taken from an infected colony at different times of the acquisition period, i.e., 1 day, 4 days, 7 days (i.e., during the presence of infected plant source in the cage), then weekly, after the removal of infected plant source, until the 8th week from the 1rst day of acquisition (1, 8). In spite of the poor survival on feeding medium of some of the leafhoppers, the data obtained showed that leafhoppers could release phytoplasma into feeding medium starting on the 3rd week after acquisition and on following weeks. At the same periods of time they could feed-inoculate broadbean seedlings, as formerly reported (1, 2, 9). However, it was shown in addition that leafhoppers from the 1st-day batch and the 4th-day batch could transmit phytoplasma to the TE-sucrose medium though they were not able to feed-inoculate plants, suggesting a mere mechanical transport of phytoplasma that had been just acquired and had remained in the stylet of the leafhopper.

Improved feeding medium and device will be developed with reared leafhoppers or planthoppers and with wild insects. The method could be used to screen for potential vectors of phytoplasma diseases among a variety of species trapped in the field. Selected species could then be assessed for actual transmission to the sensitive crop. The method would also be useful to assess the actual inoculative status of wild populations of known vectors trapped in particular areas or periods of time in the year.

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LATENT INFECTION BY PHLOEM-LIMITED VIRUSES IS LINKED TO LOWER FRUIT QUALITY IN FRENCH-AMERICAN HYBRID GRAPEVINES

Kovacs, L.G., Hanami, H., Fortenberry, M., and Kaps, M.L.

Missouri State Fruit Experiment Station, Southwest Missouri State University, Mountain Grove, MO 65711, USA.
E-mail: lgk378t@mail.smsu.edu

Infection of French-American and American *Vitis* hybrid cultivars by leafroll viruses is latent and has received little scientific and viticultural attention. Recently, however, several laboratories have reported that grapevine leafroll-associated virus 3(GLRaV-3) is widespread in mid-western and eastern North America where these hybrid grapes are commonly cultivated (1, 2, 3). The latent nature of the infection does not necessarily mean that the agronomic performance of the vines is not affected. There is experimental evidence that symptomless *Vitis vinifera* cv. Riesling vines infected by a mild leafroll agent accumulate slightly less carbohydrates in their fruit than their healthy counterparts (4). The purpose of this survey study was to test if there is a linkage between natural infection by GLRaV-3 and reduction in grape performance indices in two French-American hybrid cultivars. It was also our objective to determine if the GLRaV-3 strains detected in these vines are virulent on a *V. vinifera* indicator.

The study was conducted in a Vidal blanc and St. Vincent commercial vineyards in south-central Missouri. These vineyards had a GLRaV-3 incidence of 59 and 51%, respectively. In addition, 55% of the GLRaV-3-infected Vidal blanc plants harbored grapevine fleck virus (GFkV) also. The sample vines tested serologically free of North American nepoviruses. The infection status of several hundred vines was tested serologically in 1998 and 1999. Of the vines that tested consistently positive or negative in both years, large samples (37 to 50 vines) of virus-free, GLRaV-3/GFkV-, and GLRaV-3-infected Vidal blanc, and virus-free and GLRaV-3-infected St. Vincent vines were selected for further study.

Canes from randomly selected sample vines of various viral infection status were rooted, and the resulting plants were green-grafted with Cabernet franc or *Vitis rupestris* St. George indicators for the expression of GLRaV-3 and GFkV symptoms, respectively. Serological tests showed that all infected test plants transmitted the virus to indicators by day 150 post-grafting. Virus transmission was associated with symptom expression with the exception of a single St. Vincent-Cabernet franc graft which failed to show leafroll symptoms. Both the leafroll and fleck symptoms were characteristic of the inducing virus. Cabernet franc indicators grafted on GLRaV-3-positive test plants developed severe leaf curling and discoloration, suggesting that the GLRaV-3 strains harbored by the French-American hybrid sample vines are virulent in *V. vinifera.*

Table 1. Berry weight and fruit quality indices of St. Vincent and Vidal blanc vines of various viral infection status

	St. Vincent		Vidal blanc		
	Virus-free	GLRaV-3	Virus-free	GLRaV-3	GLRaV-3/ GFkV
Average Berry Weight (g)	2.57 ± 0.15[a]	2.43 ± 0.16***	1.63 ± 0.11	1.55 ± 0.09***	1.52 ± 0.08***
Soluble Solids of Juice (°Brix)	16.34 ± 1.34	15.69 ± 1.56*	18.38 ± 0.99	17.97 ± 1.03*	17.31 ± 0.77***
Titratable Acidity of Juice (g/L)	11.34 ± 0.68	11.95 ± 0.74***	8.12 ± 0.76	8.87 ± 0.65***	9.26 ± 0.75***
Jucie pH	3.00 ± 0.04	3.02 ± 0.05	3.10 ± 0.08	3.08 ± 0.07	3.05 ± 0.09**
Number of Plants in Sample	50	37	41	47	50

[a] mean ± s.d.
***, **, and *, significantly different from the corresponding virus-free Vidal blanc or St. Vincent statistic in two-sample t-test at the 99.9%, 99%, and 95% probability level, respectively.

During the 1999 growing season, the sample vines were balance-pruned and individually studied for pruning weight, yield components, and fruit quality indices. No relationship was found between the presence of GLRaV-3 and indices of pruning weight, number of clusters per vine, clusters per shoot, and berries per cluster in either of the two cultivars. None of the above indices differed between GLRaV-3/GFkV-co-infected and virus-free Vidal blanc either. Average berry weight and fruit quality

indices, however, were found to be statistically different in virus-infected and virus-free plants (see Table 1 for these results). The average berry weight in GLRaV-3-infected plants was 5% lower ($P<0.001$) in GLRaV-3 positive Vidal blanc and St. Vincent plants than in their virus-free counterparts. Titratable acidity was 9 and 5% higher in the fruit of GLRaV-3-positive Vidal blanc and St. Vincent plants, respectively, and 14 % higher in GLRaV-3/GFkV-co-infected Vidal blanc plants ($P<0.001$). The 14% increase in titratable acidity was accompanied by a 6% decrease in fruit soluble solids in GLRaV-3/GFkV-co-infected Vidal blanc ($P<0.001$). GLRaV-3-infection alone was associated with only a slight decrease in soluble solids that was significant only at $P<0.05$ in both cultivars. Juice pH was slightly lower only in GLRaV-3/GFkV-co-infected Vidal blanc fruit.

The results of the graft-transmission experiments indicate that the lack of symptoms in GLRaV-3-infected French-American hybrid grapevines is not due to the weak virulence of the viral strains, but rather, to the resistance of the plants. This conclusion is supported by the findings that GLRaV-3 infection is associated with only a slight (albeit statistically significant) reduction in performance indices in two of these cultivars.

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MOLECULAR EVIDENCE OF PHYTOPLASMA TRANSMISSION TO GRAPEVINE BY *METCALFA PRUINOSA* (SAY) IN ITALY

Guadagnini M.[1], Mori N.[2], Alberghini S.[2], Carturan E.[2], and Girolami V[2]., Bertaccini A.[1]

[1]UCI-STAA, Patologia Vegetale, University of Bologna, Italy.
[2]Istituto di Entomologia Agraria, University of Padova, Italy.

Objectives

Metcalfa pruinosa (Say) is a Flatid widespread in Italy since more than ten years on all cultivated and wild plants. Preliminary investigations carried out on insects collected on diverse wild and cultivated plants, showing phytoplasma symptoms, have demonstrated that *M. pruinosa* can carry phytoplasmas belonging to different subgroups such as 16SrI-B, 16SrI-C-related, 16SrX and 16SrXII-A, usually the same phytoplasmas that were detected on infected plants (3) Considering that in the recent outbreak of yellows epidemic in grapevine in Veneto region (Italy) several molecularly distinguishable phytoplasmas were identified (2) The relationships between phytoplasmas and the possible role of *M. pruinosa* as vector were investigated. Two researches were carried out: A) detection and identification of phytoplasmas in *M. pruinosa* samples collected in vineyards with yellows diseases or reared under laboratory conditions to verify phytoplasma presence in different life-stages (i.e. eggs, nymphs and adults); B) transmission trials with *M. pruinosa* from grapevine with yellows to phytoplasma-free grapevine potted vines derived from micropropagation.

Materials And Methods

A) Phytoplasma detection. Template nucleic acid from eggs (batches of three), nymphs (batches of two) and adults (single insect) of *M pruinosa* was obtained following the procedure described in (8) with slight modifications (method a). Eggs were also extracted by boiling at 64°C for 2 minutes (method b). Template nucleic acid from adults (batches of 100 insects) and plant material was extracted also as described by Prince *et al.* (6) (method c). Phytoplasma control strains employed were from periwinkle (CHRY, "Chrysanthemum yellows" group 16SrI-A; AY, "Aster yellows" and AAY "American aster yellows" group 16SrI-B; CPh, "clover phyllody" group 16SrI-C; IPVR, "Italian periwinkle virescence" group 16SrXII-A; and EY, "Elm yellows" group 16SrV-A). Phytoplasma detection in *M. pruinosa* and in the different plants species used for the research was performed by nested PCR with universal primer pairs R16F1/R0, R16F2n/R2 and 16R758f/16R1232r (M1/M2) (4, 5) under conditions previously described (7). To control non-specific priming, nested-PCR using primers R16 (I) F1/R1 and R16 (V) F1/R1 specific for groups 16SrXII, 16SrI and 16SrV respectively were also carried out on positive samples. Sterile water as negative control was included in each PCR experiment. RFLP analyses of amplified products were performed with *Mse*I endonuclease.

B) Several transmission trials to different plant species were carried out during two years in late spring and summer (from May to August) using *M pruinosa* samples captured on symptomatic grapevines in a limited area. The insects used in the transmission experiments belonged to the same groups employed for the phytoplasma detection described above.

In 1998 more than two hundred *M. pruinosa* insects were used to inoculate caged phytoplasma-free plants of periwinkle and sunflower, the insects were allowed to feed until they die. Further transmission was carried out in four grape plants (see below) single caged with 100 insects each collected at the end of August.

In 1999 four transmission tests were carried out using micropropagated potted vines cv Chardonnay, kindly provided by M. Borgo (MiPAF, Conegliano, TV, Italy) and *Rubus* spp. plants: all the material was tested with nested-PCR (as described above) before transmission. I) *M. pruinosa* eggs collected in infected vineyards were caged together with two grapevines and a *Rubus* sp. plant. II) Eggs laboratory lied on *Acer campestris* in 1998 were caged with a grapevine and a *Rubus* sp. plants. III) Nymphs of different ages (L_3 - L_5) field collected, were caged with a grapevine plant: two repetitions were done with 25 insects and four repetition were done with 50 insects. IV) Adults of *M. pruinosa*, field collected at the beginning of July, were caged separately on 2 grapevine plants using 20 and 100 insects respectively.

Results And Conclusions

A) Results on phytoplasma detection in *M. pruinosa* field collected and laboratory grown are summarized in table 1. Eggs tested in 1998 showed the presence of mainly 16SrI-B or C phytoplasmas, while in those tested in 1999 only 16SrXII-A phytoplasmas were detected. Phytoplasma presence was statistically estimated in 3.4% of the egg tested (9.8% of batches of 3 eggs each). In the nymphs the same phytoplasmas detected in the eggs were identified with very lower percentages (2%). Phytoplasma presence in one laboratory reared nymph batch confirms the viability of the prokaryotes detected in the eggs as reported for phytoplasma of the same groups in *S. titanus* (1). In *M. pruinosa* adults field collected, phytoplasmas of the same groups were detected together with other belonging to 16SrV group. The percentage of phytoplasma detection was in agreement with the extraction method adopted: method a) give a 10.5% of phytoplasma detection while method c) allow detection in 30% of tested samples. It is interesting to underline that 50% of positive insects were carrying 16SrV phytoplasmas.

B) The plants insect-inoculated in 1998 and repeatedly tested during 14 months resulted always phytoplasma-free. Three of the four transmission experiments carried out in 1999 i.e. I), II) and III), give negative results in the tests carried out till now. Experiment IV) provides evidence of phytoplasma transmission by *M. pruinosa:* a first nested PCR with template from a

batch formed by the two inoculated grapevines show positive results in September 1999. RFLP analyses identified the detected phytoplasmas as belonging to 16SrI group, in particular the patterns resulted to be identical to the one 16SrI-C- related described in *M. pruinosa* (3) (Fig. 1a).

Further amplifications were carried out at the end of October out templates from single grapevine insect-inoculated and revealed that the grapevine inoculated with 100 *M. pruinosa* insects was the positive plant. RFLP analyses show a pattern identical to 16SrI-C phytoplasmas (Fig. 1b). Phytoplasmas belonging to the 16SrI group were identified not only in the adults of *M. pruinosa* tested but also in grapevine plants in the area where the insects were collected (Bertaccini *et al*, unpublished). Further studies are necessary to verify transmissibility of the other phytoplasmas detected in *M. pruinosa* and to define its epidemiological importance as phytoplasma vector in vineyards.

Table 1: Phytoplasma detection and identification in the different life stages of *M. pruinosa* derived from laboratory (L) and from field (F) in nested PCR.

Life stage	Sample	From	Extraction method	M1/M2	16Sr(I)F1/R1	16Sr(V)F1/R1	Phytoplasmas identified
Egg	M.p. 1-98	L	b	+	+	-	16SrI-B
	M.p. 18-98	L	b	+	-	-	not identified
	M.p. 2-98	L	a	+	-	-	16SrI-B or -C
	M.p. 19-98	L	a	+	-	-	not identified
	M.p. 20-98	L	a	+	-	-	not identified
	M.p. 21-98	L	a	+	-	-	not identified
	M.p. 3-99	L	a	+	+	-	16SrXII-A
	M.p. 4-99	L	a	+	+	-	16SrXII-A
	M.p. 5-99	L	a	+	+	-	16SrXII-A
	M.p. 6-99	L	a	+	+	-	16SrXII-A
Nymph	M.p. 7-98	L	a	+	+	-	16SrXII-A
	M.p. 8-99	F	a	+	+	-	16SrI-B
	M.p. 9-99	F	a	+	+	-	16SrXII-A
Adult	M.p. 10-98	F	a	+	-	-	16SrV
	M.p. 11-98	F	a	+	-	-	16SrV
	M.p. 12-98	F	a	+	-	-	16SrXII-A
	M.p. 13-98	F	a	+	+	-	16SrXII-A
	M.p. 14-98	F	a	+	-	+	16SrV
	M.p. 15-98	F	c	+	-	+	16SrV+16SrI-B or-C
	M.p. 16-99	F*	a	+	+	-	16SrXII-A
	M.p. 17-99	F	c	+	-	+	16SrV

*, Sample from Tuscany

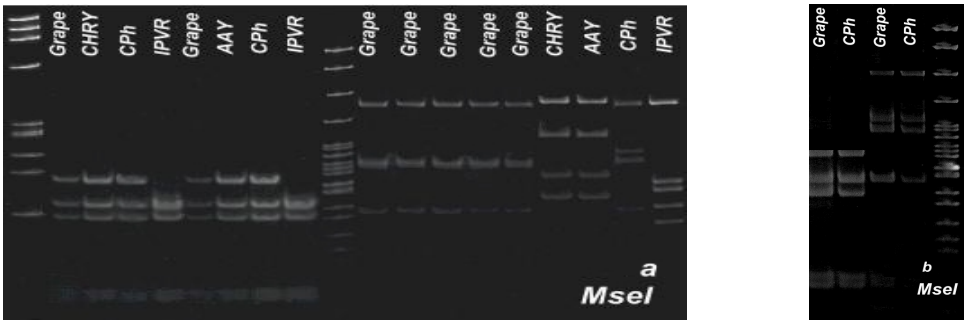

Fig.s 1a and b. RFLP profiles of phytoplasmas detected in grapevines (grape) after inoculation with *M. pruinosa*, in a) test carried out in September and in b) test carried out in October; primers M1/M2 on the left and R16 (I) F1/R1 on the right, controls are described in the text.

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EFFICIENCY OF MOLECULAR TESTS TO CONTROL PHYTOPLASMA ELIMINATION

Bertaccini A.[1], Borgo M.[2], Pondrelli M.[1], Murari E.[1], Sartori S.[1] and Bonetti A.[1]

[1]UCI-STAA, Patologia Vegetale, University of Bologna, Italy, e-mail Bertaccini_A@biblio.cib.unibo.it,
[2]Istituto Sperimentale per la viticoltura, MiPAF, Conegliano Veneto, TV, Italy

Objectives

Several experiments and a lot of experiences are available about the grapevine thermotherapy to eliminate phytoplasmas (2, 3) since it is necessary to rescue genetically important varieties of grapevine propagation materials. Recently in Italy some researches were carried out to verify the effectiveness of this method and of chemotherapy in phytoplasma elimination. During three years ungrafted vines and grafted cuttings of different cultivars were maintained, after therapy in different conditions in an insect proof screenhouse. None of the plants shows symptoms therefore molecular tests based on PCR/RFLP analyses (1, 4) were carried out periodically, to verify phytoplasma elimination.

Materials And Methods

Cultivars employed were Chardonnay, Prosecco, Manzoni Bianco and Garganega. Cuttings were collected during the winters 1996, 1997 and 1998 and after several weeks (4 to 8) of storage at 4°C were treated for thermotherapy following the systems described in (1). Ungrafted and grafted vines, after treatments, were maintained in woody cases for 40 to 50 days, then planted under insect-proof greenhouse in soil covered with plastic film to avoid weed growth. The plants were periodically controlled for field performance and for phytoplasma symptoms presence. Different types of heat treatments were tested (tables 1 and 2) in order to control the fitness of plants after treatment (fig. 1); tests were also performed by using immersion of cuttings in a 0.5% solution of Virkon (Antec International ltd, Kilton Industrial Estate, Sudbury, England, EU) for different periods (table 2).

Phytoplasma detection tests were performed on single plants (table 2) or on batches of plants varying from 1 to 6 (table 1), following the schedules described under the tables. Identification was performed on nucleic acid chloroform/phenol extracted from 1 gr. of fresh phloematic material as described in (4) and amplified in nested PCR using primers R16F1/R0 followed by R16F2n/R2, R16(I)R1/R1 and R16(V)F1/R1 for detection of phytoplasmas belonging to groups 16SrI, 16SrXII and 16SrV respectively (1). To distinguish between phytoplasmas belonging to groups 16SrI and 16SrXII RFLP analyses were performed using *Mse*I endonuclease.

Results And Conclusions

Field performance during four years is summarized in fig. 1: temperatures higher than 50° C for long periods increased dramatically the mortality percentage, 50°C for 40 min, as reported in the literature (2, 3), show the best performance of heat treated material, lower temperatures for long periods (up to 3 hrs) allow the best survival. Grafted plants show a lower percentage of survival in both cases ungrafted and grafted plants, Virkon treatment shows not severe losses in both theses tested. No important differences were observed on agronomic behavior among the different varieties treated, only Prosecco shows an apparently higher tolerance to the heat treatment.

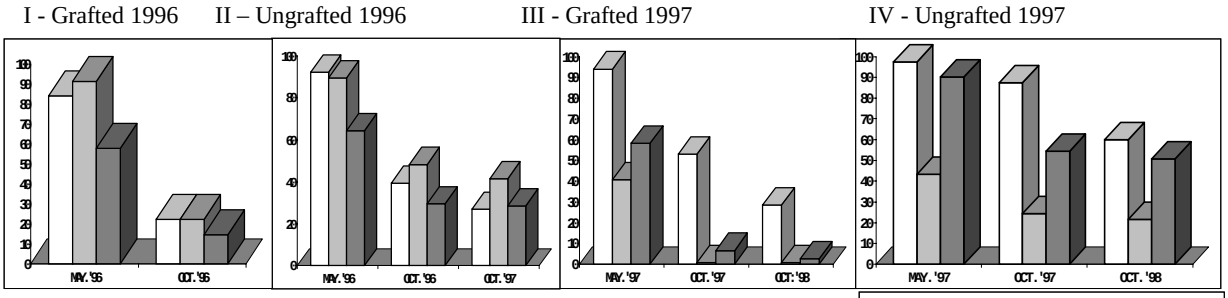

Fig. 1. Histograms representing the survival of grapevine plants listed in tables 1 and 2: white is always A; in I and II grey is B and dark grey is C; in III and IV grey is E and dark grey is D; in V black is F; dark grey is G and grey is H.

All the grape material was asymptomatic for the period of the tests; the first experiments carried out 5 months after planting in soil with nested PCR (samples 1996 in table 1) provide negative results for all the plants (treated and untreated). Since no symptoms were expressed from untreated plants, molecular tests resulted to be only system to verify phytoplasma elimination; these tests appear to be reliable only after at least one year from the planting.

The comprehensive results show that the treated material was not always free from phytoplasmas. Moreover a percentage of untreated plants, varying from 20 to 30%, was also negative, confirming the uneven phytoplasma distribution in the canes employed for treatment. Flavescence dorée (FD) phytoplasmas (16SrV) were detected in only two of the treated samples while Bois Noir and aster yellows (16SrXII-A and 16SrI-B) phytoplasmas resulted to be more difficult to eliminate. In tests carried out on 1998 heat-treated material mainly 16SrI-B and 16SrI-C phytoplasmas were identified. The percentage of phytoplasma elimination by thermotherapy was comprised between 60 and 70%. Higher temperatures increased the percentage of phytoplasma free material but were associated with high mortality of the plants (fig. 1).

Table 1: Results of molecular tests to verify phytoplasma elimination after thermotherapy carried out in 1996 and 1997.

Mother plant/ phytopl. Detected	10. 96	03. 97	05. 97	09. 97	01. 98	03. 98	12. 98	*
Prosecco 1996								
27	-	+	Nt	+	Nt	+	/	A
16SrI-B	Nt	-	Nt	Nt	Nt	Nt	-	B
16SrV	-	/	/	/	/	/	/	C
29	-	-	+	+	Nt	+	/	A
16SrI-B	-	-	-	-	-	-	/	B
	-	+	-	+	-	+	/	C
30	-	Nt	Nt	+	Nt	/	/	A
16SrI-B	-	Nt	Nt	Nt	+	-	-	B
	-	Nt	-	Nt	-	-	-	C
40	-	-	Nt	Nt	-	-	-	A
16SrXII-A	Nt	-	Nt	Nt	-	-	-	B
16SrV	-	/	/	/	/	/	/	C
41	-	+	-	-	Nt	+	/	A
16SrI-B	Nt	+	-	+	-	-	/	B
16SrV	Nt	+	-	-	-	-	/	B
42	-	Nt	Nt	+	Nt	-	-	A
16SrI	Nt	-	Nt	-	-	-	-	B
	Nt	-	Nt	-	-	-	-	C
Chardonnay 1996								
33	-	-	-	-	Nt	-	-	A
16SrI-C	Nt	-	+	+	-	-	-	B
16SrV	Nt	-	-	+	-	-	/	C
36	-	+	-	-	Nt	+	Nt	A
16SrI	-	-	-	-	-	-	-	B
	-	-	-	+	-	-	/	C
37	-	+	-	+	-	/	/	A
16SrI-B	-	Nt	-	-	-	+	/	B
16SrV	-	Nt	-	-	-	-	-	C

Mother plant/ phytopl. detected	04. 98	06. 98	10. 98	12. 98	*
Prosecco 1997					
14	-	-	Nt	+	A
16SrV	-	Nt	Nt	+	D
	Nt	Nt	Nt	+	E
19	+	Nt	Nt	-	A
?	Nt	Nt	Nt	-	D
	-	-	-	-	E
40	Nt	Nt	Nt	/	A
16SrV	-	Nt	Nt	/	D
	Nt	nt	Nt	Nt	E
Chardonnay 1997					
2	-	Nt	Nt	+	A
16SrI-B	-	Nt	Nt	-	D
16SrV	-	Nt	Nt	-	E
3	+	Nt	Nt	-	A
16SrI	-	Nt	Nt	-	D
16SrV	+	-	Nt	+	E
7	Nt	Nt	Nt	+	A
16SrV	Nt	Nt	Nt	/	D
	+	-	Nt	+	E
15	-	Nt	Nt	-	A
16SrI	-	Nt	Nt	-	D
	-	+	-	-	E
17	Nt	-	Nt	-	A
16SrI	-	Nt	Nt	-	D
	-	-	-	-	E
Garganega					
10	Nt	Nt	Nt	/	A
16SrV	-	-	Nt	Nt	E

Nt, not tested; +, positive; -, negative; /, plant death; ?, unidentified phytoplasma
*A, control untreated; B, 45°C/3 hrs; C, 50°C/40 min; D, 53°C/20 min; E, 48°C/40 min.

Table 2. Preliminary results of molecular tests to verify phytoplasma elimination after thermotherapy (F) and chemotherapy (G and H) carried out in 1998.

Mother plant	A		F	G		H	
	06. 99	10. 99	10. 99	06. 99	10. 99	06. 99	10. 99
Prosecco							
4	+	+	+	+	-	+	-
5	-	+	+	-	-	-	+
Manzoni Bianco							
24	-	+	+	+	-	-	Nt
25	+	+	-	-	-	-	Nt

Nt, not tested; +, positive; -, negative. A, control untreated; F, 52°C/40 min.;

Temperatures that resulted to be more efficient in phytoplasma elimination (E, F) cause severe losses of materials (fig. 1), while tests carried out on Virkon-treated plants show no consistent results in phytoplasma elimination in the two cultivars employed. It is important to perform molecular test on the treated material considering the persistence of some phytoplasmas after all therapy tested. However it appears that all treatments were able to eliminate FD from the majority of infected plants.

G, Virkon 0.5% for 3 hrs; H, Virkon 0.5% for 5 hrs.

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EXPERIMENTAL TRANSMISSION BY *SCAPHOIDEUS TITANUS* BALL OF TWO FLAVESCENCE DOREE-TIPE PHYTOPLASMAS

Mori N.[1], Martini M.[2], Bressan A.[1], Guadagnini M.[2] ,Girolami V.[1] and Bertaccini A.[2]

[1]Istituto di Entomologia Agraria, University of Padova, Italy.
[2]UCI-STAA, Patologia Vegetale, University of Bologna, Italy.

Objectives

During the last five years the "Flavescence dorée" disease (FD) has been expanding from the first outbreaks foci in Veneto region (1) to the major viticultural areas of Northern Italy showing the higher increase of infection percentages in 1999. The FD vector is *Scaphoideus titanus* Ball (3, 4, 5, 6). Recently it has been demonstrated that in Veneto the FD disease is associated with two molecularly distinguishable types of phytoplasmas both belonging to 16SrV group (Elm yellows and related strains) (7). The two phytoplasma types appeared to be geographically separated in 1997: FD-16SrV-D in provinces of Verona, Vicenza and Padova, infecting mainly cultivars Garganega and Chardonnay; while FD-16SrV-C in provinces of Treviso and Venice infecting cultivars Prosecco, Chardonnay and Perera. During 1998 the two types started to merge in Treviso province (2) showing that 16SrV-D was more actively and long distance transmitted than the other type. To achieve more informations about epidemiology of the two types of agents during 1998-1999, trials were carried out to verify the transmissibility by *S. titanus* of both phytoplasmas.

Materials And Methods

(A) Phytoplasma transmission. During the summers 1998 and 1999 (June-September) batches of *S. titanus* nymphs and adults were collected on symptomatic grapevine plants located in five different vineyards where the surveys carried out allowed the detection of the two types of FD (2). The insects were caged for phytoplasma transmission until they died on micropropagated potted vines cv Chardonnay, kindly provided by M. Borgo (MiPAF, Conegliano, TV, Italy) and tested by nested-PCR as described below before their use. The experimental scheme of the transmission trials is reported in table 1; the experiments were carried out in cages under an insect proof greenhouse.

(B) Phytoplasma detection. Nucleic acid extraction from insects was performed using the procedure described in (9), slightly modified for centrifugation times and speeds. Plant nucleic acid was extracted following the chloroform/phenol procedure described in (10). Template nucleic acids were diluted to a final concentration of 20ng/µl; 1µl of this dilution was employed for PCR assays. Reaction mix contained 10 pmol of each primer, 200µM of d-NTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 10mM Tris-HCl (pH 9) e 0.625 U of *Taq* Polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden, EU) for a total volume of 25µl. The amplification cycle was the one described by (11). All the samples were tested by nested-PCR, using universal primers R16F1/R0 followed by R16F2n/R2 and 16R758f/16R1232r (M1/M2). All samples positive with these primers were further amplified with group 16SrV-specific primers R16 (V) F1/R1 (=V) in the same conditions described above.

On samples positive for EY-type phytoplasmas, another PCR procedure was adopted using primers 16R723f/P7 followed by primers 16R758f/M23SR1804r in nested-PCR or primers 16R758f/V1731r in seminested-PCR in order to amplify the 3' end of the gene coding for 16S RNA and most of the spacer region (7). RFLP analyses of amplified products were carried out with *Taq*I endonuclease incubation overnight at 65°C. Control samples used in PCR/RFLP analyses for group 16SrV were grapevine FD strain B1872 (kindly provided by E. Boudon-Padieu, INRA, Dijon, France) and strain 19TV (7) for groups 16SrV-D and 16SrV-C respectively; elm yellows (EY), (H. Griffith and W.A. Sinclair, Cornell University, Ithaca, NY, USA), elm witches'-broom (ULW) (E. Seemüller, BBA, Dosseheim, Germany) for 16SrV-A group, and Italian periwinkle virescence (IPVR) for 16SrXII-A group.

The micropropagated grapevine plants tested before and after transmission were 28; 393 insects batches of 2 to 6 individuals were also tested by sampling in the total of collected insects.

Table 1: Experimental plan of *S. titanus* transmission trials.

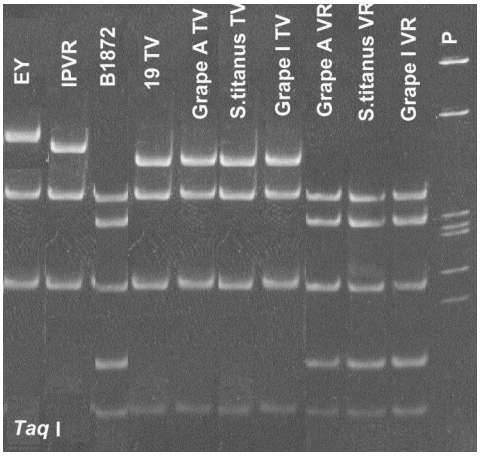
Year	Location	(province)	Cultivars used for acquisition	Inoculation time	Insect stage	n° insects/ inoculated plant	n° inoculated plants
1998	Soave	(Verona)	Garganega	24 August	Adult	20	4
	Farra di Soligo	(Treviso)	Prosecco	12 August	Adult	40	3
1999	Affi	(Verona)	Garganega	08 July	L_4 L_5	30	2
	Affi	(Verona)	Garganega	18 July	Adult	30	2
	Montecchio M.	(Vicenza)	Cabernet Franc	02 July	L_4 L_5	50	6
	Montecchio M.	(Vicenza)	Cabernet Franc	14 July	Adult	60	3
	Farra di S.	(Treviso)	Prosecco	28 July	Adult	50	6
	Torreglia	(Padova)	Chardonnay	30 June	L_3 L_4	30	2

Results And Conclusions

The results of transmission tests are summarized in table 2. It was confirmed that two types of FD are detected in Veneto. Symptomatic grapevines employed for feeding the vectors and *S. titanus* samples tested were infected by 16SrV-D phytoplasmas in Verona and Vicenza vineyards, and by 16SrV-C phytoplasmas in the Treviso vineyard (table 2 and fig. 1). Previous preliminary investigations did not allow the detection of 16SrV-C phytoplasmas in *S. titanus* (8). The molecular tests carried out on insect-inoculated grapevine plants show the ability of *S. titanus* to transmit both FD-types (fig. 1). In particular FD-16SrV-D phytoplasmas were transmitted to 6 out of 19 grapevine plants inoculated while FD-16SrV-C phytoplasmas were transmitted on 2 out of 9 grapevine plants inoculated. Among the grapevines insect inoculated and resulted positive to the molecular analyses 3, infected with 16SrV-D phytoplasmas, showed typical FD symptoms in the same year of the inoculation (table 2). Further tests will be carried out during the next year to verify FD transmission to the plant negative during the first year of tests and to verify symptom expression from the other type of FD-related phytoplasmas.

Table 2: Results of FD phytoplasmas transmission by *S. titanus*

Year	Location (province)	Phytoplasma detected on symptomatic grapevines used for acquisition	Phytoplasma transmitted	Infected/ inoculated plants	Symptoms on infected plants
1998	Soave (Verona)	FD-16SrV-D	FD16SrV-D	¼	No
	Farra di Soligo (Treviso)	FD-16SrV-C	-	0/3	No
1999	Affi (Verona)	FD-16SrV-D	FD-16SrV-D	½	Yes
	Affi (Verona)	FD-16SrV-D	-	0/2	No
	Montecchio M. (Vicenza)	FD-16SrV-D	FD-16SrV-D	3/6	Yes
	Montecchio M. (Vicenza)	FD16SrV-D	FD-16SrV-D	1/3	No
	Farra di S. (Treviso)	FD-16SrV-C	FD-16SrV-C	2/6	No
	Torreglia (Padova)	FD-16SrV-D	-	0/2	No



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PHYTOPLASMA IDENTIFICATION IN HUNGARIAN GRAPEVINES BY TWO NESTED-PCR SYSTEMS

Varga, K.[1], Kölber, M.[1], Martini, M.[2], Pondrelli, M.[2], Ember, I.[1], Tökés, G.[1], Lázár, J.[3], Mikulás, J.[3], Papp, E[4], Szendrey, G[5], Schweigert, Á[6] and Bertaccini, A.[2]

[1]PHSCS of MARD, Budapest, Hungary, e-mail: viruslab@mail.elender.hu.
[2]UCI-STAA, Patologia Vegetale, University of Bologna, Italy, e-mail Bertaccini_A@biblio.cib.unibo.it.
[3]RIVE of MARD, Kecskemét, Hungary.
[4]PHSCS of county Bács, Kecskemét, Hungary.
[5]PHSCS of county Heves, Eger, Hungary.
[6]PHSCS of county Somogy, Kaposvár, Hungary

Objective

In 1993 a several-year survey was begun to determine presence of grapevine yellows (GY) disease in the main grapevine growing regions of Hungary. Grapevine samples, showing typical GY symptoms were collected from 7 cultivars of 4 grapevine growing regions in 1993 and 1995/96; the samples were tested for phytoplasma presence in direct and nested PCR with phytoplasma universal and specific primer pairs (1, 3). Phytoplasma DNA was amplified from samples of cultivars Chardonnay, Pinot Grey, Merlot and Zweigelt and identified as belonging to phytoplasmas of Stolbur-type or subgroup 16SrI-G (2); according to the recently revised classification of phytoplasmas (4, 8) they belong to subgroup 16SrXII-A. Since phytoplasma infection was detected only in samples from Northeast Hungary, in 1998/1999 further surveys were conducted to determine geographical distribution of GY as well as to identify other phytoplasmas using PCR/RFLP analyses comparing two different nested-PCR systems.

Materials And Methods

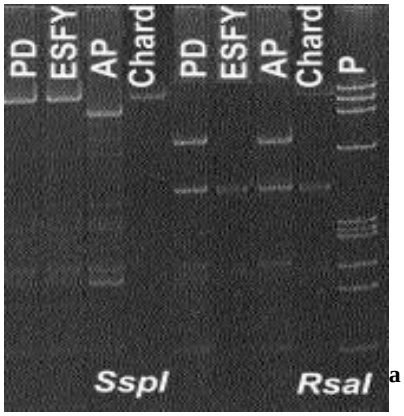
In 1998 presence of severe GY symptoms was observed in 14 vineyards of 8 counties. Fifty plants/cultivar/vineyard were visually inspected. In October laboratory tests were carried out on cane, shoot or leaf samples of seven symptom-showing plants from three different cultivars. An asymptomatic Chardonnay plant was used as healthy control (table 1). In 1999 tests were repeated using the same PCR systems on two different types of materials i.e. fresh phloem from canes (I) and dry leaves (II) from the same areas as 1998 plus other two counties (table 2); four cultivars were examined. PCR on total nucleic acids, extracted with a chloroform/phenol procedure (7) from 2 g/sample of leaf midribs or phloem tissue was carried out using two different nested systems: „A" and „B", to verify sensitivity of phytoplasma detection and identify phytoplasmas. In the first PCR assays phytoplasma-specific universal primers from 16S (System „A") and 16S - 23S (system "B") ribosomal DNA were used. Nested PCR assays using primers, specific for groups 16SrI and 16SrXII, 16SrIII, 16SrV and 16SrX (3) were carried out in „A" and using other general primers in „B" (5). In order to determine subgroup affiliation of the detected phytoplasmas, RFLP analyses were then conducted with *Mse*I, *Rsa*I, *Ssp*I or *Taq*I according to the DNA sequence amplified in PCR assays.

Results And Conclusions

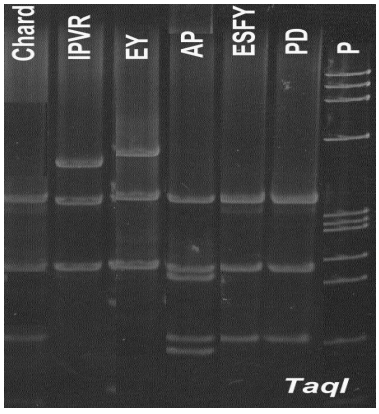
In 1998, two thousand-seven hundred-fifty-three of more than 9900 grapevine plants inspected showed GY symptoms. They belonged to 13 cultivars grown in 8 counties. Leaf yellows, severe triangle-shaped leaf rolling, dropping of shoots due to uneven ripening as the most common symptoms were observed on the following cultivars: Alicante Bouchet, Aligote, Cabernet franc, Chardonnay, Ezerfürtü, Kerner, Merlot, Muscat lunel, Pinot blanc, Pinot Grey, Pintes, Zöld Veltelini and Zweigelt.

Results of phytoplasma detection/identification in the grapevine samples selected for laboratory analyses using two nested-PCR systems are shown in tables 1 and 2. Both systems appeared to be able to detect phytoplasmas; nevertheless in RFLP analyses on PCR products obtained following system B, appeared that some bands were aspecific. This suggests that amplification of DNA containing the spacer region, must be checked to verify phytoplasma specificity.

In 1998 the asymptomatic plant and one of those showing symptoms were negative in both tests. In six of the seven samples found positive, the identified phytoplasmas belonged to the 16SrXII-A group, already reported in Hungary on grapevine (2). In some cases they were found in mixed infection with phytoplasmas of the 16SrV and 16SrX groups: phytoplasmas not having been reported on grapevine in Hungary. Results of RFLP analyses showed that phytoplasmas of the 16SrX group belonged to subgroup –B (apricot chlorotic leafroll= ACLR or European stone fruit= ESFY) (Figs. 1a and b). This is a new



a



b

Fig. 1. Results of RFLP analyses on grapevine samples in which phytoplasmas of 16SrX-B group were identified. In a) amplification with primers R16(X)F1/R1 ("A") in b) with primers $16R_{758f}$/$M23SR_{1804r}$ ("B). PD, pear decline (16SrX-C); ESFY, European stone fruit yellows (16SrX-B); AP, apple proliferation (16SrX-A); IPVR, Italian periwinkle virescence (16SrXII-A); EY, elm yellows (16SrV-A), Chard, grapevine cv Chardonnay; P, marker phiX174 *Hae*III digested

finding for grapevine, as phytoplasmas belonging to this group were detected only on grapevine in Veneto region, Italy (6), but no subgroup was reported.

Tests carried out in 1999 show that the dry leaf material could also be employed for successful phytoplasma detection with results more sensitive than those obtained with nucleic acid extracted from fresh phloem tissue (table 2). In fact many cases of mixed infections were identified where the 16SrXII-A phytoplasmas were detected together with phytoplasmas of different subgroups in the aster yellows group (16SrI-B and 16SrI-C). The presence of 16SrXII-A and ESFY (16SrX-B) phytoplasmas was generally confirmed in the same locations of the previous year, at least when the phytoplasma detected could be identified. Phytoplasmas of the 16SrV group were detected only in the 1998 samples: and it was not possible to identify their subgroup affiliation. Further epidemiological studies are necessary to identify the importance in Hungarian grapevines yellows of the newly detected 16SrX-B phytoplasmas as well as to exclude the presence of phytoplasmas related to the elm yellows.

Table 1. Results of phytoplasma detection/identification in grapevine samples in 1998.

Location (County)	Serial number/Cultivar	PCR system		Phytoplasma(s) identified
		„A”	„B”	
Kecskemét (Bacs)	11. Chardonnay	+	+	16SrXII-A+16SrV
Balatonszarszo (Somogy)	12. Chardonnay	-	+	16SrI-B
Balatonszarszo (Somogy)	13. Chardonnay	-	+	16SrX-B
Balatonszarszo (Somogy)	14. Zöld veltelini	+	+	16SrV
Balatonszarszo (Somogy)	15. Zöld veltelini	-	+	16SrI-B+16SrV
Katonatelep (Bacs)	16. Kerner	-	+	16SrXII-A
Andornaktalya (?)	17. Chardonnay	-	-	-
Kecskemét (Bacs)	18. Chardonnay asym.	-	-	-

"A": Position of the products, obtained in nested-PCR, were inside 152-1503 bp in 16S DNA, when R16(I)F1/R1, R16(III)F2/R1, R16(V)F1/R1 and R16(X)F1/R1 (3) primers were used.
"B": Position of the products, obtained in nested-PCR, was inside 723-1832 in 16S/spacer/23S DNA, when $16R_{758f}$/$M23SR_{1804r}$ (5) primers were used.

Table 2. Results of phytoplasma detection/identification in grapevine samples in 1999 (footnotes as in table 1).

Location (County)	Serial number/Cultivar	(I) PCR system		Phytoplasma(s) identified	(II) PCR system		Phytoplasma(s) identified
		„A”	„B”		„A”	„B”	
Kecskemét (Bacs)	1. Chardonnay	+	-	16SrI-B	+	-	16SrX-B
Katonatelep (Bacs)	2. Kerner	+	+	16SrXII-A	+	+	16SrXII-A
Kisapati (Veszeprem)	3. Chardonnay	+	+	16SrXII-A	+	+	16SrXII-A+16SrI-C
Balatonszarszo (Somogy)	4. Chardonnay	-	-	-	-	+	?
Balatonszarszo (Somogy)	5. Zöld veltelini	-	+	?	-	+	?
Balatonszarszo (Somogy)	6. Zöld veltelini	-	+	?	+	+	16SrXII-A
Fertoszentmiklos (Somogy)	7. Chardonnay	-	-	-	-	+	16SrXII-A
Kecskemét (Bacs)	8. Chardonnay	+	+	16SrXII-A+16SrI-B	+	-	16SrXII-A+16SrI-B
Lengyeltoti (Somogy)	9. Zweigelt	+	-	16SrI-B	+	-	?
Vas (Vas)	10.Chardonnay	-	+	16SrV???	+	-	16SrXII-A+16SrI-C

(I) DNA extraction performed from fresh phloem; (II) DNA extraction performed on dry leaves.

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THE APPEARANCE AND DISTRIBUTION OF GRAPEVINE FANLEAF VIRUS AND ARABIS MOSAIC VIRUS IN YUGOSLAVIA

Delibasic, G., Babovic, M., Jakovljevic Dragana
Faculty of Agriculture, University of Belgrade, Yugoslavia

Introduction

The appearance and distribution of grapevine virus complex being cause of the infective degeneration was tested on 25 sorts in eight most significant regions of vine grow in Yugoslavia. The preliminary identification of the cause of these changes (the infectious degeneration) was made by serology analysis of ELISA test. Out of 9.200 tested stocks the virus infection was detected on 1.716. In the infected stocks the presence of the following viruses was detected: Grapevine fanleaf virus, Arabis mosaic virus, Tobacco mosaic virus and Sowbine mosaic virus. Among the identified viruses the most spread was the Grapevine fanleaf virus (1.189) and the Arabis mosaic virus (427). The other two viruses were detected in only 73 tested grapevines.

Due to their large presence, the Grapevine fanleaf virus and the Arabis mosaic virus were selected for further more detailed testing. Their complete identification was carried out by studing the possibilities of their spreading to herbaceous host, and graft transmissible plants. Morphology and serology features of the purified isolates were studied, too. The hydrolysis of the viruses (identification of the molecular weight of the protein coat, and the nucleic acid of the viruses) was done, too.

Materials And Methods

During this research work detailed examination of a large number of various grapevine sorts was carried out on the sites of the more significant regions of vine grow in Yugoslavia, like: Bela Crkva, Negotin, Zajecar, Krusevac, Nis, Leskovac, Vranje and Podgorica. The following sorts of the grapevine were tested: Cabernet Frank, Merlot, Game bojadiser, Zupski bojadiser, Rkaziteli, Vranac, Rajnski Riesling clone 21B, Black burgundy, White burgundy, Zupljanka, Cabernet sauvignon, Julski biser, Rizvanac, Italija, Tajfi rozovij, Smederevka, Game black, Frankovka, Talijanski Riesling, Neoplanta, Halili white, Krstac, Chardonnay, Kings rubi and Afuz ali. Samples have been taken of grapevines on which the characteristic changes were noticed by being manifested on leaves, branches and bunches. Samples of leaves and branches were taken of these grapevines, and the nature of their disease was afterwards tested through herbaceous hosts and graft transmissible plants in the green house conditions.

Purification of virus isolates was carried out by Lester & Hadidi (1971) and Martelli (1984) methods.
Imunobiology tests by ELISA method were carried out by application of DAS ELISA as described by Clark and Adams (1977). The morphology features of the purified virus isolates were tested by electronic microscopic methods (coloration and ISEM). The extraction of the protein coat and identification of its molecular weight, as well as the extraction of the nucleic acid and identification of their molecular weight was carried out by Leammli method (1970).

Results

On grapevines selected for further testing, symptoms of diseases were clearly manifested on leaves, branches and bunches. Out of 9.200 tested stocks, the visible changes appeared on 1.716 and their symptoms were indicative for the virus nature of disease. Among the tested samples by ELISA method, the biotest on the herbaceous and graft transmissible plants and electronic microscopy, the virus infection was detected on 1.689 stocks. The isolated virus survey by the regions in Yugoslavia has been shown in the Table 1. Out of 1,716 infected and tested grapevines the presence was proved on 1,689 of them. As Tobacco mosaic virus and Sowbine mosaic virus were detected only in few sites, and in a small number (73 grapevines), and are in the virus group whose economy perniciousness on vine has not been known, these two viruses were eliminated of further tests.

Table 1: Review of isolated grapevine viruses

SITES	NUMBER OF STOCKS WITH SYMPTOMS OF INFECTIVE DEGENERATION	ELISA (405 nm) NUMBER OF INFECTED STOCKS				NUMBER OF NONINFECTED STOCKS
		GFLV	ArMV	TMV	SoMV	
BELA CRKVA	72	40	21	6	2	3
NEGOTIN	114	80	29	3	0	2
ZAJECAR	79	43	31	2	1	2
KRUSEVAC	167	105	53	5	0	4
NIS	536	428	93	8	3	4
LESKOVAC	332	232	78	14	5	3
VRANJE	227	138	68	10	7	4
PODGORICA	189	123	54	6	1	5
TOTALL:	**1716**	**1189**	**427**	**54**	**19**	**27**

For virus isolation of the infected grapevines, and their mechanical transfer to herbaceous hosts the *Chenopodium quinoa* was used. During further testing on the possibility of the mechanical transfer of isolates to the other sorts of the herbaceous hosts, and with the intention of studing the host circle, *Ch. amaranticolor, Nicotiana occidentalis, N. bentamiana, N. clevelandi, N. rustica, N. tabacum cv. "Samsun", N. glutinosa, Gomphrena globosa, Petunia hybrida, Cucumis sativus, and Phaseolus vulgaris* were used, too.

Reactions of the tested plants were similar, and the only difference was noticed with *N. glutinosa and G. globosa*. While *G. globosa* reacted only to GFLV isolates, *N. glutinosa* positively reacted only to isolates ArMV.

Testing of both isolate groups of the examined viruses was carried out by the green graft transmission method on the graft transmissible plants: LN_{33}, *Vitis rupestris "St. George"* and *Mission*. The first symptoms of disease were usually manifested on all graft transmissible plants 7–8 weeks after graft transmission. The positive reaction on the graft transmissible plants LN_{33} and *Vitis rupestris "St. George"* was provoked by both tested virus isolates: Grapevine fanleaf virus and Arabis mosaic virus. However these viruses have not provoked any kind of visible reactions on the graft transmissible plants *Mission*. By repeated graft transmission on the plants *Mission* which negatively reacted to GFLV and ArMV, to *Vitis rupestris "St. George"* and LN_{33}, there were none of reactions.

Partially purified isolate suspensions of both viruses were tested by negative coloring method. By the electronic microscope examination of the prepared slides spherical form particles were detected with Grapevine fanleaf and Arabis mosaic viruses. The average size of these particles was 28nm–31nm.

Purified suspensions of both virus isolate groups were tested by spectrophotometer at the wavelength of 220 to 320nm. The following results was obtained: A 260/280 = 1,50.

The molecular weight of both viruses protein coat, counted by computer processing of the known values, and by implementing the regression equation, is both for the Grapevine fanleaf virus and the Arabis mosaic virus 54–55kd.

By examining the molecular weight of the nucleic acids (RNA_1 and RNA_2) carried out by the computer processing of the known values and by anti logarithmic computing of the newly obtained value it was found out that they are $2{,}4x10^6$ for RNA_1, and $1{,}4x10^6$ for RNA_2.

Conclusions

The following conclusions might be drawn by the results obtained during this research:

-Besides application of ELISA test and the biotest methods on the herbaceous and graft transmissible plants, the presence of these viruses was proved and verified also by implementation of the electronic microscopy;

-Only the Grapevine fanleaf viruses provoked the positive reaction on the plants *Gomphrena globosa*, and it is the specific selective host of this virus;

-*Nicotiana glutinosa* is the specific, selective host of the Arabis mosaic virus;

-Grapevine fanleaf virus and the Arabis mosaic virus isolates provoked the symptoms on the graft transmissible plants LN_{33} and *Vitis rupestris "St. George"*;
-In the tested Grapevine fanleaf virus and the Arabis mosaic virus isolates by the electronic microscopy, the uniformed spherical form particles were detected of the average size 28nm to 31nm;
-On the basis of these tests, it is possible to conclude that the Grapevine fanleaf virus and the Arabis mosaic virus have been identified as the economy significant viruses in Yugoslavia while the others were detected only in few cases.

CONFIRMATION OF THE PRESENCE OF STOLBUR TYPE YELLOWS IN SWISS VINEYARDS BY MOLECULAR DIAGNOSIS OF GRAPEVINE

Bourquin L.[1], Schmid A.[2], DeMeyer J. [3], Cazelles O.[1], Ramel, M. E. [1] and Gugerli P.[1]

[1] Swiss Federal Research Station for Plant Production, Changins (RAC), CH-1260 Nyon 1, Switzerland
[2] Station cantonale de protection des plantes Châteauneuf, 1950 Sion, Switzerland
[3] CIMMYT-Zimbabwe, Maize Research Station. P.O. Box MP 163 Mount Pleasant Harare Zimbabwe

Grapevines affected by yellows type symptoms were occasionally observed in Swiss vineyards in the past (1) and more precisely recorded in the Valais and Geneva vineyards since 1991 (2). First samples collected in 1992 were serologically assayed in Dijon and all found negative for Flavescence dorée (3). The symptoms were therefore likely to be due to some other yellows. Indeed, half of the same samples revealed in ulterior tests to be infected by a phytoplasma of the stolbur type (4, 5). The affected vines, mostly Chardonnay, were scarcely spread in several vineyards and many of the marked plants recovered to some extend whereas new ill vines appeared elsewhere over the years. More recently, yellows occurred also on Pinot noir and some other red varieties in the Valais near Sion. This incited us to put up a survey using polymerase chain reaction (PCR) based diagnosis. The first results clearly indicated the presence of Stolbur type yellows and the absence of Flavescence dorée, confirming the earlier analysis. Two universal and four subgroup specific primers were used, either directly or in nested PCR. The results were further confirmed by analysis using RFLP.

Material And Methods

Leaf samples were collected in September 1999 in vineyards near Sion from vines with typical yellows symptoms or vines that were recorded ill before. Tissue from the leaf base, comprising major veins, was retained and frozen at -20 °C for about three weeks. DNA extraction was carried out according to (6) with minor modifications. Samples of 0.5 g tissue were ground in plastic bags in 5 ml ice cold grinding buffer (125mM potassium phosphate, 30mM ascorbic acid, 10 % sucrose, 0.15 % BSA and 2 % PVP, pH 7.6), using a Homex 6 plant tissue homogenizer from BIOREBA AG, Reinach, Switzerland. Extracts were then centrifuged at 4000 g for 10 min and the pellet suspended and incubated for 30 min at 60°C in 1.5 ml warm (60°C) extraction buffer (2 % CTAB, 1.4 M NaCl, 20mM EDTA, 0.2% 2-mercaptoethanol, 200mM Tris-HCl, pH 8.0). DNA was then purified by treating the homogenate with an equal volume of chloroform / isoamyl alcohol (24:1, v/v). Following centrifugation, the DNA was precipitated from the aqueous fraction with a two-third volume of -20 °C isopropanol. The pellet was washed with 70 % ethanol, dissolved in 100 µl of distilled water and used for amplification.

All the PCR reactions were carried out with the TAQ PCR Master Mix of QIAGEN, following the manufacturers instructions, using primers at 50 nmol/l.

Primers	Designation	Fragment	Reference
Universal primers for phytoplasmas	P1/P7	1750 bp	(7)
	fU5/rU3	882 bp	(8)
Stolbur specific primers	STOL11-f2/STOL11-r2	900 bp	(9)
	rStol/fStol	570 bp	(10)
Elm-Yellows specific primers	FD9-F1/FD9-R1	1300 bp	(9)
	fB1/rULWS1	1500 bp	(11)

More sensitive nested PCR was performed from P1/P7 amplification products, using rStol/fStol and fB1/rULWS1 primers for further amplification. All the PCR reactions were performed at an annealing temperature of 55°C except for the primer pair rStol/fStol that was run at 58°C. RFLP analysis, using the enzyme *TrU9*I according to (5), was done on the fU5/rU3 PCR product.

Results And Discussion

Both universal primer pairs (P1/P7; fU5/rU3) amplified DNA fragments of the expected length, indicating the presence of phytoplasmas in many of our samples, as reported in table 1. The Stolbur-subgroup specific primers revealed the stolbur type nature of this DNA. Indeed 12 out of 38 samples were STOL11 positive and nested PCR, using primers rStol/fStol, led to the detection of five further infected samples (fig. 1). Elm-yellows group-specific primers FD9 and fB1/rULWS1, including nested reactions, yielded only negative results. The digestion of fU5/rU3 amplification product by means of *Tru9*I enzyme showed a Stolbur type profile as published (5), confirming herewith a single stolbur type infection (Figure 2).

The results of this study indicate that only phytoplasmas belonging to the STOL group were detected in the samples collected in 1999. It can be concluded that the surveyed vineyards near Sion are not affected by Flavescence dorée but by Stolbur-subgroup yellows. The methods applied are now well established in our laboratory and can be used for future diagnosis in order to detect early enough eventual cases of FD and other grapevine yellows.

We thank Dr E. Boudon-Padieu (INRA-Dijon, France) and T. Oberhänsli (BIOREBA AG, Reinach, Switzerland) for Stolbur and Flavescence dorée type control samples.

Table 1 : Results obtained with the diverse primers.

Origine	Source	Samples	P1/P7	fU5/rU3	stol11	nPCR fStol/rStol	FD9	nPCR fB1/rULWS1	Digestion *TrU9*I
Noes	Pinot noir 2/45	1,4,6,7	-		-				
Noes	Pinot noir 2/45	2,3,5	+	+	+		-		
Noes	Gamay	8,10,12	-		-				
Noes	Gamay	9,11	+		+		-		
Conthey	Chardonnay	13-17, 19-25	-		-				
Conthey	Chardonnay	18	+	+	+		-		Stol
Conthey	Pinot noir	26	-		-				
Salgesch	Chardonnay	30,32	+	+	+		-	-	Stol
Salgesch	Chardonnay	28,31	+	+	+		-	-	
Salgesch	Chardonnay	27,29,33	+		-	+	-	-	
Venthâne	Garanoir	34	-	-	-		-	-	
Venthâne	Garanoir	35	+	+	+		-	-	
Venthâne	Gamaret	36	+	+	+		-	-	
Venthâne	Gamaret	37b,38	+	+	-	+	-	-	

(+): Positive reaction; (-) : negative reaction; empty cells: not tested

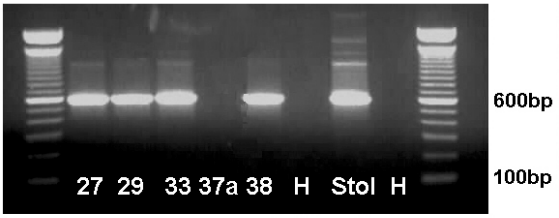

Figure 1. PCR product (570 bp) obtained with primers rStol/fStol (Between markers: 5 grapevine samples, H: healthy grapevine; Stol: positive grapevine control).

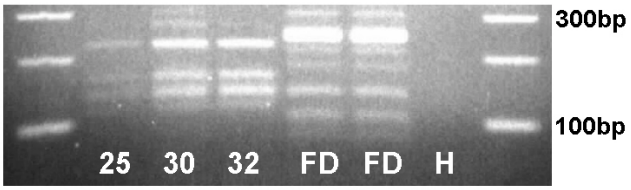

Figure 2. TrU9 I digestion of fU5/rU3 PCR product (Between markers: 3 grapevine samples; FD: positive grapevine control of Flavescence dorée, H: healthy grapevine control).

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DIAGNOSIS OF GRAPEVINE VIRUS DISEASES : AN OVERVIEW AND A PRACTICAL IMPLEMENTATION

P. Gugerli, Swiss Federal Research Station for Plant Production, Changins (RAC), CH-1260 Nyon -1 , Switzerland

A survey on progress of diagnosis of grapevine virus diseases revealed that most of the recently published data have been outlined during the last meeting of ICVG in 1997. This overview will consequently focus on the consolidation of these results and on their partial, practical implementation at RAC, Nyon. Since the development of new technology depends primarily on our understanding of viruses and their interactions with grapevine, which is the theme of an other chapter, several related papers are not referred here.

Biological Indexing

Today, the high sanitary standard of most important grapevine clones is still due to biological indexing. Classical indexing has therefore recently been revised by Garau *et al.* (1) and the state of the art of rapid indexing in the greenhouse through green-grafting by Kassemeyer *et al.* (2). Green grafting allows detection of leafroll, fleck, corky bark, vein mosaic and vein necrosis in less than 2 to 3 months, compared to at least two seasons by indexing in the field. Reliable detection of stem pitting and stem grooving syndromes in the greenhouse is however only possible within 8 - 12 months. Controlled variation of the growth conditions (temperature, light, humidity) is heretofore most important. Vindimian *et al.* (3) propose a further modification of the greenhouse bioassay. The technique uses micropropagated and acclimatized indicator plants for green-grafting with leaf petiole or secondary shoot from plants grown in the field. The material is manually grafted on the predisposed indicator plants, which are pruned to two buds to allow development of sprouts and leaves showing possible symptoms. The assembled plants are first kept in the greenhouse under mist for 15 days and the controls are carried out after 40 days. Best results are seemingly obtained by using secondary shoots of infected vines from rootstocks and leaf petioles from *Vitis vinifera* cultivars. Leaves or secondary shoots are available during the whole growing season and can always be of the right size with respect to the indicator plant.

Our partial, practical implementation at RAC-Nyon (4,5). Indexing by means of the green grafting procedure developed in France, revealed that fleck symptoms appeared as rapidly in the field as in the greenhouse, whereas leafroll symptoms, associated with grapevine leafroll associated virus 1, 2, 3 and 6 (GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-6), developed faster in the greenhouse. However, we were not able to diagnose stem-pitting nor stem grooving after extended periods of incubation in the greenhouse. The control of optimal incubation conditions seems not to be straight forward. The cultivation of the indicators in the greenhouse also requires much care. As a consequence, we still continue indexing in the field for large-scale indexing.

Immunoassays

Enzyme-linked immunosorbant assays (ELISA) are widely applied to diagnose grapevine for virus diseases. They have recently been reviewed by Boscia *et al.* (6). Different approaches are presently used to produce the necessary antibodies. Schieber *et al.* (7) described the production of monoclonal antibodies to grapevine fleck virus (GFkV). Gugerli *et al.* (8) have now also produced monoclonal antibodies to GLRaV-6, which are commercially available. The new antibody is well suited for the detection of the virus in petioles, leaf blades and canes by immuno-electron microscopy, immunoblot and ELISA procedures. Goszynski *et al*. (9) produced useful antibodies starting with electrophoretically separated capsid protein of grapevine virus B (GVB). Ling *et al*. (10) used a fusion coat protein containing a sequence of GLRaV-3 coat protein to produce viral antigen in *E.coli*. The antigen was purified by SDS-polyacrylamide gel electrophoresis and finally applied to animals as immunogen for obtaining antibodies. The latter were shown to be virus-specific by Western blot and ISEM. In ELISA, they were efficient together with an enzyme-labeled secondary monoclonal antibody. Large quantities of highly effective antibodies can be produced in this way. In a similar approach, Rubinson *et al*. (11) cloned and expressed in Escherichia coli the putative movement protein (MP) gene of grapevine virus A (GVA) in order to produce antiserum. Applied to virus detection, the forthcoming antibodies allowed to detect the infection at a very early stage after inoculation, proving that detection of the nonstructural MP may be most efficient for serological detection of GVA infection in grapevines. Rowhani *et al.* (12) compared ELISA and biological indexing for the detection of GLRaV-1, GLRaV-2 and GLRaV-3. Perfectly concurring results were obtained for GLRaV-1 and GLRaV-2, whereas for GLRaV-3 more infections were detected by ELISA than by indexing, using Cabernet franc as an indicator. The presence of the virus was further confirmed by reverse transcription polymerase chain reaction (RT-PCR).

Our partial, practical implementation at RAC-Nyon: Double-antibody sandwich ELISA (DAS-ELISA), mainly with monoclonal antibodies, is our method of choice for the routine detection of nepo-viruses, leafroll associated viruses, especially GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-6, as well as grapevine fleck virus (GFkV). Reliable detection of GLRaV-2 is obtained later in the season than for the other GLRaV's. Leaf blade, petioles and cane tissue can be used for all of them. For high accuracy, two to three repeated samples per vine are necessary to compensate variation of the virus titer, especially when testing American rootstock vines. Vitiviruses (GVA, GVB) are however not reliably detected by ELISA because of very low virus concentration in some cultivars. ELISA can nevertheless serve the purpose of a preliminary screening followed by more reliable biological or molecular indexing. Electrophoresis and Western blot, combined with regular and immuno-electron microscopy, is our method of choice for small-scale testing, e.g. important new accessions of mother vines for propagation

programs. Western blot can be more precise than DAS-ELISA. This joins results described by Pollini (13) where a dot-immunobinding assay was furthermore improved by enhanced chemiluminescence, which employs luminol, a cyclic diacylhydrazide, as substrate for horseradish peroxidase conjugated with a secondary antibody, for the detection of GLRaV-1 and GLRaV-3.

We obtained a very significant improvement of ELISA with monoclonal antibody, when we exchanged chromatic substrates with chemiluminescent (1,2-dioxetanes) substrates (LUMINO-ELISA) (14), as shown elsewhere in these proceedings (15). The sensitivity (detectability) was increased by a tenfold factor at least. The luminescence reaction has a surprising wide dynamic range, which facilitates quantitative measurements. The reaction is extremely fast. Maximum luminescence is reached, depending on the substrate, in less than hour. Luminescence amplifies the advantages of ELISA based on monoclonal antibody or any immunoassay with maximized target specificity.

Nucleic acid based assays

Nucleic acid type molecular virus detection by polymerase chain reaction that amplify viral genes offers a new dimension in sensitivity and accessibility to diagnostic reagents, although simple hybridization reactions have still a future, especially in some automated and miniaturized formats (microarray). As mentioned above, a first level of improvement of the molecular diagnosis of virus diseases of the grapevine is inherent to the biochemical, physical and biological characterization of novel viruses and their interaction with grapevine (Zhu et al. 16, Ling & Zhu et al. 17). A second field of improvement concerns sampling and sample processing. La Notte *et al*. (18) described a rapid and easy sampling method, using leaf petiole to express crude sap, which is spotted on a nylon membrane. The nucleic acid of the virus is then dissolved and amplified by RT-PCR. The release of the viral template was improved by a thermal treatment of 10 minutes at 95 °C. The method was used successfully with samples from GVA, GVB, and GLRaV-3 infected grapevines with a sensitivity comparable to that of standard PCR technique. The membranes can be processed up to one month after spotting. Duplex PCR (amplification of two viruses from a mixed-infected grapevine source was possible with GVA and GLRaV-3, using a mixture of specific primers in the same reaction. Abou-Ghanem *et al*. (19) used the technique as well for the detection of GLRaV-2 in infected grapevines. MacKenzie *et al.* (20) described an efficient method for the extraction of high-quality RNA from woody plants without the use of phenol, organic solvents, or alcohol. The method employs the commercially available spin-column "RNeasy" from Qiagen Inc., Chatsworth, California, USA, and allows the elimination of inhibitory plant polysaccharides and phenolic compounds. It was applied successfully for RNA preparation from GFLV, arabis mosaic virus (ArMV) and GLRaV-3 in grapevine. The third level of improvements includes the design of suitable primers. Exciting work has been done in this field, taking advantage of the rapidly growing data bases of known viral genes. A good example is given by Meng *et al*. (21,22). DsRNA of about 8 kb was isolated from rupestris stem pitting (RSP)-infected grapevines, cloned and sequenced. The viral genome revealed precisely 8726 nucleotides, excluding the poly-A tail. The structure is similar to that of apple stem pitting virus and potexviruses and contains five open reading frames. The authors proposed to call this virus rupestris stem pitting associated virus (RSPaV). An RT-PCR method for detecting the virus in grapevine was developed and it helped to diagnose consistently grapevine infected with rupestris stem pitting (23). Zhang *et al.* (24) obtained very similar results, using again modern molecular techniques to clone and sequence the entire viral genome. The authors used these data to design suitable primers for the detection of the postulated virus associated to grapevine stem-pitting. The use of degenerated primers on dsRNA has allowed to perform RT-PCR to amplify gene fragments of GLRaV-4 and GLRAV-5 (Routh *et al*. 25) and to develop a suitable immunocapture RT-PCR virus detection protocol (IC-RT-PCR), based on a gene fragment from the heat shock protein 70 homologue of the known closteroviruses, capable of detecting both GLRaV-4 and GLRaV-5.

Our partial, practical implementation at RAC-Nyon. As mentioned above, we find the immuno-chemical detection of vitiviruses unreliable. Therefore, we adapted to our needs a PCR-based approach for the detection of GVA and GVB. RT-PCR and IC-RT-PCR were used and found more sensitive than ELISA. However, the published primers were not "universal" enough. We found isolates of GVA with differing sequences and had to generate new primers, as shown in an accompanying presentation (de Meyer *et al*., 26). We prefer RT-PCR to IC-RT-PCR. RNA extracts obtained by means of Qiagen RNeasy procedure, as described by MacKenzie, yields most reliable preparations and they allow multiple PCR runs. Concerning laboratory diagnosis of rupestris stem-pitting, we were very much excited to simply use two published primer pairs in PCR work and to correlate in this way molecular detection with field indexing results. We detected indeed by RT-PCR, using primer pairs RSP2/21 and RSP 8/13 according to Zhang *et al.* (24), corresponding sequences in 44 out of 46 grapevine clones that were indexed stem-pitting positive on *Vitis rupestris* St-George. Nevertheless 3 out of 12 healthy clones, as judged by indexing, were also positive by RT-PCR. Our excitement was however distressed when one important origin of imported certified "healthy" indicator material reacted positively by RT-PCR. A careful further analysis of all indicators at our institute showed however that the other indicator vines, imported long ago from California, were RSPV-negative by RT-PCR. This gives further confidence to the published data. Our experience with PCR based detection of GLRaV-1 and GLRaV-3 is to limited to see a rapid abandon of the fairly reliable and simple serological detection.

Conclusion

Only the most reliable or promising future techniques have been briefly discussed. There is good hope that laboratory assays can soon replace biological indexing to a great extend. More universal reagents will however be necessary. These new tests will be much faster but not necessarily cheaper depending on whether laboratory facilities and trained staff exist or are accessible nearby.

Nucleic acid based techniques have the great advantage of being more sensitive and reagents can be easily generated from published data. Simpler immunoassays might nevertheless be sufficient for several applications. Therefore, I believe that a combination of biological, serological and nucleic acid based diagnosis of grapevine for virus diseases will mark the next decade, respectively the beginning of the new millenium. I emphasize a stepwise process. Biological indexing will also remain useful to reveal new diseases and virus strains. We might even adapt the test conditions and the range of indicators in order to integrate bioassays intelligently into the diagnostic arsenal. There is still plenty of room for innovation.

A survey in the plenum may complete the authors view.

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DETECTION OF GRAPEVINE LEAFROLL ASSOCIATED VIRUSES BY CHEMILUMINOMETRIC ENZYME-LINKED IMMUNOSORBENT ASSAY (LUMINO-ELISA)

P. Gugerli, Swiss Federal Research Station for Plant Production, Changins (RAC), CH-1260 Nyon -1, Switzerland

Enzyme-linked immunosorbent assay ELISA (1) is now broadly used for the detection of grapevine nepoviruses, leafroll associated viruses (GLRaV's) and fleck virus. Results can be fairly reliable if the collection, number and processing of samples and test parameters are optimal. Serological detection of vitiviruses in general and leafroll associated viruses in American rootstock vines is however more critical. Multiple samples and repeated testing are required here to compensate variation due to low virus concentration and to irregular virus distribution. In spite of the development of more sensitive nucleic acid type detection methods, the simplicity of immunoassays makes them still attractive, especially if their detection limit (detectability) can be improved. The use of highly target-specific antibody, e.g. monoclonal antibodies, is obviously a first step and essential to enhance the signal-to-noise ratio of any immunoassay. In this work, I report on the use of chemiluminescence enhanced ELISA (LUMINO-ELISA) in comparison with classical ELISA with chromatic substrate (CHROMO-ELISA). The use of 1,2-dioxethane enzyme substrate permits indeed an ultra-sensitive detection of analytes by ELISA, thanks to the low background emission of the substrate and the ease of detection of the chemiluminescent signal (Bronstein et al. 1989) (2).

Material And Methods

Double antibody sandwich ELISA (1) with phosphatase-labeled secondary antibody, adapted to grapevine (3, 4), was used with substrates of either p-nitrophenyl-phosphate (Sigma) (CHROMO-ELISA) or 1,2 dioxethane / CDP-Star™ enhancer (Tropix PE Applied Biosystems) (LUMINO-ELISA). All tests were carried out in 96 well microtiter ELISA plates (BIOREBA AG, Reinach, Switzerland). Crude leaf sap from infected grapevine, stabilized in 1 : 20 (w/v) extraction buffer (0.5 M Tris pH 8.2, 137 mM NaCl, 2 % PVP 24k, 1 % PEG 6k and 0.05 % Tween 20), was serially tenfold diluted in buffered (1 : 20 w/v) healthy grapevine leaf extract. The volume of chemiluminescent substrate was 100 µl per well. All other test parameters were unchanged and identical in the comparative tests. No particular blocking was used in LUMINO-ELISA, in order not to bias the comparison. Monoclonal antibodies to GLRaV-1 and GLRaV-6 were described in (5,6). Absorbance and light emission readings were recorded with a Dynex MRX microtiter plate reader and Dynex MLX luminometer, respectively.

Results and discussion

Light emission in the form of the "glow" type LUMINO-ELISA was extremely rapid and reached a maximum at around 45 to 55 minutes after adding the CDP-Star™ / Sapphire II™ chemiluminescent substrate, compared to over 12 hours for classical CHROMO-ELISA. As shown in fig.1 and table 1, LUMINO-ELISA revealed a wide dynamic range, which can be up to eight decades with a Dynex-MLX reader. This sensitivity facilitates quantitative measurements. The lower noise level of the luminescent substrate also increased here the detectability of GLRaV-1 and GLRaV-6 to the remarkable endpoint dilution of 1/40'000 compared to 1/4'000 for CHROMO-ELISA.

Applied to the detection of GLRaV-1 in 3309 rootstock vines, the increased detectability of LUMINO-ELISA allowed to trace more infected vines than CHROMO-ELISA, as shown by some typical data in table 2. Since all these

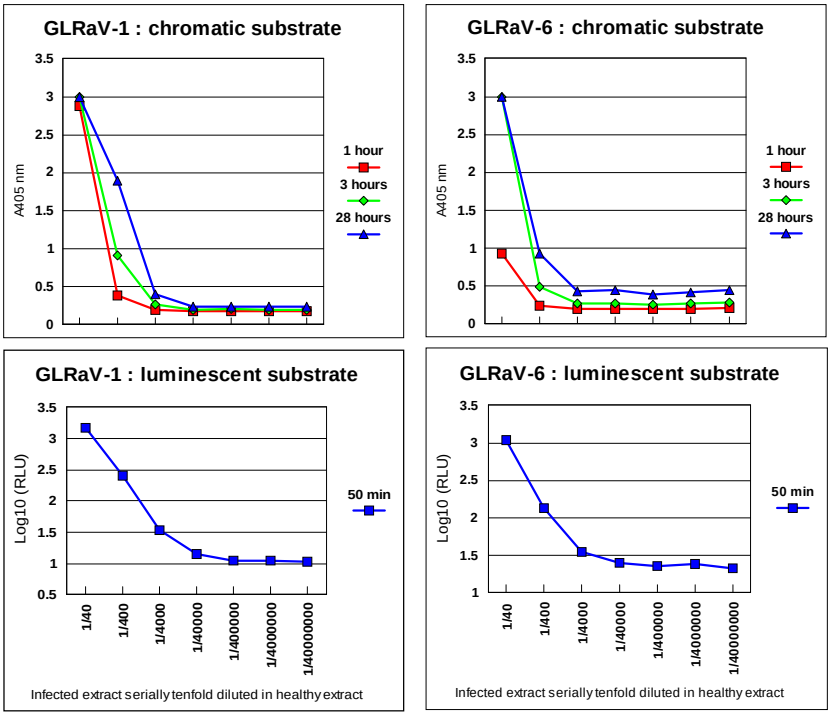

Fig1. GLRaV-1 (left) and GLRaV-6 (right) serially diluted and detected by CHROMO-ELISA (top) and LUMINO-ELISA (bottom).

vines were clonally derived from a healthy initial material, these results point out the possibility of a natural GLRaV-1 contamination in a rootstock plot. The possible vector remains however unknown.

Dilution	RLU	Log10
1/40	1475	3.17
1/400	253	2.40
1/4000	35	1.54
1/40000	14	1.15
1/400000	11	1.05
1/4000000	11	1.05
1/40000000	11	1.04

Table 1. Serially diluted crude leaf sap from GLRaV-1 infected Räuschling grapevine detected by LUMINO-ELISA: RLU (relative luminescence unit) readings after 50 minutes of substrate incubation. Values printed in printed in bold represent positive reactions. Log10: logarithmic transformation of RLU values.

	CHROMO	CHROMO	LUMINO
Sample	ELISA	ELISA	ELISA
	A 405 nm	A 405 nm	RLU
	moy. 2 h	moy. 18 h	65 min
3309 c2 cane	0.20	0.25	5
3309 c3 cane	0.29	0.60	31
3309 c4 cane	0.20	0.25	5
3309 d1 cane	0.21	0.26	6
3309 d2 cane	0.25	0.35	9
3309 d3 cane	0.21	0.26	4
3309 d4 cane	0.22	0.26	5
3309 e1 cane	0.21	0.27	6
3309 e2 cane	0.21	0.27	10
3309 e3 cane	0.21	0.25	4
3309 e4 cane	0.21	0.30	8
3309 f1 cane	0.21	0.25	5
3309 f2 cane	0.20	0.26	5
3309 f3 cane	0.21	0.26	4
3309 f4 cane	0.23	0.30	5
GLR + cane	0.57	2.42	264
GLR + leaf	2.64	3.00	734
healthy cane	0.20	0.28	5
healthy leaf	0.19	0.26	5

Table 2. Typical results of GLRaV-1 detection in 3309 rootstocks by CHROMO- and LUMINO-ELISA. Shaded cells indicate positive reactions.

Conclusions

LUMINO-ELISA clearly underlines the advantage of ELISA based on highly target specific monoclonal antibodies. The assay revealed here both an increased sensitivity and at least a tenfold higher detectability than CHROMO-ELISA. In analogous experiments with other antibody/virus combinations, the gain can be up to two decades. Most polyclonal antibody preparations are however not suited for LUMINO-ELISA, because of lower target specificity. Compared to CHROMO-ELISA, the luminometric assay requires only a different substrate solution. The fast substrate reaction is also an advantage. A disadvantage of LUMINO-ELISA is nevertheless the absolute need of more an expensive luminescence reader. Light emission is indeed to weak for being detected by the human eye.

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NEW PRIMERS FOR THE MOLECULAR IDENTIFICATION AND DETECTION OF GRAPEVINE VIRUS A (GVA)

De Meyer, J. [1], Gaudin, M.[2], Bourquin L.[2], Jakab, G.[3], Malnoe, P. [2] and Gugerli, P. [2]

[1] CIMMYT-Zimbabwe, Maize Research Station. P.O. Box MP 163 Mount Pleasant Harare Zimbabwe
[2] Swiss Federal Research Station for Plant Production, Changins (RAC), CH-1260 Nyon-1, Switzerland
[3] Plant Biology Institute, University of Fribourg, 3, rue Albert Gockel, CH-1700 Fribourg, Switzerland

Low concentration of viral nucleoprotein frequently impairs the serological detection of grapevine virus A (GVA) in crude extracts of grapevine tissue. Hence, more sensitive nucleic acid based detection of this vitivirus, associated with Kober stem grooving (KSG) (1), could improve routine grapevine diagnosis. We therefore evaluated and adapted to our needs molecular diagnosis by polymerase chain reaction (PCR). Prior to this work, we had differentiated by means of monoclonal antibodies two serologically distinct groups of GVA's (2), designated GVA-1 and GVA-2. Here we report about primers, which were well suited to detect GVA-2 but failed to recognize GVA-1. We also present the design of further primers with a broader specificity for the identification and detection of both types of GVA's from California, Canada, France, Italy, Israel and Switzerland.

Material And Methods

Swiss and Italian grapevine culivars served as main virus sources. They were either maintained on herbaceous plants (*N. clevelandii and N. benthamiana*), grapevine or both. Cloning and partial sequencing of the genomes of four of these isolates were done by standard techniques and the results will be published elsewhere (3). Other isolates were from Canada, France, Israel and California. For PCR applications, RNA extracts were mostly obtained according to the Qiagen RNeasy procedure, as described by MacKenzie (4). Basic protocols of PCR were as described by Barbara *et al*. (5) but were subsequently modified and adapted to our needs (6). A one step procedure with AMV RT and Tth polymerases from Promega was generally used, with the following PCR conditions (programme A-4): 45'/48°C, 2'/94°C, 35 cycles of (44''/94°C, 40''/52°C, 90''/72°C), 10'/72°C. Immuno-capture reverse transcription PCR (IC-RT-PCR) was carried out as described by (7) with polyclonal immunoglobulin from BIOREBA AG, Reinach, Switzerland.

Results And Discussion

The first primer pair, designated G1/G4 (table 1), was derived from a partially sequenced genome of GVA-2 isolate 825, originally isolated from a Sicilian origin of grapevine cv. Inzolia (8). These primers allowed us to amplify a fragment of 398 base pairs (bp) from GVA-2 isolates (fig. 1 a and table 2). They failed however to amplify a homologous fragment of GVA-1 isolate 873.

Table 1: Primer names and sequences

Name	Sequence	Position	Size of the Amplicon
G1	5' ATACTCTCTTCGGGTACATCGC 3'	6532-6553	398 bp
G4	5' GTGCATGGCCTGTATCACAGT 3'	6909-6929	
MP	5' TGCCAGAGGTGTTTGAGACAAT 3'	6369-6390	986 bp
CPdt	5' TTTTTGTCTTCGTGTGACAACCT 3'	7332-7354	
G5	5' CCAGAGGAGTTTGAGACAATA 3'	6371-6391	195 bp
G6	5' GTCCCGACCAAGGCGATGTACCC 3'	6543-6565	

We then designed primers for sequencing purposes, considering a few available sequences of vitivirus (9). A new homologous primer (MP) was defined on the basis of homologies at the amino-acid level, i.e. the sequence "LVMPEVFETIKK", coded by nucleotides 6362 to 6397 of the GVA genome (9). The complementary second primer (CPdt) corresponded to sequences in the poly-A tail of the viral genome, comprising the 18 last nucleotides from nucleotides 7332 to 7349. This primer set MP/CPdt amplified a segment of the expected length of 987 bp from GVA-1 isolate 873, GVA-2 isolate 939 and GVA-2 isolate 1001 (fig. 1d). Other primer combinations (G1/CPdt, MP/G4) allowed us to produce suitable DNA for sequencing purposes (fig. 1 b and c). Long amplification products were only generated from samples of infected herbaceous plants and not from infected grapevine, especially for primer MP/CPdt reaction.

Alignment of the new sequence with published data (9), helped us finally to define a suitable primer set for diagnosis purposes, i. e. homologous primer G5, from the overlap between the movement protein and of the coat protein, and the complementary primer G6, corresponding to the nucleotides reported in table 1. This primer set G5/G6 generated a short fragment (195 bp) of all available isolates of GVA (table 2), including GVA-1 isolate 873, as shown in fig. 2.

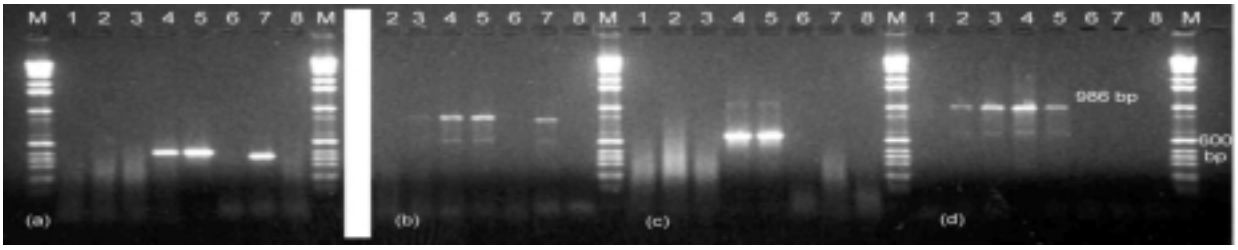

Figure 1. IC-RT-PCR with the following primer pairs: (a) G1/G4, (b) G1/CPdt, (c) MP/G4, and (d) MP/CPdt. Assayed samples from left to right: (1) healthy *N. benthamiana*, (2 and 3) GVA-1 isolate 873 on *N. benthamiana*, (4) GVA-2 isolate 825 on *N. benthamiana*, (5) GVA-2 isolate 1001 on *N. benthamiana*, (6) healthy grapevine Païen (7), KSK diseased Païen KSG and (8) water control.

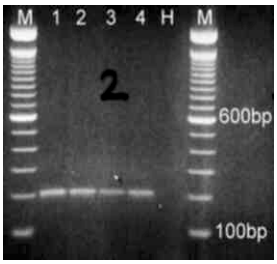

Figure 2. Typical RT-PCR with primers G5/G6. M: DNA markers; 1) and 2) GVA extract 508 from grapevine Savagnin rose (France); 3) GVA-1 isolate 873 from *N. benthamina*, 4) GVA extract 472 from Pinot noir (California)

GVA isolate	Origin	Primers G1/G4	Primers G5/G5
GVA-1 873	Italy	-	+
GVA-2 825	Italy	+	+
GVA-2 934	Switzerland	+	+
GVA-2 935	Switzerland	+	+
GVA-2 939	Switzerland	+	+
GVA-2 1001	Switzerland	+	+
GVA-2 1027	Israel	+	+
GVA-2	Canada	+	nt
GVA	France	+	+
GVA	California	+	+

Table 2. GVA isolates identified by RT-PCR with primers G1/G4 and G5/G6. (+) positive, (-) negative, nt not tested.

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Conclusion

The results obtained at the molecular level with GVA-1 and GVA-2 isolates confirmed the antigenic heterogeneity of isolates of GVA. The results emphasize that the selection of molecular probes, as well as of monoclonal antibodies, needs careful investigations. The primers G5/G6 revealed promising for diagnosis purposes, especially because they generate a short fragment and recognize both GVA-1 and GVA-2 isolates.

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