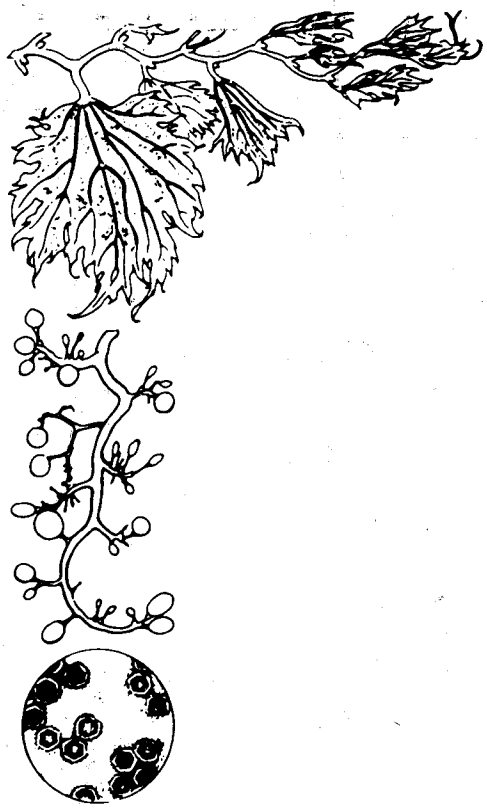
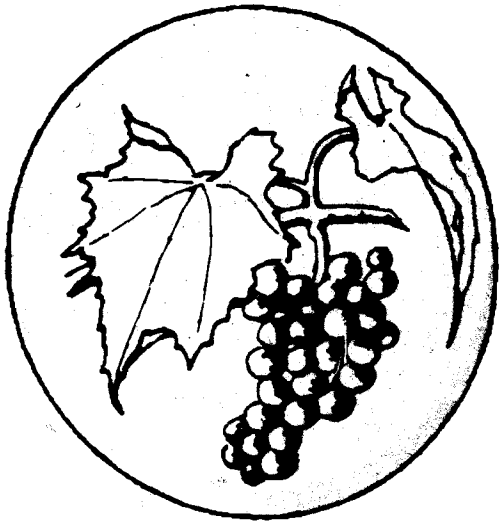


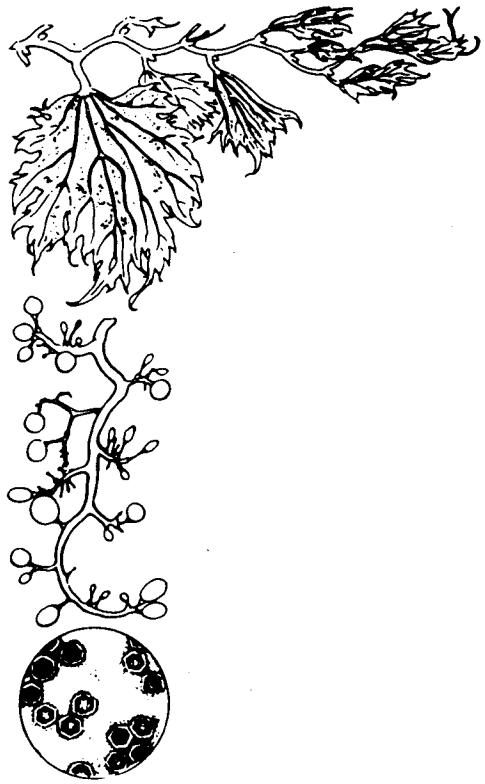
12th MEETING OF THE INTERNATIONAL COUNCIL FOR THE STUDY OF VIRUSES AND VIRUS-LIKE DISEASES OF THE GRAPEVINE

(ICVG)

EXTENDED ABSTRACTS



28th September-2nd October, 1997
LISBON, PORTUGAL



12th MEETING OF THE INTERNATIONAL COUNCIL FOR THE STUDY OF VIRUSES AND VIRUS-LIKE DISEASES OF THE GRAPEVINE

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EXTENDED ABSTRACTS

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OEIRAS, PORTUGAL
1997

PLACE OF THE MEETING

The place chosen by the Organising Committee for the 12th ICVG Meeting is the excellent complex of the Calouste Gulbenkian Foundation. This complex is surrounded by beautiful gardens and includes the Modern Art Museum, the Gulbenkian Museum and theatres for congresses and musical performances. In the museums, Mr. Gulbenkian's private art collections are exhibited as well as other pieces and works of art.

The Organising Committee are much indebted to the Administration of Calouste Gulbenkian Foundation for providing these excellent facilities for the 12th ICVG Meeting and to the Director of the Central Services, Engº João Lucas and Mr. J. Lourenço for all the help.

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AKNOWLEDGMENTS

The Organising Committee are indebted to several Institutions and private enterprises that supported the Meeting and are listed below.

Special thanks are due to the Minister of Agriculture of Portugal who authorised the printing of this abstract book in "Oficina Gráfica da Secretaria Geral do MADRP and also to Engº Domingos Soares Franco (J.M. da Fonseca, Succ^{s}, Vinhos S.A.), Engº António Avillez (J. P. Vinhos, S.A.) and Engº António Guedes (Sociedade Agrícola e Comercial da Quinta da Aveleda, S.A.) for generous support and for making their facilities available during the technical visits and post-conference tour of the participants.

Particular gratitude is expressed to Dr. René Bovey for suggestions and for supplying lists of addresses.

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CORRECT CITATION

Extended abstracts 12th Meeting ICVG, Lisbon, Portugal, 29 September-2 October 1997, pg. - pg.

Ficha Técnica:

Título: 12th Meeting of the International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine – Extended Abstracts.
Editor: Óscar A. de Sequeira, José C. Sequeira Margarida T. Santos; Departamento de Fitopatologia, Estação Agronómica Nacional (EAN) / INIA – MDRP
Impressão, encadernação e acabamento: Oficina Gráfica da Secretaria Geral do Ministério da Agricultura, do Desenvolvimento Rural e das Pescas.
Depósito Legal: 114767/97
ISBN: 972-97465-0-8
Tiragem: 250 ex

PREAMBLE

It is Portugal's turn to organise the 12th ICVG Meeting, the last of this century. It is interesting to remember that it was also in Portugal (Oeiras) where the first preparatory meeting took place in 1961 and where this already long series of ICVG meetings originated. It was organised by the late and well remembered Humberto Dias who attracted few delegates from several wine producing countries from the mediterranean region, England and United States.

Over the intervening years, the number of participants in these meetings has increased tremendously. This has contributed to the great scientific progress that has been achieved in the field of grapevine virology, which has also had major repercussions in several other fields. In particular they have been contributing to the increasing improvement of viticulture products in general, with implications in the legislation at the european and international level. It is expected that this trend will be maintained.

This extended abstract book has been prepared with great care. We have done our best to eliminate errors or misprints during the editing to standardise the presentation of the texts. However, some may have escaped our attention, for which we apologise.

The Organising Committee hope that this meeting will contribute towards maintaining the high standards already achieved by this series. It is expected that it will be both fruitful and rewarding for all participants. We wish you a pleasant and enjoyable stay in Portugal.

The Organising Committee

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TOPIC 6 - EPIDEMIOLOGY

TOPIC 7 - NEW STRATAGIES FOR INTRODUCING RESISTANCE TO VIRUSES

GRAPEVINE VIROLOGY HIGHLIGHTS 1994-97

G.P. Martelli

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Reviewing the 400 or so papers published since the 11th Meeting of ICVG on various aspects of grapevine virology is far beyond the scope of this presentation, which is centred on some of the noteworthy advances made since 1994.

A. Nomenclature and updated list of grapevine viruses with their taxonomic affiliation

In 1994, Martelli (1) compiled a list of 40 different viruses identified in grapevines. This list has now grown to 44 species (Tab. 1), relevant additions being three filamentous and one isometric virus.

Grapevine leafroll-associated virus 7 (GLRaV-7) was first detected in an Albanian grapevine accession (2), but it has now been recorded from several Mediterranean countries (Italy, Greece, Turkey, Palestine) and in Armenia (unpublished information). GLRaV-7 is a closterovirus serologically unrelated to the other six viruses of the series, from which it also differs because of the unusual modifications induced in the chloroplasts. When present singly in grapevines, GLRaV-7 elicits typical leafroll symptoms (3).

Grapevine virus D (GVD) is a member of the newly established genus *Vitivirus* (5, see also these Proceedings). Morphologically, physico-chemically, and molecularly GVD resembles the other viruses of the series, but was reported to be serologically unrelated to them (6). However, a very distant serological relationship with GVA and GVB was more recently discovered (7), due to the presence of at least one common internal (cryptotope) antigenic determinant (8).

An unnamed and still uncharacterised capillo-like virus was observed in Canada in vines affected by Rupestris stem pitting (RSP) and LN33 stem grooving (9). Whether this virus has anything to do with the unusual RNA found in RSP-affected vines (10) is unknown, but this will be one of the issues to be addressed for a further approach to the ultimate definition of the aetiology of the rugose wood complex.

A non-mechanically transmissible isometric virus 30 nm in diameter with particle morphology strikingly similar to that of grapevine fleck virus (GFkV) was found in vines affected by asteroid mosaic (11). The studies have not progressed further. Recently, however, a pair of primers designed on the sequence of the polymerase cistron of GFkV, amplified a fragment of the asteroid mosaic-associated virus RNA (S. Sabanadzovic, personal communication), indicating a phylogenetic relationship between the two viruses.

Grapevine leafroll-associated virus 2 (GLRaV-2) was mechanically transmitted in two laboratories (12, 13) and shown to possess two biological variants inducing differential reactions in *Nicotiana benthamiana* (13). The outcome of comparative serological studies co-operatively carried out in four laboratories was: (i) the recognition that the formally described grapevine corky bark-associated virus was the same as GLRaV-2; (ii) the re-naming as GLRaV-6 of the virus formerly denoted GLRaV IIa; (iii) the designation of the French isolate of GLRaV-2 as the authentic reference species (12). Besides setting order in the GLRaVs nomenclature Boscia *et al.* (12) pointed out that GLRaV-1 and GLRaV-3 are *"so consistently and intimately connected with leafroll disease that they can be regarded as genuine agents of leafroll"*, concluding that "*The word "associated" may soon be dropped from their name*". This suggestion was taken up by Belli *et al.* (14), who, based on graft-transmission tests using sources containing either GLRaV-1 or GLRaV-3 as inoculum, reproduced the leafroll disease syndrome and, without further ado, erased the word "associated" from the name of both viruses.

The question now is, are we prepared to proceed along this way, i.e. modifying current closterovirus names whenever there is reasonable evidence that any of them, by itself, can induce leafroll symptoms? If yes, also GLRaV-2 and GLRaV-7 should be changed into GLRV-2 and GLRV-7. It should kept in mind, however, that this is not a mere nomenclatorial exercise for it implies the recognition of a defined aetiological role.

The presence of seven (for the time being) serologically unrelated putative closteroviruses in the same host is puzzling and the cause of searing doubts. Are really all these viruses serologically distinct from each other, or do they have some sort or relationship (think of the common "hidden" determinants in vitiviruses)? Is serology alone sufficient for demarcating a virus species? For practical detection purposes whether the seven closteroviruses are different species or different strains of the same species does not make a bit of difference. This however, would have a bearing on taxonomy and, in the long range, on the predictability of the epidemiological behaviour of individual viruses.

Table 1. Grapevine viruses and their taxonomic affiliation (from 1, modified)

FAMILY	GENUS	VIRUS SPECIES	
A. Virus belonging to genera included into families			
BROMOVIRIDAE	*Alfamovirus*	Alfalfa mosaic	(AMV)
	Cucumovirus	Cucumber mosaic	(CMV)
	Ilarvirus	Grapevine line pattern	(GLPV)
BUNYAVIRIDAE	*Tospovirus*	Tomato spotted wilt	(TSWV) (?)[a]
COMOVIRIDAE	*Fabavirus*	Broadbean wilt	(BBWV)
	Nepovirus	Artichoke Italian latent	(AILV)
		Arabis mosaic	(ArMV)
		Blueberry leaf mottle	(BBLMV)
		Bulgarian latent	(GBLV)
		Grapevine chrome mosaic	(GCMV)
		Grapevine fanleaf	(GFLV)
		Grapevine Tunisian ringspot	(GTRV)
		Peach rosette mosaic	(PRMV)
		Raspberry ringspot	(RRV)
		Tobacco ringspot	(TRSV)
		Tomato ringspot	(ToRSV)
		Tomato black ring	(TBRV)
		Strawberry latent ringspot	(SLRSV)
TOMBUSVIRIDAE	*Tombusvirus*	Petunia asteroid mosaic	(PAMV)
		Grapevine Algerian latent	(GALV)
	Carmovirus	Carnation mottle	(CarMV)
	Necrovirus	Tobacco necrosis D	(TNV-D)
CLOSTEROVIRIDAE	*Closterovirus*	Grapevine leafroll-associated 1	(GLRaV-1)
		Grapevine leafroll-associated 2	(GLRaV-2)
		Grapevine leafroll-associated 3	(GLRaV-3)
		Grapevine leafroll-associated 4	(GLRaV-4)
		Grapevine leafroll-associated 5	(GLRaV 5)
		Grapevine leafroll-associated 6	(GLRaV 6)
		Grapevine leafroll-associated 7	(GRLaV-7)
B. Viruses belonging to floating genera			
	Sobemovirus	Sowbane mosaic	(SoMV)
	Potexvirus	Potato X	(PVX)
	Tobamovirus	Tobacco mosaic	(TMV)
		Tomato mosaic	(ToMV)
	Vitivirus	Grapevine A	(GVA)
		Grapevine B	(GVB)
		Grapevine C	(GVC)
		Grapevine D	(GVD)
	Capillovirus	Unnamed	
	Trichovirus	Grapevine berry innner necrosis	(GINV)
C. Taxonomically unassigned viruses			
		Grapevine fleck	(GFkV)
		Grapevine asteroid mosaic	(GAMV)
		Grapevine ajinashika	(GAV)
		Grapevine stunt	(GSV)
		Grapevine labile rod-shaped	(GLRSV)

a. Infections by a TSWV-like virus were reported from Taiwan, but this record has never been confirmed. In fact, Stobbs and Broadbent (4) were unable to infect 8 *Vitis vinifera* cultivars, 34 *Vitis* hybrids, 7 *Vitis labrusca* and 3 *Vitis rupestris* by mechanical inoculation with an isolate of TSWV or by exposure to viruliferous *Frankliniella occidentalis*.

B. Aetiology

Convincing evidence was obtained that at least three distinct closteroviruses (GLRaV-1, GLRaV-3, and GLRaV-7) are genuine agents of leafroll. A fourth closterovirus (GLRaV-2) was shown to be the cause of a graft incompatibility revealed by Kober 5BB (15). This, however, does not conflict with the alleged aetiological role played by GLRaV-2 in the genesis of leafroll. The same study (15) and another by Bonavia *et al.* (16) provided additional evidence of the involvement of GVB in the aetiology of corky bark, whereas GVA was convincingly linked with Kober stem grooving (17,18,19,20). It seems then plausible to conclude that the current knowledge indicates closteroviruses as the primary agents of leafroll and vitiviruses, and, perhaps, other morphologically similar viruses, as the agents of rugose wood.

The general properties of grapevine clostero- and vitiviruses were recently reviewed (21,22,23), one of the questions addressed being the origin of these viruses. The following speculative answer was provided for closteroviruses:

"American Vitis *species used as rootstocks and their hybrids, undergo symptomless GLRaV infection and impair virus multiplication in their leaves, and these vine thus exhibit resistance of some sort. Comparable situations with other viruses were interpreted as deriving from the co-evolution of host and pathogen in the same centres of origin. If this were the case with GLRaVs, the conclusion could be drawn that their centre(s) of origin might be in the American continent. This, however, contrasts with two established facts. First, records of leafroll in the European literature predate the introduction of American roostocks. Second, GLRaVs [especially GLRaV-1 and GLRaV-3* (now also GLRaV-7)] *are profusely present in the self-rooted European grapes that are still grown in scattered enclaves in some Mediterranean and Middle Eastern areas, and they also occur extensively in Phylloxera-free countries like Cyprus, Afghanistan, and Yemen* (now also Armenia). *It thus seems more plausible to suggest that GLRaVs, rather than with American* Vitis *species, had a long association with* V. vinifera, *with which they have travelled from common centre(s) of origin to far away places"* (23), and for trichoviruses (now vitiviruses):

"Similarly, lack of information makes it difficult to theorise on the possible site of origin of the trichovirures, except perhaps for GVA. This virus occurs in the same areas and conditions, and appears to have followed the same dissemination routes as the closteroviruses. One can thus speculate that GVA, like the closteroviruses may have the same centre of origin as Vitis vinifera *The presence of GVA in countries like Yemen* (and Armenia). *the site of a highly traditional millennial viticulture impervious to the introduction of foreign germplasm, seem to support this hypothesis* (21).

C. Epidemiology

Interesting advances were made in the knowledge of the epidemiology of some grapevine viruses, whose current vector situation is as outlined in Tab. 2.

As to nematode-transmitted viruses, a survey of *Xiphinema index* in Spain revealed that this nematode occurs in 14% of the vineyards sampled and in 50% of those with grapevine fanleaf virus (GFLV) infections (31). GFLV was recorded from Nigeria (32) and was isolated in Hungary from *Aristolochia clematitis* and *Lagenaria siceraria turbinata* with vein clearing and bright chrome yellow mosaic (33). This constitutes the first substantiated record of a natural infection by GFLV to hosts other than grapevines. Grapevine chrome mosaic virus (GCMV) was recorded from Austria (34) and tomato ringspot virus (ToRSV) was apparently isolated in Slovakia from Cabernet Sauvignon vines with leafroll symptoms (35). The last record is unusual, based on what known on the presence of American nepoviruses in Europe (24), and may requires confirmation. Not so the long suspected transmission of the so-called grapevine strain of raspberry ringspot virus by *Paralongidorus maximus* which was experimentally confirmed (26).

New ideas were put forward with reference to the mechanism of virus retention in their associated vector species. Similarly to tobraviruses, the C-terminal part of the coat protein of nepoviruses may have a protruding amino acid sequence which could serve for linking virus particles to the carbohydrate-containing material that lines the surface of the areas where virions attach in the nematode (36).

Several new vectors of filamentous viruses were identified: *Pseudococcus affinis* for GVA, GVB (27), and GLRaV-2 (28); *Ps. calceolariae* for GLRaV-3 (29); *Pulvinaria vitis* for GLRaV-3 (30). The latter record is of special interest because it constitutes the first evidence that coccid mealybugs can be virus vectors. The lack of transmissibility of GLRaV-1 by pseudococcid mealybugs was confirmed in New Zealand (29), whereas natural spreading of this virus, and of GFkV, was reported from Northern Italy in vineyards where mealybugs other than pseudococcids (*Neopulvinaria innumerabilis, Parthenolecanium corni, P. persicae*) were present (37). By converse, in Southern France field spread of GLRaV-1 and GLRaV-3 was observed in vineyards where only *Pseudococcus bohemicus,* the most northerly pseudococcid species, occurs (38). An exception to general trend came from Australia, where natural spread of GLRaV-3 could not be linked with the presence of mealybugs of any type (39).

The relationship of filamentous viruses to their vectors begins to be unravelled. It was experimentally demonstrated that GLRaV-3 and GVA are both semipersistently transmitted by *Pl. citri* and *Ps. longispinus*, respectively (40,41), a behaviour consistent with what postulated by Tanne *et al.* (42) based on statistical analysis of field spread of corky bark. The transmission parameters of GVA were: 15 min acquisition access time, 12 to 48 h retention time, 30 min inoculation access time, and no latent period (41).

Table 2. Experimentally recognised grapevine virus vectors and the viruses they transmit.

VIRUS	VECTOR	REFERENCE
A. Soil-borne viruses		
Grapevine fanleaf	*Xiphinema index*, *X. italiae*	24
Arabis mosaic	*X. diversicaudatum*	25
Tomato black ring	*Longidorus attenuatus*	25
Raspberry ringspot	*Paralongidorus maximus*	26
Tomato ringspot	*X.californicum*, *X. rivesi*	24
Tobacco ringspot	*X. americanum*	24
Peach rosette mosaic	*X. americanum*, *L. diadecturus*, *X. rivesi*	24
B. Viruses transmitted by aerial vectors		
Grapevine virus A	*Pseudococcus longispinus*, *Ps. affinis*, *Planococcus citri*, *Pl. ficus*	21, 27
Grapevine virus B	*Ps. longispinus*, *Ps. affinis*, *Pl. ficus*	21, 27
Grapevine leafroll-associated virus 3	*Pl. ficus*, *Ps. affinis*, *Ps. longispinus*, *Pulvinaria vitis*, *Ps. calceolariae*	22, 28, 29,30
Grapevine leafroll-associated virus 2	*Ps. longispinus*, *Ps. affinis*	28

D. Molecular Biology

Molecular biology studies have progressed a great deal, especially with filamentous viruses. The GVA and GVB genome was completely (43, 44) and that of GVD (6) partially sequenced, showing differences that substantiated the retention of a separate species status for the three viruses. Likewise, the molecular analysis of GLRaV-3 (39, 45) and the sequencing of the 3' end of GLRaV-2 (46), demonstrated that both are genuine closteroviruses for they possess genome structure and organisation typical of the genus, and the genes coding for the HSP70 analogue and the diverged copy of the coat protein. The molecular study of GLRaV-1 has just been initiated (47). Partial sequencing of the genome of grapevine berry inner necrosis virus (GINV) demonstrated that it is a genuine trichovirus (48).

As to nepoviruses, the coat protein gene of several GFLV isolates of different geographical origin was sequenced showing limited variations (49, 50). By contrast, remarkable differences were found in the coat protein composition of strawberry latent ringspot (SLRSV) compared with that of other nepoviruses, so as to suggest that SLRSV may be the representative of a distinct virus group (51). This likelihood was strongly supported by another study in which a thorough comparative computer-assisted analysis of como- and nepoviruses showed that their coat proteins share a common evolutionary origin. Como- and nepoviruses clustered quite nicely but separately in a phylogenetic tree, from which SLRSV branched off quite clearly (52).

The putative 38K GFLV movement protein was searched for in infected *Chenopodium quinoa* plants, and found, primarily in the cytosol, from the early to the late stages of infection. This behaviour, though unusual, is not incompatible with the biological function suggested for this protein (53).

The RNA-1 of a grapevine isolate of arabis mosaic virus (7330 nt in size) was totally sequenced (54), completing the molecular information on this virus. Unlike the serologically related GFLV (and other nepoviruses) ArMV was shown to contain two RNA-2 species of different length encoding two polyproteins more than 95% identical, except in their N-terminal domains (55).

Le Gall *et al.* (56) demonstrated that the RNA-2 of an isolate of an English serotype of tomato black ring virus (TBRV) probably arose by an RNA recombination event resulting in the exchange of the putative movement protein gene between TBRV and grapevine chrome mosaic virus (GCMV). In pseudo recombinants of the same two viruses (GCMV RNA-1 and TBRV RNA-2) the 3' non translated region of RNA-1 was promptly transferred to RNA-2, thus restoring the 3' identity. This was interpreted as a mechanism whereby randomly appearing mutations can spread from one genomic RNA to the other (57).

Molecular investigations had a great bearing on diagnosis and production of transgenic plants, two facets of grapevine virology that are gaining increasing momentum. Probes (cDNA and cRNA) and primers for genome amplification (PCR) are major by-products of molecular studies. Such reagents were produced and used for the sensitive detection of a number of isometric (nepoviruses, GFkV) (58, 59, 60,61,62,63) and filamentous (closteroviruses, vitiviruses) (19,39,41,64,65,66,67,68, 69,70,71) viruses, so as to become competitive with immuno-enzymatic assays. There is little doubt that detection and identification of grapevine viruses will rely more and more on molecular assays which, in the medium range, are likely replace indexing, the traditional and still indispensable bioassay.

As to transgenic resistance, several projects are under way, primarily in Europe, for the introduction of pathogen-derived genes into *Vitis*. Successful transformation was obtained of *Nicotiana* plants with the coat protein (CP) genes of GFLV (72),, GVA, and GVB (73) and the putative movement proteins of GVA and GVB (74). More advanced is the work with nepoviruses (GFLV, ArMV and GCMV, in particular) which has already yielded rootstocks and *V. vinifera* cultivars expressing either the viral CP (75, 76,77,78) or the polymerase (77,79) genes.

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PHYSICO-CHEMICAL AND MOLECULAR CHARACTERIZATION OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2

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Grapevine leafroll-associated virus 2 (GLRaV-2) is the only mechanically transmissible closterolike virus infecting grapevines (1). *Nicotiana benthamiana*, the sole infectible host, was used for purification and characterisation of the virus. In cesium sulphate gradients, partially purified virus preparations sedimented as two bands, the lowest of which contained virus particles. Virus yields were exceedingly low so that all attempts to extract and resolve the nucleic acid in agarose gel electrophoresis failed. The mol. wt of the viral RNA, estimated from its full genome double stranded form, was about 6 x 10^6 daltons. The coat protein consisted of a single polypetide with a mol. wt of 21.5 kDa.

CYTOPATHOLOGY

Tissue fragments from leaves and petioles of GLRaV-2-infected grapevine accessions were processed for embedding, thin sectioning and immunogold labelling as described (1). Parenchyma cells were apparently unaffected and contained no virus particles. These, however, were present in the nuclei and cytoplasm of sieve tubes and companion cells. Virions could be differentiated from P-protein filaments by immunotagging with colloidal gold. The cytoplasm contained also accumulations of vesicles with fibrillar content, derived from the peripheral vesiculation of mitochondria, a feature common to GLRaV-1 and GLRaV-3 infections (2).

MOLECULAR ANALYSIS

Complementary DNA was synthesised by hexanucleotide random priming denatured viral dsRNA and was cloned and subcloned in the plasmid pUC18. Sequencing was performed on PEG-purified plasmids using a Thermo-Sequenase kit and 35S-dATP, and filling gaps between adjacent clones by polymerase chain reaction (PCR). To do so, three sets of primers were designed, that amplified sequences of 2470 bp, 821 bp, and 3500 bp, respectively. A total of 7 clones accounting for 7944 nt in the 3' end region of the viral genome were obtained and sequenced. Eight open reading frames (ORFs) were identified in the 5'->3' direction in the sequenced genome fragment (Fig. 1). ORF 1a, which was only partially sequenced, encoded a polypeptide with conserved motifs of viral helicases. The ORF 1b product was a 48K polypeptide with the eight conserved motifs of RNA dependent RNA polymerase. ORF 1b overlapped the preceding ORF 1a by 120 nt. This overlapping area lacked an initiation codon, thus suggesting that the RdRp protein was expressed via a +1 ribosomal frameshifting. ORF 2 coded for a small 6K hydrophobic protein containing a transmembrane motif. The 65K polypeptide encoded by ORF 3 contained the eight conserved motifs of the heat shock proteins of the HSP70 family. ORF 4 encoded a protein with some homology with the HSP90-like proteins expressed by other closteroviruses. ORF 5 and ORF 6 coded the coat protein and its diverged copy. ORF 7 and ORF 8 coded for products (19K and 24K, respectively) with a low homology with the comparable proteins coded by other closteroviruses. The phylogenetic analysis of GLRaV-2 RdRp revealed a high affinity with the polymerases of beet yellows (BYV), citrus tristeza (CTV), and beet yellow stunt (BYSV) closteroviruses, and a more distant relationship with those of species of the *Capillovirus* and *Trichovirus* genera. The structural organisation of the GLRaV-2 genome is strikingly similar to that of BYV (Fig.1). This fact and the results of computer-assisted comparative analyses with the sequence of taxonomically relevant cistrons of BYV and other definitive closteroviruses, leave little doubt that also GLRaV-2 is a *bona fide* closterovirus species.

DIAGNOSIS

One of the cDNA clones (paV38) was used for the production of 300 bp DNA digoxigenin-labelled probe which proved virus-specific and successfully detected GLRaV-2 in total nucleic extracts from infected vines and *N. benthamiana* . A set of GLRaV-2 specific primers were designed to amplify a cDNA fragment of 821 bp and successfully applied in RT-PCR analysis of infected vines.

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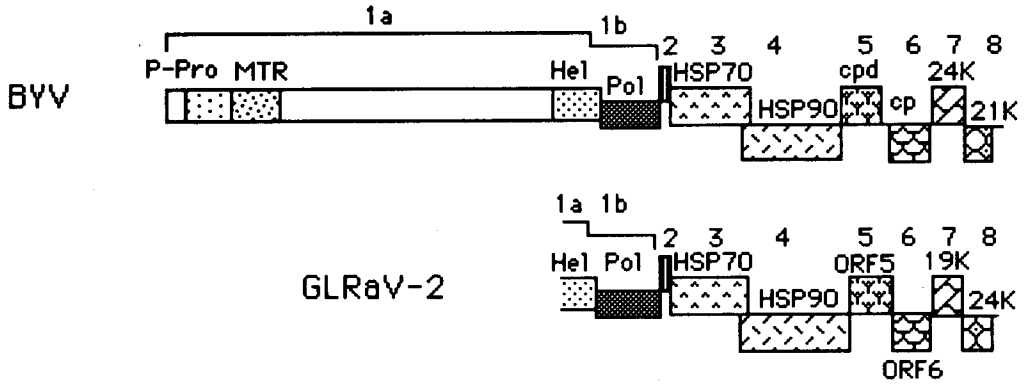

Fig.1. Organization of the 3' end of the GLRaV-2 genome compared with the full genome of beet yellows closterovirus.

NUCLEOTIDE SEQUENCE AND GENOME ORGANIZATION OF GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUS 2

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A cDNA library was constructed from a 16 kb dsRNA that was consistently extracted from grapevine leafroll associated closterovirus 2 (GLRaV 2) infected grapevines. Thus, 14.536 nucleotides at the 3' region of GLRaV 2 have been sequenced. The sequenced portion of the GLRaV 2 genome encompasses nine open reading frames (ORFs) which include, in the direction 5' to 3', an incomplete ORF 1a and eight ORFs encoded proteins of 48 kDa (ORF 1b), 6 kDa (ORF 2), 65 kDa (ORF 3), 63 kDa (ORF 4), 25 kDa (ORF 5), 22 kDa (ORF 6), 19 kDa (ORF 7), 24 kDa (ORF 8), and 216 nucleotides of 3' untranslated region. An incomplete ORF 1a region potentially encodes a large polyprotein containing the domains characteristic of papain-like protease (P-PRO), methyltransferase (MTR) and helicase (HEL). Based on the alignment of the P-PRO surrounding region between GLRaV 2 and beet yellows virus (BYV), cleavage site in GLRaV 2 polyprotein was predicted to be at Gly-Gly bond, where autoproteolysis in its N-terminal portion may occur similar to other closteroviruses. The downstream ORF 1b potentially encodes a putative RNA-dependent RNA polymerase. Alignment of the frameshift region of GLRaV 2 with BYV suggests that the expression of ORF 1b may also be via a +1 ribosomal frameshift, which probably occurs at TAGC similar to BYV, beet yellows stunt virus (BYSV) and citrus tristeza virus (CTV). ORF 2 encodes a small hydrophobic protein of 6 kDa. ORF 3 (65 kDa) represents a homologue of the cellular HSP70 heat shock proteins, and the ORF 4 (63 kDa) with unknown function shows some degree of homology to other closteroviruses. ORF 5 (22 kDa) and ORF 6 (25 kDa) encode the capsid protein and its diverged duplicate, respectively. Identification of the ORF 6 (22 kDa) as the coat protein gene of GLRaV 2 was further confirmed by in vitro expression in *Escherichia coli* and immunoblotting. Two other ORFs (ORF 7 and ORF 8) are present at the 3' end. These have similar molecular mass tc corresponding ORFs of BYV but have no significant sequence similarity. Phylogenetic analysis of various protein domains along with the gene array demonstrates a close relationship with other closteroviruses: BYV, BYSV and CTV. The genome organization of GLRaV 2 is most similar to BYV, but is quite different from GLRaV 3, which has a coat protein of 36 kDa.

NUCLEOTIDE SEQUENCING AND GENOME ORGANISATION OF GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUS 3 AND DEVELOPMENT OF TRANSGENIC PLANTS EXPRESSING ITS COAT PROTEIN AND OTHER GENES

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With particle length ranging from 1,800-2,000 nm long, grapevine leafroll associated closterovirus 3 (GLRaV 3) consists of a ssRNA and a coat protein of 43 kDa. A high molecular weight dsRNA (~18 kb) is observed in GLRaV-3 infected plants. In the present study, we cloned this dsRNA and sequenced 16,038 nucleotides of the genome. Sequenced portion contains 12 open reading frames (ORFs). Incomplete ORF1a encoded a polyprotein which contained domains of methyltransferase (MTR) and helicase (HEL). ORF1b (61 kDa) encoded a RNA-dependent RNA polymerase (POL) which may be expressed via a +1 frameshift mechanism. ORF2 (6 kDa) and ORF3 (5 kDa) were small proteins that separated with a long intergenic region (1,065 bp). ORF4 (59 kDa) was shown to be a heat shock 70 related protein (HSP70). Function of ORF5 (55K) was unknown. ORF6 (35 kDa) and ORF7 (53 kDa) were identified the coat protein gene and its diverged copy, respectively. Four other ORFs (21 kDa, 20 kDa, 20 kDa and 7 kDa) presented on the 3′ terminal region were similar in size and location to citrus tristeza virus (CTV) but had no significant sequence homology. General genome organisation of GLRaV 3 was similar to other closteroviruses, particularly to CTV. Phylogenetic analysis using different domains or genes of GLRaV 3 with respective sequences from other closteroviruses placed GLRaV 3 into a separate but closely related branch.

Availability of genome sequence of GLRaV 3 made it possible to use coat protein gene and other gene sequences to exploit the pathogen-derived resistance. Coat protein-mediated protection, a form of pathogen-derived resistance has been successfully used to control several economically important plant virus diseases. However, the mechanism of the resistance is not well understood. Both protein-mediated and RNA-mediated resistance have been reported to be responsible under certain circumstances. Thus, several versions of GLRaV-3 coat protein gene, including sense translatable, antisense and nontranslatable were constructed into a plant expression vector pEPT8 and further mobilised into a plant transformation vector pGA482G. Also, a truncated GLRaV-3 gene (43K from ORF5) was engineered in the pBI525 expression vector and cloned into the pBIN19 transformation vector. Initially, *Agrobacterium* transformation were employed to transform *Nicotiana benthamiana*. As expected, transgenic plants containing the sense translatable coat protein gene were shown to express detectable levels of the coat protein in ELISA and Western blot. For the other constructs, NPT-II ELISA and PCR were used to evaluate their respective transgenic lines. After evaluation in *N. benthamiana* for their expression, these same constructs were used to transform several economically important grapevine rootstocks, including Riparia Gloire, 3309 C, 110 Richter, MGT101-14 and SO4. So far we have obtained transgenic 3309 C plants containing 43K or coat protein genes of GLRaV-3, and transgenic Riparia Gloire plants containing the antisense coat protein gene.

NEW DATA ON GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 7

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The studies on grapevine leafroll-associated virus 7 (GLRaV-7), the last of the putative closteroviruses found in *Vitis vinifera* (1), were furthered by investigating the cytopathology of infected vines, the relationships of the virus with leafroll disease, the development of new diagnostic procedures, and the effect of *in vitro* culture for virus elimination.

ULTRASTRUCTURAL INVESTIGATIONS

Tissue fragments from leaves and petioles of four grapevine accessions infected by GLRaV-7 alone or in mixture with GVA were processed for embedding and thin sectioning as described (2). In all cases, virus particles scattered or in bundles were localised in the cytoplasm and nuclei of sieve tubes and companion cells. Cytoplasmic virus aggregates were sometimes surrounded by a membrane and gave rise to intranuclear invaginations. The accumulations of fibril-containing vesicles typical of closterovirus infections (2) were not seen and the mitochondria from which these vesicles may originate (3) were apparently unaffected. Chloroplasts were profoundly altered in shape and internal organisation. Their outer border was lined with double-membraned, flask-shaped vesicles with fibrillar material, derived from the invagination of the envelope. This very unusual feature differentiates GLRaV-7 from all closteroviruses. Peripheral vesiculation of chloroplasts is typical of tymovirus infections (2) and may occur also in vines infected by fleck virus (GFkV) (4). However, the vines used for ultrastructural studies indexed negative for fleck, and GFkV was not detected with repeated ELISA and molecular (dot blot, PCR) assays.

PROGRESS IN GLRAV-7 DIAGNOSIS

Immunoprinting assays (5) were successful and very promising. Rolled up leaves and petioles from a field-grown GLRaV-7-infected grapevine and graft-inoculated LN 33 and Cabernet sauvignon indicators were cut with a scalpel and gently pressed on a nitrocellulose membrane, which was then exposed in succession to biotinilated virus-specific IgGs, alkaline phosphatase-streptavidin conjugate, and BCIP/NBT substrate. Immunoprints of infected tissues were clear-cut and readily distinguished from the controls. A time-course study with ELISA on the accumulation of virus in the leaves confirmed that tissues collected in autumn are the best antigen source for diagnosis.

Complementary DNA was synthesised by hexanucleotide random priming denatured viral dsRNA, and was cloned in pUC18 (6). One of the clones (pGLR-17) 650 bp in size, was subcloned in the transcription vector pGEM-4Z and utilised for the production of digoxigenin-labelled riboprobes in both orientations (7). The probe was virus-specific and successfully hybridised total nucleic acid extracts from leaf petioles from July onwards, before symptom appearance.

GLRAV-7 AND LEAFROLL DISEASE

Budwood from four different grapevine accessions thought to contain no closteroviruses other than GLRaV-7 was bench- or green-grafted in 1995 on six plants each of the standard indicator series (8). Readings made a year after grafting showed that typical and strong leafroll reactions had developed on Cabernet sauvignon and LN33. All of 30 symptomatic indicators were ELISA positive for GLRaV-7. Nine of these indicators chosen at random, were serologically checked also for the presence of GLRaVs 1 to 5, GVA, GVB, GVD, GFLV, and GFkV and were found to contain none of these viruses. This was taken as evidence that GLRaV-7 is a genuine agent of leafroll and is a good candidate for the suppression of the word "associated" from its name.

SANITATION TRIALS

Meristem tips 0.5-0.8 mm in size were excised in spring 1995 from six field-grown grapevine accessions infected by GLRaV-7 and grown *in vitro* following the protocol and under the conditions described by Bottalico *et al.* (9). After three months the explants were transplanted and rooted in Jiffy pots. After two months growth under glass, the 18 surviving plantlets were checked by ELISA for the presence of GLRaV-7. Only one plantlet was still infected.

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DEVELOPMENT OF METHODS TO STUDY INTERACTIONS BETWEEN NEPOVIRUSES AND THEIR VECTORS

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Two Nepoviruses GFLV (grapevine fanleaf virus) and ArMV (arabis mosaic virus), both specifically transmitted by *Xiphinema* species are responsible for severe losses on grapevine in all viticultural countries. Today sanitary selection and soil desinfection are the only ways to control this grapevine degeneration. Soil desinfection using chemical nematicides remains inefficient in compact and deep soil. Moreover these chemicals are dangerous pollutants for the soil inhabiting fauna and dripping water and their use becomes more and more restricted .

Understanding nematode/Nepovirus interaction could lead to new strategies for controlling Nepovirus transmission by nematodes. In this view, several tools were developed : virus detection inside the vector, acquisition of virus-free and viruliferous *Xiphinema* rearings, collection of nematode populations from different areas in the world, quick and efficient transmission of GFLV using the grapevine as a common host for the nematode and for the virus.

1. DETECTION OF GFLV INSIDE THE *Xiphinema index*

Three methods based on serology and on molecular biology of the virus namely ELISA, RT-PCR and Immunocapture RT-PCR (IC RT-PCR) were developed to detect the GFLV inside the nematode.

Biotin-streptavidin DAS-ELISA was adapted to detect GFLV from nematode populations. Using polyclonal serum raised against virus particles, increasing number of nematodes isolated from infected fields were compared to a nematode virus-free population used as a reference. ELISA required a minimum of 10-20 nematodes to differentiate a virus-free population from a viruliferous population (1).

For RT-PCR, total RNA extracted from viruliferous nematodes were used as template for cDNA synthesis by reverse transcription. The cDNA was amplified by PCR using primers of 25 mers corresponding to a Coat Protein region. This technique allows the detection of the virus in single individuals(2).

For Immunocapture RT-PCR, virus particles extracted from viruliferous nematodes were trapped in a polycarbonate tube with a polyclonal serum raised against the virus particles. The trapped particles were disrupted with a detergent and free viral RNA used as a template for the synthesis of a cDNA followed by an amplification by PCR as described in the RT-PCR method. GFLV was detected from 5 viruliferous nematodes.

2. NEMATODES REARINGS AND EVALUATION OF THE VIRAL INFECTIOUS POTENTIAL OF VIRULIFEROUS NEMATODE POPULATIONS

Xiphinema index populations from GFLV infected vineyards were multiplied on fig plants at 20-25°C in 10 litter containers filled up with a steam-sterilised mixture of 1/3 sand and 2/3 loess. After one year multiplication on fig the nematode population has increased 100 to 150 times. ELISA values from several lots of 30 nematodes fed on fig plants show no difference with a virus free population indicating that the recovered populations are virus free.

To get viruliferous populations about 400 virus-free nematodes were added in the soil around the roots of GFLV-F13 infected grapevines grown in 10 litter containers. After one year the population has increased about 25 to 50 times and lots of 30 nematodes were positive in ELISA compared to virus free nematodes.

Soil sampling were made in France and other countries from infected vineyards. The viral infectious potential (VIP) of the collected populations was estimated using the ELISA method. A field population from Mesnil (Champagne) with a high ELISA value was chosen to study the persistence of GFLV in the absence of any host plant. Soil samples from this infected vineyard were stored at 20°C and 7°C. Lots of 30 adult or pre-adult nematodes were isolated after different storage periods (0, 6 and 12 months) and tested by ELISA. Comparison of field nematodes with virus free control nematodes showed an important decrease of the ELISA values after 6 and 12 months compared to those obtained for the 0 month storage date. Nevertheless GFLV is still clearly detected by RT-PCR with lots of 5 nematodes even after 12 months.

3. GFLV TRANSMISSION BY *Xiphinema index*.

Healthy and GFLV-F13 infected grapevine 5BB (*Vitis berlandieri x V. riparia)* plants were produced *in vitro* and acclimatised for 4 weeks in a greenhouse. After acclimatisation one healthy plant and two infected plants were cultivated in plastic pots filled up with a steam sterilised mixture of 2/3 loess and 1/3 sand. About 300 virus free nematodes reared on fig plants were added around the roots of the plants. Pots were kept in the greenhouse at 20-25 °C. Two months later roots were washed, inspected for the presence of swelling or galls and tested by ELISA for the presence of the virus. All the bait plants have shown galls on the roots and were positive in ELISA indicating that the virus was efficiently transmitted from infected to healthy plants.

A similar experimentation was also undertaken with healthy and infected GFLV-F13 5BB green cuttings produced in climate chambers. Only 45% of the bait cuttings were ELISA positive after a 3 month cultivation in the presence of virus free nematodes. This lower rate of transmission is probably due to the bigger size and the hardness of the roots of the cuttings which are inadequate to the nematode for feeding.

The methods we have developed to detect GFLV inside *Xiphinema index* are complementary tools that allowed us to evaluate the viral infectious potential of the nematode population and to isolate genes from virus particles present in nematodes. Acquisition of an efficient and reliable transmission test of GFLV offers the possibility to study the molecular mechanism of the GFLV transmission using the GFLV infectious transcripts(3).

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A CRITICAL APPRAISAL OF THE TAXONOMIC POSITION OF GRAPEVINE VIRUS A, B, AND D, AND THEIR ASSIGNEMENT TO A NEW GENUS

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When first established (1) the *Trichovirus* genus embraced two definitive [apple chlorotic leafspot (ACLSV) and potato virus T (PVT) viruses] and three tentative species [grapevine virus A (GVA), grapevine virus B (GVB) and heracleum latent virus (HLV)]. Recent studies, however, have shown that four additional viruses, i.e., grapevine virus C (GVC), grapevine virus D (GVD) (2), grapevine berry inner necrosis virus (GINV), and cherry mottle leaf virus (CMLV) (3) have properties that qualify them as possible members of the genus.

BIOLOGICAL PROPERTIES

Trichoviruses infect dicotyledonous plants and have geographical distribution and host range from restricted to wide. All species are experimentally transmitted by inoculation of sap, some readily (ACLSV, PVT, HLV, CMLV), others with great difficulty (GVA, GVB, GVC, GVD). Natural spread is mediated by mealybugs (GVA, GVB), which transmit in a semipersistent manner (4), by aphids (HLV), or by an unidentified vector (GINV). No vectors of ACLSV and PVT are known. Some of the viruses are phloem-restricted in the natural host (GVA, GVB, GVC, GVD); others (ACLSV, PVT) multiply primarily in parenchymas.

MORPHOLOGICAL AND PHYSICO-CHEMICAL PROPERTIES

Virus particles are helically constructed flexuous filaments 640-825 nm long, showing distinct cross-banding. The nucleic acid is a single molecule of linear, positive sense, ssRNA capped at the 5' end and polyadenylated at the 3' end, accounting for *c.* 5% of the particle weight. The coat protein (CP) consists of a single polypeptide with M_r of 20.5-27 kDa.

MOLECULAR PROPERTIES

Two definitive [ACLSV (5) and PVT] and two tentative [GVA (6) and GVB (7)] trichovirus species have been totally sequenced. Their genome structure is shown in Fig. 1. The sequence of the 3' end of GVD and GBINV RNAs is also available (2, 8). ACLSV, PVT, and GINV have three open reading frames (ORFs) encoding, respectively, a 216K polypeptide with the conserved motifs of viral replicases (ORF1), a 40-50K polypeptide identified as the movement protein(ORF2), and the 21-28K CP (ORF3). GVA and GVB have five ORFs. ORF1 is the replicase cistron encoding a 195K protein, ORF2 codes for a 20K polypeptide with unknown function, for which no significant homology was found in protein databases, ORF3 encodes a polypeptide possessing the conserved motifs of the 30K superfamily movement proteins, ORF4 (21.5K) is the CP cistron, and ORF5 codes for a small (10-14K) polypeptide sharing homology with the 3' terminal putative nucleic acid binding proteins of different plant viruses. In the 3' terminal region of GVD genome two ORFs were identified encoding, a 10K polypeptide and the 17.6K CP, respectively.

PHYLOGENETIC ANALYSIS

Computer-assisted comparative analysis of virus-coded proteins of definitive (ACLSV and PVT) and tentative (GVA, GVB, GVD) trichovirus species showed that they are phylogenetically related. However, GVA, GVB and GVD are much closer to each other than to ACLSV, PVT, and GINV with respect both to coat protein and polymerase (RdRp) domain. The difference with the RdRp of ACLSV is especially noticeable, as this virus does not even cluster with GVA and GVB.

CONCLUSIONS

There are distinct differences between definitive (ACLSV, PVT, and GINV) and some tentative (GVA, GVB, and GVD) members of the *Trichovirus* genus in biological behaviour, tissue tropism, epidemiology, genome structure and composition. The genome of GVA, GVB and, likely, GVD contains two extra cistrons (ORF2 and ORF5) encoding polypeptides that are not represented among the expression products of ACLSV, PVT, and GINV genome. Moreover, GVA and GVB, seem to constitute a phylogenetically more coherent cluster than ACLSV, PVT, and GINV. These molecular and biological differences seemed to warrant a re-definition of the taxonomic position of GVA, GVB, and GVD. Thus, in May 1997 the proposal for the establishment of a new genus denoted *Vitivirus* was submitted to and approved by the Executive Committee of the International Committee on Taxonomy of Viruses.

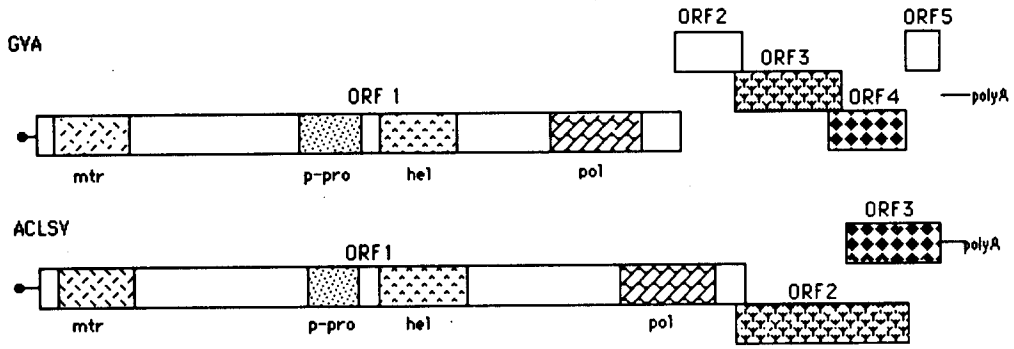

Fig. 1. Comparative genome organization of a vitivirus (GVA) and a trichovirus (ACLSV)

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PHYSICO-CHEMICAL AND MOLECULAR CHARACTERIZATION OF GRAPEVINE FLECK VIRUS

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The agent of grapevine fleck disease (GFkV) is a phloem-limited non mechanically transmissible virus with isometric particles *c.* 30 nm in diameter exhibiting a rounded contour, and a surface structure suggestively similar to that of tymoviruses (1). The physico-chemical properties of four GFkV isolates of different geographical origin (Italy, Bulgaria, Russia, USA) were compared among themselves and with those of a strain of eggplant mosaic tymovirus (EMV). A partial molecular characterisation of GFkV was also made.

PHYSICO-CHEMICAL PROPERTIES

No differences were observed among GFkV isolates. In density gradient centrifugation the sedimention pattern of virus particles was same, differing from that of EMV. Both viruses had two centrifugal components, but the relative sedimentation rates were distinct. Coat protein subunits of both viruses were of a single type but differed in size, i.e. 28 kDa and 22 kDa for all GFkV isolates and EMV, respectively. The nucleic acid was a single-stranded RNA of *c.* 8,800 nt (GFkV) and *c.* 6,300 nt (EMV). Previous estimates had assigned a size of *c.*7,400 nt to GFkV RNA (1). Double-stranded GFkV RNA consisted of a single species with mol. wt of *c.* 5.6 kDa, thought to represent the full-size genome dsRNA. Dissociated coat protein of the four GFkV isolates was recognised equally well in Western blot by a polyclonal antiserum to GFkV which, however, did not react with dissociated EMV coat protein.

MOLECULAR ANALYSIS

Complementary DNA was synthesised by hexanucleotide random priming viral RNA and was cloned and subcloned in the plasmids pGEM-4Z or pUC18. Sequencing was performed on PEG-purified plasmids using the Thermo-Sequenase kit and ^{35}S-dATP and filling gaps between adjacent non overlapping clones by polymerase chain reaction (PCR). To this effect, three sets of primers were designed, that amplified sequences of 1932 bp, 806 bp, and 501 bp, respectively. A total of 13 clones accounting for 6222 nt in the 5' end region of the viral genome (i.e. over 70% of the whole genome) were obtained and sequenced. A very peculiar feature of the GFkV RNA was the extremely high frequency of cytosine residues, which represented about 50% of total residues. The other bases accounted for 19% (U), 17%(G), and 15% (A). In the sequenced genome fragment five open reading frames (ORFs) were identified in the 5'->3' direction (Fig. 1). ORF 1 encoded a 204K polypeptide with conserved motifs of methyltranferase, helicase and RNA dependent RNA polymerase. ORF 2 coded for a proline rich (32%) 94K product with some homology with the movement protein of tymoviruses. A second proline rich (25%) protein about 20K in size was coded by ORF 3, whereas ORF 4 was identified as the coat protein cistron. Its product, a 27K polypeptide, had a size compatible with that of the GFkV CP, as estimated electrophoretically (28 kDa), and showed homology and two conserved motifs with the CP of tymoviruses. A fifth possible ORF, overlapping with ORF 4 in a different frame, was partially sequenced. Computer-assisted comparative analysis of the products of ORF 1(RdRp domain) and ORF 4 (CP) showed a clear-cut relationship with the comparable proteins of tymoviruses. Thus, it seems that besides particle morphology, presence of two centrifugal components, and type of cytopathic structures (2,3) also molecular traits approximate GFkV to tymoviruses (4). However, differences in biological, physico-chemical and molecular properties (i.e. genome size and organisation) are wide enough to justify the conclusion that GFkV is separate from tymoviruses and worth a taxonomic position of its own.

DIAGNOSIS

A side-effect of molecular investigations was the widening of the range of GFkV detection methods. One of the cDNA clones (GFkV20) was used for the production of 270 bp digoxigenin-labelled riboprobe which proved virus-specific and successfully detected GFkV in alkaline extracts of grapevine roots and leaves (dot-spots assays) and in tissue blots on NaOH-EDTA presaturated Hybond N+ membranes. RT-PCR and spot-PCR assays (5) were also successful on infected vine tissue extracts. In these tests two different sets of primers were used that amplified cDNA fragments of 248 bp and 117 bp respectively.

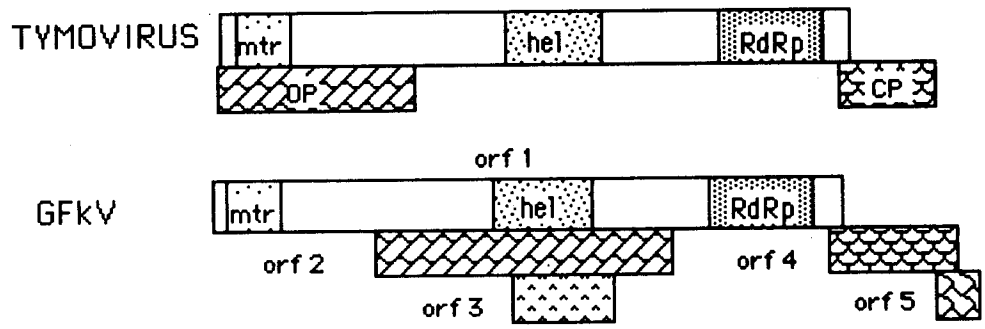

Fig. 1. Organization of the 5' end region of the GFkV genome compared with the whole genome of tymoviruses

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RECOMBINANT RNA OF GFLV AND ArMV: AN APPROACH TO STUDY TRANSMISSION SPECIFICITY OF NEPOVIRUSES BY NEMATODES

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The molecular determinants of vector specificity for transmission of nepoviruses are not known and no non-transmissible isolates are available. It was shown that serologically diverse strains of raspberry ringspot nepovirus (RRV) are transmitted efficiently by two distinct species of *Longidorus* nematodes (1). The ability to be transmitted by specific nematodes has been associated with RNA2. We therefore assume that at least one of the 3 proteins (2a, 2b, 2c) encoded by RNA2 is a determinant of vector specificity. To find out which proteins are involved, several recombinants of Grapevine Fanleaf Virus (GFLV) and Arabis Mosaic Virus (ArMV) were constructed and their ability to replicate and encapsidate the genomic RNAs was evaluated prior to start transmission experiments.

GFLV and ArMV are two serologically related nepoviruses causing the widespread disease affecting grapevines. These two viruses are transmitted by two species of nematodes, *Xiphinema index* (2) and *X. diversicaudatum* (3), respectively. Genetic informations are divided over two single-stranded positive sense RNA molecules. The genomic organisation (4, 5), the proteolytic processing of the polyprotein P1 and P2 (6) encoded by RNA1 and RNA2, respectively, and the function of several proteins (6, 7) have been determined. Biologically active full-length transcripts of RNA1 and RNA2 have been obtained for GFLV strain F13 (GFLV-F13) (8). Polyprotein P2 of GFLV-F13 and ArMV strain Syrah (ArMV-S) show significant homologies except for the N-terminal 2a protein encoded by RNA2 (5, 9). Sequence modifications were introduced in the cDNA clone used to produce full-length transcripts of GFLV RNA2 in order to synthesise recombinant transcripts. Exchanges from GFLV to ArMV, ranging from 240 to 2550 nts were carried out in the movement (2b) and coat protein (2c) regions as shown in figure 1. For ArMV-S, the two RNA2 species, cloned and sequenced (9), were alternatively used for domain exchanges.

The biological properties of the resulting recombinant isolates were evaluated by their ability to replicate in *Chenopodium quinoa* protoplasts in the presence of RNA1 transcripts (trRNA1). Therefore, 2.10^5 protoplasts were electroporated with 5 mg of trRNA1 and 10 mg of modified RNA2 transcripts. Three days post inoculation, the protoplasts were collected and total protein extracts of 2.10^4 protoplasts were analysed for the presence of movement protein, by Dot or Western blotting with a polyclonal antiserum specific for movement protein (7). Total RNA extracted from the remaining protoplasts, were electrophoresed through agarose-formaldehyde denaturing gel and blotted to a Hybond-N membrane. The viral RNAs were detected using digoxigenin-labelled riboprobes specific to movement protein sequence for RNA2 and to VPg-proteinase sequence for RNA1. Analysis of proteins or RNAs showed that recombinant RNA2 or wild type RNA2 leaded to similar accumulation of movement protein and viral RNAs indicating that replication was efficient for all recombinant tested.

Proteolytic processing of mutated RNA2 by GFLV-F13 proteinase was checked by *in vitro* translation of mutated RNA2 transcripts in the presence of proteinase transcripts and [^{35}S]-methionine. The translation products were analysed by SDS-PAGE and autoradiographed. Recombinant RNA2 produced protein patterns similar to those of wild type RNA2, except if the sequence upstream of the Arg/Gly cleavage site between 2b movement and 2c coat protein is an ArMV sequence. The presence of a 2bc protein, indicates the absence of proteolytic processing at the Arg/Gly site between protein 2b and 2c.

The ability of modified coat protein to assemble and produce stable virions containing genomic RNAs in the protoplasts was investigated. If stable virions are formed the viral RNAs will be protected from degradation. Conditions for degradation of unprotected RNA depend on the RNA extraction procedure used.

The standard RNA extraction procedure was modified as follows: 2.10^5 *C. quinoa* transfected protoplasts were pelleted, resuspended in 1% SDS, 10mM EDTA, 100mM LiCl, 100mM Tris-HCl pH 8 before total RNA was extracted. The conditions for extraction of encapsidated viral RNAs only were incubation of protoplasts in 0.1% Tween 20, 50 mM PIPES pH 6.5, during 30 min at 37°C followed by phenol extraction. Transcripts of GFLV-RNA2 were used as positive control and 66K transcripts coding for 2ab protein, *i.e.* RNA2 devoid of CP sequence, as negative control. Northernblot analysis showed that exchanges of the total CP sequence (CPU) or of nts 2048 - 3334 (NBxU) lead to stable virions, while recombinant CP in which the ArMV fragment corresponded to nts 2852 - 3078 (SmSbU) was exchanged appeared inefficient for RNA protection (Fig.1). RNA encapsidation assays for all recombinant RNA2 have to be completed with ISEM observations to determine for which constructs virion assembly occurred before attempting nematode transmission assays.

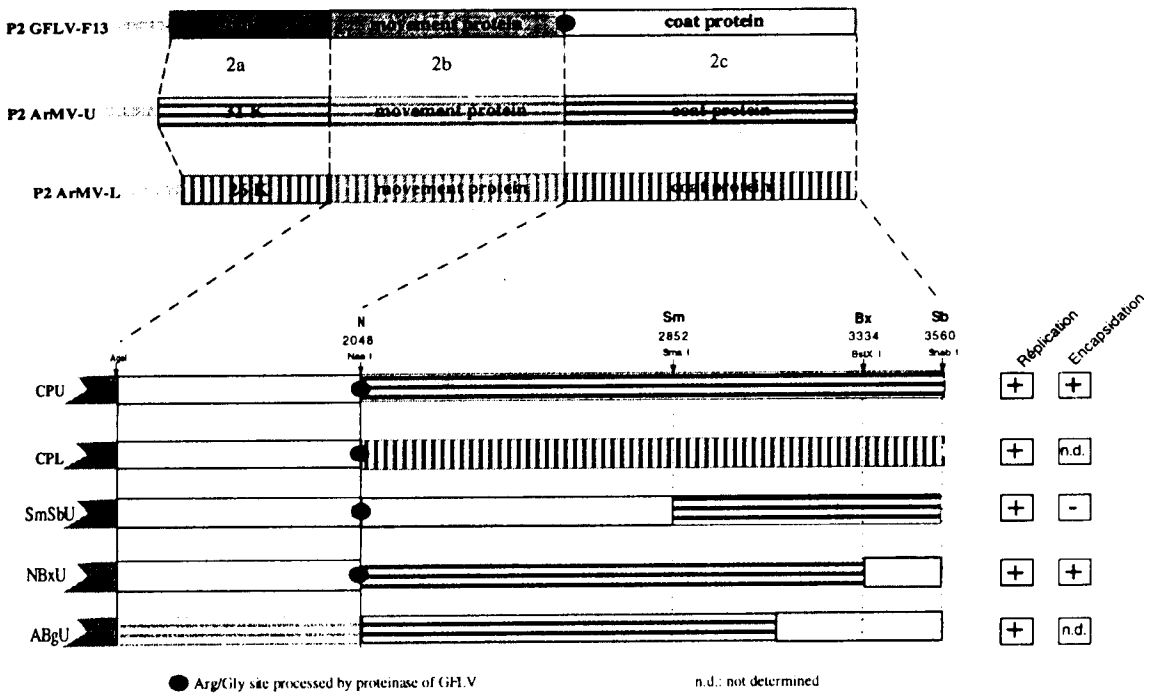

Fig.1 Schematic representation of cDNA clones used for synthesis of recombinant transcripts. Results of replication and encapsidation assays are indicated in the boxes on the right. The genomic organisation of RNA2 GFLV-F13 and ArMV-S are indicated in the upper part.

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MOLECULAR VARIABILITY OF GRAPEVINE FANLEAF VIRUS COAT PROTEIN

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Grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV) are two nepoviruses responsible for an economically important disease in vineyards all over the world. Though the coat proteins present more than 70% homology, the two viruses are specifically transmitted by two different nematode species, respectively *Xiphinema index* and *X. diversicaudatum* (1).

The understanding of the mechanisms involved in recognition between nematode and virus could lead to the emergence of new strategies to inhibit viral dissemination.

Even if several viral proteins are implied in the interaction virus-nematode, the coat protein is the factor which is most probably involved. If so, the structural feature recognised by *X. index* should be conserved in the various isolates of GFLV, but should be absent from the ArMV capsid protein.

To identify such regions in GFLV coat protein, a molecular analysis was initiated, using eight isolates originating from various vineyards in the world, and maintained in infectious plants or nematode rearings. The regions coding for the coat protein were reverse- transcribed and the resulting DNAs were amplified by polymerase chain reaction. A second PCR reaction allowed amplification of shorter overlapping fragments which were directly sequenced.

METHODS

Viral isolates originating from several infected vineyards located in various parts of the world were first maintained either in infected grapevines (European collection of INRA Colmar) or in viruliferous nematode rearings (INRA Antibes). Eight isolates were used for the analysis; four originating from Switzerland, France, Greece, and Italy were extracted from infected vineyards and the other four, coming from Switzerland, Cyprus and France, from nematode rearings. One of the two French isolates extracted from nematodes was the reference strain F13 (2).

For one nematode isolate (Switzerland), the total RNAs of 10 nematodes were extracted as described before (3). For the other isolates from infected plants or viruliferous nematodes, viral particles were first extracted by immunocapture using a polyclonal serum raised against GFLV F13. Viral particles were then disrupted by Triton X100 treatment just before the reverse transcription.

The reverse transcription was performed with Boehringer Expand Reverse Transcriptase according to the manufacturer's recommendations, using a primer specific from the 3' non coding end of GFLV RNA2.

All the PCR were carried out with GFLV-specific primers, with a number of cycles reduced to 25. The first PCR amplified a 1,617 pb fragment, corresponding to the whole coat protein sequence. To ensure fidelity of the replication, Vent Polymerase was added to the reaction mix. The second PCR amplified overlapping fragments of 250 to 550 pb, which were directly sequenced. Sequence analysis was made using the DNAsis V2.1 package (HITACHI Software).

RESULTS

To improve the method, a cloned F13 sequence was used as a template. The sequence obtained after the two PCR was 100% homologous to the F13 reference sequence (2).

The complete nucleotide sequences of the coat protein gene were determined for the two French isolates extracted from nematodes (F13 and Frejus). For the six other isolates, only the two thirds of the C-terminal end were sequenced.

The analysis of nucleotide sequences showed more than 90% homology between the eight isolates. The mutations were distributed all along the sequence.

The peptide sequences derived from nucleotide sequences were compared to the F13 coat protein sequence. Few mutations were observed, but none of the isolates was 100% homologous to the reference strain. The F13 isolate extracted from nematodes varied from the reference sequence by only two amino-acid changes, both located in the first third of the coat protein sequence.

To compare all the isolates, the alignment was then limited to two thirds of the coat protein sequence. In this part, the F13 isolate extracted from nematodes was identical with the reference F13 strain. All the other isolates presented 3 to 11 mutations (for 330 amino acids aligned). Most of these mutations were conservative and present on at least two sequences. Two regions of the coat protein, located between amino acids 40 to 70 and 270 to 300 of the aligned sequence (corresponding to amino acids 208 to 238 and 438 to 478 of the coat protein) seemed to be the most variable.

The phylogenetic tree derived from peptide sequences showed the relationships between the isolates that were analysed. No clear organisation of the segregation could be deduced from that tree, neither regarding geographical origin of the isolates nor the biological source (plant or nematode).

CONCLUSION

The results show that immunocapture can be a suitable method to extract viral particles from nematodes.

As proved by the positive test, the methodology (including reverse transcription, two PCR reactions and direct sequencing of PCR products) does not induce artifactual changes in the sequences.

The phylogenetic tree does not reveal clear relationship between strains, and therefore does not give any information about the spreading of infection. It is clear that viral dissemination by vegetative propagation and diversity of commercial networks complicate such an analysis.

The high conservation of the coat protein sequence suggests that GFLV coat protein is submitted to high selection pressure. As there are several strictly conserved regions, it is not possible for the moment to locate a particular region possibly involved in the viral coat protein recognition process by nematodes.

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CYTOLOGICAL ALTERATIONS ASSOCIATED WITH AN UNIDENTIFIED ISOMETRIC GRAPEVINE VIRUS (UIGV)

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The presence of isometric virus particles of about 24 nm and double-membraned vesicles formed by invagination of the boundary membrane of mitochondria and plastids are a typical cytopathic feature induced by grapevine fleck virus (GFKV) in grapevine phloem cells (1). However, in the course of ultrastructural observations carried out in the past years on numerous grapevine accessions we found that similar cytopathic effects were sometimes present also in plants ELISA-negative for GFKV. This discrepancy was at first attributed to the possible failure of ELISA test in detecting GFKV in particular circumstances (2), but the isolation of an unidentified isometric virus (UIGV) with the same morphology of GFKV from Chasselas vines (3) suggested the possibility that these two viruses could be responsible for similar cytological alterations as well.

To verify this hypothesis an ultrastructural study has been carried out on two grapevine clones, cv. Gamay Rouge de la Loire (GRL), in which the presence of UIGV had been previously assessed by ISEM. These clones contained also the closteroviruses GLRaV 4 and 5, respectively, and showed leafroll symptoms, but they were free from GFKV and the most frequent nepoviruses, as assessed by ELISA and indexing on *Vitis rupestris* St. George. Main veins from leaves with symptoms were excised, cross cut in 0.5-1 mm pieces while dipped in a mixture of 2% glutaraldehyde and 3.3% paraformaldehyde in 0.1 phosphate buffer, pH 7.4. Samples were fixed for ½ h under slight depression at room temperature and for 2 h at 4°C, then carefully washed in the same buffer and postfixed in 1% osmium tetroxide for 2 h. Dehydration was performed in ethanol and embedding either in Epon-Araldite or in London Resin White. Samples of leaf main veins from symptomless LN-33 plants grafted with the above mentioned GRL clones were also fixed and embedded, as well as samples from healthy plants, as control.

In all the GLR and LN-33 infected plants the most striking cytopathic feature was the presence of vesiculated plastids in phloem parenchyma and companion cells (Fig.1). These organelles were swollen, showed disorganised thylakoid membranes and contained double-membraned peripheral vesicles, 50-150 nm in diameter. Peripheral vesicles contained fine strands and were sometimes connected to the external envelope by an invagination from which they appeared to originate. The vesiculation process apparently did not involve mitochondria, though in GRL samples also containing GLRaV 5 some of them showed single membraned vesicles typical of grapevine closterovirus infection (4). Isometric virus-like particles of about 24 nm were rarely seen in sieve tubes (Fig. 2), or in parenchyma phloem cells. In the latter virus particles were easily confused with ribosomes because they did not show a clear cut profile. UIGV apparently infected only phloem cells; however, a more exhaustive observation of the surrounding tissues is needed to confirm this limited localisation.

Although it is not possible to draw a definitive conclusion because of the presence of closteroviruses in the examined samples, it is very likely that UIGV produces cytopathic effects similar to those found in GFKV infected cells. However, the former induces double-membraned vesicles from plastids, while the latter mainly from mitochondria (5,6). If this discriminating feature can be confirmed, it will be of a great diagnostic value for differential diagnosis of these viruses.

On the basis of the above results we have re-examined the ultrastructure of some of the Barbera and Croatina clones coming from different provenance and in which cytopathic features similar to those produced by GFKV had been previously observed. All these clones were ELISA-negative to GFKV and positive to GLRV 3. These further observations showed that the double- membraned vesicles found in the phloem cells were originated only from plastids as in GRL and LN-33 clones, but not from mitochondria. Thus it is possible that also the Barbera and Croatina plants were infected by UIGV, suspecting that this virus, though in a latent form, could be largely diffused in grapevine.

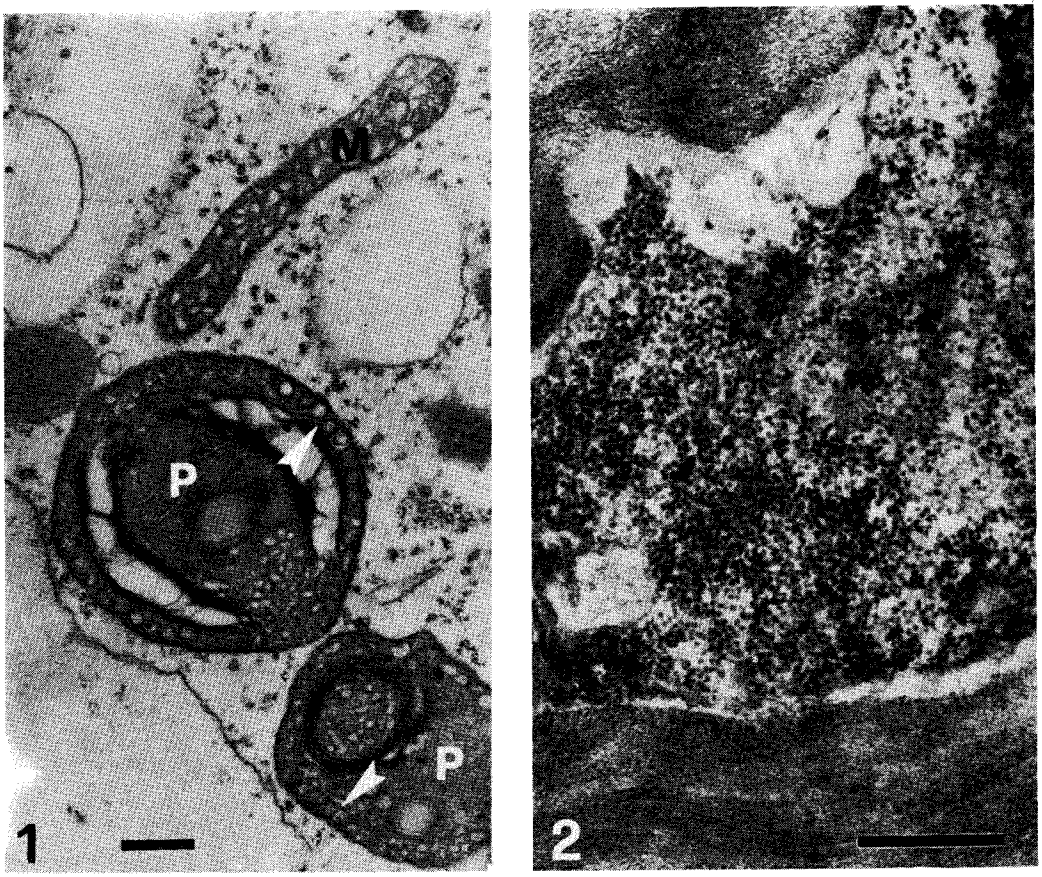

Fig. 1. LN-33 phloem parenchyma cell showing degenerating plastids (P) with double-membraned vesicles (arrowheads) located at their pheriphery ; M = mitochondrion; bar = 500 nm.
Fig. 2. GLR sieve tube containing numerous isometric virus-like particles 24 nm in dia; bar = 500 nm.

AKNOWLEDGEMENTS

Authors are indebted to Prof. A. Fortusini and Dr. J.-J. Brugger for indexing and to Mr. S.Cinquanta, Dr. S. Prati and Dr.G.Scattini for performing ELISA tests.

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IMMUNO-CHEMICAL AND BIOLOGICAL DISTINCTION OF GRAPEVINE LEAFROLL ASSOCIATED VIRUSES 2 AND 6 IN COMPLEX INFECTIONS WITH OTHER KNOWN AND UNIDENTIFIED VIRUSES

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Grapevine is commonly affected by complex viral infections which determine symptom type and severity. Leafroll type II (LR II) on Swiss Chasselas is a typical case (5). This inoculum induces on the indicator Gamay Rouge de la Loire (Gamay RdL) atypical symptoms characterised by the absence of typical green tissue zones along the veins (4). Grapevine leafroll associated viruses (GLRaV's) IIa and IIb, now called GLRaV-6 and GLRaV-2 (1), were shown to be involved. The double infection was first substantiated thanks to the monospecific antibody 29-1 to GLRaV-2 (MCA 29-1) (7) and more recently confirmed with a new MCA to GLRaV-6 (6). In order to comprehend the atypical leafroll symptom and possible interaction with other known and yet unidentified viruses as well as with other grapevine diseases, such as corky bark (CB), we further analysed the immuno-chemical and biological distinction of GLRaV-2 and GLRaV-6 and opposed various complex infections with biological indexing.

Viruses were maintained on vines in the greenhouse or in the experimental vineyard of Changins: GLRaV-1 on Räuschling 24; GLRaV-2 on Chasselas 8/7 and Semillon V.C.A 3v7 Davies; GLRaV-3 on Gamay RdL; GLRaV-4 on Thompson seedless V.C.A. 2v22 Davis; GRaV-5 on Emperor V.C.A 2v18 Davis; GLRaV-6 together with GLRaV-2 on Chasselas 8/22; healthy Chasselas 2/13. GLRaV-2 (isolate 973) was also maintained on herbaceous plants (*Nicotiana benthamiana)* after its isolation by mechanical inoculation of partially purified virus extract from leaves of Chasselas 8/22. Partial virus purification, production of antiserum and of monoclonal antibodies, immuno-precipitation electron microscopy (IPEM), ELISA, electrophoresis and Western immunoblot were described in (6). Molecular weight markers were provided by Bio-Rad (art. 161-0304). Indexing was carried in the field over three seasons using Gamay RdL as an indicator vine for LR, LN33 for CB and *Vitis rupestris* cv. St George for grapevine fleck (Fk).

New antiserum to GLRaV-2 was produced using immunogen purified from *N. benthamiana* infected with GLRaV-2, whereas antiserum to GLRaV-6 (as 25) was obtained with immunogen prepared from leafroll diseased Chasselas 8/22 infected by both GLRaV-2 and GLRaV-6. One out of several rabbits yielded antiserum with dominant specificity to GLRaV-6. By means of IPEM, the latter antiserum aggregated all closterovirus-like particles of Chasselas 8/22 but distinguished them by the intensity of decoration. In addition, this antiserum also aggregated isometric virions, with an approximate diameter close to 30 nm, in enriched extracts of Chasselas 8/22 and 2/13 (Fig. 3). This unidentified isometric grapevine virus (UIGV) was not serologically recognised by antibodies to GFkV or 5 common nepoviruses. UIGV was not mechanically transmissible to common herbaceous host plants. Indexing was negative for Fk, as shown below. Similar viruses with the same characteristics were also observed in Thompson seedless and Emperor used as virus sources for GLRaV-4 and GLRaV-5 but not in the well characterised GLRaV references such as Räuschling 24. The new antisera also decisively improved the screening procedure for the selection of hybridoma producing monoclonal antibodies (MCA's) to GLRaV-2 and GLRaV-6, in particular MCA 36-117 against GLRaV-6. Using Western immunoblots (Fig. 1), the latter allowed us to determine for the coat protein (CP) of GLRaV-6 an apparent molecular weight of 32 kDa, a value well distinct of those recorded for GLRaV-1 to 5 (GLRaV-1: 38 kDa, GLRaV-2 : 25 kDa, GLRaV-3: 43 kDa, GLRaV-4 : 34 kDa and GLRAV-5 : 36 kDa). By means of IPEM, MCA 36-117 also strongly decorated and aggregated closterovirus-like particles from Chasselas 8/22 (Fig. 2). Decoration of particles was always homogenous, contrasting clearly with the reaction of MCA 29 -1 and GLRaV-2, where one particle end, 50 to 100 nm long, frequently remained non-decorated (Fig. 4). There were no undecorated particles left when both antibodies were used simultaneously, except the undecorated short ends of one particle type. Pure GLRaV-2 particles from *N. benthamiana* were also decorated in the same manner by MCA 29 :1. The two monoclonal antibodies therefore clearly distinguished GLRaV-2 and GLRaV-6 from Chasselas 8/22. The partial decoration observed with GLRaV-2 might be explained by a genomic structure similar to that of citrus tristeza closterovirus (CTV), i.e. genes coding for two serological distinct CP's (3). We did not observe such partial decoration with any other GLRaV and its respective MCA's. This together with the similar molecular weights of their respective CP's indicates a potentially closer relationship between CTV and GLRaV-2 than for the other GLRaV's. GLRaV-2 also seems to infect a distinctive host range, since it was selectively transmitted to herbaceous plants from the doubly infected (GLRaV-2 and GLRaV-6) grapevine Chasselas 8/22. Symptoms (vein clearing and necrosis, followed by apical death) appeared 4 to 8 weeks after inoculation.

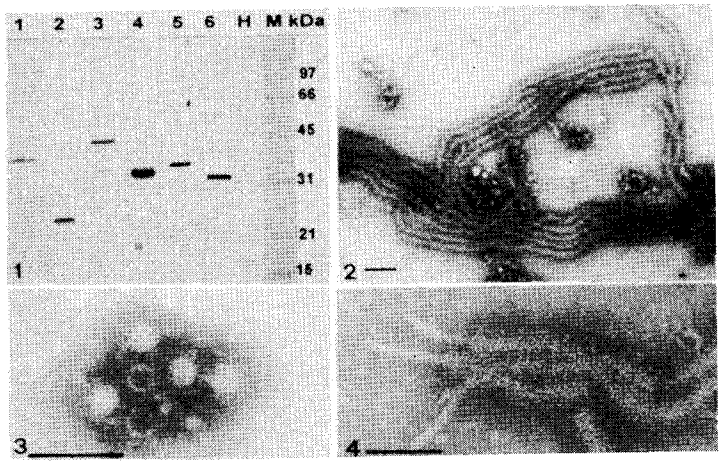
In order to dissect complex infections of GLRaV-2, GLRaV-6 and other viruses, we carefully analysed the virus and disease status of a few representative vines by the most sensitive available methods: direct electron-microscopy, IPEM, Western immunoblots and mechanical transmission with partially purified and enriched extracts, PCR for the detection of GVA and hop stunt viroid (HSVd), ELISA as well as biological indexing. The screening included the following agents: GLRaV-1 to 6, GFkV, GVA, GVB, UIGV

and HSVd. The results are summarised in Table 1. From them we conclude that GLRaV-2 is associated with LR type II symptoms and that GLRaV-6 is not associated with CB. Although GLRaV-2 was detected in all three CB sources its presence is not sufficient to induce CB (Chasselas 8/22) unless an other virus would interfere as an inhibitor of symptom expression. We did not detect GVB in all of the CB sources, possibly due to low virus titre. GFkV and UIGV were variably present and are not strictly associated with a disease (except GFkV with Fk on *V. rupestris*). Nevertheless they are likely to alter tissue ultra-structure as shown in an accompanying abstract in this volume (2).

We conclude that GLRaV-2 differs significantly in physical and biological traits from all other GLRaV's. Differential elimination of either GLRaV-2 or GLRaV-6 from vines with complex infections is presently undertaken in order to study single virus/host plant interactions.

Table 1

Clone	Virus status (10 viruses + HSVd)	Indexing (LR, CB, Fk)
Chasselas 8/22	GLRaV-2, GLRaV-6, UIGV-1, HSVd	LR II
Chasselas 8/7	GLRaV-2, GFkV, (not tested for HSVd)	LR II, severe CB, Fk
LN 33 / Chasselas 306	GLRaV-2, GVB(?)(not tested for HSVd)	LR II, mild CB
Semillon V.C.A 3v7	GLRaV-2, (not tested for HSVd)	LR II, mild CB
Chasselas 2/13 healthy	UIGV, (not tested for HSVd)	none



Figures 1 - 4. (1) Western immunoblot of GLRaV's 1 to 6 and healthy Chasselas 2/13 control (H) detected by their respective homologous monoclonal antibodies (MCA 2-4; 29-1; 8-3; 3-1; 43-1 and 36-117 (MCA 36-117 also on healthy control extract) (M: mol. wt markers). (2) IPEM of GLRaV-6 using MCA 36-117. (3) IPEM of UIGV from healthy Chasselas 2/13 using antiserum 25. (4) Undecorated particle ends shown by IPEM of GLRaV-2 using MCA 29-1(bar = 100 nm).

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RUPESTRIS STEM PITTING OF GRAPEVINES: NUCLEOTIDE SEQUENCE, RT-PCR DETECTION, AND VIRAL ORIGIN OF ASSOCIATED DsRNA

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Rupestris stem pitting (RSP) is one of the major virus diseases on grapevines worldwide. It is widespread in Europe and USA and probably in other countries as well. RSP is among the viruses to be eliminated in grapevine clean stock programs in several countries (2, 3, 7).

Since its recognition in the 1970s, the only method for RSP diagnosis is indexing on woody indicator–*Rupestris* St. George which develops a narrow strip of pitting on its xylem just below the chipbud after graft inoculation (3). However, this method takes three years to complete. Despite many efforts being made toward the etiology of RSP, no viruses have ever been associated with this disease yet. These problems hinder the accurate estimation of its real negative impact on grapevine industry. Finding out the causal agents and establishing rapid and practical disease diagnostic methods have been the major interests of RSP research.

Walter & Cameron (8) isolated high molecular mass double-stranded RNA (dsRNA) from the stem phloem tissue of several Pinot Gris and Pinot Noir clones which were indexed positive for RSP. While dsRNA of 5.5 X 10^6 Da was detected from all clones tested, two smaller ones with molecular mass of 5.0 and 3.6 X 10^6 Da were observed in some of the tested clones. Azzam et al. (1) reported dsRNA species B and C, 5.3 and 4.4 X 10^6 Da respectively, from the phloem of RSP-infected grapevines collected from California and Canada. However, results for New York samples were not consistent.

We hypothesize that specific dsRNAs, which we assume to be the replicative forms of the RSP-specific genomic viral RNAs, are consistently associated with RSP. Cloning of the dsRNAs will make it possible to discover the causal agents of RSP and to develop rapid and practical diagnostic methods. The objectives of this study are: (i) cDNA cloning of the RSP-specific dsRNA; (ii) unraveling the nucleotide sequence and genomic organization of RSP-associated viral RNA through sequencing overlapping cDNA clones and RT-PCR amplified cDNA fragments; and (iii) establishment of RT-PCR for rapid diagnosis of this disease.

MATERIALS AND METHODS

Phloem tissue of 15 grapevine selections with known RSP status were collected from the germplasm repository of the Plant Genetic Resources Unit (PGRU), USDA-ARS at Geneva. DsRNA isolation and analysis followed the procedure of Hu et al. (5). cDNA synthesis and cloning followed the methods of Gubler & Hoffman (4) modified by Jelkmann et al. (6). Lambda ZAP II was used as vectors. Northern blot and plaque hybridization were conducted according to the manufacturer's recommendations. RT-PCR was performed based on our own procedure. Nucleotide sequencing was done on ABI automated sequencer model 373 using either recombinant plasmids or purified RT-PCR products amplified from dsRNA. Genomic organization and open reading frames (ORF) were analyzed with DNASTAR program (DNASTAR Inc., Madison, Wisconsin).

RESULTS

DsRNA analysis: DsRNA species, approximately 8.7 kbp in length, were observed in 12 of the 15 grapevine selections tested. Three selections, Aminia, Canandaigua, and Freedom did not reveal dsRNA in repeated experiments. Although Colobel 257 and Seyval had two dsRNA species, the remaining 10 selections had only one. However, the intensity of dsRNA bands on agarose gels varied greatly among different selections (Table 1, unpublished data).

DNA synthesis and cloning: A cDNA library was constructed using purified dsRNAs as templates and lambda ZAP II as vectors. Upon plaque hybridization with ^{32}P labeled, first-strand cDNA probes, a total of 182 positive clones were selected. Two of them, RSP95 and RSP149, hybridized with dsRNAs isolated from RSP-infected grapevine, indicating that they were RSP-specific (Table 1, unpublished data).

Sequence and genomic organization: A total of six plasmids containing RSP-specific cDNAs and four cDNA fragments amplified from dsRNAs by RT-PCR with specific primers were sequenced in both directions and used to construct the apparent complete nucleotide sequence of this viral agent associated with RSP. It contained 8,762 bps excluding the poly (A) tail. The 3' and 5' nontranslated regions contain 176 and probably 61 nucleotides, respectively. Similar to apple stem pitting virus (ASPV) and potexviruses, the genome of RSP consists of five open reading frames. ORF1 encodes the putative replicase with molecular mass of 244 kDa; ORF2–ORF4 encode three proteins with molecular mass of 24.4, 12.8, and 8.4 kDa, respectively. These three proteins form the triple gene block, a common feature for a number of plant virus groups; ORF 5 encode the putative coat protein of 28 kDa (Fig. 1). This implies that a virus, which resembles some plant viruses in both nucleotide sequence and genomic structure, is associated with RSP. We propose to call this virus "*Rupestris* stem pitting associated virus" (RSPaV).

RT-PCR: Two pairs of oligo nucleotide primers, RSP95F1/R1 and RSP149 F1/R1, were used in RT-PCR. A total of 129 grapevine samples with known RSP status were collected from USA, Canada, and Italy and tested by RT-PCR. Results of RT-PCR correlated to those of indexing (Table 1, unpublished data).

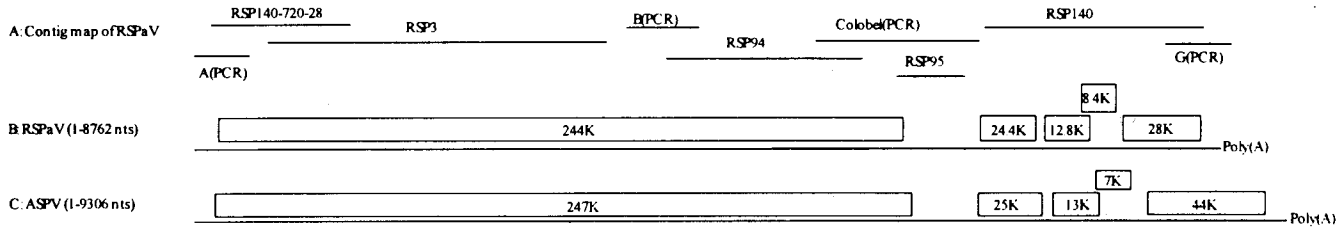

Fig. 1. Contig map and genomic organization of RSPaV and comparison with Apple stem pitting virus (ASPV). RSP140-720-28, RSP3, RSP94, RSP95, and RSP140 are cDNA clones. A(PCR), B(PCR), Colobel(PCR), and G(PCR) are cDNA fragments amplified by RT-PCR from dsRNA templates.

Table 1: Correlation of dsRNA analysis, RT-PCR, northern blot hybridization and indicator indexing for *Rupestris* stem pitting from grapevine selections collected from PGRU at Geneva

Variety	Indexing	DsRNA	RT-PCR(RSP95F1R1)	RT-PCR(RSP149F1/R1)	Northern
Aminia	+	-	-	-	-
Bertille Seyve 3408	+	NT	+	NT	+
Bertille Seyve 5563	+	+	+	+	+
Canandaigua	+	-	-	-	-
Colobel 257	+	+	+	+	+
Couderc 28-112	+	+	+	+	+
Freedom	-	-	+	+	-
Grande Glabre	+	+	+	+	+
ILL 344-1	+	+	+	+	-(?)
Joffre	+	+	+	+	+
Ravat 34	+	+	+	+	+
Seyval	+	+	+	+	+
Seyve Villard 14-287	+	+	+	+	+
Seyve Villard 3160	+	+	+	+	+
Verdelet	+	+	+	+	+
Thompson seedless*	+	NT	+	+	NT
Pinot Noir*	+	NT	+	+	+

Notes: 1. NT: not tested. 2. Probe used in northern blot hybridization was insert from cDNA clone RSP149.
3. * These two cultivars were used as positive controls in RT-PCR and northern blot hybridization.

CONCLUSIONS

(a) DsRNA molecules of ca. 8.7 kbp were consistently associated with grapevines which were indexed on St. George positive for RSP. (b) A cDNA library was constructed using these dsRNA as templates and lambda ZAP II as cloning vectors. (c) The apparent complete nucleotide sequence of this dsRNA was achieved. It had 8,762 and a poly (A) tail and consisted of five open reading frames encoding the putative replicase, capsid protein, and three other nonstructural proteins. (d) RT-PCR had the potential use in rapid and practical RSP diagnosis.

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RELATIONSHIP BETWEEN RUGOSE WOOD COMPLEX SYMPTOMS AND GRAPEVINE VIRUS A.

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In Italy the analysis for Rugose Wood complex (RW) is also scheduled in grapevine clones selection procedures, in addition to testing for Degeneration complex, Leafroll complex and Fleck. Indexing tests are carried out by grafting indicators *Vitis Rupestris*, *Vitis Berlandieri* x *Vitis Riparia* cv. Kober 5BB and LN 33 hybrid to assess respectively: Rupestris Stem Pitting (RSP), Kober Stem Grooving (KSG), LN 33 Stem Grooving Virus (LNSG) and Corky Bark (CB). Recent availability of commercial antisera for detection of Grapevine Virus A (GVA), now known as *Trichovirus* genus, has given the possibility to perform immunoenzymatic tests by ELISA method also for such virus that some Authors think linked to KSG (2, 3).

In the beginning the use of commercial kits of antisera for GVA complicated the diagnosis of such virus because ELISA tests did not grant the detection of surely infected samples; only later, more reliable results were achieved using antisera from different origins. Such opportunity triggered a comparison trial between ELISA and biological tests (indexing) for Rugose Wood complex.

The trial started in 1993 and was carried out on plants of a collection of "Istituto Sperimentale per la Viticoltura (ISV)" and mainly concerned varieties grown in Veneto region. Samples of vines of other cultivars grown in Central Italy were tested as well. As for some biotypes of Torbato variety, trial included their offspring as well. Such descendance was obtained by *in vitro* culture of shoot tips of 1-1,5 mm with the target of sanitising materials from other closteroviruses affecting mother plants (1). ELISA tests for GVA diagnosis were done by double antibody sandwich (DAS-ELISA); antigene extraction was done on leaves, collected at summer end, or cortical scrapings from mature canes, harvested in autumn - winter time. Antibodies from Bioreba (CH), from Agritest (Bari-I) and from "Istituto di Fitovirologia Applicata" (IFA, Turin, I) were used and often compared on materials under test.

Indexing tests were done by "omega grafting" on indicators Rupestris du Lot, Kober 5BB (as hypobion) for detection of RSP and KSG, on LN33 (as epibion) for detection of CB and LNSG. Grafted plants were kept in open field for three years; since the first year analysis for CB was performed. At the end of the third year the plants were uprooted to check for Rugose Wood complex.

Number of samples affected by GVA depends on the kind of materials considered (table 1). Biotypes and varieties from ISV collection have shown low occurrence of Trichovirus A (only 8,2%) where compared with populations of Vermentino and Ansonica of which 25,7 and 39,7% of tested samples were found infected.

Biological tests, carried out on testing materials from ISV collection and on some biotypes of the other two varieties, have underlined that plants affected by GVA are also mainly affected by RSP (62%) and only partially by KSG which has been found linked to RSP. Occurrence of KSP has also been high (57%) in plants GVA free, while KSG has been detected only in four plants of Vermentino, three of them affected by the two viruses. All samples have not shown CB and LNSG symptoms.

Table 1. Relation between rugose wood complex and GVA in different cultivars and biotypes.

Cultivar	BIOTYPES			GVA +						GVA-					
	Total	GVA		Total	RSP		KSG		RPS KSG	Total	RSP		KSG		RSP KSG
		+	-		+	-	+	-			+	-	+	+	
different-veneto	73	6	67	6	4	2	1	5	1	67	39	26	0	66	0
Vermentino	113	29	84	8	5	2	0	5	0	45	26	16	4	39	3
Ansonica	73	29	44	2	1	1	1	1	1	5	1	4	0	5	0
Total	259	64	195	16	10	5	2	11	2	117	66	46	4	110	3

+ positive ; - negative

The outcome of trial carried out on 13 plants (biotypes) of Torbato cultivar has highlighted the occurrence of GVA in 12 mother plants (MP), while during trunk inspection step on July, only three plants showed Rugose Wood complex symptoms. Indexing tests accounted for 11 biotypes affected by RSP and 4 by KSG while CB and LNSG were not detected (table 2). ELISA and indexing tests carried out on samples of plants obtained by *in vitro* culture have given the opportunity of a more detailed analysis of phytosanitary status of samples that have been grouped according to original mother plant. Some biotypes, derived from different mother plants, appeared to be sanitised as for GVA. Also in this case, materials were assembled in two groups: - one that consisted of samples affected by GVA; percentage of RSP is very high (70,1% of whole population), while occurrence of KSP is confirmed for the 5 biotypes already affected by origin (26,3% of whole populations); - the other one that enlisted samples GVA free where percentage of RSP is lower and accounts for 31%, while RSG concerned two plants only.

Table 2. Occurrence of rugose wood complex in relation to GVA in different biotypes of cv Torbato.

BIO-TYPE	Phytosanitary Status of MP					Phytosanitary Status of Offspring *in vitro* Propagated									
						GVA +					**GVA -**				
	GVA	RW	RSP	KSG	CB	Sampl.	RSP		KSG		Sampl.	RSP		KSG	
						N°	+	-	+	-	N°	+	-	+	-
187	+	-	+	+	-	11	11	0	6	5	0	0	0	0	0
188	+	-	+	-	-	2	2	0	0	2	7	4	3	0	4
189	+	-	+	+	-	6	5	1	5	1	0	0	0	0	0
190	+	-	+	-	-	8	5	3	0	0	0	0	0	0	0
191	+	-	+	-	-	10	8	2	0	10	4	0	4	0	4
192	+	-	+	+	-	14	11	3	2	12	7	6	1	2	5
193	+	-	+	+	-	10	7	2	10	0	0	0	0	0	0
195	+	+	+	-	-	9	4	5	0	9	8	0	8	0	8
196	-	-	-	-	-	0	0	0	0	0	9	0	9	0	9
197	+	-	+	-	-	8	8	0	0	8	9	4	4	0	9
198	+	+	+	-	-	9	7	1	0	9	0	0	0	0	0
199	+	-	-	-	-	9	0	9	0	9	0	0	0	0	0
202	+	+	+	-	-	3	3	0	3	0	0	0	0	0	0
Total						99	70	26	26	65	44	14	29	2	39
%						-	70,1	26,3	26,3	65,6	-	31,8	65,9	4,5	88,6

Low occurrence of GVA in vineyards of Veneto is surely to be considered in relation with selection activity carried out at an early stage with the aim of excluding plants with clear symptoms of viruses including Rugose Wood complex. Since in such area Kober 5BB is the more common rootstock variety, it was possible to nearly exclude at all the occurrence of KSG; this could be helpful in excluding occurrence of GVA as well.

The other three considered vine varieties that are typical of Central Italy, where rootstocks other than Kober 5BB are utilised, showed occurrence of GVA in an higher percentage. But KSG has not always been detected in samples affected by GVA; KSG instead was found in indexing tests of some biotypes of Ansonica variety free from GVA.

Trial started on Torbato variety also underlines that GVA is not always linked to KSG. The linkage seems due mostly to biotype and this is confirmed also in the offspring obtained by in vitro culture.

Results show that there is no relation between GVA and RSP because RSP has been found either with and without GVA; similarly occurrence of CB and LNSG is not related to GVA. It must be said that immunoenzymatic test ELISA for GVA is not yet a reliable tool of analysis because sometimes to confirm the outcome it took several repetitions of tests and comparisons of different antisera. Trial has in addition underlined that *in vitro* culture of vegetative tips can, even if partially, remove GVA trichovirus, Rupestris Stem Pitting and Kober 5BB Stem Grooving symptoms as well

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FURTHER EVIDENCE THAT GRAPEVINE VIRUS A IS THE AGENT OF KOBER STEM GROOVING

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INTRODUCTION

Kober stem grooving (KSG), one of the syndromes of the rugose wood complex (1), is thought to be caused by grapevine virus A (GVA). This notion rests on ample circumstantial and experimental evidence of the very close association of GVA with KSG (2, 3), and the fact that its elimination from affected vines by heat treatment was accompanied by the loss of GVA detection (4). The conclusive proof of a genuine cause-effect relationship between GVA and KSG as, for example, that provided by reproducing KSG symptoms on the specific indicator Kober 5BB through the introduction of a pure inoculum of GVA is, however, still lacking. To this aim, "pure" sources of GVA to serve as inoculum were searched for in nature, or were experimentally obtained.

MATERIALS AND METHODS

More than 60 grapevine accessions of different geographical origin known to be infected by GVA were analyzed for the presence of other trichoviruses (GVB), closteroviruses (GLRaV-1, -2, -3) and isometric viruses (GFLV, GFkV). The screening was done by: (i) ELISA, using virus specific polyclonal antisera and monoclonal antibodies; (ii) micropurification of tissue extracts followed by electron microscope observation and ELISA testing (except for the accessions from Yemen); (iii) mechanical inoculation to a standard series of herbaceous hosts; (iv) dsRNA banding pattern (1). Some of the tests (micropurification, ELISA) were repeated several times in different seasons, using young or aged leaves, petioles, and cortical scrapings.

Rooted cuttings of healthy LN33 were exposed to *Pseudococcus longispinus* instars (c. 50 individuals per plant) that had fed on GVA-infected *Nicotiana clevelandii* acquiring the virus. Mealybugs were killed after three weeks and the vines were placed and kept under screen.

RESULTS AND DISCUSSION

Of the grapevine accessions analyzed, two from Italy (SS6 and BR V6), three from Yemen (Y20, Y21,Y22) and one each from Albania (AA42), Hungary (H25), Bulgaria (BU12), and Cyprus (CY3), responded consistently for the presence of GVA, but were negative for the other viruses. Assuming that these accessions were putative pure sources of GVA, budwood from all of them was top grafted in 1995 on the standard series of woody grapevine indicators (1).

No cases of fanleaf or fleck were detected, but three of the accessions (YE20, YE21, and AA42) induced leafroll symptoms in Cabernet sauvignon and LN33. Symptomatic indicators were shown to contain GLRaV-1 (YE20 and YE21) and GLRaV-7 (AA42). This latter virus had not yet been discovered when the original screening was done.

For ascertaining the presence of the diseases of the rugose wood complex and their identification, the indicators were uprooted two years after grafting, carefully peeled, and their responses recorded.

As shown in Tab. 1, three (SS6, Y22 and BU12) of the nine accessions presumed to be pure sources of GVA actually contained no other viruses, and all indexed positive for KSG. The same disease was detected to a very high rate also in the remaining six accessions, half of which (YE20, YE21, and AA42), however, were also infected by closteroviruses and half by Rupestris stem pitting (RSP) (H25 and BR V6) or LN stem grooving (LNSG) (CY3). Because closteroviruses are not known to induce wood pitting and the still undetermined agents of RSP and LNSG elicit specific responses in indicators other than Kober 5BB, it is plausible to conclude that GVA, as the sole responsible for Kober 5BB reactions, is the agent of stem grooving disease. Of the 22 LN33 rooted cuttings exposed to viruliferous mealybugs, five became infected by GVA, as ascertained serologically and by polymerase chain reaction. After two years, infected LN33 did not show leafroll symptoms nor alterations of the woody cylinder. Early in 1997, shoots from infected plants were green-grafted onto Kober 5BB rooted cuttings, but the results are not yet available.

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Table. 1. Diseases of the rugose wood complex identified by graft transmission to Kober 5BB, *Vitis rupestris* and LN33

Accessions	Kober stem grooving	Rupestris stem pitting	LN stem grooving
SS6	2/4[a]	0/7	0/3
Y20	15/16	0/15	0/12
Y21	14/16	0/11	0/10
Y22	3/8	0/9	0/12
AA42	1/2	0/5	0/5
BU12	2/4	0/6	0/4
H25	3/3	6/6	0/3
BR V6	3/3	5/7	0/3
CY3	5/5	0/5	5/5

[a] symptomatic indicators/ surviving indicators

IDENTIFICATION OF GRAPEVINE RUGOSE WOOD COMPLEX IN HUNGARY: OCCURRENCE OF RUPESTRIS STEM PITTING, KOBER STEM GROOVING AND LN 33 STEM GROOVING

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Alterations resembling rugose wood symptoms were described in the French literature in the early 1990's, but their possible infection nature were identified by Italian researchers in 1961 (1). The disease is often latent in most of the *Vitis* rootstocks varieties as well as in European grapes. Specific symptoms are apparent only on a few varieties, especially when the plants have been grafted: pits and/or grooves show up on the woody cylinder of the rootstock or of the scion or in both.

Grapevine rugose wood is now considered to be a complex disease caused by four apparently different disorders and can be distinguished only by graft transmissions (indexing with chip transmission) onto indicators: *Vitis rupestris* St. George, LN 33 (Couderc 1613 x *Vitis berlandieri*) and Kober 5BB (*Vitis berlandieri* x *Vitis riparia*). Based on symptoms developed on these specific indicators, the disease is identified as rupestris stem pitting (RSP), kober stem grooving (KSG), LN 33 stem grooving (LSG) and corky bark (CB) (1).

In Hungary the typical symptoms of rugose wood were described from own-rooted grape stocks by Lehoczky et al. (1968) (3). Visual selection had been used in that time for the establishment of virological status of wine regions. Regular virological screening of grape varieties started in 1972 (2) by visual selection and by indexing on herbaceous and woody indicators. At the beginning three (FS-4 /Siegfriedrebe/, *V. rupestris* St. George, *V. vinifera* cv. Pinot noir), later six woody indicators (*V. vinifera* cv. Chardonnay, *V. riparia* Gloire, 110 R /*V. rupestris* x *V. berlandieri*/) were applied in the indexing. *V. rupestris* St. George, one of the special indicators for rugose wood, proposed by Bovey and Martelli (1992) (1) was used from the beginning for identification of fanleaf and fleck, the other two indicators (LN 33 and Kober 5BB) were introduced in the national screening system in 1992.

MATERIAL AND METHODS

In the regular virological screening all 27 symptomless stocks of 13 different rootstock varieties and 107 symptomless stocks of 42 European varieties were indexed by chip-transmission from 1992. The standard 8 grapevine indicators, including *V. rupestris* St. George, Kober 5BB and hybrid LN 33 have been inoculated since 1992. After growing, a minimum of five indicators were planted in a nursery plot and the symptoms of graft-transmitted diseases were recorded. Rugose wood was finally checked on indicators at the end of the third season after planting, by uprooting the single plants and removing the bark from the stem.

RESULTS AND CONCLUSIONS

Results of biological indexing are summarised in Table 1. Single infections and different combinations of rugose wood components were identified. No rugose wood on rootstocks was observed. The percentage of plants affected by rugose wood on European grapes was moderately high: the frequency of RSP (22,4%) and KSG (19,6%) was near, while the LSG was slightly lower (13,0%). The occurrence of mixed infections is possible, as found in some plants in this study. Corky bark (CB) occurrence was not definitely ascertained, though could be seen some kind of cracks on a few LN 33 indicator plants. Not only rugose wood were also found, but symptoms of fleck, vein necrosis, vein mosaic and leafroll on the specific indicators.

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

Origin of samples	Years	Cultivars	Candidate plants	Healthy	Infected						
					RSP	KSG	LSG	RSP + KSG + LSG	RSP + KSG	RSP + LSG	KSG + LSG
		nr.	nr.	nr.	nr.	nr.	nr.	nr.	nr.	nr.	nr.
Rootstocks											
Selected varieties	1992-1994	2	6	6	0	0	0	0	0	0	0
Mother plant coll.	1992-1994	9	17	17	0	0	0	0	0	0	0
Selected varieties	1993-1995	2	4	4	0	0	0	0	0	0	0
	Total	**13**	**27**	**27**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	%			100,0	0	0	0	0	0	0	0
European grapes											
Selected varieties	1992-1994	14	34	27	4	5	4	1	0	1	1
Selected varieties	1993-1995	18	51	27	17	12	8	2	4	4	0
Selected varieties	1994-1996	10	22	15	3	4	2	0	0	0	2
	Total	**42**	**107**	**69**	**24**	**21**	**14**	**3**	**4**	**5**	**3**
	%			64,5	22,4	19,6	13,0	2,8	3,7	4,7	2,8

DETECTION OF AN UNUSUAL RNA IN GRAPEVINES INDEXING POSITIVE FOR RUPESTRIS STEM PITTING

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To obtain a cDNA library from GLRaV-1 RNA to be used for diagnostic purposes and sequencing, dsRNA was extracted from vines of cvs. Chiavennasca and Cortese thought to be infected only by GLRaV-1. dsRNA preparations were denatured and used as template for cDNA production by hexanucleotide random priming (1). Some of the synthesised cDNA fragments were cloned in a pUC18 plasmid, sequenced and found to possess virtually no homology with published sequences of known closteroviruses. Unexpectedly, data base responses gave significant homology with apple stem pitting virus (ASPV) and carlaviruses. In particular, clone p48 (716 bp in size) showed a high level of identity with the replicase gene of ASPV (69%) and blueberry scorch carlavirus (66%).

Extracts from cortical scrapings of 186 vines that were either apparently healthy or affected by fanleaf, leafroll, or rugose wood were analyzed by RT-PCR (2) using primers designed on the p48 clone sequence, that amplified a 355 pb fragment. Of these samples, 95 came from plants indexing positive for Rupestris stem pitting (RSP), one of the rugose wood syndromes, the causal agent of which is still unknown (3). The expected 355 bp fragment was amplified at an intriguingly high level from more than 86% of the RSP-positive samples (82 of 95). A confirmatory indication of the specific relationship between the p48 sequence and the unusual RNA present in RSP-affected vines, was given by Northern blots assays in which a digoxigenin-labelled riboprobe transcribed from clone p48, hybridised dsRNA extracts from RSP-affected vines.

The present results suggest that besides GLRaV-1, the vines used as dsRNA source for molecular cloning contained also a graft-transmissible agent, likely a virus, whose dsRNA was inadvertently used as template for cDNA production. The nature of this virus is undetermined and its involvement in the aetiology of RSP needs to be further investigated.

A virus with filamentous particles 600-700 nm long with distinct cross banding but lacking the flexibility of trichoviruses was recently found in Canada in a vine affected by RSP and LN33 stem grooving. This virus was serologically unrelated to a number of filamentous viruses, including ASPV, and was tentatively identified as a possible capillovirus (4). Moreover, RSP-infected vines were shown to contain a dsRNA with a mol. wt of 5.3-5.5 x 10^6 Da (5, 6), in the range of the values reported for capilloviruses. ASPV has particles morphologically similar to those of the virus from the Canadian vine accession, but is not a capillovirus (7).

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DOUBLE STRANDED RNA STUDIES ON SHIRAZ DISEASE IN SOUTH AFRICA

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Grapevine Shiraz disease (SD) is a local virus-like disease of the cultivars Shiraz, Gamay, Merlot and Malbec. It is graft transmissible and field spread has been reported (1). A number of symptoms indicate the presence of SD. Budburst is delayed, canes do not mature properly and appear droopy with a rubbery texture. Infected vines have fewer bunches with very bad fruit set and shed their leaves later than leafroll infected plants of the same cultivar in spite of leaf symptoms appearing leafroll-like.

Grapevine leafroll associated virus-1 (GLRaV-1), -2, -3 and GVA were also found to be associated with SD in vines, but the causative agent has not yet been found (2). The objective of this study was to investigate the use of dsRNA for detection of the causative agent of SD.

A simplified procedure was used for the isolation of dsRNA from SD infected plants (3). This involved the grinding of phloem shavings to a powder in liquid nitrogen; recovery of dsRNA using 4% p-aminosalicylic acid; purification of the dsRNA on cellulose columns and precipitation with 30% ethanol. 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 flourescence at 302nm.

Extractions were made from Merlot and Shiraz infected with SD and dsRNA bands were compared to dsRNA extracted from the cultivar LN-33 infected with GLRaV-3 only. Most of the SD vines showed only one high molecular weight band. Some vines also showed a second band with a lower molecular weight. The presence of this band seems to depend on the concentration of the virus in the vines. However these bands both correspond with bands of GLRaV-3.

The procedure on its own is clearly not sensitive enough to identify the agent causing SD, probably due to the high concentration of polyphenols in grapevine tissue and the low concentration of the viruses sometimes present in grapevines.

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CURRENT STATE OF THE RESEARCH ON THE VEIN YELLOWING LEAFROLL IN THE CHAMPAGNE REGION.

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A graft transmissible « Vein Yellowing Leafroll » (VYLR) has been described in 1983 in the Champagne region. Most of the symptoms, close to that of leafroll, appeared two years after planting, but epidemiology suggested a fast spreading. However, the plants newly diseased in the following years showed only weak symptoms (1). Healthy periwinkles exposed in the field in July-August appeared to be infected by a «dwarfing with small flowers», but the identity with VYLR has not yet been confirmed (2). Thread-like structures similar to closteroviruses completely invaded some mature sieve elements. Indexing indicated that « Roll on Pinot noir » was the best candidate for playing a role in the VYLR. Elisa tests showed the occurrence of the Strain NY1 in most of the samples (3). The aim of this paper would be to bring further informations concerning epidemiology.

The notations in the infected fields proceeded as already described (1), with three levels of the disease 1, 2, and 3 (note 3 for the stronger symptoms). Groups were constituted by the plants having the same notes in the first notation in 1981. All the plants in the groups were followed year after year. Fig. 1 shows the distributions of the notes in 1983 and 1985 for each of the three groups, and also for the plants which had no symptoms in 1980-1981 but gave their first symptoms either in 1982 or in 1984 (right part of the figure). These first symptoms were very weak in both cases (most note 1). The survey of the four distributions 1983 (fig. 1) showed that they could be considered to belong as a whole to a « family of distributions 1983 » (family of curves) with a majority of notes 3 in the distribution of group 3 (note 3, 1981) to a majority of note 0 in the distribution of the group 1 (note 1 in 1981). As already said (1), one can see that the higher notes in 1981 tend to give high notes in 1983 and the lower notes 1981 tend to give low notes in 1983. Furthermore, the distribution in 1983 of the group of plants which showed their first symptoms in 1982 (group 0) appeared to belong to the same « family of distributions », with a further slipping towards note 0. The same results were obtained on the following years. As an example, the plants showing their first symptoms in 1984 (group 00) gave a new distribution in 1985 which clearly belonged to the « family of distributions 1985 », but which further slipped downwards note 0 (fig. 1).

One can then consider that the totality of the disease symptoms may in fact have appeared in 1980 and that the plants which showed their first symptoms later in 1982 or in 1984 had merely too light symptoms to be detected in the conditions of the autumn of 1980. The plants which showed their first symptoms in 1984 had too light symptoms to be revealed either in 1980 or in 1982 and may represent the lighter strains of the disease. This fact could be taken into accounts if a premunition process were to be experimented in the future for VYLR. These results give a good explanation for the rapid extinction within a few years of the « fast spreading » of the disease as it was first suspected (1). The VYLR cannot be longer considered as a fast spreading leafroll.

The best hypothesis concerning the outcome of VYLR remains that of a virus disease (probably of a leafroll type) brought as a contaminant in the rootstock material. The new type of symptoms (vein yellowing) would then suggest either a new virus or a synergy with a latent virus occurring in the *V. vinifera* clones used.

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Plants showing first symptoms in 1980			Plants showing first symptoms later than 1980	
group 3	group 2	group 1	group 0	group 00

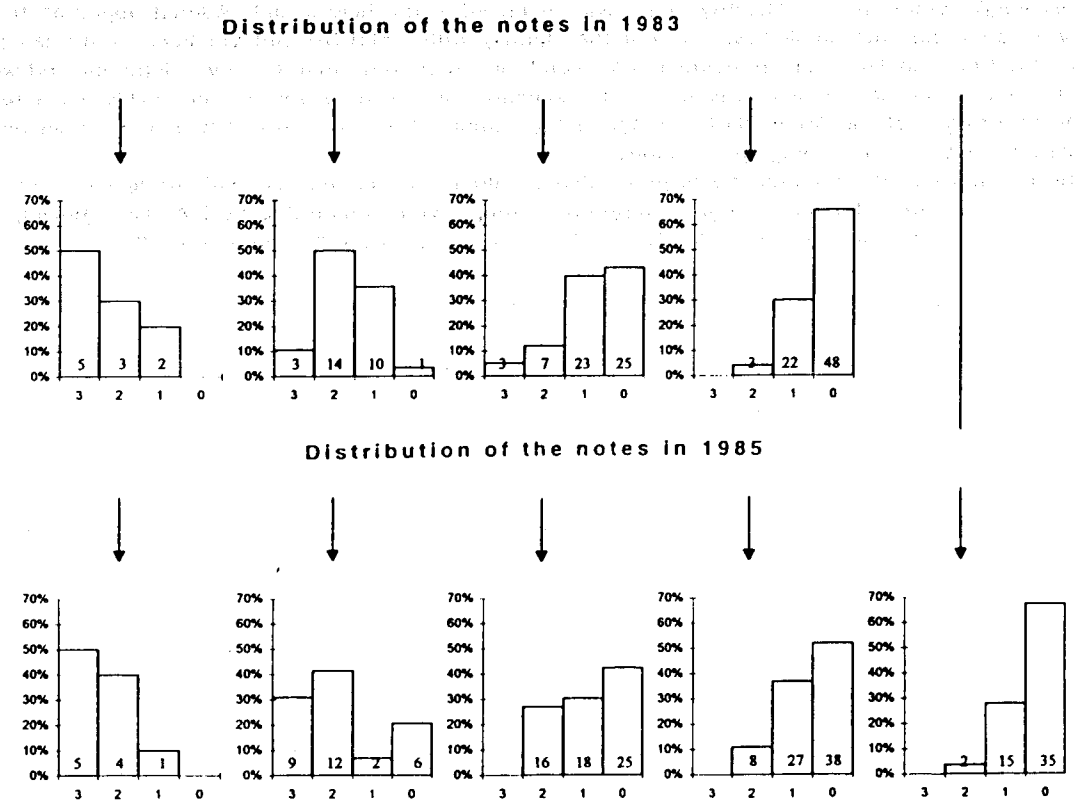

Figure 1. Comparison of the distribution of the notes in 1983 and 1985 for the following groups of plants :
group 3, 2 and 1 : plants with first symptoms in 1980 and noted 3, 2 and 1 respectively in 1981, according to the severity of symptoms;
group 0 : plants with first symptoms in 1982;
group 00 : plants with first symptoms in 1984.
The distributions are shown in percent, and numbers of plants in numerals.

OCCURRENCE OF ENATION DISEASE IN TUNISIA

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In the course of surveys for virus diseases in Tunisia, striking symptoms were observed in 1993 on a few vines of the wine grape cv. Grenache in the Cap Bon area (south of Tunis) and, in the years that followed, in a single vine of the native table grape variety Testouri in the north west of the country. Affected plants of both varieties showed the same symptomatology which, however, was more severe in cv. Testouri.

Symptomatic vines exhibited delayed and bushy growth in spring, shoots were short and bore strongly malformed basal leaves with a thick blade and prominent veins. Outgrowths of various size and shape (enations) were present on the underside of the leaf blade along the main veins. In cv. Grenache only the four basal leaves of some of the shoots bore enations, whereas in cv. Testouri outgrowths were visible up to the 12th leaf from the base of many shoots. Some of the rooted cuttings propagated from the symptomatic cv. Testouri plant showed enations on the basal leaves of the first flush of vegetation. In cv. Grenache the crop was reduced by c. 30% by comparison with apparently normal vines. Disease expression in the field was erratic, for out of five cv. Grenache vines kept under observation for three years in a row, only one consistently exhibited symptoms each year.

The similarity of the field syndrome observed in Tunisia with that characterising enation disease (1) was impressive. Also other traits of the disorder, such as the erratic appearance of symptoms from year to year, their perpetuation by vegetative propagation, and the extent of crop reduction, tallied with what reported in the literature for enation disease (1, 2, 3).

To prove the infectious nature of the Tunisian syndrome, transmissions were attempted by green-grafting rooted cuttings of LN33 with material from symptomatic vines. Only one grafted indicator out of 20 produced leaves with a few enations over two months after inoculation, a response which is in line with the low graft-transmission efficiency reported for enation disease (4).

It is clear that the disease observed in Tunisia is comparable to enation disease, one of the grapevine disorders of unknown aetiology. Records of this disease exist from many different countries but, except for Israel, it has never been reported from the viticultural areas of the southern Mediterranean rim (5), this representing its first record in this region.

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GRAPEVINE ENATIONS DISEASE OF GRAPEVINE IN MURCIA (SPAIN)

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In this work it is exposed the symptomatology of the syndrome of Grapevine Enations Disease (GED) as observed in wine and table grapes in the Region of Murcia (Spain). The presence of the symptoms has been observed exclusively in the table grape cultivar Italia. Moreover it has been observed that the symptoms do not appear every year in the affected vines. This could be explained by considering that the manifestation of the symptoms is closely related to the cultivar and the climatic conditions in which it develops.

In order to see if the syndrome could be an atypical expression of any of the usual virus disorders of vines a test was made by ELISA against GFLV, GFkV, ArMV and SLRV. The results were negative. However, some plants with GED symptoms and other without the symptoms chosen at random, were positive for GLRaV-3. Indexing with the indicators Rupestris de Lot, Cabernet Sauvignon and LN 33 are in process.

The practical incidence of GED is small. It appears in a few vines, without temporal or spatial progression. The production of the affected plants is normal in quality and quantity. From the comparison of symptomatic plants, it seems that there is not synergism between GED and GLRaV-3.

THE MYSTERY DISEASE: EMERGENCE OF YELLOW SPECKLE-VEIN BANDING SYNDROME IN CALIFORNIA?

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Surveys of grapevine viroids found in California vines have reported the presence of the grapevine yellow speckle viroid (GYSVd) in about 85% of vines tested (1). Nevertheless, the expression of the yellow speckle disease symptoms in the field has been historically rare. From an initial observation in 1988, an increase in vine symptoms termed "mystery disease" but resembling yellow speckle (YS) and vein banding (VB) syndrome has been observed principally in Napa County (2). Testing of a wide range of biotic and abiotic factors to establish a direct causal association for the disorder have been unsuccessful except for the presence of viroids. Detection and characterization of the viroids associated with "mystery disease" vines to determine the relationship with viroids inducing the syndrome is the subject of this report.

Field samples were collected from a number of cultivars including Chardonnay, Sauvignon blanc, Semillon, Cabernet franc, Cabernet Sauvignon, Merlot, Petite Verdot grafted onto the rootstocks 5C, 101-14, 3309, 420A, 5BB, 039-16, and 1103P. Plant materials reflected both certified as well as non-certified vines. Nucleic acid extraction and viroid detection by sPAGE were as previously described (3, 4). Viroids from mystery disease vines were compared by nucleotide sequence and DNA single strand conformational polymorphism (SSCP) analysis with grapevine yellow speckle viroid-1 (GYSVd-1) from a known YS vine of the cultivar Mission.

The initial observations of "mystery disease" foliar symptoms characterized by veinal and interveinal patterns suggested a nutritional disorder or pesticide damage. Tissue and soil analysis and the application of soil supplements have discounted these possibilities. Similarly, testing for the presence of transmissible agents of disease, such as viruses (fanleaf, leafroll, or yellow vein) and phytoplasmas (Pierce's disease) has not established a convincing causal relationship.

Viroid analysis has demonstrated the presence of GYSVd-1 in all "mystery disease" samples. Heterogeneity of the GYSVd-1 population has been demonstrated by nucleotide sequence and SSCP. Nevertheless, the GYSVd variant associated with known yellow speckle sources has also been identified from "mystery disease" samples.

With the known widespread occurrence of the grapevine viroids, detection of GYSVd-1 in field samples is not surprising and therefore a correlation with symptomatic tissues is not unexpected. The more intriguing question is why the disease symptoms have only recently become more prevalent and what are the specific factors governing this emergence. The frequency and occurrence of yellow speckle symptoms in Australia has been associated with periods of sustained heat thus suggesting a "stress" related disorder. In addition, the expression of vein banding symptoms has been demonstrated by the synergistic infection of symptomless viroid-containing vines with grapevine fanleaf virus (GFLV) (5). Thus, it would appear that a range of biotic as well as abiotic factors might be effective in the induction of yellow speckle-vein banding symptoms.

The site of the major appearance of the "mystery disease", the Napa Valley, has also been subjected to extensive replanting due to vine loss by *Phylloxera*. Therefore, this region is currently characterized by a higher than normal percentage of young and not well established vines. It is suggested that the combination of predisposed plant materials with yet undefined "stress factors" has resulted in the expression of disease symptoms induced by otherwise latent viroids.

The emergence of yellow speckle-vein banding-like disease symptoms in California suggests that it may be prudent to monitor all transmissible molecules present in vegetatively-propagated plant materials even in the absence of apparent disease symptoms. The possibility of regulatory restrictions for the movement of viroid-containing plant materials should also be considered.

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CHARACTERIZATION OF YELLOW SPECKLE VIROID VARIANTS

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INTRODUCTION

Grapevine viroids have been shown to be ubiquitous in *Vitis* varieties and rootstock selections from California and Europe (6). There are five known grapevine viroids, two reported to induce symptoms of yellow speckle, GYSVd-1 and GYSVd-2 (1, 4). Both GYSVd-1 and -2 are members of the apple scar skin viroid group (ASSVd). Symptoms of yellow speckle are extremely diverse, ephemeral and most evident at the end of the summer. Yellow speckle viroid has also been reported to cause vein-banding symptoms in a synergistic reaction with fanleaf virus.

Variants of GYSVd-1 have been reported within a single vine and among vines within a vineyard (2, 3). Rigden and Rezaian (1993) reported sequence variations within clones of GYSVd-1 in a single vine and separated the clones based on the absence (type 1) or presence (type 2) of eight sequence variations in the pathogenic domain.

In an attempt to attribute a defined biological activity to these "type" variants, characterization of GYSVd-1 among field samples were examined by reverse transcription (RT), polymerase chain reaction (PCR), single stranded conformational polymorphism (SSCP) and sequence analysis utilizing primers from conserved regions of ASSVd .

MATERIAL AND METHODS

Yellow speckle, vein-banding, and non-symptomatic tissues from *Vitis vinifera* cultivars of Pagadebit, Sauvignon blanc, and Zinfandel were collected from vineyards in Italy and California. Fresh tissues were macerated in liquid nitrogen and either maintained at -20°C or lyophilized. A Mission cultivar, known to be yellow speckle positive and Zinfandel-IA, which was non-symptomatic were used as references. Tissue extraction and detection of viroids were performed as previously reported (6).

For RT and PCR, both complementary and homologous primers were synthesized from the upper (82-97) and lower conserved region (225-241) (CRU and CRL, respectively) of ASSVd. Degenerate bases were synthesized in positions 228 and 229 thus producing "generic" primers to ensure optimal priming of all possible viroids within the ASSVd group. Reverse transcription and PCR were performed essentially as previously reported (5), except 1.5 mM Mg^{2+} was employed for amplification of the dsDNA products in PCR protocol. Utilizing a different combination of primer pairs, double-stranded PCR products analogous to the left and right sides of the viroid molecule were amplified.

For SSCP analysis, double-stranded PCR products from the left and right side of the molecule were denatured and applied to a 80 x 100 x 0.75 mm 14% polyacrylamide gel containing 5% glycerol and electrophoresed at 4°C until xylene cyanole was approximately 60 mm from the origin. The SSCP gels were silver stained for conformational analysis. Conformational variations among the grapevine field samples were compared to the migration patterns of the Mission and Zinfandel references.

Grapevine yellow speckle viroid-1 specific primers were also employed for amplification of full length products from the two reference sources. Full and partial length PCR products were sequenced using the 5' end-labeled primer protocol by the Promega *fmol* sequencing system.

RESULTS

Sequential PAGE analysis of all field samples, as well as the references, indicated the presence of GYSVd-1 and HSVd-g. Grapevine yellow speckle -2 was not detected.

Products of approximately 185 bp were amplified utilizing the complementary CRU and the homologous CRL primer pair which delineated the left side of the molecule. If the reciprocal primers were employed, complementary CRL and homologous CRU, products of approximately 140 bp were amplified, delineating the right side of the molecule.

Two different migration patterns were observed in SSCP for the Mission and Zinfandel samples on the left side of the molecule. These two patterns also predominated in the field samples using these same primers. However, the dsDNA products which were amplified from the right side of the molecule from Mission and Zinfandel gave similar patterns in SSCP.

Sequence analysis revealed the Mission cultivar to be a "type II" and Zinfandel to be "type I" variant as reported by Rigden and Rezaian (1993) utilizing both GYSVd-1 specific and the left side "generic" primer pairs. Representative field samples were also sequenced which displayed similar SSCP patterns to either Mission or Zinfandel on the left side of the molecule. A positive correlation was maintained between the SSCP patterns and the GYSVd-1 "type" sequence.

DISCUSSION

Of the three principal occurring grapevine viroids only GYSVd-1 and HSVd-g were detected by sPAGE in all symptomatic and non-symptomatic materials tested while GYSVd-2 was not found. However, since CEVd-g, AGVd, and GYSVd-1 are similar in size and extremely difficult to separate by electrophoresis, CEVd-g and AGVd could be present but not detected by sPAGE.

Using the "generic" primer pairs for the ASSVd group, partial length products were produced from the left and right side of the molecule. This provided the opportunity to detect by SSCP analysis the variants within GYSVd-1 from field samples and could potentially amplify any viroid of ASSVd family. The major differences were observed on the left side of the molecule.

Partial and full length products of Mission and Zinfandel references were sequenced to determine the variation between these two sources which was observed in SSCP and to ascertain if the same nucleotide sequence was obtained using the "generic" primer pairs. Both the partial and full length products resulted with Mission a "type II" and Zinfandel a "type I" variant. Using the SSCP as a screening protocol, field samples were also shown to be either "type I" or "type II" by sequence analysis.

A correlation between symptoms and the "type" GYSVd-1 variant was attempted. Even given the heterogeneity of GYSVd-1 population reported in field samples, a trend was found correlating the "type II" variant with symptom expression. However, since yellow speckle symptoms are extremely diverse, ephemeral and seem to be correlated to certain climatic conditions or "stress" factors, the appearance of symptoms may be difficult to predict.

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GRAPEVINE YELLOWS DISEASES: NEW PERSPECTIVES ON DETECTION AND IDENTIFICATION OF ASSOCIATED PHYTOPLASMAS

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Grapevine yellows diseases have long been distinguished from one another on the basis of biological characteristics and/or geographical occurrence (3). Recent studies have provided molecular criteria to separate the different grapevine yellows diseases by distinguishing among the phytoplasma pathogens presumed to be their respective causal agents. At least four major grapevine yellows diseases have been recognised: Flavescence doree (FD) *sensu stricto* (3), attributed to a group 16SrV (elm yellows and related phytoplasmas) phytoplasma; Bois Noir (BN), Vergilbungskrankheit (VK), and Mediterranean grapevine yellows (MedGY), attributed to phytoplasma strains in group 16SrI, subgroup G (stolbur and related phytoplasmas) (1,3,7); Australian grapevine yellows, associated with a phytoplasma representing subgroup 16SrI-J and recently named "*Candidatus* Phytoplasma *australiense*" (4); and Virginia grapevine yellows disease, associated with a phytoplasma belonging to group 16SrIII, subgroup E (R.Jomantiene, R.E. Davis, and E.L. Dally, unpublished data). Additional phytoplasmas have also been found in diseased grapevines in the field (2,8).

Results from molecular genetic characterisations of phytoplasmas have underscored the importance of recognising distinctions among phytoplasmas. For example, the use of multiple (6 or more) enzymes in restriction analyses of 16S rDNA makes possible distinctions among members of different groups or among subgroups within a 16S rRNA gene group (6), whereas, the use of one or few enzymes in RFLP analyses may fail to make such distinctions. Furthermore, analyses of additional gene sequences, for example ribosomal protein (rp) gene operon sequences, have unveiled distinctions among various phytoplasmas that were not previously distinguished by the analyses of 16S rRNA gene sequences alone (5). Developments such as these provide important new bases for specific pathogen detection and identification. Cautious and thorough analyses are required for definitive identification of grapevine yellows phytoplasmas, for accurate disease diagnosis, and for development of a more thorough understanding of the epidemiology of grapevine yellows diseases.

Recent results in our laboratory also underscore the importance of judicious interpretation of results from use of phytoplasma "group- or subgroup-specific" primers in polymerase chain reactions (PCRs). Primer pair fSTOL/rSTOL was designed for specific detection of the stolbur phytoplasma in PCRs (7) and has become a valuable tool in the diagnosis of BN, VK, and MedGY (7). However, recent work in our laboratory has shown that the use of primer pair fSTOL/rSTOL in PCRs can result in the amplification of DNA from, and the consequent detection of, several mutually distinct phytoplasmas other than stolbur.

In our study of Australian grapevine yellows, DNA from the presumed causal phytoplasma, "*Candidatus* Phytoplasma *australiense*", was amplified in PCRs primed by primer pair fSTOL/rSTOL (4). In subsequent work with diseased strawberries (*Fragaria* X *ananassa* Duch.) in Florida, we have found that DNA sequences from two other mutually distinct phytoplasmas were amplified in PCRs primed by fSTOL/rSTOL (Jomantiene et al., unpublished data) (Table 1). These data indicate that four distinct phytoplasmas shared sequence homologies that enabled amplification of DNA in PCRs using primers previously designed as stolbur-specific (R. Jomantiene, R.E. Davis, J. Maas, and E.L. Dally, unpublished data). This result clearly indicates that phytoplasma identification should not be based solely on the use of PCRs primed by fSTOL/rSTOL.

When primer pair fSTOL/rSTOL was designed (7), AUSGY, MPV, and STRAWB1 phytoplasmas were unknown or unavailable for study. Since the degree of specificity determined for any given PCR primer pair is a function of the breadth of diversity represented by the microbial genotypes incorporated in the determination, results obtained from the use of a phytoplasma-specific primer pair should be interpreted with caution. In the identification of grapevine yellows phytoplasmas, it seems advisable to incorporate additional assays. In the light of our new data, it may be worthwhile to re-examine grapevine yellows phytoplasma identifications previously based principally or only on the use of fSTOL/rSTOL in PCRs.

Table 1. Phytoplasmas detected in PCRs primed by primer pair fSTOL/rSTOL.

Phytoplasma	16S rRNA subgroup	rp subgroup	Ref.
Mediterranean grapevine yellows (BN, VK)	16SrI-G	---	1,7
"*Candidatus*" Phytoplasma *australiense* (AUSGY)	16SrI-J	---	4
Mexican periwinkle virescence (MPV)	16SrI-I	16SrI-H(rp)	Unpubl. data
Strawberry phytoplasma (STRAWB1)	16SrI-I	16SrI-I(rp)	Unpubl. data

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DIFFERENTIATION OF GRAPEVINE PHYTOPLASMAS IN THE ELM YELLOWS AND THE STOLBUR GROUP WITH THE USE OF RFLP OF NON-RIBOSOMAL DNA

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Grapevine yellows diseases (GY) have been associated in Europe to phytoplasmas belonging to two different groups, the elm yellows (EY) group and the stolbur group (1, 4, 7, 10). To the elm yellows group belong the phytoplasma causing Flavescence dorée (FD), a GY which was first described in France and which is transmitted by the ampelophagous leafhopper *Scaphoideus titanus* Ball. Conversely, the phytoplasmas associated to Bois noir (BN) in France, to Vergilbungskrankheit (VK) in Germany, and to similar diseases in Italy, Spain, and Israel belong to the stolbur group. The stolbur phytoplasma was experimentally transmitted to grapevine using naturally stolbur-infected specimens of the fulgore species *Hyalesthes obsoletus* Sign (7, Sforza et al. submitted). *S. titanus* can be found only in vineyards of regions with a long warm summer and a fairly cold winter. It is not a vector for BN. On the contrary *H. obsoletus* is widespread in Western and Eastern Europe and around the Mediterranean sea.

Surveys in vineyards conducted from 1992 to 1996 included the sampling and analysis of leaf tissues from several hundred GY-diseased stocks in 21 viticultural regions of Western Europe and Israel. The stolbur phytoplasma could be detected in vineyards from all the regions investigated. In contrast, an EY-type phytoplasma was identified in samples from Southern France, Northern Italy and Northern Spain where *S. titanus* is present, and also in the German Palatinate where the latter species has not been recorded (6, 8).

The severity and intensity of GY epidemics obviously depend on the presence of specified vectors. For these reasons, the shipping and planting of grapevine material latently infected with phytoplasmas might result in an epidemics outbreak if an efficient vector is present on the place. Thus, methods for the characterisation of phytoplasma strains are important to search for vectors and control the diseases.

The diversity of grapevine phytoplasmas has previously been investigated in numerous reports using PCR amplification and restriction analyses of 16S rDNA. However, rDNA is highly conserved and its degree of polymorphism may not be suited for differentiating phytoplasma strains within a given group. Alternatively we developed PCR primer pairs for non-ribosomal DNA fragments from a FD strain and a stolbur strain, and we investigated the polymorphism of the amplified DNA fragments in periwinkle-maintained reference strains of the EY and stolbur groups and in GY-affected grapevines from different regions.

Leaf samples of various cultivars of *V. vinifera* showing symptoms of GY were collected in several regions of France, Italy, Spain, Israel and in Palatinate (Germany). Fifteen periwinkle-maintained strains of phytoplasmas assigned to different ribosomal groups were used in the study. In the elm yellows group were included two strains isolated from elm in the USA and in France and two FD strains (FD70 and FD88) (3) isolated from naturally infected *S. titanus* in Southern France. In the stolbur group were included strains STOL from pepper in Croatia, STOLF from tomato in France, CH1 from grapevine in Italy and MOL from cherry in France. Certified healthy grapevine material and symptomless grapevines as well as periwinkle seedlings were used as healthy control.

Standard procedures were used for DNA extraction and isolation. Grapevine DNA was extracted from a phytoplasma-enriched fraction of leaf main veins, according to Daire et al. (3).

PCR primers were constructed from DNA fragments which had been randomly cloned from strain FD70 (FD9 fragment) or from strain STOL (STOL4 and STOL11 fragments) (Daire, unpublished) and used as hybridisation probes. These DNA fragments were partially sequenced from both ends and specific oligonucleotides of about 20 mers in length were selected. Consequently the expected length of the PCR product was close to the length of the DNA fragment from which each pair had been constructed.

PCR amplification was conducted as already described for periwinkle or grapevine DNA (4, 6). Native PCR products were visualised with agarose electrophoresis. For restriction analyses, 10 enzymes were used including enzymes with AT-rich sites and the digested DNA was electrophoresed in 10% acrylamide gel.

The results showed that the primer pair FD9f/r amplified a 1.3 kb fragment in reference strains of the elm yellows group in periwinkle and not in any other reference strain or the healthy control. A fragment of the same size was amplified from naturally infected grapevines collected in Alt Empordà (Catalonia), in Southern France, in Northern Italy and in Palatinate (Germany). The restriction enzymes *Alu*I and *Tru9*I appeared as best suited for RFLP analysis of the FD9 fragment. Six restriction profiles were obtained with *Alu*I and eight profiles with *Tru9*I. With either enzyme the two phytoplasmas from elm were clearly different from the FD phytoplasmas in periwinkle and from all the grapevine samples. Among the latter, all the

samples from Southern France and Catalonia and 3 samples out of 4 from Northern Italy belonged to the same RFLP group as FD88. With both enzymes the profiles in samples from Palatinate showed profiles which were different from elm yellows and FD reference strains and also from FD isolates.

Previous work (3) had shown with Southern hybridisation data that strains FD70 and FD88 in periwinkle presented dissimilarities and that these strains were related to, though distinct from elm yellows strains isolated from elm. Several authors have reported sensitive detection of EY-group phytoplasmas in grapevine in France and Italy using universal or group-specific 16S rDNA primers. However, no differentiation between these phytoplasmas had been reported up to now, probably due to the high degree of conservation of rRNA genes. In contrast, we demonstrated that the polymorphism of a non-ribosomal DNA sequence allowed to differentiate phytoplasmas within the EY group. The results strongly suggest that FD *sensu stricto* (i.e. the GY transmitted by *S. titanus*) is present in Northern Italy and in Catalonia. This is consistent with the reports on the presence in large numbers of *S. titanus* populations in France, Italy and Spain, and with data obtained on *S. titanus* specimens which tested FD-positive in serological (2, 9) and DNA-based assays (Daire, Thesis). On the contrary FD-type phytoplasma previously detected in grapevine samples from Palatinate in Germany (8) were shown to be different both from elm phytoplasmas and FD phytoplasma. This suggests that it was not imported via infected stocks from France or Italy. As *S. titanus* is not present in Germany, another vector must be responsible for transmission.

The FD primers might be used to assess the relationships between EY-group phytoplasmas in various plant species and to search for their vectors. Trials to experiment the transmission ability of *S. titanus* for different EY-group phytoplasmas will help in the study of the biological relationships within this group.

The results with STOL primers showed that the pairs STOL4f/r and STOL11f2/r1 were specific for the stolbur group, as they allowed the amplification of fragments of the expected size (1.7 kb and 0.9 kb respectively) solely from members of the stolbur ribosomal group. In grapevine, STOL4f/r and STOL11f2/r1 amplified similar fragments with DNA of diseased samples originating from several regions of France and Italy, and from Spain and Israel, thus confirming the results previously obtained with rDNA analysis. Restriction analysis of STOL4 and STOL11 fragments amplified in stolbur reference strains and in grapevine isolates from France, Italy, Spain and Israel. None of these analyses could demonstrate any RFLP among these phytoplasma DNA fragments. Thus, in contrast with elm yellows phytoplasmas, the results of restriction analyses suggest a rather low variability within the stolbur group. Stolbur phytoplasma is an ubiquitous pathogen agent which can cause Yellows in a wide range of wild and cultivated plant species. A link between these diseases and BN occurrence in vineyards is most probable since *Hyalesthes obsoletus* is a polyphagous species which was successfully used to inoculate such a phytoplasma to grapevine.

The present study also confirmed the occurrence of GY caused by different phytoplasmas within the same viticultural areas. As vector transmission and sources of inoculum are obviously different, control regulations must be established for each disease with respect to the biology of their potential vectors.

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GEOGRAPHICAL DISTRIBUTION OF ELM YELLOWS-RELATED PHYTOPLASMAS IN GRAPEVINE FLAVESCENCE DORÉE OUTBREAKS IN VENETO (ITALY)

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A severe grapevine disease with symptoms indistinguishable from Flavescence dorée (FD) and grapevine yellows (6) has been found over the past years in some vineyards of the Veneto region in the north east of Italy (1, 8) where phytoplasmas belonging to the elm yellows group (EY or 16SrV) have been detected very often in mixed infection with other phytoplasma groups (2, 4). All the grapevine cultivars were severely affected: 2-3 years old vineyards were destroyed, while plants in older vineyards reduced in yield up to 50%. During the past two years a survey was carried out to verify the spreading of the yellows/FD epidemic in different provinces of the region: the phytoplasma identification was carried out using PCR/RFLP (3, 4, 7, 10, 11, 12). The results of this survey together with further molecular characterisation of some EY-related phytoplasma strains detected in grapevines and in *Scaphoideus titanus* Ball samples are reported.

During the Fall 1995 and 1996, a total of 120 samples from symptomatic and asymptomatic grapevines belonging to different cultivars were collected in vineyards mainly located in Verona, Treviso and Padova provinces. Nucleic acid from 1 g of freshly cut leaf midribs and some individual samples of *S. titanus* collected near Verona, were extracted following described procedures and used for direct and nested polymerase chain reaction (PCR). Tubes with the reaction mixture devoid of DNA templates were included in each experiment as negative controls; positive controls in periwinkle were representative of major phytoplasma groups described in Italy: AY (16SrI-B), IPVR (16SrI-G), CX (16SrIII), EY (16SrV-A), AP (16SrX-A). A sample of rubus stunt (3) in rubus was also employed. Amplification reactions were carried out on 16S rDNA by using primers R16F1/R0 followed by R16F2/R2 for a total of 35 cycles each run, under conditions previously described (13). PCR products were analysed with electrophoresis through a 1% agarose gel followed by staining in ethidium bromide. The DNA sequences amplified were compared by restriction fragment length polymorphism (RFLP) analysis with *AluI*, *MseI*, *HhaI* and *KpnI* followed by electrophoresis in 5% polyacrylamide gels. Phytoplasma group-specific primers R16(V)F1/R1 (10) were employed to amplify only phytoplasmas belonging to the EY group: the products were digested with *BfaI* (4). Only on samples positive to this amplification other PCR assays were carried out using primers that amplify from middle of 16S rDNA to the beginning of 23S rDNA designated M1/B6 (9, 12): the products were then digested with *TaqI* (11). Three of these isolates (from cultivars Corvina, Garganega and Perera) and rubus stunt were used for spacer region sequencing with primer P3 (14). The sequencing was performed on an ABI Prism DNA sequencing machine; alignment of sequences was done by Clustal method with DNAStar using FD strain (7) (EMBL Data Library: X76560) from bases 1494-1792 as comparison.

When direct PCR was performed with primers R16F1/R0, no visible amplification products were obtained with any of the templates from field-collected grapevine samples, but only from phytoplasma control strains. The tests carried out with nested-PCR with primers R16F2/R2 followed by RFLP analyses allow to detect the 1.2 kb DNA phytoplasma product in 40 of the 46 (87%) grapevines tested in 1995 and in 47 of the 74 (64%) grapevine tested in 1996. RFLP identification show that predominant phytoplasma groups detected were aster yellows (16SrI-B), stolbur or bois noir (16SrI-G) and elm yellows or flavescence dorée (FD, 16SrV) many times in mixed infections. The amplification with specific EY-primers show that in the two years the 53% of phytoplasma positive samples were infected with EY-related strains. RFLP with *BfaI* shows identities of all these isolates with the rubus stunt strain (Fig. 1A). PCR/RFLP assays on the spacer region (amplification with M1/B6 and digestion with *TaqI*) show the presence of two different patterns in the grapevine samples: one identical to the rubus stunt and one peculiar of grapevines (Fig. 1B). *S. titanus* positive sample was carrying EY-type phytoplasmas with pattern identical to the one detected only in grapevine. No amplification products were observed in negative controls. Alignment of spacer region sequences from the grapevine 3 isolates and from rubus stunt shows identity of the major part of the sequence with FD strain from France. The differences between the strain from Perera and FD were 14%, while those between the other phytoplasma strains and FD were about 6-7%.

The molecular test confirmed that phytoplasmas are widespread in Veneto vineyards: FD and aster yellows type phytoplasmas were often detected in mixed infections (2, 4); the decrease percentage of infected samples in 1996 is related to the fact that the survey was carried out on the border of the epidemic areas. The RFLP study of 16S and spacer regions of FD-type phytoplasmas, distinguishes two types of them: one identical to rubus stunt and one detected only in grapevines. Two types of 16SrV phytoplasmas (similar to rubus stunt and to elm yellows) were already reported in grapevine in Italy but in different regions (5). The differences detected in the present work were in the spacer region (*TaqI* digestion) and were confirmed by sequencing data: elm yellows strain was never detected (Fig. 1): the two subgroups

identified are preliminary called 16SrV-B (rubus stunt and related strains) and 16SrV-C (FD and related strains). They were detected in two different provinces, respectively Treviso and Verona and in *S. titanus* from Verona. The spacer sequencing data confirm the close genetic relationship among 16SrV phytoplasmas infecting grapevines in Veneto and in France; in some strains i.e. Perera the differences from FD are consistent and more precise sequencing data will provide informations about usefulness of specific primers to routinely detect Italian FD strains. The epidemiological importance of the two subgroups of 16SrV phytoplasmas infecting grapevines in geographically distinct areas will be clarified when *S. titanus* from Treviso or other areas of Veneto will be studied since *S. titanus* is rarely found because of the strict control from growers.

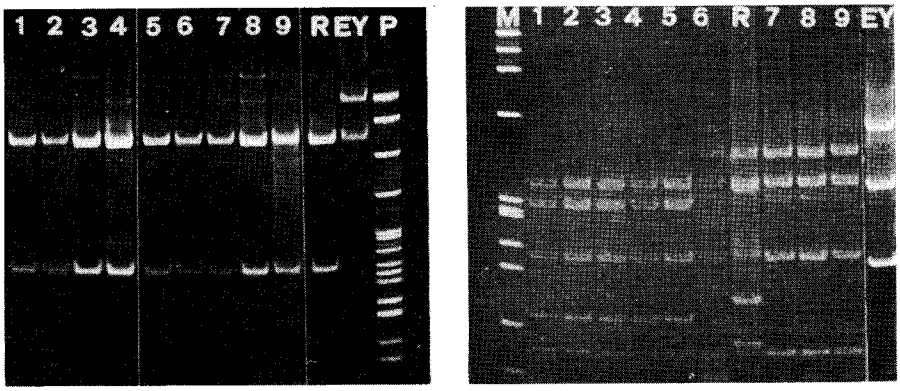

Figure 1. A. Polyacrylamide 5% gel showing RFLP of phytoplasma 16S rDNA fragment of 1.1 Kb amplified by nested-PCR with primers R16(V)F1/R1 and B. of 16S rDNA plus spacer region amplified with primers M1/B6. P, marker pBR322 *Msp*I digested. M, marker phiX174 *Hae*III digested. Samples 1 through 9 grapevine; R, rubus stunt; EY, elm yellows. The enzymes used are: left *Bfa*I and right *Taq*I.

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EXPERIMENTAL TRANSMISSION OF 16SrV PHYTOPLASMAS BY *SCAPHOIDEUS TITANUS* BALL.

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The grapevine yellows (GY) have been reported in several countries and diverse phytoplasma groups were found associated with these diseases (7). Among the GY, Flavescence dorée (FD), the epidemic form transmitted by the leafhopper *Scaphoideus titanus* Ball (2), is considered one of the most severe threat for the grapevine crop. In Italy, where the presence of FD has been reported since 1973 (3), experimental transmission of the causal agent was carried out using *S. titanus* reared on infected grapevine plants (8). Moreover in 1992-1993 a severe outbreak was observed in some areas of north-eastern Italy and phytoplasmas belonging to 16SrV group were detected in several grapevine samples (4, 6). In order to obtain further information about the role of *S. titanus* in disease spreading, transmission trials with this insect were conducted.

Experiments have been carried out during summer 1996 in two vineyards, cv Cabernet franc and Chardonnay, located in Vicenza province where most of the plants showed typical symptoms of the disease. In the same vineyard, high populations of *S. titanus* were observed during the previous four years. Leaf samples of different symptomatic vines were collected in June and July, while adults of *S. titanus* were captured from symptomatic grapevines on July 25th and August 20th. These insects were partly used for phytoplasma detection tests and the remaining for experimental transmission trials to grapevine and broadbean seedling plants grown in insectproof greenhouse. Groups of these adults were allowed to feed one week long on single seedlings with two or three leaves, immediately frozen by using liquid nitrogen and then stored at -30 °C until the total nucleic acid extractions, conducted as elsewhere described (12).

Two months after the inoculation, leaf tissues from symptomatic plants were collected and total nucleic acid extractions were conducted as previously described (4). Polymerase chain reaction experiments were conducted adding separately 30 ng of DNA template of each sample to a reaction mixture containing 0.4 mM of each primer of a primer pair, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50mM KCl, 0.001% gelatine, 0,625 units of Taq DNA polymerase (Pharmacia™) and 200 μM each of dATP, dCTP, dGTP and dTTP, in a total volume of 25 μl. Primer pair named 16SrF2/16SrR2, designed for universal amplification of DNA 16S RNA gene from all known phytoplasmas (9), were used for a first round of PCR tests. A second round of PCR test (nested PCR) was conducted for all the examined samples, using primer pairs designed for specific amplification of 16SrI (aster yellows, AY) and 16SrV (elm yellows, EY) phytoplasma groups (10). Direct PCR amplification tests were conducted using primer pair for amplification of Vergilbungskrankeit (VK) phytoplasmas (11).

Almost all the leaf samples collected from grapevine plants showing symptoms in the two vineyards were found to be infected by phytoplasmas belonging to 16SrV group, while few of them contained phytoplasmas related to 16SrI group. However, in transmission tests, inoculated grapevine and broadbean plants contained only phytoplasmas belonging to 16SrV group. The same phytoplasma group was detected in some *S. titanus* adults collected in the two vineyards, including the insects used for transmission experiments. Further analyses based on RFLP technique, conducted on products of specific amplification for 16SrV group, showed that all the samples from plants and insects contained the same phytoplasma which was shown to be a particular subgroup of the 16SrV group (5). Despite phytoplasmas belonging to 16SrI-B subgroup were found in some of the *S. titanus* insects, we never detected this latter phytoplasma in inoculated plants. On the light of previous work (1) and of these results, we plan to further investigate the role of *S. titanus* in the epidemiology of GY in northern Italy and the meaning of the presence of different phytoplasma subgroups in vineyards affected by GY and FD diseases.

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EPIDEMIOLOGY OF GRAPEVINE DIE-BACK DISEASE IN LIGURIA, NORTHERN ITALY

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Although the area devoted to viticulture in Liguria (Italian Riviera) is small compared with that in other Italian regions, grapevines are important locally as they are the main crop on many farms in the eastern part, produce some typical wines of good quality, and play a crucial role in preventing erosion in mountain or hilly areas.

Since the beginning of the nineties, vineyards in the eastern Riviera have been affected by a severe phytoplasma disease (1; 6) named 'Moria della vite' (grapevine die-back disease, GDD). From 1992 to 1995 it spread rapidly causing dramatic losses, and is still serious representing a threat to the survival of viticulture in that area. Following discovery of the etiological nature of GDD (6), research was continued to identify in more detail the pathogens involved and to investigate some epidemiological aspects of the disease.

Surveys were carried out in the hills close to the sea in eastern Liguria, where vines are grown either in small fields or in rows between crops such as broadbean, potato, lettuce or cabbage. The grape cultivars mostly grown are Bianchetta genovese, Vermentino (about 40%), Albarola (about 13%) and Bosco but a few others such as Barbera, Bonarda, Ciliegiolo, Sangiovese, etc. are also present (1-3%). Twenty vineyards and the surrounding cultivated or wild vegetation in eight different localities were inspected regularly. Samples were collected at monthly intervals from both -infected grapevines and crops or wild species showing symptoms suggesting phytoplasma infection. Two molecular probes constructed on fragments of chromosomal DNA from one isolate of European aster yellows (EAY) phytoplasma were used for diagnosis: one (EAY 352) reacting with phytoplasmas of the EAY, stolbur and elm yellows types, and the other (EAY 335) reacting only with those of the EAY group. DNA extraction and analysis, labelling, prehybridisation, hybridisation and posthybridisation conditions were as described previously (6). The identification of phytoplasmas from grapevine was done by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified products (4,7) of the 16S RNA gene of the pathogens. Field observation concerned the appearance and development of GDD symptoms on various vine cultivars, the occurrence of infections in other plant species growing in their proximity, and the presence of leafhopper potential disease vectors. In addition, an experiment was conducted during 1995 to check the load of air-borne phytoplasma inoculum from June to October in three vineyards using bait-plants. To this purpose, young seedlings of *Catharanthus roseus* were grown in single pots in the glasshouse up to the 8-10 leaf stage, and moved to a screen house one week before use. Twenty bait plants were brought to each vineyard, planted in the soil in their pots, and taken back to the laboratory after one month exposure. They were then maintained in a screenhouse and checked periodically for the appearance of phytoplasma - like symptoms. Plants which developed symptoms were checked for phytoplasma presence as described above.

In about 40 grapevine samples tested two different phytoplasmas were detected, one belonging to the aster yellows group, and another to the elm yellows group (4) which includes the agent of 'Flavescence dorée' (FD) originally described in France (3). Both such pathogens had already been reported to infect grapevine in Italy in either single or mixed infections (2; 5). In the vines from Liguria they were found only in single infections so far.

The symptoms in affected plants did not allow to distinguish between the two types of phytoplasma. In both cases they consisted of typical bright yellow or bright purple leaf discoloration which appeared in April-May in cultivars with white or red fruit respectively. These were followed by leafrolling of increasing severity, frequently accompanied by necrosis. Growth was considerably depressed and canes matured irregularly or not at all, thus remaining unusually flexible. Yield was drastically reduced or lost completely. Many vines with severe GDD infection collapsed during summer and died, 1-2 years after appearance of first symptoms. Mortality appeared to be correlated with sudden changes of either humidity or daily soil temperature. It mostly affected plants grown either in colloidal soils with high clay content, or close to the stone terrace walls used in the area (M. Boselli, personal communication).

Of 14 herbaceous or shrub species tested, the following were found to be infected by phytoplasmas: *Linaria* sp., *Plantago media*, *Prunus spinosa*, *Rubus fruticosus* and *Spartium junceum.* All phytoplasma isolates belonged to the aster yellows group. Infections were by far the most numerous in the last two species, which appear to be the main sources of infections for the grapevines, as suggested by the fact that highest GDD incidences occurred in vineyards growing close to large masses of infected bushes. It was observed, in particular, that a new plantation of Vermentino grapevine established in proximity to many such infected plants became nearly totally infected by GDD in 3 years.

Insect catches ascertained the presence of either nymphs or adults of the following leafhopper species, putative phytoplasma vectors: *Euscelis incisus*, *Euscelidius variegatus* and *Scaphoideus titanus.* In

agreement with previous findings (8) only *S. titanus* was observed to infest continuously grapevine from late June to October, while the other species were seen to infest the herbaceous vegetation and to feed on vines only occasionally. Both *E. incisus* and *E. variegatus* are vectors of EAY, and the latter can also experimentally transmit the agent of FD of which *S. titanus* is the specific vector.

The cases of infection in bait-plants exposed at monthly intervals in three different vineyards were as follows: June 3/58 (5.1%); August 0/55; September, 5/57 (8.7%); October, 1/60 (1.6%). Four plants with symptoms of infection, checked at random from different groups, all contained phytoplasma of the aster yellows group.

The above results suggest the following practices to attempt the control of GDD in Liguria:

- keep vineyards and surrounding fields free from weeds and shrubs during the whole vegetative period;
- vineyards which are no longer cultivated must be eradicated;
- avoid growing vines in mixture with herbaceous crops whenever possible;
- remove and destroy all pruning cuttings;
- avoid planting new vines to replace individual plants that died due to GDD infection;
- spray to control leafhopper infestation. The early appearance of hoppers in crops can be monitored (e.g. by yellow sticky traps) to decide the best time for spraying.

ACKNOWLEDGEMENTS

The authors thank Giampaolo Gaviani, Istituto di Fitovirologia Applicata- Consiglio Nazionale delle Ricerche, and Domenico Peirano, Coldiretti Genova, for skillfull help in field surveys.

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The research was supported by a grant from Regione Liguria, Genova.

ON THE TRANSMISSION OF GRAPEVINE YELLOWS DISEASES BY BENCH-GRAFTING

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Regarding to polymerase chain reaction (PCR) technology and serological analysis (ELISA) both Flavescence dorée *sensu stricto* (FD) and Bois noir(BN) have been found to be present in north-eastern Italy (1,2,3,4). The two diseases besides natural vectors(5,6,7) are transmitted by grafting (8,9). This paper presents the results obtained in trials conducted in Trento and Friuli provinces regarding the propagation of BN by machinery-bench-grafting in particular when following the ordinary procedures adopted by nurseryman in grapevine propagation.

In both localities all the grapevine test plants were obtained by grafting single bud-scions of cv Chardonnay onto healthy roostocks. According to the standard method used by nurseryman the scions were collected from asymptomatic grapevines in vineyards (ten in our case) characterised by having a low percentage (about 5%) of infected plants. These grapevines are cited as standard. Controls were grapevines either obtained with healthy (negative control) or infected buds (positive control).After rooting ("cartonage" procedure) the grapes were planted inside two insect-proof greenhouses and observed for a period of five years for symptom expression. The total number of grapevines tested under screenhouse were 2278: 1686 standard, 206 infected and 204 healthy.

Grapevines of the same groups above quoted were planted also in vineyards of different areas of north-eastern Italy and periodically observed for seven years for symptom expression. Grapevines chosen at random were analysed by PCR. Only in Friuli the test grapevines were selected after rooting following the national grade-size regulation, .in order to discard the less vigorous.

The percentage of taking of the graftings was 90, 55 and 25 for the healthy, standard and infected scions respectively. According to the results achieved in both localities (Table 1) BN is poorly propagated by bench-grafting. Using infected scions the highest propagation level of BN was 2,8%. Utilising standard material one out of 720 grapevines (0,14%) and one out of 966 (0,1%) developed yellows symptoms respectively in the Trento and Friuli greenhouses.

Based on the average time for symptom appearance inside the screenhouses the minimum incubation period of the disease in young grapevines is about five months, while the maximum does not exceed two years. The percentage of infected grapevines planted in the field is highly variable mostly depending on the year and the location (Table 1). Until 1996 both in Trento and Friuli areas the yearly repeated PCR analyses constantly revealed the presence of yellows referable to BN but never to FD.

On the grounds of the above results the following conclusions can be drawn: the success of graftings is strongly influenced by the health conditions of the scions; BN is poorly propagated by bench-grafting, this confirming previous results (9); by selection the percentage of infected grapevines put on the market is further decreased; according to the experimental data acquired on the maximum length of the incubation period of BN, only few of the grapevines that become symptomatic in the field have been infected by grafting (the ones that exhibited symptoms during the first year of planting).In contrast, the plants that exhibit the first symptoms of the disease after the first year of planting are attributable to natural inoculations occurred in the field.

The first results obtained in a trial still in progress in the Veneto region indicate that also FD is very poorly propagated by bench-grafting.

These results do not negate the fundamental role played by the propagating material in diffusing the disease especially in areas not yet contaminated.

ACKNOWLEDGEMENTS
The authors are indebted to Dr. E. Boudon-Padieu,. and Dr. G.Firrao, for the genetical and serological test.

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Table 1. Behaviour of grapevines cv Chardonnay transplanted after rooting ("cartonage" procedure), either inside insect-proof screenhouses or in the field in different locations of northeastern Italy.

Location	Plants (*) compared	Incidence of GV (**) in the various years									
		1988	1989	1990	1991	1992	1993	1994	1995	1996	Total
Trentino screenhouse	standard	0,14	0	0	0	0					0,14
Trentino screenhouse	infected	1,4	1,4	0	0	0					2,8
Trentino screenhouse	healthy	0	0	0	0	0					0
Trentino Varone field	standard		0,6	4,6	2,0	2,3	5,2	4,9	1,6		21,2
Trentino Navicello field	standard		0,3	0	0	0	0	0	0		0,3
Friuli (***) screenhouse	standard			0,1	0	0	0	0			0,1
Friuli (***) screenhouse	infected			0	0	0	0	0			0
Friuli (***) screenhouse	healthy			0	0	0	0	0			0
Friuli Casarsa field	standard			0	0	0,4	0,7	0	0	0,7	1,8
Friuli Casarsa field	healthy			0	0	0	0	10	18	40	68
Friuli S.Vito field	healthy			0	0	0	0	2	2	8	12

(*) All the grapevines were obtained by grafting, on healthy rootstocks single bud scions of Chardonnay of different state of health as follows: buds collected from asymptomatic grapevines of vineyards with a low percentage of infected plants (standard); buds from symptomatic plants (infected); buds from heatly isolated mother plants (healthy).

(**) Percentage of grapevines that showed GY-symptoms for the first time.

(***) In Friuli the grapevines were selected by discarding the less vigorous ones before planting, following the national grade-size regulation.

EPIDEMIOLOGICAL STUDIES ON A NEW GRAPEVINE YELLOWS IN GERMANY

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A new type of grapevine yellows (GY) has been detected in Germany. Most of the vines known to be infected by this disease are cv. Scheurebe and are growing in the viticultural area of Palatine. While Vergilbungskrankheit, the common GY in Germany, belongs to the stolbur-subgroup of phytoplasmas, the second type of GY was found to be member of the elm yellows (EY) group (4). Although this disease is closely related to Flavescence dorée, it was shown recently that the two diseases are not identical (2). Despite of the restricted distribution and the still low incidence of the disease, information is required about its epidemiology since our data suggest an endemic source of the pathogen in the affected regions. *Scaphoideus titanus*, the vector of FD, is not present in Germany.

Samples were collected from various plant species in and around two vineyards in the southern Palatinate and Mittelhaardt regions of Palatine, where most of the grapes infected by the EY-type GY are growing. The samples were tested for infection with phytoplasmas of the EY-group by PCR with the group specific primers fAY/rEY (1). The relationship of positive samples to GY was analyzed by RFLP analysis of the complete 16S rRNA gene and the adjacent spacer region, amplified using the primers P1/P7 (5). Furthermore, insects collected from infected plants were tested for infection with EY-group phytoplasmas. The periwinkle isolate of elm yellows EY (New York) was used as a reference strain.

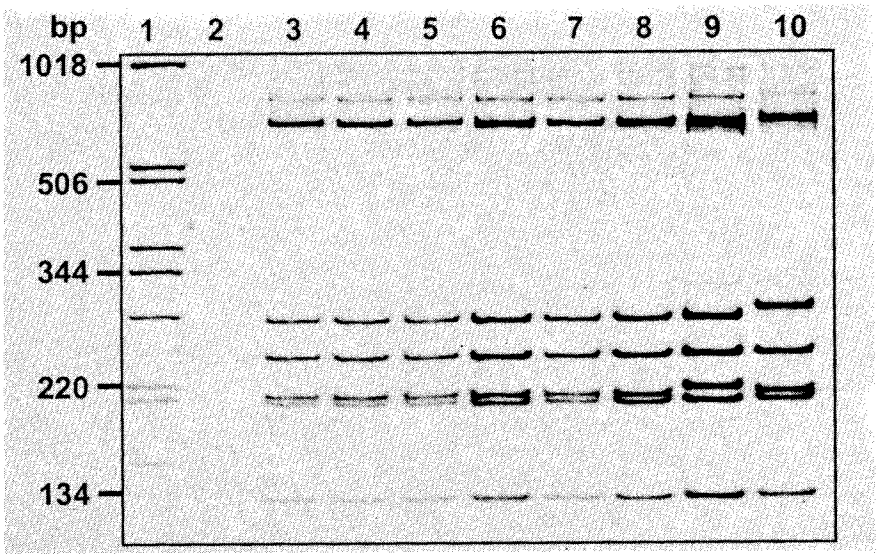

Figure 1. *Alu*I restriction profiles of PCR-products amplified with primers P1/P7. 1 - Molecular weight markers. 2 - Healthy grapevine seedling. 3 and 4 - *Alnus glutinosa*. 5 - *Psylla alni*. 6 to 8 - Grapevine with symptoms of grapevine yellows. 9 - Reference strain, periwinkle isolate EY. 10 - *Rubus sp.*

EY-type phytoplasmas were detected in three plant species beside grapevine: *Alnus glutinosa* (alder), *Rubus sp.* (blackberry), and *Prunus amygdalus* (almond), all growing in the vicinity of affected vineyards. While we found only one diseased almond that may be casually infected by EY, 15 of 18 alders tested positively. The widespread infection of alder with an EY-type phytoplasma has been reported previously (Mäurer).Three different profiles could be distinguished after RFLP analysis with the enzymes *Alu*I (figure 1), *Tru*1I and *Hae*III: a) grapevine, alder, and almond; b) *Rubus*; c) EY. *Taq*I digest of the same fragments did only allow to differentiate between EY and the other isolates. All grapevine isolates showed identical restriction profiles, although three different patterns could be distinguished after digest of a fragment amplified with non-ribosomal primers (2). The infected alders tested so far exhibited only one of these patterns that is also predominant in the grapevine samples.

If the EY-type GY is transmitted to grapevine from one of the tested plants mentioned above, alder should be the most likely source. Therefore, Auchenorrhyncha and psyllids feeding on alder trees in the vicinity of vineyards were collected and tested for the presence of EY-type phytoplasmas (table 1). A considerable proportion of *Psylla alni* was infected. Restriction profiles achieved from the *Psylla* isolates were identical to the patterns detected in alder and grapevine (figure 1, lane 5). These results justify to perform transmission trials to grapevine with *P. alni*. Although this monophagous psyllid is specialized to the genus *Alnus*, we found it also on sticky traps in the vineyards affected by GY. Under experimental conditions, it is surviving on grapevine for approximately one day.

Table 1. Results of PCR detection of EY-group phytoplasmas in insects collected from *Alnus glutinosa*.

Species tested	No. positive / No. tested
Oncopsis alni	1 / 25
Idiocerus stigmaticalis	0 / 32
Allygus sp	0 / 15
Macropsis fuscula	0 / 2
Psylla alni	34 / 47

Different isolates of EY-group phytoplasmas are present in the area where phytoplasmas of this group have been found associated with grapevine yellows. Infected *Alnus glutinosa* trees growing in the vicinity of vineyards could be a potential source of the pathogen. Although a considerable proportion of *Psylla alni* was found to be infected by the phytoplasma, transmission experiments are necessary to prove the ability of this insect to transmit the disease. These trials are in progress.

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MONITORING OF FIELD POPULATIONS OF THE VECTOR *HYALESTHES OBSOLETUS* FOR INFESTATION WITH 'VERGILBUNGSKRANKHEIT'

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Vergilbungskrankheit (VK), the most widespread grapevine yellows in Germany, is transmitted by the cixiid *Hyalesthes obsoletus* (1). Although this xerotherm plant hopper was thought to be rare in Germany, we found it in all viticultural areas that are affected by VK. Unlike Flavescence dorée, VK is not transmitted from grape to grape but from alternative hosts to grape, due to the vector's feeding preference for herbaceous plants. VK is widespread as a pathogen of bindweed (*Convolvulus arvensis*), a common weed in vineyards. Moreover, this plant is also an important host for the vector, whose larval instars acquire the phytoplasma through feeding on the roots of infected *C. arvensis*. Occasional feeding by adults of *H. obsoletus* on grapevine leads to inoculation and infection of the vines with VK. Infected vectors are therefore the propagative units of VK. The risk of infection which depends on the density of inoculum, can be estimated from the abundance of *H. obsoletus* and the proportion of infected individuals of this species. Assessment of disease pressure is particularly necessary in advance of planting and re-planting of vineyards, since VK is destructive for young vines. A high risk of infection may justify control measures against weeds to reduce inoculum of VK.

Planthoppers were collected in four vineyards which were affected by VK. Adult *H. obsoletus* planthoppers were caught by sweep net or removed from yellow sticky traps exposed in the vineyards to monitor the activity of the vector. Individual insects were cut longitudinally and the two halves were used separately for ELISA or PCR detection of VK phytoplasma respectively (4). This method allowed us to compare ELISA and PCR results for individual insects. Mixtures of crude extracts and batches of up to 25 planthoppers were used for DNA extraction and subsequent PCR testing, and the possibility to use those batch samples to estimate the infestation of vector populations was investigated.

The ELISA readings of female and male planthoppers were significantly different. OD readings of females were higher than the results of males in the PCR positive (mean OD 0.49 versus 0.16) but also in the PCR negative (mean OD.14 versus 0.05) test population. ELISA results that corresponded sufficiently with the outcome of PCR tests were only achieved, when positive/negative thresholds were calculated and applied separately for both sexes. With these thresholds, 63 % of 79 PCR positive insects tested positive in ELISA. More detailed investigations with laboratory cultures of the vector would be necessary to prove a possible biological significance of this difference between the detection methods, such as the difference between just infected and already inoculative vectors.

High proportions of *H. obsoletus* carried the VK phytoplasma in three of the four vineyards (Table 1). The infestation of more than 30 % of the vectors could provide sufficient inoculum for an epidemic spread of the disease, which is only prevented by the feeding preference of *H. obsoletus* for herbaceous plants. The importance of alternative hosts of the phytoplasma for the epidemiology of VK is emphasised by the comparison of the infestation levels of the vector populations in the four vineyards. High proportions of infected insects correspond with the prevalence of *C. arvensis* (BK, KS, EK), while only 7 % of the planthoppers carried the phytoplasma in the vineyard PL, where *Ranunculus bulbosus* was the most common weed and preferred host of *H. obsoletus*. In contrast to *C. arvensis*, the latter plant was never found to be infected by VK.

PCR tests of *H. obsoletus* caught by sweep net or sticky traps resulted in lower proportions of positive insects in the groups caught by sticky traps, but Chi-square tests revealed no significant differences for the samples from three of the four vineyards. In the vineyard EK, however, only 14 % of the planthoppers removed from sticky traps tested positive compared to 30 % of the insects caught by sweep net, probably because of the higher temperatures that were recorded in this vineyard. In spite of this problem, sticky traps are the most favourable measure for monitoring the activity of *H. obsoletus* and for the collection of samples for PCR tests. However, due to our experience, the traps should not exposed in the field for more than one week.

Testing of sufficient numbers of planthoppers to estimate the proportion of infected individuals is cost and labour intensive, particularly if the infestation is low. The number of tests required for reliable results could be reduced by testing batches of insects instead of individual vectors. Methods to estimate the infestation of the vector population from the proportion of positive batch tests have been reported (2, 3). We tested the reliability of batch samples with mixtures of crude extracts of infected and healthy *H. obsoletus*. The VK phytoplasma could be detected in all samples in a dilution of 1:50. Consequently, all samples which were prepared from batches of 25 halves of planthoppers including one half of an infected insect tested positive. The reliability of a combination of batch samples of variable size will be tested by comparing the results with test of individual insects of the same populations.

Table 1. Results of PCR tests of individual *H. obsoletus* collected by sweep-net or sticky-trap in four different vineyards.

Vineyard	Collection of planthoppers by ...			
	Sweep-net		Sticky-trap	
	No. positive / no. tested	Infestation	No. positive / no. tested	Infestation
BK	49 / 150	32 %	11 / 50	22 %
KS	42 / 123	34 %	14 / 50	28 %
EK	49 / 166	30 %	7 / 50	14 %
PL	11 / 165	7 %	4 / 50	8 %

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IDENTIFICATION OF GRAPEVINE YELLOWS PHYTOPLASMAS IN THE NORTHERN SPAIN

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Grapevine yellows (GY) diseases occur in various viticultural areas of the America, Australia, Israel and Europe (1) causing several damages. Grapevine yellows diseases are almost identical in symptomatology inducing severe and mild symptoms on grapevine organs. However they differ in epidemiology and cultivar susceptibility. The diseases affects the quality and quantity of the grapes produced and can result in the death of the vine stock. Flavescence dorée (FD) and Bois noir (BN) phytoplasma are the more frequent GY diseases in vineyards. FD phytoplasma belongs to the elm yellows group and BN phytoplasma belongs to the stolbur group .These phytoplasma appear to be ubiquitous pathogens that can be hosted by herbaceous plants of several families. Weeds, vegetables and trees are known hosts for the stolbur phytoplasma. FD is transmitted from grapevine to grapevine by an efficient vector, *Scaphoideus titanus* Ball. an ampelophagous leafhopper, and BN is transmitted by at least one polyphagous planthopper *Hyalesthes obsoletus* Sign. The vector of FD cannot transmit the BN phytoplasma.

Over the five last years grapevine yellows have been observed in vine in several areas of northern Spain. From 1994 to 1996 a survey was conducted in three regions, Aragon, Catalonia and Navarra, during September and October. The cicadellid *S. titanus* were studied in all sampled areas, other vectors were not studied.

Leaf samples showing phytoplasmas symptoms of various cultivars: Carignan, Chardonnay, Grenache and Tempranillo were collected in order to characterise the phytoplasma involved. An epidemiological study of the cv. Chardonnay infected by BN phytoplasma in the Catalonia region. was conducted at the same time using a microplot with 500 plants distributed in 20 rows with 25 plant per each. The position of all infected plants in each plot was recorded in plot maps on each sampling date. The spatial pattern of the disease occurrence was calculated with the PATCHY program (5).

Serological and DNA methods have been applied to identify the phytoplasmas.

Serology : BN was detected by a double antibody sandwich ELISA, using an stolbur monoclonal antiserum (Sanofi, Cedex France). The extraction buffer used was 0.5 M Tris, 0.8 % NaCl, pH 8.2 containing 2% PVP 30 and 3% Tween 40.

DNA extraction, amplification and restriction analysis : DNA was extracted from phytoplasma enriched fraction from grapevine leaf veins as previously described (2). PCR and nested-PCR were used with specific primers for a 16S rDNA region in all known phytoplasma. The primer used in PCR was fU5/rU3 (4) and the primers used in nested-PCR were in the first amplification P1 (3) and P7 (6) and for the second amplification was fU5/rU3. PCR was conducted according to Daire *et al* (2)

Not all affected plants reacted positively to the ELISA. This procedure was very useful for a first screening of the BN disease. PCR was more sensitive than ELISA and detected a higher number of positive plants, nested-PCR increased the sensitivity of the detection.

S. titanus was present in all vineyards sampled, often in high numbers (five to seven individuals per leaf).

In the vine sampling done in 1994 and 1995, only BN phytoplasma was detected in five plots out of fifteen in Catalonia and in two plots out of ten in Navarra on the cvs. Chardonnay, Grenache and Tempranillo. In a survey realised in 1996, FD was identified in several plots in the Northeast of Catalonia near to the French border. The cultivars affected were Carignan and Grenache .

The results of the epidemiological study indicate that the BN infection in 1994 was 3.4%. The infection was aggregated in clusters of 35 plants (7 plants x 5 rows) with a significative Lloyd's index of 1.727. In 1995 the incidence increased to 7.2 % and in new infected cluster with a size of 12 plants appeared with a Lloyd's index of 1.548. In 1996 the incidence of plants with BN was 14 % an the size of the new clusters increased. The results show that the diseases increased along the rows. from plant 1 to plant 25. Following an analysis of the spatial distribution (1995), the increase of cluster infections tended to create larger overall areas of infection, as found in 1996. In the latter year a tendency towards random distribution of infected plants was observed and the Lloyd's index was near to 1

ACKNOWLEDGE

The authors wish to acknowledge the collaboration in this study to E.Boudon-Padieu, J. Larrue and D.Clair. Station de Recherches sur les Phytoplasmes, INRA Dijon, France.

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PHYTOPLASMA DISEASES OF GRAPEVINES IN SARDINIA

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The occurrence of phytoplasma diseases not transmitted by *Scaphoideus titanus,* thus differing from flavescence dorée, has been repeatedly reported from various Italian regions in the last 15 years or so (1). Unconfirmed possible records of comparable disorders exist also from Sardinia (Southern Italy), where occasional and atypical yellows-like symptoms were observed in the late 80s in the north of the island, in cvs Torbato and Trebbiano toscano (2). Similar symptoms had also been detected earlier in central Sardinia during surveys for clonal and sanitary selection in a cv. Cannonau vineyard which was uprooted, thus impairing further investigations. In the last few years, field syndromes recalling those typically induced by phytoplasma infections were repeatedly observed in cvs Italia and Pascale di Cagliari in the south, cvs Semidano and Vernaccia in the centre, and again in cv. Torbato in the north of the island. Occasionally, plants clonally propagated from affected vines showed symptoms.

The type of symptoms varied with the cultivar. White-berries varieties showed chlorotic to bright yellow discolorations of interveinal leaf tissues that could extend to large sectors of the blade, whereas red-berries varieties exhibited sectorial leaf reddening. Chromatic alterations were often accompanied by necrosis of the veins and leaf rolling but, apparently, not by delayed or irregular wood ripening. A clear-cut adverse effect on the crops was not observed. Symptoms recurred every year and were at their peak at harvest time or later. Their severity varied with the year and the variety, cv. Semidano being the most affected. No severe disease outbreaks were even observed, symptomatic plants in each single vineyards being few and scattered or in small groups.

To ascertain the aetiology of the disorder graft-transmission assays have been made, but their results are not yet available. In addition, petioles and main veins from apparently healthy and symptomatic vines of five different varieties were collected and shipped to Torino to be analysed for the presence of phytoplasmas by molecular hybridisation. In these tests a probe (EAY 352) constructed onto a fragment of European aster yellows DNA was used (3). DNA was extracted from grapevine tissue samples as described (4), spotted *in vacuo* ontò nylon membranes, cross-liked by u.v. irradiation, denatured *in situ* with 0.4 NaOH, and hybridised with the radiolabelled probe.

As shown in Tab. I, clear-cut hybridisation signal were obtained in six cases out of eight, indicating that phytoplasmas of the aster yellow group are associated with, and are the likely agents of the disease observed in Sardinian grapevines. This finding is in agreement with what reported from other southern Italian areas (5; 6) where, as in Sardinia, *S. titanus* does not occur.

The surveys made lately in Sardinia seem to indicate that phytoplasma infections of grapevine are on the increase. Although up to now the outbreaks have never taken an epidemic form, the potential threat remains. Thus to prevent the problem met in a recent past by several regions of the Italian mainland because of the severe phytoplasma epiphytotics, it would be essential to known where the inoculum comes from. An introduction of phytoplasmas with the rootstocks that have been imported in great amounts from the mainland, although possible seem unlikely. In any case, this would be little epidemiological significance because infected grapevines are thought not be sources of inoculum for further spreading in the vineyards, except in the case of flavescence dorée. Natural phytoplasma reservoirs, like native vegetation or vegetable crops are therefore more likely sources of infection.

Table 1. Results of dot blot hybridisation of DNA extracts from symptom-showing grapevines with a probe to European aster yellow phytoplasma.

Cultivar and clone number	^ Hybridisation signal
Italia 8/14	+ + +
Vernaccia 13/12	+ + +
Vernaccia 12/74	+
Torbato Bn	+
Semidano 6/6	+
Semidano 8/55	+
Semidano 6/50	?
Pascale di Cagliari 5/36	?

^ The number of crosses indicate the intensity of the hybridisation signal.
Question marks indicate doubtful signals.

It is known that recurrent and very serious outbreaks to tomato stolbur occur in Sardinia (7) where there are also several leafhoppers vectors such as *Euscelidius variegatus, Euscelis incisus, E. lineatus* (Arzone, pers. comm.). As yet, however, there is no evidence of a relationship between the stolbur agent and the potential vectors with the grapevine yellows detected in the island.

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OCCURRENCE OF GRAPEVINE YELLOWS DISEASE IN GRAPEVINE GROWING REGIONS OF HUNGARY

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INTRODUCTION

In Hungary, symptoms resembling those of grapevine yellows disease were first observed on a few plants of grapevine cultivars 'Aligote' and 'Rajnai rizling' as early 1970'. Indexing for infection of these plants, by grafting onto the indicator cultivar Baco 22 A, resulted in development of severe stunting of the indicator plants. Such an indicator plant reaction is seen in the case of positive reactions when indexing for grapevine flavescence dorée disease, now attributed to a phytoplasma (formerly mycoplasmalike organism), but infection of the Hungarian grapevines by phytoplasma has never been demonstrated.

Occurrence of grapevine yellows disease in Hungary was not reported again until the 1990's (1). At the beginning of the 90's occurrence and geographic distribution of grapevine leafroll associated viruses (GLRaV types) and of GVA in Hungarian grapevine growing regions were surveyed. In the course of this survey, certain plants with severe leaf roll symptoms were observed in plants in which viruses could not be detected either by use of ELISA or by graft indexing on woody indicators. Moreover, physiological causes of the leaf rolling were also excluded (1). Since the affected grapevine plants exhibited additional symptoms similar to those of Flavescence dorée disease, it was hypothesised that phytoplasma may be responsible. The present work was initiated to test the hypothesis that phytoplasma was associated with disease in grapevines in Hungary.

MATERIALS AND METHODS

In the autumn of 1993, a survey was begun to determine the possible presence of grapevine yellows (GY) disease in the main grapevine growing regions of Hungary. The geographic distribution of plants exhibiting possible grapevine yellows disease symptoms, and the cultivars of symptomatic grapevines were recorded. During 1993, and again in 1995/1996, leaf and cane samples were collected from symptomatic stock plants of different grapevine cultivars for laboratory analyses to detect the possible presence of phytoplasma in the diseased plants. Study of the samples that were collected in 1993 and in 1995/96 started in collaboration with the U.S. laboratory. Detection and identification of phytoplasmas were accomplished through the use of nested PCR assays developed previously (2).

For phytoplasma detection and identification in samples collected during 1993, DNA was extracted as previously described (3). for assays of samples collected in 1995/1996, extracted dna was further purified by using Gene Clean III kit before its use as template in PCR. In nested PCRs, products amplified in reactions primed by phytoplasma-universal primer PAIR R16mF2/R16mR1 or by phytoplasma-universal primer pair R16F1/R16F0 were diluted 1:50 with sterile deionized water and used as templates in PCRs primed by phytoplasma-universal primer pair by R16F2n/R16R2 (3,4). Identification of detected phytoplasmas was accomplished by restriction fragment length polymorphism (RFLP) analyses of DNA amplified in PCRs primed by R16F2n/R16R2. Stolbur phytoplasma-specific primer pair fstol/rstol was used to prime direct (non-nested) PCR as previously described (4).

RESULTS AND DISCUSSION

Grapevine plants of several cultivars in Hungary were found to exhibit disease symptoms characteristic of grapevine yellows diseases found in other countries. More than 6700 grapevine stock plants were surveyed for the presence of GY in four grapevine growing regions in Hungary between 1993 and 1996. Symptoms typical of or closely resembling those of grapevine yellows disease were observed in 2328 plants. Severe triangle-shaped leaf rolling, leaf yellowing with or without necrosis of leaf blade, drooping of shoots and drying of flowers and fruit, clusters were frequently found on the same plants. High percentage of plants was observed in the Eger grapevine growing regions (county Heves, north-east Hungary), especially on cultivar Chardonnay (Table 1).

In the initial experiments with all samples, nested PCR primed by R16mF2/R16mR1 (4) or R16F1/R16R0 (2) followed by R16F2n/R16R2 (4) was used. No phytoplasma DNA was detected in plant samples collected during 1993. Similarly, PCR assays yielded no phytoplasma DNA amplification from any of the samples with typical symptoms collected in autumn 1995. In the initial assays of samples collected during both years, the same DNA template extraction method was applied as was successfully used to detect phytoplasmas in diseased grapevines in other work (3). Following subsequent use of the Gene clean kit III for further purification of template DNA extracted from samples collected during 1995, amplification of phytoplasma DNA was observed in PCR reactions containing template DNA from Samples collected in

1995. Phytoplasma DNA was amplified from samples collected from diseased plants of cultivars Chardonnay, Szürkebarát (Pinot grey), Merlot, and Zweigelt in the Eger grapevine growing region (county Heves) in Northeast Hungary (5). Collective RFLP patterns of DNA amplified in PCR primed by R16F2n/R16R2 were characteristic of phytopoplasmas previously classified n 16S rRNA gene group 16SrI, Subgroup G (Stolbur and related phytoplasmas), and they were indistinguishable from those of 16SrDNA amplified from a known reference strain of the Stolbur phytoplasma (Fig I). On the basis of the RFLP data, we conclude that all symptomatic grapevine plants sampled in 1995 were infected by a Subgroup 16SrI-G phytoplasma. DNA was amplified in PCRs containing Stolbur-specific primer pair fstol/rstol and template DNA from the diseased grapevine plants, confirming the identities of detected phytoplasmas as members of Subgroup 16SrI-G. Diseased plants infected by Subgroup 16SrI-G phytoplasmas belonged to cultivars Chardonnay, Szürkebarát (Pinot grey), Merlot and Zweigelt growing in the Eger grapevine growing region (county Heves) in north-east Hungary (5).

In the present work, typical symptoms of grapevine yellows were observed on more than two thousand grapevine plants belonging to seven cultivars growing in four different grapevine growing regions in Hungary, but molecular assays revealed the presence of phytoplasma DNA - and therefore phytoplasma infection - only in symptomatic grapevine plants in north-east Hungary. Since we have thus far not detected phytoplasma infection of diseased grapevines growing in districts other than north-east Hungary, we are continuing surveys of the geographical distribution of grapevine yellows disease symptoms and efforts to detect and identify phytoplasmas infecting grapevines in all the grapevine growing regions of Hungary.

Table 1. Relationship between detection of phytoplasma and symptoms observed in diseased grapevine plants in various grapevine growing regions of Hungary

Grapevine growing region cultivar	Leaves rolled to triangular shape	Yellowing of leaves	Drooping of shoots	Dried flower and/or fruit clusters	Phytoplasma detected genomic subgroup #
Szekszárd region					
Chardonnay*	x	x	x		not detected
Alicante Bouchet*	x	x			not detected
Dél-Balaton region					
Chardonnay**	x	x	x	x	not detected
Semillon***	x	x			not detected
Eger region					
Chardonnay***	x	x	x	x	+ (16SrI-G)
Szürkebarát*	x	x	x	x	+ (16SrI-G)
Merlot*	x	x	x		+ (16SrI-G)
Zweigelt*	x	x			+ (16SrI-G)
					+ (16SrI-G)
Kecskemét region					
Chardonnay**	x	x	x	x	not tested
Ezerfürtû**	x	x	x		not tested

Note: No. of grapevine plants surveyed/No.of symptom-showing grape plants observed: * = 50 / 1 - 10, ** = 50 / 11 - 20, *** = 6000 / 21 - 2000, # = Phytoplasmas were detected through use of direct and nested phytoplasma-universal and stolbur-phytoplasma-specific polymerase chain reacrtions and analyses of DNAs amplified inphytoplasma-universal PCRs primed by oligonucleotide pair R16F2n/R16R2 ## = 16SrI-G genomic subgroup containing stolbur and related phytoplasmas

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SPATIO-TEMPORAL ANALYSIS OF THE DISTRIBUTION OF GRAPEVINE YELLOWS IN GERMANY

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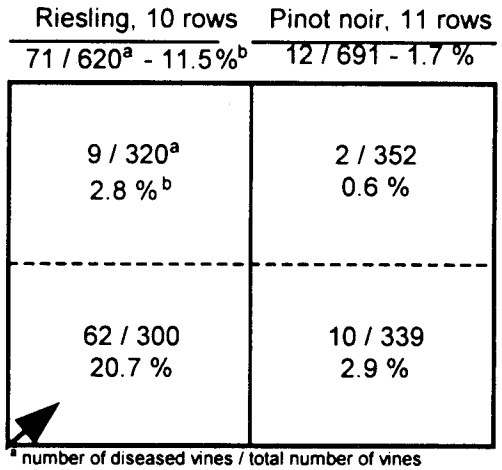
The spatial distribution of grapevine yellows (GY) and the temporal dynamics of these diseases provide valuable information on their epidemiology. Conclusions can be drawn from the patterns of infected vines about the ways and directions of dissemination. Disease patterns are also influenced by the occurrence and distribution of alternative hosts of the pathogens and the structure of adjacent areas. Temporal changes of distribution patterns and the status of individual vines are influenced by disease-host interactions such as infection and recovery. In addition, information about the possibility and significance of latent infections can be derived from the observation of the phytosanitary status of individual vines over consecutive years. Together with the monitoring of vector populations, these data provide the information that is needed to predict disease pressure and to justify activities to control the spread of GY.

Data about the occurrence of Vergilbungskrankheit (VK) were collected and analyzed from 28 vineyards of the viticultural regions of Mosel-Saar-Ruwer and Mittelrhein. The program PATCHY (2) was used to calculate disease levels and gradients, to estimate indices of dispersion (1) and to perform ordinary-runs analyses. Temporal changes were analyzed by comparing the occurrence and position of symptomatic vines over several consecutive years. Data of at least three years were used to estimate the rates of new infections, remission, and re-occurrence of symptoms.

The proportion of infected vines increased from 18 % in 1990 (n = 6537) to 26 % in 1995 (n = 12896). However, these data do not represent the overall incidence in the areas under investigation, since only affected vineyards were included in the study. The disease incidence varied between individual vineyards from 5 % to 65 %. It was increasing in seven of 12 vineyards and decreasing in three vineyards. The increase in the number of symptomatic vines was highest in young plantings (≤ five years), which often provide favorable conditions for the vector *Hyalesthes obsoletus* and the alternative host of the phytoplasma, *Convolvulus arvensis*. Those vineyards are severely damaged, since young grapes develop symptoms systemically and die or have to be replaced. Figure 1 gives an example of such a vineyard, where approximately 6 % of the vines exhibited symptoms in the year following the planting. The inoculum is apparently spreading from an adjacent uncultured area through the lower left corner of this plot, where already 20 % of the vines appeared infected by VK.

Significant aggregations were detected in most of the vineyards either by runs analysis or by calculation of distribution parameters. Random distribution was associated with high disease incidence or detected in young vineyards, where infected vines were replaced continuously. Clusters of infected vines were frequently found associated with patches of *Convolvulus arvensis*. The average cluster size extended with increasing incidence of VK.

The expression of symptoms of individual vines (n = 3912) was recorded in four vineyards over a period of three or four years. This allowed us to calculate the rate of new infections and the proportions of vines that apparently recovered from VK. The data show (table 1), that the incidence of VK depends on the interactions between new infections and recovery. Another group of vines seemed to be recovering, but symptoms re-occurred after one or two years. Although it is not impossible, that some of these vines were inoculated again, they probably remained latently infected. This hypothesis is supported by the observation, that the probability for an individual vine to exhibit symptoms is influenced by its history. The proportion of symptomatic grapes was significantly lower in the population of vines that were never found infected before, compared to grapes which were infected in the previous year, but also compared to vines that were found free of symptoms in the previous year bud had shown symptoms of VK before.



[a] number of diseased vines / total number of vines
[b] incidence of VK

Figure 1. Incidence of Vergilbungskrankheit in a two year old vineyard. Arrow: Direction of spread of VK into the vineyard.

Table 1. Incidence of Vergilbungskrankheit and rates of new infection, remission and re-occurrence of symptoms in four vineyards of the valleys of Mosel- and Rhine-river.

Vineyard	Number of plants	Years of observation	% Incidence of VK [a]	% New infections [b]	% Remission of symptoms [c]	% Re-occurrence of symptoms [d]
Boppard	1011	4	19.3 → 29.0	21.2	34.5	22.2
Enkirch	708	4	29.4 → 15.1	8.4	61.1	21.2
Senheim	611	3	11.5 → 39.4	34.6	31.4	25.7
Platten	1582	3	25.7 → 25.6	15.9	37.3	19.2

[a] Symptomatic vines versus total number of vines; Data from the first year to the end of observation
[b] Newly symptomatic vines versus number of vines which previously stayed free of symptoms
[c] Number of previously symptomatic vines, which stayed free of symptoms till the end of the observation (at least two years), versus number of infected vines
[c] Number of vines with symptoms re-occurring after at least one season without symptoms versus number of symptomatic vines

The high levels of recovery recorded by this study were not expected for VK. Almost one third of infected vines recovered from the disease and stayed free of symptoms for at least two years, even in vineyards, where the incidence of VK was still increasing. However, the recovery could have been supported by pruning, which probably is a considerable factor for the remission of symptoms. In the vineyard of Enkirch (table 1), all canes that carried symptomatic shoots were cut off during two winters. Consequently, 61 % of the symptomatic vines recovered from the disease. This treatment, in combination with a low level of new infections, led to a decrease of the proportion of symptomatic grapes from 29% to 15%.

Even types of grapevine yellows like Vergilbungskrankheit which are not spreading epidemically exist in a dynamic equilibrium in the vineyards. While the distribution of infected vines is influenced by the occurrence and distribution of vectors and alternative hosts, the actual level of disease incidence represents the antagonistic effects of new infections, retaining of pathogens and recovery. More detailed studies are necessary to prove the hypothesis, that the VK-phytoplasma could infect vines without causing symptoms.

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MOLECULAR DETECTION OF PHYTOPLASMAS INFECTING GRAPEVINES IN SLOVENIA AND CROATIA

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Several studies have indicated that grapevine yellows disease is present in many regions of the world (1, 2, 3, 4, 5, 8, 9, 10, 11). The disease has been recorded in the western littoral part of Slovenia (13, 14) and only personal communications have, so far, been indicating the appearance of the disease symptoms in Croatia. No molecular studies have been carried out. In order to verify phytoplasma presence and identify them a molecular study was performed on symptomatic grapevines from both countries. The symptoms appear at the beginning of summer as downward rolling of the leaves. Blades assume yellow colour but the veins remain green. Wood does not lignify regularly and in the year following symptoms appearance, grapevine plants dye due to the winter frost. None of the affected plants recovered. In this presentation, data confirming association of phytoplasmas with grapevine yellows are reported in Slovenia and Croatia.

Nucleic acid samples from phloem of winter cuttings of grapevine cultivars Chardonnay and Rebolla from Slovenia and Chardonnay and Pinot gris from Croatia were extracted in spring of 1995 and 1997 and used for direct and nested polymerase chain reaction (PCR). Tubes with the reaction mixture devoid of DNA templates were included in each experiment as negative controls. Polymerase chain reactions (PCR), using an oligonucleotide general primer pair designed R16F1/R0 (7), followed by nested PCR with other general primer pairs R16F2/R2 or other primer pairs R16(I)F1/R1, R16(III)F2/R1, R16(V)F1/R1, R16(X)F1/R1 (6) specific for four phytoplasma groups (16SrI, 16SrIII, 16SrV, and 16SrX) were used for amplification of phytoplasma 16S rDNA. Further PCR experiments were carried out using primers specific for stolbur phytoplasma isolate (8). Control samples employed were AY1 (Maryland aster yellows, I.-M. Lee, USDA, Beltsville, MD, USA), CPh (Clover phyllody, Chiykowski, Agriculture Canada, Ottawa, Ontario, Canada *via* I.-M. Lee), IPVR (Italian periwinkle virescence), CX (Peach x disease, B.C. Kirkpatrick, University of California, Davis, CA, USA *via* I.-M. Lee), EY (Elm yellows, W.A. Sinclair, Cornell University, NY, USA *via* I.-M. Lee), AP (Apple proliferation, L. Carraro, Università di Udine, Italy). All the PCR experiments were carried out for a total of 35 cycles for each run, under conditions previously described (12). PCR products were analysed with electrophoresis through a 1% agarose gel followed by staining in ethidium bromide and visualisation of DNA bands using an UV transilluminator.

The results revealed amplification of a 1.2 kb DNA fragment after using primers R16F2/R2 in reaction mixtures containing template DNA from some of the sample tested (table 1). These results were confirmed by the positive amplifications using primers specific for the aster yellows group phytoplasmas [16Sr(I)F1/R1]. Negative results were obtained with the other group specific primer pairs as well as with primers specific for stolbur. Only the correspondent control samples resulted positives. The negative results of some samples could be explained with the reported uneven distribution of such prokaryotes in the infected plants. Further research are currently carried out to further classify the detected phytoplasmas that result to belong to the aster yellows group (16SrI).

These findings will probably affect the choice of grapevine cultivars in both countries and development of the grapevine sanitation programs. This is the first report of grapevine phytoplasmal infection in Croatia.

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Table 1. Results of molecular analyses for phytoplasma presence on grapevines samples from Slovenia and Croatia.

Grapevine samples	Origin	Year	Symptoms	Phytoplasma detection
Pinot gris	Croatia	march 1995	dying vine	aster yellows
Pinot gris	Croatia	march 1995	2 years symptoms	aster yellows
Chardonnay	Slovenia (Ormoz)	march 1995	3 years symptoms	negative
Pika red variety	Slovenia (Nova Gorica)	march 1995	dying vine	aster yellows
Chardonnay 15	Slovenia	april 1997	symptomatic	negative
Chardonnay 12	Slovenia	april 1997	symptomatic	aster yellows
Rebolla 13/12	Slovenia	april 1997	symptomatic	aster yellows
Pinot grisPGC1	Croatia	april 1997	symptomatic	negative
Pinot grisPGC2	Croatia	april 1997	symptomatic	negative
Pinot grisPGC3	Croatia	april 1997	symptomatic	negative
Pinot grisPGC4	Croatia	april 1997	symptomatic	negative
Pinot grisPGC5	Croatia	april 1997	symptomatic	aster yellows
Pinot grisPGC6	Croatia	april 1997	symptomatic	negative
Pinot grisPGCH	Croatia	april 1997	asymptomatic	negative
Chardonnay	Croatia	april 1997	dying vine	aster yellows

MOLECULAR DETECTION OF PHYTOPLASMAS ASSOCIATED WITH GRAPEVINE YELLOW DISEASE IN ISRAEL.

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Flavescence Dorée was the first grapevine yellows disease described in the 1950s in France by Caudwell (3) Since then, various yellows diseases such as Bois Noir and Vergilbungskrankheit have been reported from several countries (2,6,11,13,14). In 1980s new outbreaks and spread of yellows diseases were described in new countries and regions in several continents (5,7,10). Table grapes grown in the Jordan valley in the 1970s showed some yellows symptoms, which at that time could not be confirmed by molecular methods. These vineyards were uprooted and have not been replanted. In the late 1980s symptoms resembling yellows diseases were observed, on few grapevines of the cultivar Chardonnay in the Golan Heights (15). The disease has spread since, to other cultivars and regions of the country.

Phytoplamas (previously called mycoplasma-like organisms - MLOs) are now known to be the causative agents of various yellows diseases (4). Molecular methods such as polymerase chain reaction (PCR) are applied for diagnosis and detection. Universal primers carrying phytoplasma-conserved sequences of the 16S ribosomal DNA, are applied to distinguish between phytoplasma and other micro-organisms. Nested PCR with type- specific primers or restriction analysis are used to classify phytoplasma type (1,8,9).

In this paper we confirm the association of different phytoplasma with grapevine yellows disease in Israel and its transmission to periwinkle by grafting.

The objectives of this work were to confirm the association of phytoplasma with yellows diseased grapevine and explore a method to transmit phytoplasma from grapevine to periwinkle.

Samples of symptomatic and asymptomatic grapevine Chardonnay, Carignane, Cabarnet franc, French colombard and Merlot were collected from vineyards growing in various geographic regions of Israel. Different tissues were tested for the presence of MLOs using PCR. Template DNA was extracted from leaf midribs, phloem scrapings and root tissue and extracted by the method described by Maixner (12). PCR was performed using universal primers and group-specific primers synthesised according to sequences published by Lee et al., 1994. Twenty ng of template DNA were used in a reaction mixture were subjected to 35 cycles in which the annealing temperature was 52°C. Products obtained from PCR performed with universal primers and primers F2/R2 were analysed by electrophoresis on 1% agarose gel.

Restriction-pattern analysis was carried out using 15 ml of the PCR product obtained from the amplification with primers F2/R2. Samples were digested by four enzymes Mse I, Alu I, Hpa II or Kpn I and fragments were separated by polyacrylamide electrophoresis.

Graft transmission experiments were carried out using infected grapevine shoot tips. These were wedge-grafted onto healthy periwinkle plants and grown under controlled conditions in the greenhouse.

The PCR products obtained using universal primers from symptom-exhibiting grapevines confirmed phytoplasma infection and absence of phytoplasma in asymptomatic grapevines. Phytoplasma was detected in the above mentioned cultivars and in various growing regions of Israel. Nested PCR assays with phytoplasma group-specific primers and restriction analysis of the PCR products indicated that aster-yellows and western-x are associated with grapevine yellows in Israel.

Graft-transmission of phytoplasma from grapevines to periwinkle provides a better source for grapevine phytoplasma studies. The phytoplasma detected by PCR in periwinkle faithfully represented those present in the donor grapevine.

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THE USE OF *IN SITU* HYBRIDISATION WITH OLIGONUCLEOTIDE PROBES TO SPECIFICALLY LOCALISE PHYTOPLASMAS IN PLANT TISSUES IN ELECTRON MICROSCOPY

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In spite of the similarity of symptoms in affected grapevines, Grapevine yellows (GY) have been shown to be associated with diverse phytoplasmas in viticultural regions in the world (1, 2, 3). In Europe, Flavescence dorée (FD) phytoplasma belongs to the Elm Yellows (EY) group. Bois noir (BN) and Vergilbungskrankheit (VK) are associated to a stolbur phytoplasma (4). The two type of phytoplasmas are vectored specifically by two different insect species. On the contrary all cultivars seem to be susceptible to both GY. However, cultivar differences in the reaction of the plant have been recorded. Methods to specifically identify and localise phytoplasmas *in situ* are thus necessary for the development of studies aimed at determining the multiplication and movement of phytoplasmas in their hosts.

In vitro assays using antibodies or DNA probes and DNA primers, and relevant extraction procedures have been developed to achieve specific diagnosis of FD and BN phytoplasmas in grapevine. On the contrary, though efficient methods were developed for *in situ* labelling of FD phytoplasma in insect and herbaceous plant hosts with specific antibodies in electron microscopy, only one source of polyclonal antibodies raised to FD phytoplasma has ever been used successfully to localise FD phytoplasma by similar methods on FD-infected grapevine (7). It is suspected that epitopes on phytoplasma membrane are not easily accessible in infected grapevine cells. *In situ* hybridisation (ISH) methods are alternative labelling procedures which direct at nucleic acid targets, and might thus be valuable to achieve labelling in woody plants. The present study reports of the first results obtained in ISH with electronmicroscopy of phytoplasma localisation in plant cells with nucleic acid probes. Preliminary assays were conducted on periwinkle plants, which develop high titres of phytoplasma when infected.

Rather than long nucleotide sequences which are available only in the 16S RNA gene of phytoplasmas and would not be enough group specific, we decided to use much shorter sequences (about 20 mers) which have been devised for group-specific amplification in PCR, though the small size of probes might result in a loss of control over the stringency of the hybridisation reactions

The periwinkles (*Catharanthus roseus* L.) were infected via graft transmission with the FD phytoplasma and the stolbur phytoplasma. The oligoprobes used were the complementary sequences (antisense or [-] strand) of fStol (8) and R16[V]F1 (9) which have been designed as primers for specific PCR amplification of a 16S rDNA fragment of stolbur phytoplasma and of phytoplasmas in the EY group respectively. The antisense strand was chosen because it should be able to hybridise to both the gene sequence and the rRNA product. The oligonucleotides were end-labelled at the 3' end with Digoxigenin-11-dUTP (for fStol[-]) or Biotin-11-dUTP (for R16[V]F1[-]).

Petioles and leaf veins of periwinkle were fixed in a PIPES-buffered solution of 4 % paraformaldehyde and 0.5 % glutaraldehyde and then embedded in LRWhite resin at low temperature or LRGold resin at room temperature. Thin sections were collected on pioloform-coated nickel grids. Enhancement of ISH efficiency with a preliminary digestion step with pepsin was evaluated with a range of concentrations of pepsin in glycine buffer (pH 3.5). The grids were subsequently submerged in hybridisation buffer containing either oligoprobe, in a closed Eppendorf tube, and immersed in boiling water to denature the target nucleic acid. After boiling, the tubes were immediately transferred to a 37°C incubator overnight to hybridise. The hybridisation buffer was according to McFadden (10) in which formamide was adjusted to a range of concentrations from 10 to 45 % (pH 7.4). The detection of the hybridised material was performed in a one step procedure with gold-labelled anti-Digoxigenin (10 nm beads) or anti-Biotin (20 nm beads) monoclonal antibodies. Sections were subsequently stained with uranyl acetate and examined with a Hitachi H600 electron microscope. The controls were no probe addition in the hybridisation mixture, the use of an unrelated probe, the use of the sense probe, and digestion with RNAse 1 prior to hybridisation.

In optimal conditions (digestion with 10 µg/ml pepsin and 20-30% formamide concentration), a specific labelling of stolbur phytoplasma was obtained on groups of phytoplasmas with mature forms as well as senescent forms in phloem cells. Almost no background labelling occurred on any cellular structure. Labelling was greatly reduced when sections were first digested with RNAse 1. A very light label occurred when the sense probe was used, and no labelling could be seen in the other controls. Both embedding medium yielded successful results.

In our hands, labelling for the FD phytoplasma using a biotin-labelled probe was not successful. The 20 nm gold beads were seen to be present in low numbers and were not labelling groups of phytoplasma specifically.

The results with stolbur phytoplasma show that oligonucleotides may be used as probes for post-embedding ISH with electron microscopy on plant tissue, and that stringency conditions can be found which avoid non specific hybridisation with short nucleotide sequences. An explanation for the negative results with FD phytoplasma can be found in the literature describing similar techniques for the detection of other prokaryotic organisms, which often reports that oligonucleotides that had functioned efficiently as PCR primers did not work as ISH probes. The reasons might be related to the secondary structure of the target sequences.

To our knowledge the present work is the first report of the use of oligonucleotides as probes for post-embedding ISH with electron microscopy in plant tissue. The methods will be improved for application to infected grapevine material, with the use of confocal microscopy and electron microscopy.

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