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Viroids of grapevines in Italy

by

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Rebviroide in Italien

Zusammenfassung: Es wird über das Vorkommen niedermolekularer RNAs bei 48 *Vitis-vinifera*-Stämmen und 15 amerikanischen *Vitis*-Arten und Kreuzungen aus Italien, Osteuropa, Mittelmeer- und Nahost-Ländern berichtet. In den Sämlingen zweier Sorten wurden keine derartigen RNAs gefunden. Aufgrund ihres elektrophoretischen Verhaltens wurden diese RNAs vorläufig identifiziert als Grapevine yellow speckle-Viroid (GYSVd), Grapevine-Viroid 2 (GVd2) und Hop stunt-Viroid (HSVd). Das letztere Viroid löste bei künstlich infizierten Pflanzen von Tomate cv. Rutgers und Gurke cv. Suyo Befallssymptome aus. HSVd, GYSVd und GVd2 wurden in 97, 92 bzw. 11 % der untersuchten Proben wiedergefunden. In der Regel lagen Mischinfektionen vor, wobei die Kombination von HSVd mit GYSVd überwog. Diese beiden Viroide kamen regelmäßig in Reben mit den Symptomen von Yellow speckle oder Vein banding vor. Es wurde keine eindeutige Beziehung zwischen dem Vorkommen eines der Viroide und Rebkrankheiten mit unklarer Ätiologie, wie Vein necrosis oder Fleck, gefunden.

Key words: disease, viroid, analysis, molecular probe, cDNA, RNA extraction, PAGE electrophoresis, variety of vine, rootstock, Italy.

Introduction

Since the first record of a viroid-like RNA in the grapevine (SHIKATA *et al.* 1984), at least six such RNA molecules have been detected in Japan, Australia, USA and Europe (FLORES *et al.* 1985; SEMANCIK *et al.* 1987; REZAIAN *et al.* 1988 a). Four of these RNAs have been identified as authentic viroids, i.e. the grapevine strain of hop stunt viroid (HSVd) in Japan, Australia, and Germany (SANO *et al.* 1985; PUCHTA *et al.* 1988; REZAIAN *et al.* 1988 a and b), citrus exocortis A viroid (CEVd) in Spain (GARCIA ARENAL *et al.* 1987), grapevine viroid 2 (GVd2) in USA (SZYCHOWSKI *et al.* 1988) and grapevine yellow speckle viroid (GYSVd) in Australia (REZAIAN *et al.* 1988 a and b). GYSVd is closely related to, but apparently distinct from grapevine viroid 1B (GVd1B) (KOLTUNOW and REZAIAN 1989). However, whether GVd1B and grapevine Australian viroid (GAVd), an additional viroidal RNA from Australia (REZAIAN *et al.* 1988 a), are in turn related to, or the same as any of the other viroids is not known.

As to Italy, three viroid-like RNAs denoted GV1, GV2 and GV3 were found by SEMANCIK *et al.* (1987) in vines of cv. Corniola from Apulia (Southern Italy), and molecules with the same electrophoretic mobility were more recently observed in a limited number of grapevine samples from the same region (MINAFRA 1989). This paper reports the results of studies whereby the occurrence of viroids was investigated in a wider range of European grape cultivars and American *Vitis* species from Italy, Eastern Europe, the Mediterranean and Middle East.

Materials and methods

Plants

All vines, most of which were growing on their own roots, were from our virus collection plot. About 65 % of the plants had been indexed and their diseases identified (Table). The apical part of actively growing shoots was collected in June and July and stored at − 20 °C.

Plants of tomato cv. Rutgers and cucumber cv. Suyo were grown in a cabinet at 30 °C and 16 h artificial illumination. Tissues were collected 4—6 weeks after inoculation and processed immediately.

Extraction of nucleic acids

Grapevine leaf tissues (5 g) were pulverized in liquid nitrogen, extracted and processed according to SZYCHOWSKI *et al.* (1988), whereas nucleic acids from tomato and cucumber tissues were extracted according to ZELCER *et al.* (1982).

Polyacrylamide gel electrophoresis

Nucleic acid extracts were analyzed by double electrophoresis in 5 % polyacrylamide gels. After an electrophoretic run under non-denaturing conditions (MORRIS and WRIGHT 1975), a strip of the gel stained with ethidium bromide and roughly delimited by the marker viroids coconut cadang cadang fast form (CCCVd) and citrus exocortis (CEVd) was excised, placed on top of a denaturing gel containing 8 M urea, subjected to a second electrophoresis in a discontinuous pH system (RIVERA BUSTAMANTE *et al.* 1986), and stained with silver nitrate (IGLOI 1983). Circular RNAs separated in denaturing gels were electroeluted with a UEA apparatus (International Biotechnologies Inc., New Haven), ethanol precipitated and resuspended in distilled water.

Preparation of a cDNA probe to GYSVd

For cDNA preparation 2 kg of grapevine leaf tissues were extracted and the eluted RNAs were further fractionated by treating overnight with 2 M LiCl at 4 °C. Complementary DNA to one of the circular RNAs (GV1) which, as specified later, was identified as possible GYSVd, was prepared essentially as described by FLORES *et al.* (1985), using M-MLV reverse transcriptase (200 U/ml, BRL) and synthetic esanucleotides (Amersham) as primers. The cDNA probe was not cloned. It was applied in a limited number of tests using either electroeluted RNA molecules, or cellulose F-11-fractionated phenol extracts, or total phenol extracts. Samples were denatured with formaldehyde at 65 °C for 15 min before application to a nitrocellulose membrane. Some samples were denatured with 10 mM NaOH and 0.5 mM EDTA for 20 min at room temperature.

Prehybridization and hybridization conditions were as described by FLORES *et al.* (1985) and FLORES (1986), i.e. the radioactive probe (ca. 0.8×10^6 cpm/ml) was heated at 100 °C for 2 min and the denatured DNA was substituted by *Nicotiana benthamiana* 2 M LiCl-insoluble rRNA at a final concentration of 250 µg/ml.

Bioassay

Groups of 3—5 tomato or cucumber seedlings were inoculated either with partially purified extracts containing one or more circular RNA forms, or with electroeluted purified molecules of each RNA. The leaves were gently rubbed with celite and a drop of inoculum suspended in TE buffer (10 mM Tris-HCl pH 7.6 and 1 mM EDTA) was placed on the wounded area (R. FLORES, personal communication).

Results

Presence of viroid-like RNAs in grapevines

Except for seedlings, all samples of European and American *Vitis* species and hybrids contained one or, more often, two or three viroid-like RNAs (Fig. 1, Table). These were also present in a cv. Mission accession whose seedlings were viroid-free.

These RNAs had the same electrophoretic mobility as those named GV1, GV2 and GV3 by SEMANCIK *et al.* (1987). Fig. 2 A shows the identity of the electrophoretic pattern of a Californian accession of cv. Cardinal and that of an Italian accession of cv. Pascale di Cagliari, both containing all three RNAs.

The size of these RNA molecules was estimated to be between 300 and 370 nucleotides based on the relative electrophoretic mobility of markers viroids (CEVd and CCCVd fast form). In particular, under denaturing conditions GV1 migrated virtually at the same rate as CEVd (371 nt.) (Fig. 2 B), GV2 moved slightly faster, whereas GV3 migrated at about the same rate as CCCVd fast form (295 nt.) (Fig. 2 A and B). Such an electrophoretic behaviour is compatible with that reported in the literature for GV1, GV2 and GV3 (SEMANCIK *et al.* 1987) and, in turn, for GYSVd (REZAIAN *et al.* 1988 a and b) and HSVd (SANO *et al.* 1985; REZAIAN *et al.* 1988 a) with which GV1 and GV3, respectively, can tentatively be identified (see also SEMANCIK *et al.* 1987; REZAIAN *et al.* 1988 a).

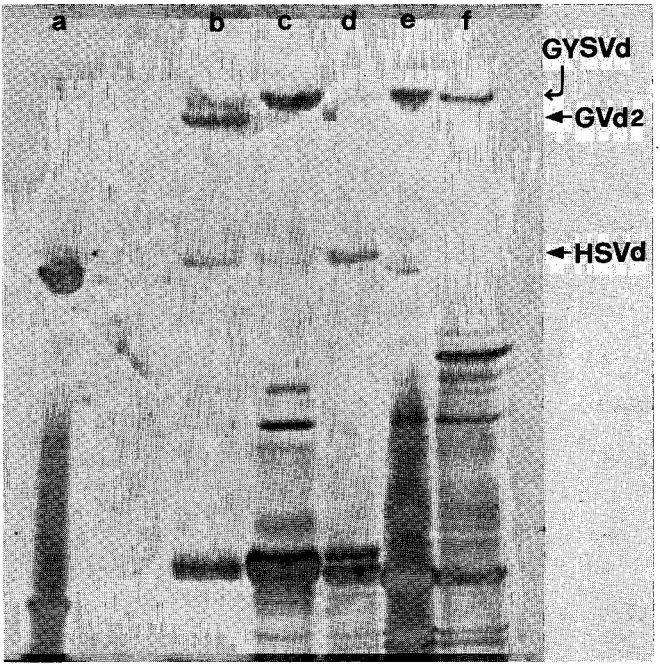

Fig. 1: Electrophoretic pattern of total RNA extracts from different grapevine accessions and Etrog citron affected by CEVd following electrophoresis under denaturing conditions. Marker CCCVd (a); cv. Cardinal (b); LN 33 (c); unknown grapevine cultivar from Malta (d); cv. Rossese (e); marker CEVd from Etrog citron (f). Staining with silver nitrate.

Elektrophoresemuster von Gesamt-RNA-Extrakten aus verschiedenen CEVd-infizierten Rebstämmen und infizierter Etrog-Zitrone. Elektrophorese unter denaturierenden Bedingungen. a) Marker-CCCVd, b) cv. Cardinal, c) LN 33, d) unbekannte Rebsorte aus Malta, e) cv. Rossese, f) Marker-CEVd aus Etrog-Zitrone. Anfärbung mit Silbernitrat.

Occurrence and relative concentration of viroids in European grapevine cultivars and rootstock accessions of various origin

Vorkommen und relative Konzentration von Viroiden in Sorten der Europäerrebe und Unterlagsstämmen verschiedener Herkunft

Cultivar	Origin	Disease	Viroids		
			GYSVd	GVd2	HSVd
1. *Vitis vinifera*					
Aglianico (seedling)		healthy	−	−	−
Al Zenzl	Middle East	nt	+ +	−	+
Baresana	Italy	Fl	+ +	−	+
Baresana rosa	Italy	Lr, Rw, Vn	+ +	−	+
Cardinal	Italy	nt	+ +	+ +	+
Cardinal	USA	healthy	+ +	+ +	+
Chiradzoum	Middle East	nt	+ +	−	+
Corniola	Italy	Cb	+ + +	−	+
Daraweeshi	Jordan	Fl	+	−	+
Kishimishi	Afghanistan	nt	+ +	+	+ +
Kokur	Middle East	nt	+ +	−	+
Krasnospit	USSR	nt	+ +	−	+
Mission 1	USA	Vn	+ +	−	+
Mission 2	USA	Ys	+ + +	−	+
Mission (seedling)		healthy	−	−	−
Montepulciano	Italy	Vn	+	−	+
Moscato bianco	Nigeria	nt	+ + +	−	+
Moscato selvatico	Italy	Fl, Fk, Vn, Vb	+	−	+
Moscato saraceno	Italy	nt	+ +	−	+
Negroamaro	Italy	Lr	+	−	+
Ohanez	Italy	Lr, Fk	+ +	−	+
Palomino	Cyprus	Fl, Lr	+	−	+
Pampanuto	Italy	Rw	+ +	−	+ +
Pascale di Cagliari	Italy	Lr	+ +	+ +	+
Perlette	Albania	nt	+ + +	+	+
Primitivo di Gioia	Italy	Lr	+ + +	−	+
Prunesta	Italy	healthy	+ +	−	+
Rcatzitelli	USSR	Fl, Ys	+ +	−	+
Rcatzitelli	Bulgaria	Ys	+ +	−	+
Regina nera	Italy	nt	+ +	−	+
Regina dei Vigneti	Italy	Lr	+ + +	−	+
Romani	Israel	Rw	+ +	−	+
S. Anna	Albania	Fl, Vb	+ + +	−	+
S. Nicola	Italy	Fl	+	−	+
S. Teresa	Italy	Fl, Fk	+ +	−	+
Sandarski	Bulgaria	nt	+ + +	−	+
Stangarone	Italy	Fl, Fk	+ + +	−	+
Sultanina	Greece	nt	+ +	+ +	+
Susumaniello	Italy	Lr	+ + +	−	+

Cultivar	Origin	Disease	Viroids		
			GYSVd	GVd2	HSVd
Tilky Raikur	Middle East	nt	+	–	+
Uva della Scala	Italy	nt	+	–	+
Valencie 1	Morocco	nt	+ + +	–	+
Valencie 2	Morocco	nt	–	–	+ +
Verdeca	Italy	Cb	+ +	–	+
Xynisteri	Cyprus	nt	+ +	–	+
Unknown	Albania	nt	+	–	+
Unknown	Greece	Fl	+ +	–	+
Unknown	Malta	Fl	+ +	–	+
Unknown	Yugoslavia	nt	+ +	–	–
Unknown	Nigeria	Fl	+ +	–	+
2. American *Vitis* species and hybrids					
a) *V. berlandieri* × *V. riparia*					
420 A	Italy	Lr, Fk	+ +	–	+
125 AA	Italy	Fl	–	–	+ +
161/49	Italy	Fl, Vn	–	–	+
34 E.M.	Italy	Rw	+ + +	–	+
225 Ru	Italy	Rw	–	–	+
b) *V. berlandieri* × *V. rupestris*					
779 P	Italy	Fl, Fk	+ + +	–	+
101/14	Italy	Lr	+ + +	–	+
140 Ru	Italy	Rw	–	–	+
3309	USSR	Yb	+ + +	+ +	+ +
c) Complex hybrids					
41 B	Spain	Fl, Vb	+ +	–	+
2 AP	Italy	Lr, Fk	+ + +	–	+
LN 33 a	Italy	Lr	+	–	+
LN 33 b	Italy	Lr	+ + +	–	+
d) *V. rupestris*	Spain	Cb, Vn, Fk	–	–	+ + +
e) *V. labrusca*	Italy	Fl	+ +	–	+

Symbols: + = presence of viroid; – = absence of viroid; nt = not tested; Fl = fanleaf; Fk = fleck; Cb = corky bark; Lr = leafroll; Rw = rugose wood; Vb = vein banding; Vn = vein necrosis; Yb = yellow blotching; Ys = yellow speckle.

In the grapevine samples analyzed in the present survey, the highest incidence was shown by HSVd and GYSVd (97 and 92 %, respectively), whereas GV2 was detected in about 11 % of the cases (Table). The lowest incidence of GV2 was in the American

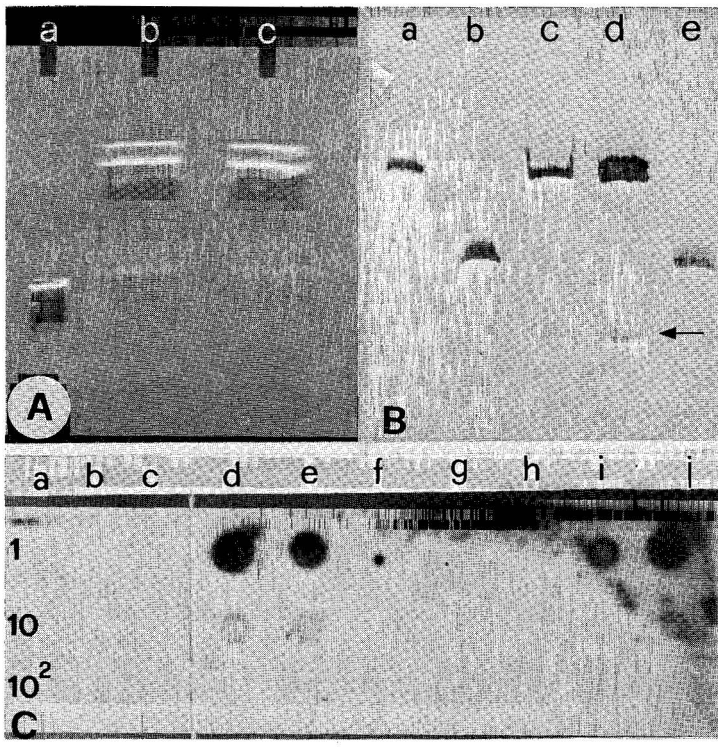

Fig. 2: A) Identity in the electrophoretic pattern of extracts from an Italian grapevine accession of cv. Pascale di Cagliari (b) and a Californian accession of cv. Cardinal (c) containing GYSVD, GVd2, and HSVd (from top to bottom). Marker viroid CCCVd fast form in lane (a). Electrophoresis in 5 % polyacrylamide gel under denaturing conditions (8 M urea and discontinuous pH). Staining with ethidium bromide. — B) Electropherogram of grapevine extracts and marker viroids: CEVd (a), HSVd (b), GYSVd (c), GYSVd and GVd2 (d), CCCVd fast form (e). Arrow indicates linear forms. Electrophoresis under denaturing conditions, staining with silver nitrate. — C) Dot-blot hybridization of a cDNA probe to GYSVd with extracts from: *Vitis rupestris* containing HSVd (a); viroid-free Mission seedling (b); cucumber cv. Suyo inoculated with all three viroids (c); LN 33 containing GYSVd and HSVd (d); cv. Corniola containing GYSVd and HSVd (e); GYSVd denatured with NaOH (f); CEVd (g); HSVd (h); GVd2 (i); GYSVd denatured with formaldehyde (j). Dilutions of the probe are given on the left end side.

A) Identität der Elektrophoresemuster von Extrakten aus einem italienischen Stamm von cv. Pascale di Cagliari (b) und einem kalifornischen Stamm von cv. Cardinal (c) mit GYSVd, GVd2 und HSVd (von oben nach unten). In Trennspur (a) Marker-Viroid CCCVd fast form. Elektrophorese in 5%igem Polyacrylamid-Gel unter denaturierenden Bedingungen (Harnstoff und diskontinuierliches pH). Anfärbung mit Ethidiumbromid. — B) Elektropherogramm von Rebenextrakten und Marker-Viroiden: a) CEVd, b) HSVd, c) GYSVd, d) GYSVd und GVd2, e) CCCVd fast form. Der Pfeil weist auf lineare Formen hin. Elektrophorese unter denaturierenden Bedingungen, Anfärbung mit Silbernitrat. — C) Dot-blot-Hybridisierung einer cDNA-Sonde für GYSVd mit folgenden Extrakten: a) *Vitis rupestris* mit HSVd, b) viroidfreier Mission-Sämling, c) mit allen drei Viroiden infizierte Gurke cv. Suyo, d) LN 33 mit GYSVd und HSVd, e) cv. Corniola mit GYSVd und HSVd, f) mit NaOH denaturiertes GYSVd, g) CEVd, h) HSVd, i) GVd2, j) mit Formaldehyd denaturiertes GYSVd. Die Verdünnungen der Sonde sind am linken Rand angegeben.

rootstocks with a single positive detection in 15 samples. Seven vines (six *Vitis vinifera* and one American hybrid) contained all three viroids. Interestingly, one of these accessions was a vine from Afghanistan collected 4 years ago, which, presumably, had never been in contact with rootstocks.

The presence and relative distribution of viroids was not related to the geographical origin of the grapevine accessions nor to the diseases by which they were affected.

Molecular hybridization

The cDNA probe to GYSVd hybridized specifically, giving a clear-cut signal, with formaldehyde-denatured extracts from vines containing both GYSVD and HSVd (Fig. 2 C, lanes d and e), or GYSVd alone (Fig. 2 C, lane j). Extract of GYSVd denatured with NaOH gave a positive but very weak signal (Fig. 2 C, lane f). There was no detectable hybridization with CEVd (Fig. 2 C, lane g), HSVd (Fig. 2 C, lane h), nor with extracts from samples that were electrophoretically negative for GYSVd (Fig. 2 C, lanes a and b) or extracts from cucumbers inoculated with all three viroids (Fig. 2 C, lane c) in which, evidently, there was no multiplication of GYSVd.

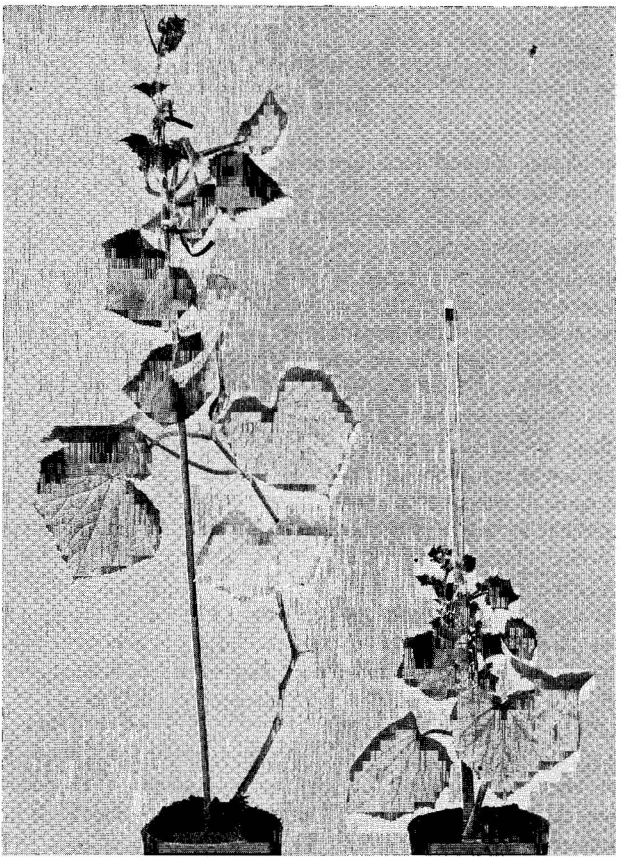

Fig. 3: Symptomatic cucumber cv. Suyo 3 weeks after inoculation with HSVd from grapevine. Healthy control on the left.

Gurke cv. Suyo mit Befallssymptomen, 3 Wochen nach der Infektion mit HSVd von der Rebe. Links: Gesunde Kontrollpflanze.

Positive hybridization was obtained with GV2 (Fig. 2 C, lane i). It was not ascertained, however, whether this was due to the existence of specific sequence homology between the two viroids or to an undetected contamination of the extract used for cDNA production.

Bioassay

Tomato and cucumber seedlings showed clear signs of infection 2—3 weeks after inoculation with partially purified grapevine extracts containing all three viroids, or with purified HSVd. Symptoms in tomato consisted of mild depression of the growth, deformation and puckering of the leaves, whereas cucumbers reacted with vein clearing, curling and deformation of the leaves, and very severe stunting (Fig. 3). From symptomatic cucumber and tomato plants an RNA with the same electrophoretic mobility of HSVd was readily and consistently isolated.

Herbaceous hosts inoculated with CYSVd and GV2 alone or in mixture, remained symptomless and no viroid-like RNAs could be recovered from them.

Discussion

The results of the present investigation support the notion that low molecular weight RNAs are widespread in *Vitis* species. In this as in previous surveys (SEMANCIK *et al.* 1987; REZAIAN *et al.* 1988 b; SZYCHOWSKI *et al.* 1988) not a single *Vitis* accession, except for seedlings, was found free from these RNAs, whose infectious nature was confirmed in one instance. In fact, the smallest of the three RNA molecules recovered from Italian grape samples successfully replicated in tomato and cucumber, thus lending further support to its identification as HSVd.

The other two RNA molecules were unable to infect herbaceous hosts in our tests, which differs from previous findings reporting that GV1, now tentatively identified as GYSVd, can multiply symptomlessly in cucumbers (SEMANCIK *et al.* 1987).

However, the viroidal nature of GYSVd and GVd2 has been convincingly established since both are able to infect grapevines (REZAIAN *et al.* 1988 a; SZYCHOWSKI *et al.* 1988) and seem to be involved in the etiology of grapevine yellow speckle, an elusive disease characterized by the erratic expression of various patterns of chrome-yellow discolorations of the foliage (TAYLOR and WOODHAM 1972).

Among the grapevine accessions of the present survey, two of cv. Rcatzitelli and one of the rootstock 3309 showed non mechanically transmissible chrome-yellow spotting of the leaves (ABRACHEVA *et al.* 1978; SAVINO *et al.* 1985) which, in the case of Rcatzitelli, strongly recalled typical yellow speckle symptoms. Furthermore, a virus-free cv. Mission accession used as indicator showed occasional minute yellow spots on the leaves, again reminiscent of yellow speckle. GYSVd was present in all the above accessions as well as in three additional vines (S. Anna, Moscato selvatico and 41B) that indexed positive for vein banding, another disease in whose etiology the involvement of GYSVd has been claimed (KRAKE and WOODHAM 1983).

The contemporary presence in all the above vines of HSVd (and in 3309 also of GVd2) does not allow to draw conclusions on the possible etiological relationship between GYSVd and the yellow discolorations affecting these vines. Likewise, no clear-cut association could be established between the presence of viroids and diseases of unknown etiology like, for instance, vein necrosis and fleck.

Summary

The occurrence is reported of low molecular weight RNAs in 48 *Vitis vinifera* accessions and 15 American *Vitis* species and hybrids from Italy, Eastern Europe, Mediterranean and Middle East countries. No such RNAs were found in seedlings of two cultivars. Based on their electrophoretic behaviour, these RNAs were tentatively identified as grapevine yellow speckle viroid (GYSVd), grapevine viroid 2 (GVd2), and hop stunt viroid (HSVd). The latter viroid caused symptoms in artificially inoculated plants of tomato cv. Rutgers and cucumber cv. Suyo. HSVd, GYSVd and GVd2 were recovered from 97, 92 and 11 % of the samples examined, respectively. Mixed infections were the rule, the prevailing association being HSVd and GYSVd. The same two viroids consistently occurred in vines with yellow speckle-like or vein banding symptoms. No clear-cut relationship was found between the presence of any viroid and grapevine diseases of unknown origin like vein necrosis and fleck.

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