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Production of monoclonal antibodies to Grapevine virus D and contribution to the study of its aetiological role in grapevine diseases

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Summary

Six stable hybridoma cell lines secreting monoclonal antibodies (MAbs) to Grapevine virus D (GVD) were obtained by fusing spleen cells of immunized BALB/c mice with mouse myeloma cell line Sp 2/0-Ag 14. In ELISA all MAbs detected the virus in *Nicotiana* leaf extracts or cortical shavings from mature grapevine canes. The use of a polyclonal antiserum for coating plates and of monoclonal antibodies and antimouse-conjugated antibodies for antigen detection, gave highly efficient and reproducible results for identification of GVD in field-grown grapevines. The reliability of the ELISA kit was confirmed by GVD-transmission tests to herbaceous hosts, using *in vitro* explants as inoculum. 223 vines affected by one or more of the 4 syndroms of the rugose wood complex (Kober stem grooving, Corky bark, LN stem grooving and Rupestris stem pitting) were tested in ELISA for the detection of Grapevine virus A (GVA), Grapevine virus B (GVB) and GVD and by Western blot for the detection of Grapevine rupestris stem pitting associated virus (GRSPaV). The possible cause-effect relationship between GVA and KSG, GVB and CB, and GRSPaV and RSP was confirmed, but no consistent association was found between GVD and any of the 4 above syndromes. Intriguingly, a reduction in the expression of stem pitting symptoms in *V. rupestris* (from 90 % to 75 %) and of stem grooving symptoms in Kober 5BB (from 95 % to 70 %) was observed when vitiviruses and GRSPaV were contemporarily present in the same indicator. Preliminary data of a survey involving 676 grapevine samples showed a high incidence (31 %) of GVD, regardless of the geographical origin of samples.

K e y w o r d s : virus diseases, rugose wood, vitivirus, foveavirus, monoclonal antibodies, ELISA, aetiology.

Introduction

Grapevine virus D (GVD), a definitive member of the genus Vitivirus (Abou Ghanem *et al.* 1997) serologically distantly related to Grapevine virus A (GVA) and Grapevine virus B (GVB) (Choueiri *et al.* 1997), was originally recovered from a grapevine showing symptoms of "corky rugose wood" (Bonavia *et al.* 1996). GVD was identified by RT-PCR in 4 % of 218 rugose wood-affected vines, but in none of 89 disease-free plants, which suggested that it could be involved, like other vitiviruses (Martelli *et al.* 1993), in the aetiology of this disease (Abou Ghanem *et al.* 1997).

The use of serology for large scale detection of GVD in grapevine tissues is difficult due to the poor immunogenic power of the virus, which has impaired the production of high quality antisera. The only antiserum available could be utilised for virus detection by immune electron microscopy (IEM) in tissue extracts from herbaceous hosts but not from grapevines, and was not appropriate for immunoenzymatic assays (ELISA) (Abou Ghanem *et al.* 1997).

To overcome this constraint, hybridoma technology was utilised for the production of monoclonal antibodies to GVD, which were used in ELISA for investigating the involvement of this virus in the aetiology of the rugose wood complex, by testing grapevines whose specific syndromes had been previously determined by indexing.

Material and Methods

V i r u s p u r i f i c a t i o n : Virus isolate Pr 13/24, originally obtained from *Vitis vinifera* cv. Primus (Bonavia *et al.* 1996), was used for the production of monoclonal antibodies. The virus was propagated in glasshouse-grown *Nicotiana occidentalis*, from which it was purified by clarification with 5 % Mg-activated bentonite, two cycles of differential centrifugation, sucrose and caesium sulphate density gradient centrifugation and dialysis against phosphate-buffered saline (PBS) (Boscia *et al.* 1993).

P r o d u c t i o n a n d c h a r a c t e r i s a t i o n o f h y b r i d o m a s s e c r e t i n g m o n o c l o n a l a n t i b o d i e s t o G V D : Three 6-week-old BALB/c female mice were immunised by injecting three times 500 µl of an emulsion of 150 µg purified virus in an equal volume of Freund's incomplete adjuvant at two-week intervals. A booster injection of 300 µg virus without adjuvant was made 75 d after the first immunisation. Fusion was performed 3 d later by mixing about 2 x 10^8 splenocytes with 2 x 10^7 SP 2/0 Ag 14 myeloma cells (American Type Culture Collection, Rockville)

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in the presence of 50 % polyethylene glycol MW 1300-1600. Fused cells in HAT medium (1 x 10^{-4} M hypoxantine, 4 x 10^{-7} M aminopterin and 1.6 x 10^{-5} M thymidine) were distributed in 100 µl aliquots in 96 well culture plates (Sterilin, Hounslow). The culture medium used (Roswell Park Memorial Institute medium, RPMI-1640) contained 2 mM L-glutamine and 20 % fetal bovine serum (FBS). One day before fusion, culture plates were seeded with two types of feeder cells (10^3 per well), *i.e.* peritoneal macrophages and splenocytes from mice primed 80 d before with 500 µl pristane. Two weeks after fusion, supernatant culture fluids were screened for the presence of GVD antibodies by TAS-ELISA. Hybridoma cells secreting virus-specific antibodies were cloned and subcloned by the limiting dilution method on a feeder layer of splenocytes (in 20 % FBS medium). Subcloning was repeated twice.

Identification of GVD-specific antibodies: TAS-ELISA was performed during early screening, cloning, and sub-cloning for selecting hybridoma cells secreting monoclonal antibodies specific to GVD. Purified IgG in 0.05 M carbonate buffer pH 9.6 (100 µl per well) from the available polyclonal antiserum (ABOU GHANEM *et al.* 1997) were used for coating polystirene plates. After incubation at 37 °C for 2 h and three washings with PBS-T (PBS 1x plus 0.02 % Tween), GVD Pr 13/24-infected *N. occidentalis* sap diluted 1:20 in extraction buffer (PBS-T plus 2 % polyvinylpyrrolidone) was added and incubated overnight at 4 °C. Plates were washed and loaded with 100 µl per well of hybridoma culture fluids (undiluted or diluted 1:2 in PBS). Alkaline phosphatase-conjugated goat antimouse IgGs were added and incubated at 37 °C for 2 h prior to addition of substrate. Controls consisted of healthy *N. occidentalis* extracts diluted 1:20 in extraction buffer. Reactions with absorbance values threefold those of controls or higher were regarded as positive.

Determination of monoclonal antibody isotypes: Isotyping was done on MAbs produced in serum-free medium. The determination of class, subclass, and light chain type of monoclonal antibodies was done using a commercial Mouse Hybridoma Sub-typing Kit (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's instructions. The method is based on the reaction of coating antibody with all immunoglobulin classes and subclasses from mouse, independently of the type of light chains. The range of class and subclass of tested antibodies was determined by specific goat anti-mouse Ig-POD conjugates.

Determination of serological relationship between GVD, GVA and GVB: To select MAbs able to identify epitopes common to different vitiviruses, TAS-ELISA was done at an early stage, using cell culture supernatants, before cloning and stabilisation of positive hybridoma cultures. Sixteen GVA and 20 GVB isolates from dried infected *Nicotiana* spp. tissues were used for testing serological relationship. GVA and GVB isolates were divided into 4 and 5 groups (4 isolates per group), respectively. Each group was used as antigen against each single supernatant. After the ascitic fluids of the 6 stable hybridoma cell lines were obtained, each of them was again checked against each single isolate of GVA and GVB.

Production of ascitic fluids and purification of monoclonal antibodies: Ascitic fluids were produced by intraperitoneal injections of 10^6 hybridoma cells in 10-month-old BALB/c mice, in which 500 µl of Freund's incomplete adjuvant had been injected one day before. MAbs were purified from ascitic fluids by affinity chromatography on protein A-sepharose columns (CLARK and BAR JOSEPH, 1984).

Selection and titration of MAbs suitable for routine ELISA tests: TAS-ELISA was done using one infected *N. occidentalis* and three grapevine accessions as antigen sources. Healthy *N. occidentalis* and grapevines were used as negative controls. For routine ELISA the optimal concentration of each MAb line was determined by adding increasing MAb dilutions to the antibody-antigen complex fixed on the plates. For this test the plates were coated with 1 µg·ml^{-1} rabbit IgGs.

ELISA protocols for GVD identification: Eight different ELISA protocols were tested (Tab. 1) using extracts from leaves of GVD-infected *N. occidentalis* and cortical shavings from mature grapevine canes. Protein A (Pharmacia, Uppsala, Sweden), and polyclonal IgGs were used at a concentration of 1 µg·ml^{-1}. Absorbance values (A_{405}) were read with a Titertek Multiskan Plus MK II photometer.

Table 1

ELISA protocols used for GVD detection in host tissues

1 PAb-Ag-MAb- GaME-S
2 PAb-Ag-CMAbs-GaME-S
3 IgG (PAb)- Ag-MAb -GaME -S
4 IgG (PAb)-Ag-CMAbs-GaME -S
5 PAb-Ag-PAbE -S
6 PAb-Ag-MAbE - S
7 MAb-Ag-MAbE-S
8 Prot A-Pab-Ag-MAb-GaME-S

Pab = Polyclonal antibodies (rabbit).
Ag = Antigen.
MAb= Monoclonal antibodies (mouse).
GaM = Goat anti-mouse antibodies.
CMAbs = Cocktail of monoclonal antibodies.
IgG (Pab) = purified globulins from polyclonal antiserum.
S = Substrate (P-nitrophenyl phosphate).
E = Enzyme (alkaline phosphatase).

Optimisation of the ELISA protocol: a) Antigen extraction: Saline (PBS 1x plus polyvinylpyrrolidone 2 % and Tween 20 from 0.05 % up to 0.5 %, with or without polyethylene glycol MW 8,000 1 %) and Tris [Tris (hydroxymethyl)-aminomethan 0.5 M, sodium chloride 0.14 M, polyvinylpyrrolidone 2 %, polyethylene glycol MW 8,000 1 % and Tween 20 0.05 %] buffers, with pH ranging from 6.0 to 8.5 (at 0.5 intervals), were compared for selecting the best extraction medium for GVD antigens.

b) Incubation of the antigen: After the addition of the antigens, the plates were stored for 2 or 4 h at 37 °C and for 12 or 24 h at 4 °C.

c) Antigen distribution in grapevine cuttings: Two cuttings from each of 5 infected grapevines were utilised. The canes were divided in 5 pieces from base to top and cortical shavings from each piece were separately assayed by TAS-ELISA.

d) Validation of the GVD-ELISA kit: To confirm ELISA results, sap from a number of ELISA-positive grape samples was mechanically inoculated onto *N. occidentalis* and *N. benthamiana* plants. Samples, extracted in phosphate buffer 0.01 M, pH 7.2, containing 0.01 M cysteine-HCl and 3 % nicotine (Boscia *et al.* 1993), were from: (i) leaves of greenhouse-forced cuttings (29 samples); (ii) flowers collected in the vineyards (19 samples); *in vitro*-grown grapevine explants (13 samples) (Bottalico *et al.* 1997)

e) GVD distribution in naturally infected vines: To assess the distribution of GVD field-grown grapevines, ELISA protocol 1 (Tab. 1) was used for a large scale survey on: (i) 433 vines from Apulia (Southern Italy), of which 133 originated from a collection plot of candidate clones of wine grape varieties, and 300 from commercial vineyards; (ii) 199 vines from Sardinia (Southern Italy), from a collection plot of the University of Sassari and commercial vineyards; (iii) 44 vines from a collection of varieties from different geographical origin. Mature canes (2-3 per plant) were collected in winter and stored at 4 °C. Cortical shavings obtained after removal of the bark were utilised as antigen source. Each test was repeated at least three times.

Investigation on the possible role of GVD, other vitiviruses (GVA and GVB), and GRSPaV in the aetiology of specific syndromes of the rugose wood complex: The association of GVD with rugose wood syndromes was investigated by ELISA testing of 223 vines affected by one or more of the rugose wood syndromes, *i.e.* Kober stem grooving (KSG), Rupestris stem pitting (RSP), LN 33 stem grooving (LNSG), and Corky bark (CB), as previously determined by indexing.

ELISA tests were extended to GVA, the putative agent of KSG (Chevalier *et al.* 1993; Garau *et al.* 1994); GVB, the putative agent of CB (Boscia *et al.* 1993; Bonavia *et al.* 1996), and Grapevine rupestris stem pitting-associated virus (GRSPaV), the putative agent of RSP (Meng *et al.* 1999).

Cortical scraping extracts were tested by TAS-ELISA (GVB and GVD) (Al Moudallal *et al.* 1984), protein A DAS-ELISA (GVA) (Boscia *et al.* 1992), and Western blot (GRSPaV) (Minafra *et al.,* 2000). Locally produced polyclonal antisera and monoclonal antibodies were used as reagents.

Results

Production of hybridomas secreting monoclonal antibodies to GVD: Although 59 positive supernatants were obtained after the second screening, only 6 stable hybridoma cell lines continued to secrete MAbs specific to GVD after cloning and subcloning. Ascitic fluids denoted D2, D3, D4, D5, D6 and D8 were produced for each MAb line.

Isotyping: MAbs D2, D5 and D8 belonged to IgG2a sub-class, MAbs D3, D4 and D6 to IgG1 sub-class. All MAbs had K-type light chain.

Determination of serological relationship between GVD, GVA and GVB: In preliminary tests, 4 out of 59 supernatants specific to GVD detected all 4 groups of GVA isolates, but none of the 5 groups of GVB isolates, thus confirming the distant serological relatedness of GVA and GVD (Choueri *et al.* 1997). Unfortunately, none of the lines that recognised GVA was comprised in the 6 GVD stable cell lines that survived cloning, thus testing for the identification of a broad-spectrum MAb was discontinued. Ascitic fluids confirmed the results obtained with supernatants of stable cell lines as they were able to recognise GVD, but none of the GVA or GVB isolates.

Selection and titration of MAbs suitable for routine ELISA tests: Of the 6 MAb lines, D3 and D5 proved little reliable when used in ELISA. Better results as to sensitivity and reproducibility were obtained with the other 4 lines, D8 in particular, which detected GVD in infected *N. occidentalis* extracts to a dilution of 1:2,048,000. The best dilution for routine ELISA of grapes was between 1:64,000 and 1:128,000.

ELISA protocols for GVD identification: The comparison among different ELISA protocols for GVD detection in infected herbaceous and grapevine tissues showed that: (i) TAS-ELISA (protocol 1-4) was more effective than DAS-ELISA (protocols 5-7) (data not shown). Protocol 1, in particular, proved to be the most sensitive and reliable, especially with grape tissues, and was routinely used for surveys; (ii) coating with polyclonal antiserum was much more effective than coating with monoclonal antibodies or purified IgG molecules from the same polyclonal antiserum; (iii) contrary to what was found with GVA (Boscia *et al.* 1992), GVD detection was not improved by pre-coating ELISA plates with protein A; (iv) in the antigen revealing step, the best results were obtained when the D8-MAb line was used alone instead of a mixture of different MAb-lines (D2+D4+D6+D8, in different combination).

Optimisation of the ELISA protocol: The composition of the extraction buffer greatly influenced ELISA detection of GVD, for sensitivity increased from two to threefold if saline buffer (pH 7.0 to 8.0) was used instead of Tris-buffer. There was no improvement by adding PEG to the buffer or increasing the amount of Tween 20. Incubation conditions of the extracts were apparently less important, although a slight improvement in sensitivity was observed when the plates were stored overnight (12 h) at 4 °C. As expected, the distribution of GVD in grapevine tissues was erratic. No gradient was observed in virus distribution along the cuttings (from base to top or vice versa) and in several internodes of infected cuttings GVD was not detected. Due to this, all subsequent testing was made on composite samples consisting of different portions from two or three cuttings from the same vine.

GVD transmission to herbaceous hosts: When ELISA-positive grapevine samples were used for mechanical inoculation of *Nicotiana* plants, GVD was recovered from leaves of one out of 29 glasshouse-forced cuttings, of one out of 19 flower samples from the field, and of 6 out of 13 *in vitro*-grown explants. These results confirmed that: (i) as with other vitiviruses, manual transmission of GVD from grapevine tissues to herbaceous hosts is

difficult; (ii) *in vitro*-grown explants are a much better source of inoculum for virus isolation, confirming previous reports (MONETTE *et al.* 1991).

GVD distribution in naturally infected vines: GVD was detected in 209 out of 676 vines tested (31 %). The level of infection was about the same regardless of the geographical origin of the sources, *i.e.* 25 %, 34 % and 30 % in vines from Sardinia, Apulia, and foreign countries, respectively. The highest levels of infection were found in some Sardinian varieties (Monica, Pascale di Cagliari, Malvasia di Bosa), but also in cvs Cardinal, Malvasia bianca, Sangiovese and Uva di Troia, where they exceeded 50 % (Tab. 2).

Possible role of GVD, other vitiviruses (GVA and GVB), and GRSPaV in the aetiology of specific syndromes of the rugose wood complex: The results of virus detection by ELISA and Western blot in vines affected by one or another of the rugose wood syndromes are summarised in Tabs 3 and 4.

Table 2

GVD infection rates of some grapevine varieties

Cultivar	Accessions tested (No.)	GVD-infected	
		n	%
Monica	8	7	87
Pascale di Cagliari	18	13	72
Malvasia di Bosa	18	10	56
Cardinal	11	6	55
Malvasia bianca	13	7	54
Sangiovese	12	6	50
Uva di Troia	18	9	50
Negroamaro	12	5	42
Primus	140	54	39
Red Globe	14	4	29
Vermentino	31	9	29
Others	381	79	21
Total	676	209	31

Table 3

Association of vitiviruses and GRSPaV, as detected by laboratory tests (ELISA and Western blot), with rugose wood syndromes, as determined by indexing

Virus	Samples	KSG		CB		LNSG		RSP	
	No.	n	%	n	%	n	%	n	%
GVA	17	16	94	2	12	1	6	6	35
+GVB	1	1	100	1	100	0	0	0	0
+GVD	3	3	100	0	0	0	0	2	67
+GRSPaV	73	49	67	2	3	15	21	55	75
+GVB+GVD	1	1	100	1	100	0	0	0	0
+GVB+GRSPaV	5	4	80	4	80	0	0	4	80
+GVD+GRSPaV	19	15	79	1	5	2	11	11	58
GVA	119	89	75	11	9	18	15	78	66
GVB	2	0	0	2	100	0	0	0	0
+GVA	1	1	100	1	100	0	0	0	0
+GRSPaV	3	1	33	3	100	0	0	2	67
+GVA+GVD	1	1	100	1	100	0	0	0	0
+GVA+GRSPaV	5	4	80	4	80	0	0	4	80
GVB	12	7	58	11	92	0	0	6	50
GVD	3	0	0	0	0	1	33	2	67
+GVA	3	3	100	0	0	0	0	2	67
+GRSPaV	34	4	12	2	6	4	12	29	85
+GVA+GVB	1	1	100	1	100	0	0	0	0
+GVA+GRSPaV	19	15	79	1	5	2	11	11	58
GVD	60	23	38	4	7	7	12	44	73
GRSPaV	51	5	10	2	4	13	25	46	90
+GVA	73	49	67	2	3	15	21	55	75
+GVB	3	1	33	3	100	0	0	2	67
+GVD	34	4	12	2	6	4	12	29	85
+GVA+GVB	5	4	80	4	80	0	0	4	80
+GVA+GVD	19	15	79	1	5	2	11	11	58
GRSPaV	185	78	42	14	8	34	18	147	79

KSG: Kober stem grooving; CB: Corky bark; LNSG: LN 33 stem grooving; RSP: Rupestris stem pitting.

Table 4

Occurrence of vitiviruses and GRSPaV in vines with or without symptoms of the 4 rugose wood syndromes

Syndromes	Samples	GVA		GVB		GVD		GRSPaV	
	No.	n	%	n	%	n	%	n	%
KSG+	101	89	88	7	7	23	23	78	77
KSG-	122	30	25	5	4	37	30	107	88
CB+	20	11	55	11	55	4	20	14	70
CB-	203	108	53	1	0,5	56	28	171	84
LNSG+	39	18	46	0	0	7	18	34	87
LNSG-	184	101	55	12	7	53	29	141	77
RSP+	166	78	47	6	4	44	27	147	89
RSP-	57	41	72	6	11	16	28	38	67

KSG, CB, LNSG, RSP: see Tab. 3.

GVD was detected in 60 out of 223 vines (27 %), with no clear-cut association with any of the 4 syndromes of the rugose wood complex. GVD presence in symptomatic vines ranged between 18 % (LNSG) and 27 % (RSP), *i.e.* infection levels were similar, if not lower, than those detected in symptomless vines (Tab. 4).

More consistent was the association of GVA, GVB and GRSPaV with specific rugose wood syndromes confirming previous reports. In particular, GVA, which occurred in 119 samples (53 %), was strictly associated with KSG (75 %); GVB, detected in 12 samples (5 %), had a 92 % association with CB, and GRSPaV, present in 185 samples (83 %), showed a 79 % association with RSP (Tab. 3).

The presence of KSG symptoms in 12 GVA-negative vines, and of CB in 9 GVB-negative accessions can be explained either by the limits of sensitivity of the diagnostic procedure adopted and/or by the use in the antigen detection step of TAS-ELISA of single MAb lines to GVA and GVB that perhaps were unable to detect all the extant strains of either virus. This, however, does not rule out the possible involvement of other viral agents in the aetiology of KSG and CB.

Latency of symptoms was observed in vines that were infected by viruses expected to induce disease expression. Thus, for instance, about 30 GVA-positive vines mostly from Sardinia, did not show KSG symptoms. This contradictory finding is likely to originate from an unexpected mealybug-mediated GVA infection that occurred in the Sardinian grapevine collection after indexing (R. GARAU, pers. inform.). Likewise, no stem pitting developed in *Vitis rupestris* indicators graft-inoculated with budwood from 38 GRSPaV-positive vines. In this case, symptom latency could be due to a sort of interference of different viruses (or groups of viruses). In fact, the association of GVA with KSG symptoms was higher than 94 % when this virus was alone or in mixed infections with other vitiviruses, but it dropped to 70 % when GRSPaV was also present. Similarly, the association of GRSPaV with RSP symptoms was 90 % in single infections, but dropped to 75 % when GRSPaV was mixed with vitiviruses (Figure). A similar apparently competitive behaviour between vitiviruses and GRSPaV was observed in the case of GVB-CB association.

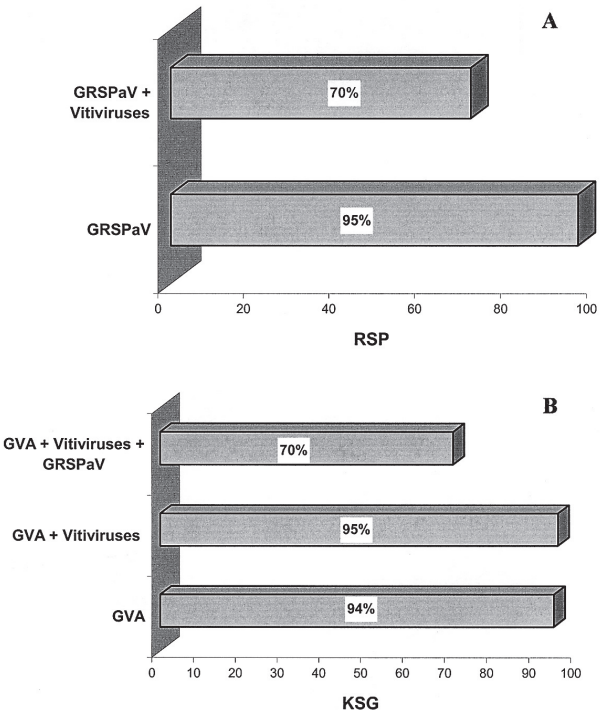

Figure: Expression of Rupestris stem pitting (RSP) and Kober stem grooving (KSG) symptoms in vines infected by GRSPaV or GVA singly or in mixed infections. **A**. Incidence of RSP symptoms in vines infected by GRSPaV alone or in mixture with vitiviruses (GVA, GVB or GVD). **B**. Incidence of KSG symptoms in vines infected by GVA alone or in mixture with other vitiviruses (GVB, GVD) and GRSPaV.

Discussion

The present investigation has shown that the problems encountered with the use of low-titre polyclonal GVD antisera can be overcome by using high-titre monoclonal antibodies in mouse ascitic fluids. At least 4 of 6 MAbs produced, all of which were GVD-specific, could reliably be used in ELISA (especially Mab D8) for the detection of this virus. Although MAbs did not give satisfactory results when used for plate coating, possibly because absorption to polystyrene reduced their activity (MARTIN and STACE-SMITH 1984),

an efficient ELISA protocol was developed, in which a polyclonal antiserum was used for antigen trapping, and MAbs for virus detection. This procedure enabled to carry out large-scale surveys of field-grown vines, with a much higher level of confidence than afforded by previous tests in which mechanical transmission to herbaceous hosts, serology or molecular probes had been employed (ABOU GHANEM *et al.* 1997).

The present survey has shown a surprisingly high incidence (approx. 31 %) of GVD infections, regardless of the geographical origin of the grapevine accessions. These data differ from those obtained by molecular assays (ABOU GHANEM *et al.* 1997), where GVD was detected only in 4 % of 218 accessions with rugose wood symptoms. The erratic distribution of GVD in grapevine tissues may have limited the number of positive detections by PCR since extracts from small amounts of tissue are assayed with this test. In this study, the problem was overcome by repeating ELISA at least three times for each single sample, which consisted of pieces from different cuttings mixed together.

The association of GVD with rugose wood (BONAVIA *et al.* 1996; ABOU GHANEM *et al.* 1997) was observed also in the present survey, but no conclusive evidence was obtained as to a specific involvement in any of the syndromes of the complex. By contrast, the close association was confirmed between GVA and KSG, GVB and CB, and GRSPaV and RSP, which had suggested aetiological relationships (BOSCIA *et al.* 1993; CHEVALIER *et al.* 1993; GARAU *et al.* 1994; BONAVIA *et al.* 1996; MENG *et al.* 1999).

The possible interference in the expression of stem grooving symptoms in Kober 5BB and of stem pitting symptoms in *Vitis rupestris* when vitiviruses and foveaviruses are contemporarily present is intriguing but unexplained, and requires further investigation.

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