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Introduction of Grapevine virus B and Grapevine leafroll-associated virus 2 testing in sanitary selection of grapevine

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ABSTRACT

To introduce testing of Grapevine leafroll-associated virus-2 (GLRaV-2) and Grapevine virus B (GVB) in sanitary selection of grapevine, commercially available antibodies were evaluated and conditions for routine ELISA testing were optimized. Extraction procedure with Granex 91 - special machine, which is used in routine testing in Slovenia, was compared with grinding samples in mortar. Three different extraction buffers were applied in order to overcome the inconvenience of using more than one extraction procedure when testing grapevine material for several viruses in a routine large-scale testing scheme. Results were verified with Western blot and immuno-electron microscopy. The best results were obtained using extraction buffer with unknown composition (pH 9.0) from BIOREBA kit for GLRaV-2. Other extraction buffers gave less positive samples and they are not convenient for routine testing where extraction with Granex is done. Both viruses, GLRaV-2 and GVB were found in Slovenia, but they couldn't be correlated with rougose wood disease that appears on indigenous cultivar Refošk grafted on 'SO4' from collection vineyard in Komen.

Key words: grapevine, selection, Grapevine virus B, Grapevine leafroll-associated virus-2

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IZVLEČEK

UVEDBA TESTIRANJA GRAPEVINE VIRUS B IN GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2 V ZDRAVSTVENO SELEKCIJO VINSKE TRTE

Z namenom, da bi uvedli testiranje virusa Grapevine leafroll-associated virus-2 (GLRaV-2) in virusa Grapevine virus B (GVB) v zdravstveno selekcijo vinske trte, smo testirali komercialno dostopna protitelesa in optimizirali pogoje za rutinsko testiranje v ELISA. Primerjali smo ekstrakcijo s strojem Granex 91, ki ga v Sloveniji uporabljajo v rutinskem testiranju, z ekstrakcijo v terilnici. Da bi poenotili ekstrakcijo različnih virusov v obsežnem rutinskem testiranju, smo preizkusili tri različne ekstrakcijske pufre. Rezultate smo preverili z imunskim pivnikom (Western blot) in imunsko elektronsko mikroskopijo. Najboljše rezultate smo dobili pri vzorcih ekstrahiranih s pufrom nepoznane sestave iz kita za določevanje GLRaV-2 proizvajalca BIOREBA. Z ostalima pufroma smo dobili manj pozitivnih vzorcev, zato menimo, da pufra nista primerna za ekstrakcijo vzorcev v rutinskem testiranju kjer se uporablja stroj Granex 91. Ugotovili smo prisotnost obeh virusov v Sloveniji, nismo pa uspeli dokazati povezave teh dveh virusov z razbrazdanjem lesa, ki se pojavlja na trsih domače sorte Refošk cepljenih na podlago 'SO4' iz kolekcijskega vinograda v Komnu.

Ključne besede: vinska trta, selekcija, Grapevine virus B, Grapevine leafroll-associated virus-2

1 INTRODUCTION

The International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG), recognizes over 70 infectious agents affecting grapevine (viruses, viroids and phytoplasmas) (ICVG, 2003). Many of them cause disorders that reduce the plant vigour and longevity or the quality and quantity of the yield. Infected propagating material is largely responsible for the spread of these diseases among and within viticulture regions. Certification of grapevine nursery stock is a powerful and effective tool to control these agents, that enables vineyards to economically and sustainably maintain quality and productivity.

According to EU directive (Council Directive 68/193/EEC) the presence of harmful organisms which reduce the usefulness of the propagation material shall be at the lowest possible level. The tehnical annex to the directive interpret this legislation as the absence of Complex of infectious degeneration (Grapevine fanleaf virus (GFLV) and Arabis mosaic virus (ArMV)), Grapevine leafroll disease (Grapevine leafroll-associated virus 1 (GLRaV-1) and Grapevine leafroll associated virus 3 (GLRaV-3)) and Grapevine fleck virus (GFkV) (only for rootstocks) (The Council of the European Communities, 1968). Additionally ICVG recommends that propagation material should be controlled on the agents that are associated with infectious degeneration and grapevine decline (nepoviruses), leafroll disease and associated closteroviruses (grapevine leafroll associated viruses 1, 2, and 3), rugose wood (Grapevine virus A, B and D (GVA, GVB, GVD)) and phytoplasmas (grapevine yellows) (ICVG, 2003).

In Slovenia grapevine selection and clone multiplication started after Second World War. The required tests were made to meet the European grapevine certification program but in order to assure better propagation material the recommendations of ICVG were also considered (Korošec-Koruza et al, 1998; Walter and Martelli,

1997). With the purpose to introduce GLRaV-2 and GVB testing to the certification scheme the optimization of ELISA procedure was done.

Both viruses, GLRaV-2 and GVB, are associated with rugose wood disease in which four different disorders participate, i.e., corky bark (CB), rupestris stem pitting (RSP), Kober stem grooving (KSG) and LN 33 stem grooving. Individual disorders can be distinguished on the basis of the differential reactions of *Vitis* indicators (Goheen, 1988; Bonavia et al. 1996; Credi, 1997).

Rugose wood is worldwide disease and it was recorded also in Slovenia. In selection vineyard of cv. 'Refošk', an old indigenous variety, 15 % of vines show rugose wood symptoms (Tomažič et al., 2005). Old indigenous varieties were not commercially interesting in the past and were not included in any type of selection. Consecutively, they are often heavily infected with viruses.

GLRaV-2 was first purified from a corky bark-affected grapevine and was designated as Grapevine corky bark associated virus (GCBaV) (Namba et al., 1991). GCBaV was later identified as identical to the GLRaV-2 isolate from France (Zimmermann et al., 1990; Boscia et al., 1995). Bonavia et al. (1996) found close relationship between corky bark disease and GVB, but not with GLRaV-2. GLRaV-2 is rather involved in leafroll symptoms, graft incompatibility and in quick decline of newly replanted vineyards (Pirolo et al., 2006).

The purpose of this study was to introduce the ELISA for detection of GVB and GLRV-2 into routine testing of grapevine and to find out correlation of GLRaV-2 and GVB with rugose wood on cv. 'Refošk' grafted on 'SO4' (*V. riparia* × *V. berlandieri*).

2 MATERIAL AND METHODS

Plant material. Samples were collected from selection vineyard of cv. Refošk in Komen – Karst region and from two grapevine germplasm collections Brda and Vipava in Primorje winegrowing region of Slovenia. Selection vineyard of Refošk was established in 1991. 76 old Refošk vines were chosen as mother plants for selection vineyard and potential clone material according to their specific, potentially interesting production characteristics and good visual sanitary status. Vines were grafted on 'SO4' rootstocks. In 1999 all of the 1680 vines from selection vineyard were visually inspected for rugose wood symptoms. 15 % (253) of vines showed rugose wood symptoms on rootstocks or on scion. Rugose wood disease could not be associated with the presence of GVA (data not shown). Vines from germplasm collections in Brda and Vipava were selected because they are heavily infected with different viruses. The collections were planted only to preserve the old indigenous cultivars and were not visually selected. Germplasm collections Brda and Vipava include 48 old cultivars. In 1999 dormant canes and leaves were collected for testing.

ELISA: For detection of GLRaV-2, two different procedures and antisera were used: 1) standard double antibody sandwich ELISA (DAS-ELISA) (Clark and Adams, 1977) was used for the detection of GLRaV-2 with specific antiserum produced by Agritest (Italy) and 2) indirect PTA (plate trapping assay) ELISA was adopted for the detection of GLRaV-2 with antiserum produced by Bioreba (Switzerland) (Lommel *et al*., 1982; Kai-Shu *et. al*. 2007). To determine which tissue is better for ELISA testing of GLRaV-2, grapevine leves and cane phloem were pulverized in liquid nitrogen and analyzed using the Bioreba detection kit.

For detection of GVB in double antibody sandwich indirect (DAS-I) ELISA (Boscia *et al.*, 1997), GVB specific antiserum from Agritest was used. Reactions were evaluated by measuring the absorbance at 405 nm. Absorbance was measured several times during the incubation with a substrate.

In order to overcome the inconvenience of using more than one extraction procedure when testing grapevine material for several viruses in a routine large-scale testing scheme, three different extraction buffers were compared:

1) Extraction buffer with unknown composition (pH 9.0) from Bioreba kit for GLRaV-2;
2) 0.5 M Tris extraction buffer (pH 8.2) containing 0.8 % NaCl, 2 % PVP (MW 24000), 1 % PEG (MW 6000), 0.02 % NaN_3 and 0.05 % Tween 20 from Bioreba;
3) 0.2 M Tris extraction buffer (pH 8.0) containing 0.8 % NaCl, 2 % PVP, 0.001 % Merthiolate and 0.05 % Tween 20 from Agritest.

The extracts were prepared mechanically from mature canes with Granex 91, special machine used in routine testing, which enables us to prepare up to 1500 samples per day.

Western blot: Phloem tissue from green shoots collected in August was ground in liquid nitrogen and homogenized with ELISA extraction buffer 2 containing 20 mM protease inhibitor phenylmethyl-sulfonyl-flouride and 0.2 % β-mercapto-ethanol (2-Me). After centrifugation (5 min at 6,000×g) samples were mixed with an equal volume of loading buffer (125 mM Tris-HCl pH 6.8, 20 % glycerol, 4 % SDS, 10 % 2-Me and 0.1 % Coomassie Brilliant Blue R-250) and denatured in boiling water for 6 minutes. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done in 5 % stacking and 12 % resolving polyacrylamide gels at a constant voltage of 75 V for two hours using Trans Blot Mini Cell (Bio Rad). A Kaleidoscope Prestained Standard (Bio Rad) was used for molecular weight determination. Proteins were electro-transferred to polyvinylidene difluoride membrane (Bio-Rad) for 1 h at 100 V. Membranes were blocked for 30 min with PBS pH 7.4 (0.8 % NaCl, 0.02 % KH_2PO_4, 0.115 % Na_2HPO_4, 0.02 % KCl) containing 0.05 % Tween-20 and 10 % nonfat dry milk. Virus specific IgG diluted 1:1000 (Bioreba) or 1:500 (alkaline phosphatase conjugated - Agritest) were added individually to the blocking buffer and incubated at 4 °C overnight. The membrane was washed three times for 15 min with PBS containing 0.05 % Tween-20. In case of using Bioreba antiserum, the membrane was incubated for 2 h with alkaline phosphatase conjugated anti-mouse antibody and washed as described above. Immuno-reactive proteins were visualized using NBT/BCIP development solution (Bio Rad).

Immuno-electron microscopy. Immuno-electron microscopy (IEM) was used to confirm the presence of GLRaV-2 and GVB in plants. The following antisera were used for IEM:
GLRaV-2 - Agritest (GLRaV-2-Agr), Bioreba (GLRaV-2-Bio), antiserum 2/16/3 donated by D.E. Goszczinsky (GLRaV-2-Gos) (Agricultural Research Council, Plant Protection Research Institute, Pretoria, Republic of South Africa),
GVB - Agritest, antiserum GVB 33-I donated by D.E. Goszczinsky (Agricultural Research Council, Plant Protection Research Institute, Pretoria, Republic of South Africa),

Phloem of dormant canes or green shoots was homogenized in 0.1 M phosphate buffer pH 7 with 2 % PVP MW 40K. Carbon-coated Formvar-filmed grids were incubated on antiserum diluted 1:1000 for 5 minutes, rinsed with phosphate buffer and incubated on plant sap extracts for 1 hour. After rinsing, grids were incubated on antiserum diluted 1:50 for 15 minutes, rinsed with distilled water, negatively stained with 1 % uranyl acetate, and viewed in a transmission electron microscope (Philips CM100).

3 RESULTS AND DISCUSSION

Detection of GLRaV-2: Positive samples from cortical scrapings grinded in mortar with buffer 1 (as recommended by antiserum producer) gave strong reaction with Bioreba antiserum. Samples are clearly divided in group of positive and group of negative samples (Table 1). Negative samples have low OD value even after 15 hours; for example OD of ‘Refošk’ 13 II/27 was 0.283 while OD of positive

samples still increase (OD of 'Sevka' IV/51 was 1.898). When samples were prepared from leaves the background was higher (after 5 hours OD of 'Refošk' 13 II/27 was 0.268, OD of 'Sevka' IV/51 was 0.548) and it was difficult to determinate threshold value, which discriminates positive results from background (Fig. 1).

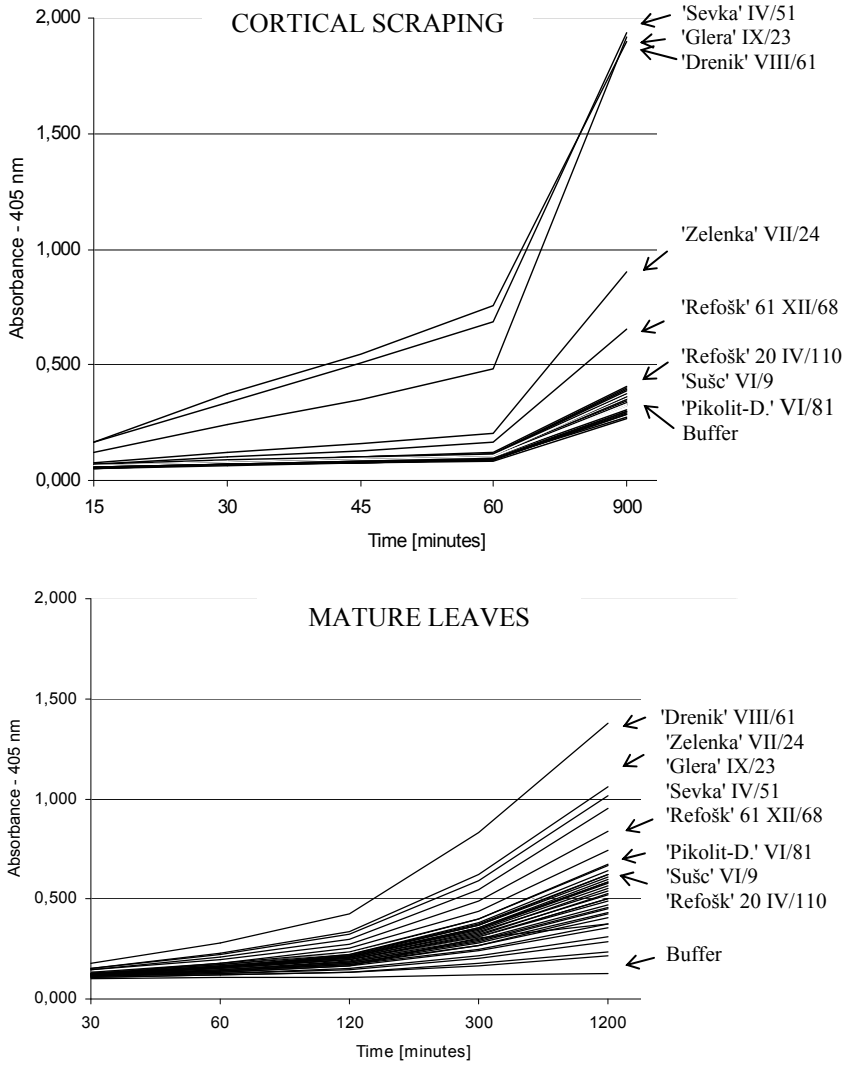

Fig. 1: Change of absorbance at 405 nm over time of the incubation with substrate in enzyme-linked immunosorbent assay (ELISA) when samples were prepared from cortical scraping in comparison with those from leaves. Each line represents one sample (all together 45 samples). Grapevine leafroll associated virus-2 Bioreba antiserum was used.

Western blot confirmed ELISA results (Fig. 2). GLRaV-2-Gos decorated virus particles well (Fig. 4) and they confirmed the presence of GLRaV-2 in samples of 'Klarnica' V/3, 'Sevka' IV/51, 'Zelenka' VII/24 and 'Refošk' 61 XII/77. In IEM GLRaV-2-Bio decorated only damaged parts of virus particles from 'Klarnica' V/3 and 'Sevka' IV/51 (Fig. 3). GLRaV-2-Agr gave no positive result in IEM or Western blot.

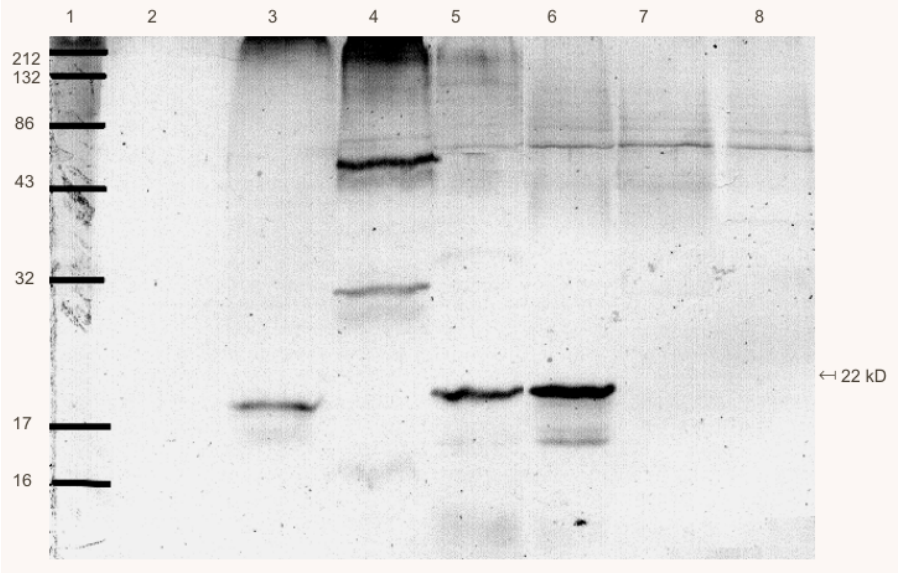

Figure 2: Detection of GLRaV-2 in grapevines by Western blot using the Bioreba antiserum. The samples are: line 2, positive control for GVB from Agritest; line 3, positive control for GLRaV-2 from Agritest; line 4, positive control for GLRaV-2 from Bioreba; line 5, 'Klarnica' V/3; line 6, 'Glera' IX/23; line 7, 'Pikolit-D' VI/81; line 8, 'Sušc' VI/9. The 22 kD coat protein of GLRaV-2 is indicated by arrow.

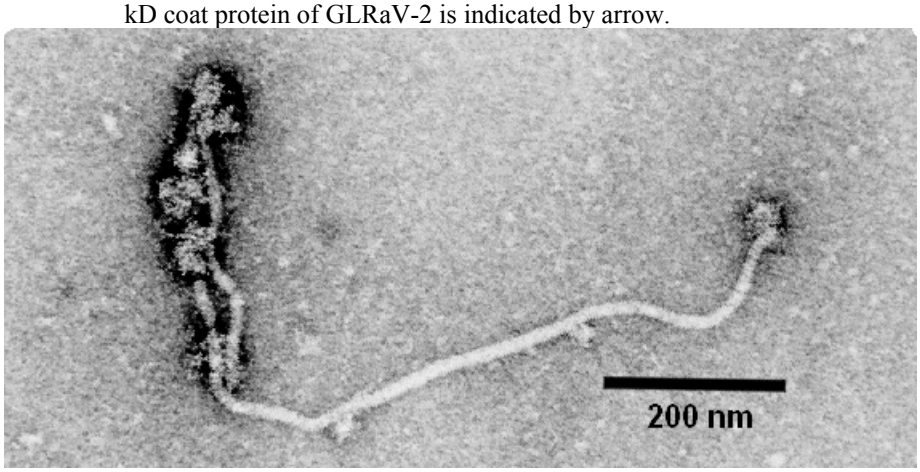

Figure 3: Electron microscopy of GLRaV-2 virus particles decorated with antiserum from Bioreba.

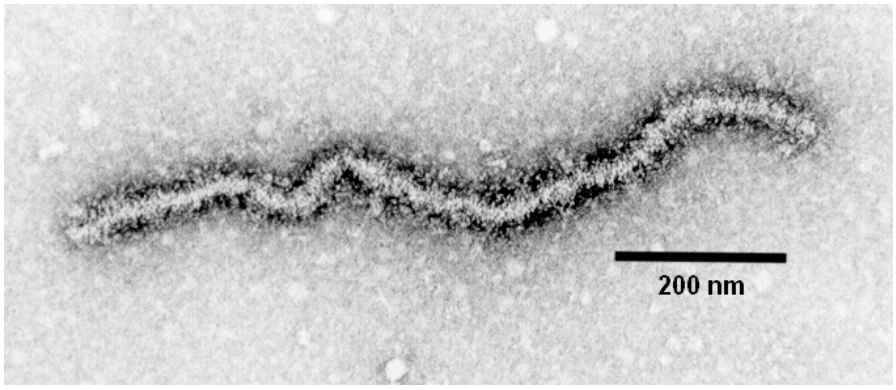

Figure 4: Electron microscopy of GLRaV-2 virus particles decorated with antiserum from Goszczynski.

To introduce GLRaV-2 testing in sanitary selection of grapevine the possibility of extraction with special machine Granex 91 was evaluated. Three different extraction buffers were used with antisera from Bioreba and Agritest. With Bioreba antiserum the same samples were positive when they were prepared in mortar or with Granex 91 with buffer 1 (Table 1), but reactions of positive samples were weaker and slower. Buffer 2 and 3 gave less positive results. Agritest antisera gave no positive results when sample extraction was done with buffer 1 and 2 and Granex 91 was used. Some samples which reacted positively in Western blot and IEM failed to be detected by ELISA when buffer 3 and the Agritest antiserum was used (Table 1).

Among 210 tested vines only nine vines were positive for GLRaV-2 and only two of them were cv. 'Refošk'. Infected vines showed no rugose wood symptoms. Rugose wood disease on the rootstocks 'SO4' or the grafted *vinifera* – 'Refošk' part of vine could not be correlated with GLRaV-2.

Detection of GVB: Two positive samples were found when samples were prepared in mortar using antiserum from Agritest and extraction buffer 2 (Table 2). Western blot confirmed ELISA results, but no particles were found in IEM when using the same antiserum from Agritest. However, GVB specific antiserum donated by D.E. Goszczynski (Goszczynski *et al.,* 1997) confirmed virus infections of the same two samples in IEM (Fig. 5). Using extraction with Granex 91 and buffer 2 or 3, we could not find any infected GVB sample. Extraction with Granex 91 and buffer 1 gave the same results as extraction in mortar with buffer 2.

Table 1: A comparison of Bioreba and Agritest antiserum and different extraction buffers for detection of Grapevine leafroll-associated virus 2 (GLRaV-2) in ELISA.

Grapevine accession / method	ELISA – I/H[a]							Western blot	IEM	RW symptoms in vineyard
antiserum	Bioreba				Agritest			Bioreba	2/16/3	
extraction buffer	Mortal	Granex 91			Granex 91					
	1[b]	1	2	3	1	2	3	2		
'Klarnica' V/3	5.8	2.3	0.9	1.0	1.7	1.1	1.3	+[c]	+	-
'Sevka' IV/51	6.7	2.7	1.3	1.4	1.4	1.0	2.3	+	+	-
'Glera' IX/23	6.7	3.0	1.4	0.9	1.2	1.0	2.1	NT	+	-
'Zelenka' VII/24	4.7	1.3	1.1	1.0	0.9	1.0	1.2	+	+	-
'Refošk' 61 XII/77	2.9	1.5	1.7	1.2	1.5	1.1	1.2	+	+	-
'Refošk' 13 III/27	0.9	0.9	1.0	0.9	1.0	1.0	1.0	NT	-	+
'Refošk' 20 IV/110	1.0	1.1	1.0	1.2	1.0	1.0	1.0	NT	-	+
'Refošk' 38 VIII/44	1.1	1.0	0.9	0.9	1.0	0.9	1.0	NT	-	+

[a] Values of OD_{405} were measured 60 minutes after adding the substrate *p*-nitrophenyl phosphate when samples were prepared in mortal and after 150 minutes when they were prepared with special machine Granex 91. The I/H (infected/healthy) ratios of tested samples were calculated based on the OD_{405} reading of each accession versus last five samples that were negative in Western blot assays.

[b] Three different extraction buffer were used in ELISA: 1 - special buffer for GLRaV-2 from Bioreba with unknown composition (pH 9.0); 2 - 0.5 M Tris pH 8,2 extraction buffer containing 0.8 % NaCl, 2 % PVP (MW 24000), 1 % PEG (MW 6000), 0.02 % NaN_3, 0.05 % Tween 20; 3 - 0.2 M Tris pH 8,0 extraction buffer containing 0.8 % NaCl, 2 % PVP, 0.001 % Merthiolate, 0.05 % Tween 20).

[c] + = presence of the decorated virus particle in IEM or the protein band reacted in Western blot; - = absence of the decorated virus particle in IEM or the protein band reacted in Western blot; NT = not tested.

Table 2: A comparison of different extraction buffers for detection of GVB with Agritest antiserum in ELISA.

Grapevine accession / method	ELISA – I/H[a]				Western blot	IEM	RW symptoms in vineyard
antiserum	AGRITEST				AGRITEST	33-I	
extraction buffer	Mortal	Granex 91					
	2[b]	1	2	3	2		
'Klarnica' V/3	2.2	1.8	1.1	1.1	+[c]	+	-
'Sevka' IV/51	7.2	2.0	1.0	1.1	+	+	-
'Glera' IX/23	0.9	1.0	1.0	1.0	-	NT	-
'Zelenka' VII/24	0.9	1.0	1.0	1.0	-	NT	-
'Refošk' 61 XII/77	1.1	1.0	1.0	1.0	-	NT	-
'Refošk' 13 III/27	0.8	0.9	1.0	1.0	-	NT	+
'Refošk' 20 IV/110	0.9	1.0	1.0	1.1	-	NT	+
'Refošk' 38 VIII/44	1.3	0.9	0.9	0.9	-	-	+

[a] Values of OD_{405} were measured 120 minutes after adding the substrate *p*-nitrophenyl phosphate when samples were prepared in mortal and after 260 minutes when they were prepared with special machine Granex 91. The I/H (infected/healthy) ratios of tested samples were calculated based on the OD_{405} reading of each accession versus those samples that were negative in Western blot assays.

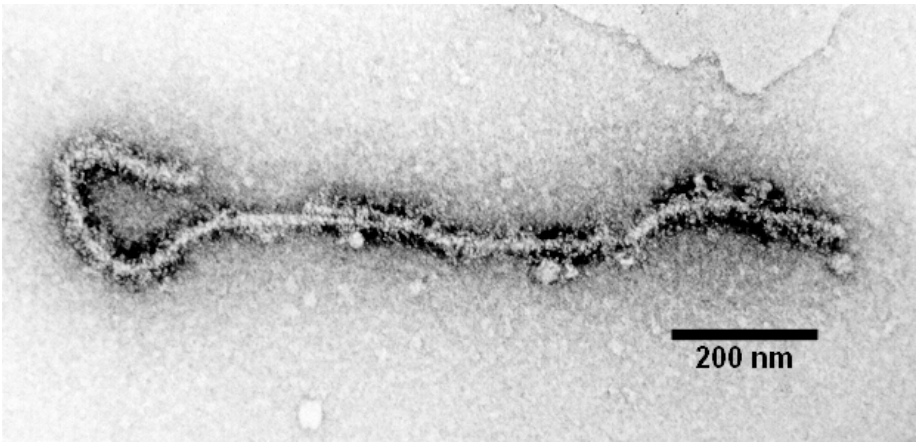

Figure 5: Electron microscopy of GVB virus particles decorated with antiserum from Goszczynski.

Among all tested vines only two vines were positive for GVB. On cv. 'Refošk' we didn't find any positive samples. Thus rugose wood disease on cv. 'Refošk' grafted on 'SO4' couldn't be correlated with GVB infection.

4 CONCLUSIONS

We found GVB and GLRV-2 in Slovenian indigenous vines using ELISA, Western blot and/or IEM.

The results of ELISA testing showed that extraction is very important step in ELISA. When samples were prepared in mortar only phloem was used while Granex 91 cut all cane in small pieces. Since the concentration of viruses is higher in phloem weaker reaction with Granex 91 is expected. Among the different buffers used for extraction with Granex 91, buffer 1 gave the best results for extraction of GLRaV-2 and GVB. Unfortunately this buffer didn't give good results in extraction of Grapevine flack virus (GFkV), Grapevine virus A (GVA) and Grapevine virus 1 (GLRaV-1) (data not shown), therefore it is not possible to use one universal buffer for extraction of all viruses.

GLRaV-2 antibodies from Agritest gave relatively weaker reactions than antibodies from Bioreba when samples were prepared with Granex 91.

Extraction of GLRaV-2 and GVB with Granex 91 is not convenient for use in sanitary selection since it is not sensitive enough and doesn't prevent propagation of virus-infected vines into new vineyards.

Infection with GLRaV-2 and GVB could not be correlated with rugose wood on cv. 'Refošk' grafted on 'SO4' (*V. riparia* × *V. berlandieri*).

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