Phytopathologia Mediterranea (2012) 51, 1, 85–90

Short Notes

Viruses of grapevine in Kosovo

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Summary. Surveys for virus and virus-like diseases were carried out in commercial vineyards in the provinces of Rahovec and Suhareka of Kosovo. Samples were collected at random from 306 individual vines for laboratory testing. Leafroll and rugose wood symptoms were commonly observed in the field, whereas fanleaf symptoms were very rare. A total of 68% of ELISA-tested vines (208 out of 306) was infected by one (40.9%) or multiple (27.1%) viruses. The highest infection rate was found in Suhareka province (75%). *Grapevine fleck virus* (GFkV) was the most widespread (52.0%), followed by *Grapevine leafroll-associated virus 3* (GLRaV-3, 18.3%), *Grapevine leafroll-associated virus 1* (GLRaV-1, 15.7%), and *Grapevine virus A* (GVA, 11.1%). Other economically relevant viruses occurred rarely, i.e. *Grapevine fanleaf virus* (GFLV, 1.6%), *Grapevine virus B* (GVB, 1.0%) and *Grapevine leafroll-associated virus 2* (GLRaV-2, 0.3%). *Arabis mosaic virus* (ArMV) was not found. Some of the most important grapevine varieties cultivated in Kosovo, i.e. Smederevka, Vranac, Prokupac, Italian Muskat, Muskat of Hamburg, and Italian Riesling, had average infection rates that ranged from 63% to 85%. In RT-PCR, *Grapevine rupestris stem pitting associated virus* (GRSPaV) was detected in 80.4% of the vines. Vein mosaic and vein necrosis symptoms were detected in graft-inoculated *V. riparia* and 110R indicators. At least one virus-tested candidate clone of 19 different grapevine varieties were identified, that could represent a potential primary source for a grapevine certification program in Kosovo.

Key words: certification programme, ELISA, RT-PCR.

Introduction

The grapevine industry is strategic for the agricultural development of Kosovo. In this country vineyards cover approximately 3,133 ha (MOAFARD, 2011), which is less than half of the total surface that was grown in the 1980s. Grapevine production is concentrated in the provinces of Rahovec (70%) and Suhareka (21%) (MOAFARD, 2011), with wine grapes prevailing over table grapes (4:1 ratio). Smederevka, Vranac, Game e Thjeshtë, Prokupac, Italian Muskat, Muskat of Hamburg and Italian Riesling are the main cultivars, representing approximately 80% of the total national grapevine crops. Numerous new varieties are being introduced from abroad in recent years, with little attention paid to their adaptability to local conditions and sanitary status. This uncontrolled introduction of vines exposes the country to the risk of epidemics by new pathogens.

This situation and the scarcity of information on the sanitary status of grapevines in Kosovo has prompted an investigation for virus diseases, and the present paper reports this study. The information presented is the first record of the sanitary status of grapevine in Kosovo.

Materials and methods

Field surveys

Field surveys were carried out in October 2009 and May 2010 in the main grapevine- growing regions of Kosovo. During the first survey, mature canes 40–50 cm in length, collected at random from individual vines, were placed in plastic bags and stored at 4°C before testing. A total of 306 vines

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ISSN (print): 0031-9465
ISSN (online): 1593-2095

were sampled from 31 commercial vineyards (22 in Rahovec and nine in Suhareka provinces). Samples were from the prevailing and promising cultivars grown in these areas, i.e. Vranac (18.3% of the samples), Italian Muskat (11%), Smederevska (10%), Pinot noir (10%), Cardinal (6.5%), Prokupac (6.5%), Game e Thjeshtë (5.6%), Muskat of Hamburg (5.2%), Afuzali (3.3%), Italian Riesling (3.3%). The other samples (20%) were from minor varieties.

Serological tests

The following ELISA protocols were used for virus detection: (i) DAS-ELISA for *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine leafroll associated virus -1, -2 and -3* (GLRaV-1, GLRaV-2 and GLRaV-3) (Clark and Adams, 1977); (ii) TAS-ELISA for *Grapevine fleck virus* (GFkV) and *Grapevine virus B* (GVB) (Al Moudallal *et al*., 1984); and (iii) Protein-A (A-DAS ELISA) for *Grapevine virus A* (GVA) (Boscia *et al*., 1992). Serological reagents were commercially available (Agritest, Valenzano-Bari, Italy). Cortical scrapings from mature canes were used as material for testing.

Biological assays

Mechanical transmissions were made from 114 accessions (approximately 40% of the total), mainly selected among the ELISA-negative samples. Cuttings were forced in a greenhouse at 22–24°C. Leaf tissues from newly developed shoots were ground in 0.1 M phosphate buffer, pH 7.2, in the presence of 2.5% nicotine, then inoculated onto a range of herbaceous hosts including species of the families Chenopodiaceae (*Chenopodium quinoa* and *C. amaranticolor*), Cucurbitaceae (*Cucumis sativus*) and Solanaceae (*Nicotiana benthamiana, N. occidentalis, N. tabacum* cv. White Burley). Three repetitions of inoculation onto each indicator species were used for testing all selected samples. Inoculated plants were grown in a greenhouse at 22–24°C for observation of symptoms.

Thirty grapevine samples, negative in ELISA and in mechanical transmission tests, were graft-inoculated during April by chip budding onto groups of five plants of *V. riparia* and 110 R indicators. Grafted plants were maintained in a greenhouse at 22–24°C for observation of symptoms.

Molecular assays

The presence of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) was assessed using nucleic acid-based techniques. In particular, RT-PCR was utilized for 46 ELISA-negative samples, representative of the different grapevine cultivars grown in Kosovo, using primers (RSP48 5′-AGC TGG GAT TAT AAG GGA GGT-3′; RSP49 5′-CCA GCC GTT CCA CCA CT AAT-3′) (Osman and Rowhani, 2006).

Total nucleic acid extraction

Total nucleic acids (TNA) were extracted from 200 mg of grapevine cortical scrapings homogenised in 1 mL grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc pH 5 and 2.5% w/v PVP-40) and purified using silica particles according to Foissac *et al.* (2001).

Virus cDNA synthesis

Eight to 10 µL of TNA extract were mixed with 0.5 µL random hexamers primer (Boehringer Mannheim, Mannheim, Germany) (0.5 µg μL^{-1}) and 2 µL of sterile water, denatured at 95°C for 5 min and kept in ice for 3 min. Reverse transcription was carried out for 1 h at 39°C in 1 µL (200 Units μL^{-1}) of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 4 µL buffer 5× (50 mM tris-HCl pH 8.3, 75 mM KCl, 3 µM $MgCl_2$), 2 µL of 100 mM DTT and 0.5 µL of 10 mM dNTPs. A final step for enzyme denaturation was conducted at 70°C for 10 min.

PCR

cDNA mixtures (2.5 µL) were submitted to PCR amplification in 2.5 µL of 10× *Taq* polymerase buffer (Promega Madison, USA), 1.2 µL of 25 µM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 0.5 µL of 10 µM primer 48d and 49d (anti-sense) and 0.2 µL of *Taq* polymerase (5 U μL^{-1}) in a final volume of 25 µL. The cDNA amplification was carried out in a Perkin Elmer Cetus Thermal Cycler apparatus with 35 cycles, after an initial denaturation at 94°C for 5 min. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec. Final elongation was carried out at 72°C for 7 min.

Electrophoresis

PCR products (7–10 µL) were electrophoresed in 6% polyacrylamide-TBE gel and visualized by silver nitrate staining. Amplicon size was determined by comparison with DNA XIV or *Eco*R1/*Hind*III markers (Promega, Madison, WI, USA).

Results

Field surveys

Fanleaf disease was rare and occurred mainly in its chromogenic form (yellow mosaic), while leafroll and rugose wood symptoms were more frequently observed in the surveyed vineyards, especially on cv. Molldavka, Smederevka and Prokupac.

Serological tests

Of the 306 samples individually tested by ELISA for eight viruses, 208 (68%) proved to be infected by one (40.9%) or multiple (27.1%) viruses. GFkV was the most widespread virus (52.0%) which occurred in almost all vineyards, with highest infection rates of 85.7% in cv. Chardonnay and 82.4% in Italian Muskat. GLRaV-3 ranked as the second most common virus, with an average incidence of 18.3%. Detection of this virus was particularly common in some of the most widespread cultivars, i.e. Smederevka (30%), Italian Muskat (29.4%) and Vranac (28.1%). The incidence of this virus was low in samples from Suhareka province (2.8%). The infection rate by GLRaV-1 was also significant (15.7%), in particular in the local cultivars Frankovke (100%), Molldavka (42.9%), Smederevka (36.7%) and Zhametna (33.3%). GVA had average incidence of 11.1%, with a prevalence in the Suhareka province (33.3%). Incidence of this virus was particularly high in the local cv. Frankovke (100%) and Molldavka (71.4%), even though only a few accessions were tested, and in the table grape cv. Cardinal and Afuzali with an infection rate of 30% each. Incidence was low for the other viruses tested, i.e GFLV, GLRaV-2 and GVB. Similarly, incidence of GFLV was low, and ArMV was not detected, which are both nematode-borne nepovirus agents of infectious degeneration of grapevine (Martelli, 1993). Results of serological tests are summarized in Table 1.

Virus incidence in different grapevine varieties ranged from 33.3% (cvs. Groqank, Demir kapi and Cabernet sauvignon) to 100% (cv. Frankovke and Molldavka). Incidence was high in cv. Italian Muskat (85.3%), Smederevka (83.3%), Muskat of Hamburg (75%), Prokupac (65%) and Vranac (63.2%) (Figure 1).

Biological assays

Transmission of viruses by mechanical inoculation was obtained only in two vine accessions which were found positive in the previous ELISA tests, and used as positive controls for this assay. Based on the symptoms shown by infected herbaceous hosts (*N. occidentalis* and *C. quinoa*) and ELISA assays made with extracts from symptomatic plants, the virus isolated was identified as GFLV.

In five 110R plants graft-inoculated with ELISA-negative materials from cvs. Vranac (2), Demir kapi, Game e Thjeshtë and Prokupac the first symptoms of vein necrosis began to appear at the end of May and became very clear in July. All the five VN-symptomatic vines were found positive for GRSPaV in RT-PCR, further confirming the association of this virus (or of some strains) to vein necrosis disease (Bouyahia *et al*., 2005; Mslmanieh *et al*., 2006a).

Vein mosaic and vein clearing symptoms, sometimes accompanied by rolling and deformation of the leaves, were observed in young leaves of *V. riparia*, a few weeks after grafting with infected buds from vines of cv. Vranac, Game e Thjeshtë, Pinot noir, Italian Muskat and Italian Riesling. With time, symptoms became more evident and severe. No viruses were transmitted from these symptomatic plants to herbaceous hosts and ELISA and PCR assays were negative.

Molecular assays

Of the 46 ELISA-negative samples tested by RT-PCR assays, GRSPaV was found in 37 samples (80.4%), in almost all tested varieties (Afuzali, Cabernet Sauvignon, Cardinal, Chardonnay, Demir Kapi, Gamay, Groqank e eershme, Rahovecit, Merlot, Italian Muskat, Muskat of Hamburg, Pinot blanc, Pinot noir, Prokupac, Italian Riesling, Vranac and Zhametna) (Figure 2).

Discussion

The present study represents a preliminary assessment of the sanitary status, relating to virus pathogens, of the viticultural industry of Kosovo. It showed that the locally grown grapevines, notwithstanding the very high incidence of certain viruses, had an overall sanitary condition less compromised than that reported in other Mediterranean countries (Digiaro *et al*., 2000). Countrywide surveys and sampling showed the presence of two major virus diseases, leafroll and rugose wood, and of some of the related viruses, i.e. GLRaV-1, GLRaV-3, GVA and GRSPaV. In addition, indexing gave positive responses

Table 1. Viruses detected by ELISA in the main grapevine varieties grown in Kosovo.

Cultivars	Samples		Viruses (%)							
	Tested	Infected								
	No	%	GFLV	GFkV	GVA	GVB	GLRaV-1	GLRaV-2	GLRaV-3	ArMV
Afuzali	10	70	-	60	30	-	-	10	-	-
Cabernet Sauvignon	3	33.3	-	-	-	-	-	-	33.3	-
Cardinal	20	85	-	55	30	5	10	-	15	-
Chardonnay	7	85.7	-	85.7	-	-	-	-	-	-
Demir Kapi	6	33.3	-	33.3	-	-	-	-	-	-
Frankovke	3	100	-	-	100	-	100	-	-	-
Gamay	17	41.2	5.9	29.4	-	-	-	-	11.8	-
Groqank	6	33.3	-	16.7	-	-	16.7	-	33.3	-
Merlot	6	50	-	33.3	-	-	-	-	16.7	-
Molldavka	7	100	-	71.4	71.4	-	42.9	-	28.6	-
Italian Muskat	34	85.3	-	82.4	11.8	-	11.8	-	29.4	-
Muskat of Hamburg	16	75	-	62.5	18.8	-	25	-	12.5	-
Pinot blanc	6	66.7	-	66.7	-	-	16.7	-	-	-
Pinot noir	29	55.2	-	55.2	3.4	3.4	6.9	-	-	-
Prokupac	20	65	-	50	-	5	30	-	15	-
Riesling I Raines	8	50	-	50	-	-	-	-	-	-
Italian Riesling	9	66.7	-	66.7	-	-	11.1	-	11.1	-
Smederevka	30	83.3	-	50	13.3	-	36.7	-	30	-
Vranac	57	63.2	3.5	42.1	7	-	14	-	28.1	-
Zhametna	6	83.3	-	66.7	16.7	-	33.3	-	16.7	-
Unknown	6	50	33.3	-	-	-	-	-	50	-
Total	306	68	1.6	52	11.1	1	15.7	0.3	18.3	0
No. of infected plants for each virus			5	159	34	3	48	1	56	0

for vein necrosis and vein mosaic, two graft-transmissible disorders previously unrecorded in Kosovo.

The low incidence of nepoviruses (sporadic records of GFLV and absence of ArMV) tallies with the observed scarcity of fanleaf symptoms in the field, and confirms that these viruses are less important than previously thought in some Mediterranean and near-east countries (Haidar *et al*. 1996; Al-Tamimi *et al*., 1998; Ahmed *et al.*, 2004; Mslmanieh *et al*., 2006b). However, whether the low nepovirus incidence in Kosovar vineyards depends on a limited occurrence of nematode vectors, remains to be established.

Notwithstanding the high infection levels of viruses detected in the vineyards, some accessions of

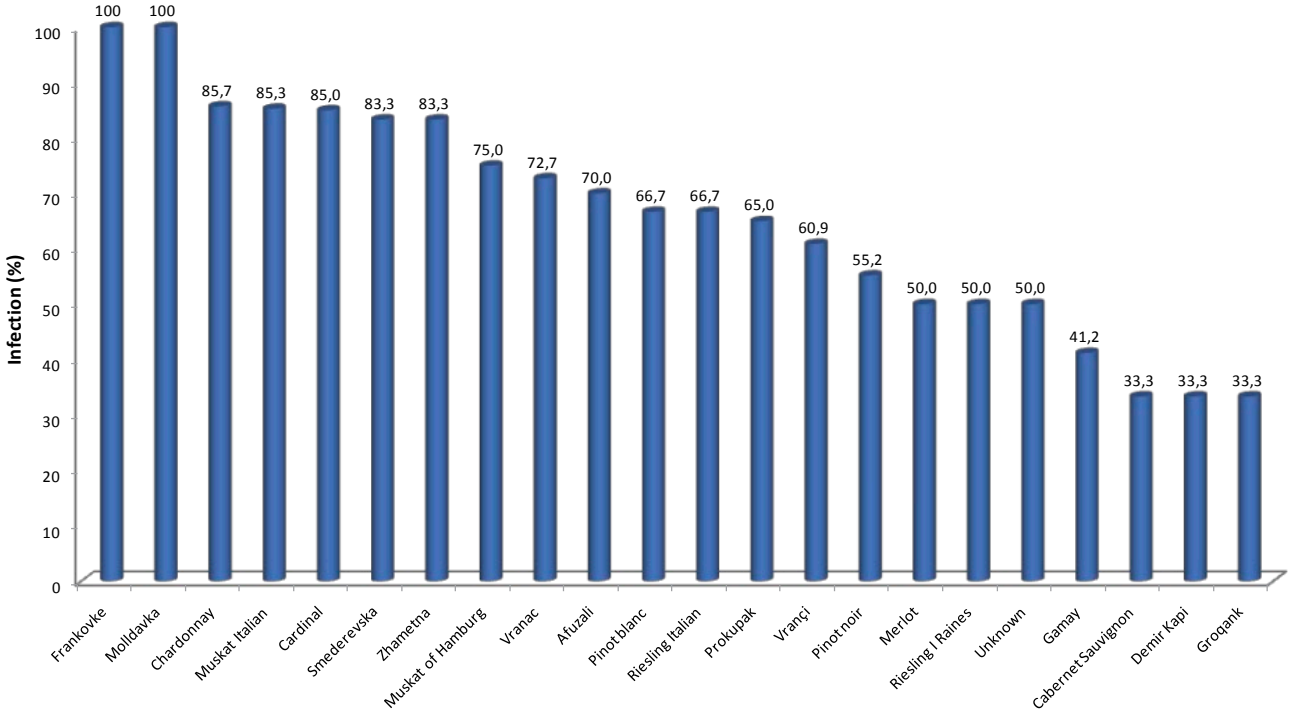

Figure 1. Incidence of viral infections in the main grapevine varieties cultivated in Kosovo, as determined by ELISA tests after analyzing 306 samples.

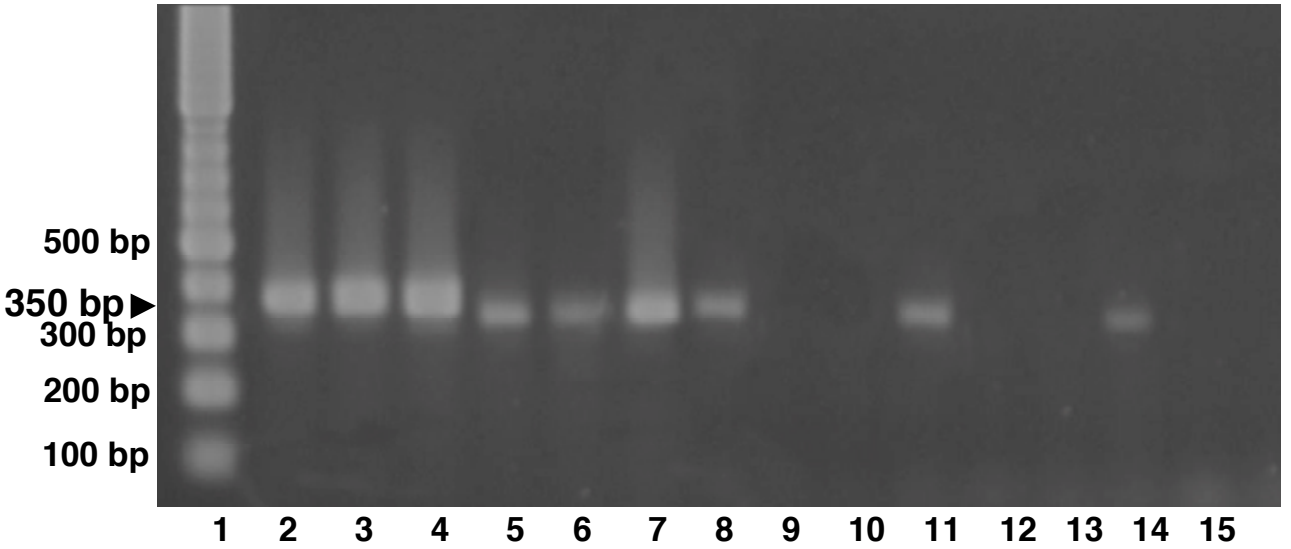

Figure 2. Agarose gel showing PCR products amplified from grapevine samples infected by GRSPaV. Lane 1, DNA ladder marker; lanes 1 to 13, tested grapevines samples (positive reactions in lanes 1–8, 11); lane 14, positive control (vine infected by GRSPaV); lane 15, negative control (healthy vine seedling).

19 different cultivars were identified that possess the minimum sanitary requirements for use as mother plants according to the European Commission Directive 2005/43/EC. These accessions represent potential sources of propagating material for sanitary improvement programs to be implemented in the country.

Acknowledgements

The authors thank Mr. Bala Sylejman and Mr. Morina Nesim, respectively Director and Researcher in the Institute of Wine & Viticulture of Rahovec (Kosovo), for their support during this study. We also appreciate the efforts of Pandeli Pasko for collecting the Kosovarian grapevine material.

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Accepted for publication: August 16, 2011