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Occurrence of grapevine leafroll-associated virus complex in the Republic of Macedonia

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Abstract. *Grapevine Leafroll-Associated Virus Complex is caused by several virus species (grapevine leafroll-associated viruses GLRaV -1, -3, -4, -5, -6, -9 and -10) belonging to the genus Ampelovirus, while GLRaV-2 is assigned to the genus Closterovirus. Because of its increasing economic importance, it is critical to determine which species of GLRaV are predominant in each region in Macedonia where this disease occurs. The laboratory test analyses used in this study consisted of a combination of two detection methods: serological test (DAS-ELISA) and RT-PCR based testing. The total number of 387 grapevine symptomatic samples from 17 regions including 27 localities, were surveyed from 2008 to 2013. All of these samples were tested for GLRaV-1, -2, -3, and -7, by using BIOREBA and SEDIAG DAS-ELISA kits, and the results showed that 55.9% (215 samples) were GLRaV positive. Out of the positive samples, 69.7% (150 samples) were single infections with GLRaV-3, 15.5% were single infections with GLRaV-1, and 14.8% were mixed infections with GLRaV-3 and GLRaV-1. Ten representative positive samples were analyzed with reverse-transcriptase polymerase chain reaction (RT-PCR) tests for GLRaV-1, GLRaV-2 and GLRaV-3. This is the first occurrence of Grapevine Leafroll-Associated Virus Complex, including GLRaV-1, GLRaV-2 GLRaV-3 and GLRaV-7 in the Republic of Macedonia.*

Keywords: *Ampelovirus*, *Closterovirus,* DAS-ELISA, RT-PCR

Introduction

One of the most widespread and economically very important complex of viral diseases on grapevine is leafroll. Grapevine Leafroll-Associated Virus Complex was first recognized around the middle of the 19th century, and since then it has gained a world-wide reputation as the most widely spread and economically important disease on grapevine (*Vitis vinifera* L.). The disease is present in all grape-growing regions of the world, including Europe, South and North America, Middle East, Africa and Oceania (Charles et al., 2009; Fuchs et al., 2009; Habili et al., 1995; Maliogka et al., 2008; Maree et al., 2008; Akbas et al., 2007; Mafoudhi et al., 2008; Fiore et al., 2008). GLRaVs complex produces distinctive symptoms in red and white grapevine cultivars. In red cultivars, affected grapevines with leafroll virus complex show symptoms consisting of green veins and interveinal reddening of mature leaves at the lower section of the canes. On the other hand, white cultivars show mild chlorosis or yellowing. In advanced stages, symptomatic leaves in both types of cultivars show rolling of leaf margins. Diseased grapevines decline slowly and the clusters suffer from berries lacking full color, delayed maturity and reduced sugar (Golino et al., 2002). Wines produced from leafroll affected grapes are inferior in quality (Komar et al., 2007; Lee and Martin, 2009).

This study was undertaken to determine the presence and distribution of grapevine leafroll virus complex in the Republic of Macedonia. The most important strategy to control viral disease in grapes is preventive and consists of planting virus-free vines during vineyard establishment. In our study, grapevine virus detection was based on serological tests (enzyme-linked immunosorbent assay ELISA) and reverse transcription-polymerase chain reaction (RT-PCR).

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Material and methods

Sample collection

The field observation carried out as part of this study showed that the symptoms that could be attributed to grapevine leafroll complex (downward leaf rolling, leaf yellowing/reddening) were detected in all marked vineyard regions in the Republic of Macedonia. For testing, leaves including their petioles and canes were sampled from 387 individual vines. Middle and basal leaf samples were collected from near the bottom portion throughout and growing season starting from July and ending in October.

Grapevine symptomatic samples were surveyed from 2008 to 2013, from 17 regions including 27 localities.

Serological assays

The source grapevine was tested with DAS-ELISA for GLRaV-1,-2,-3 and -7, and with RT-PCR for GLRaV-1, -2 and -3. For DAS-ELISA test detection for GLRaV-1, -3 and -7, a kit obtained from BIOREBA (Reinach, Switzerland) was used following the manufacturer's protocol. For GLRaV -2, polyclonal antibodies produced at SEDIAG, France were used following the manufacturer's protocol. The samples for DAS-ELISA tests were prepared by collecting ten leaf petioles from each vine and extracting them as described above (Rowhani, 1992). Ten different phloem-limited filamentous viruses, identified as Grapevine Leafroll-Associated Virus Complex (GLRaVs) (GLRaV-1 – GLRaV-10), have been isolated and characterized from leafroll infected grapevines (Martelli and Boudon-Padieu, 2006).

RNA extraction

Petioles were stored at -80ºC and total RNA was extracted

using the protocol described in MacKenzie et al. (1997). 5 µl of the dsRNA preparation with 1 µl random primers (50 µM) (Promega) and 1 µl dNTPs (10 mM) were denatured at 90ºC for 5 min, cooled on ice and then used in reverse transcriptase reaction. 20 µl reverse transcription reactions were prepared from 4 µl 5 x M-MLV RT reaction buffer, 1 µl MDTT 0.1 M (40U/µl), 1 µl RNasin (30U/µl) (Promega), 1 µl Super Script III reverse transcriptase. Reverse transcriptase was performed at 25ºC for 5 min, 50ºC for 60min and 70ºC for 15 min. RT was used in 25 µl PCR reaction containing 14.3 µl water, 2 µl $MgCl_2$ (25 mM), 5 µl 5 x buffer, 0.5 µl dNTPs, 0.5 µl each of forward and reverse primers and 0.2 µl Taq DNA Polymerase. The thermal cycling parameters were as follows: 1 cycle at 94ºC for 2 min, next 35 cycles at 94ºC for 30 s, 55ºC for 1 min and 72ºC for 1 min 30 s and a final elongation at 72ºC for 5 min.

PCR products were analyzed with 1% agarose gel (with Midory green visualization color), in 1xTBE buffer and visualized under UV transilluminator.

In order to compare the results, Italian controls were used as positive samples (Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, University of Milan, Italy).

Results and discussion

Symptoms

In almost all infected plants, the symptoms were expressed as leaf reddening, slightly downward leaf rolling and remarkable difference in rootstock and scion diameter (Figure 1a and 1b). Also, on Figure 1 we showed the presence of grapes but they are with small quality and small sugar units. These symptoms were detected in all the collected samples for analysis, but the laboratory tests with DAS-ELISA showed negative results for some of the samples.

GLRaV-1 and GLRaV-3 induced leaf roll and interveinal discoloration on vine leaves.

Vineyards inspected regularly showed considerably fewer symptoms.

Laboratory analyses

The initial screening to determine which GLRaV species were present in the surveyed vineyards detected that Grapevine leafroll associated virus 1 (GLRaV-1) and Grapevine leafroll associated virus 3 (GLRaV-3) are the most important ones as they are present in almost every vineyard in the Republic of Macedonia.

The total number of 387 grapevine symptomatic samples from 17 regions including 27 localities, were surveyed from 2008 to 2013. Detection of the viruses was carried out by DAS-ELISA and RT-PCR. In Table 1 we presented the infection rate (%) according to the results of all tested samples and the DAS-ELISA results. All of these samples were tested for GLRaV-1, -2, -3, and -7, by using BIOREBA and SEDIAG DAS-ELISA kits, and the results showed that 55.9% (215 samples) were GLRaV positive. GLRaV -2 and -7 were not detected in any of the investigated samples. Out of the positive samples, 69.7% (150 samples) were single infections with GLRaV-3, 15.5% were single infections with GLRaV-1, and 14.8% were mixed infections with GLRaV-3 and GLRaV-1. High percentage of infection is available in Stip (Krivi dol), Kocani, Argulica, Amzabegovo and Kavadarci (in all regions up to 70%) and low presence of the infection is available in Kumanovo, Demir Kapija and Gevgelija (low than 20%). Vranec variety (red vine variety) is the most widely spread in Macedonian vineyards, and also a good host plant for leafroll viral complex (GLRaV 1+3).

The data on incidence and distribution of viruses detected in randomly collected grapevine leaf samples are presented in Table 2 where a significant impact of GLRaV -1 and -3 was shown in almost every investigated variety in all the regions. About the reason why GLRaV -1 and -3 are the most widely spread in our vineyards, one of the possibility is transmission through vegetative propagation and grafting.

Ten representative ELISA positive samples for GLRaV -1 and -3, were also tested with reverse transcription-polymerase chain reaction RT-PCR using primer pairs: GLRaV1-M3/GLRaV1-M4, GLRaV2-CP1/GLRaV2-CP2, GLRaV3-M3/GLRaV3-N2 (Table 3). The results confirm the presence of GLRaV-1 and GLRaV-3 on the investigated samples before confirmed with DAS-ELISA (Figure 2). Also with RT-PCR we tested the samples for GLRaV-2, and our results compared with positive grapevine sample from Milano's lab were all negative.

The results of the surveys conducted in the collection of Macedonian grapevine cultivars revealed presence of important

a. Vranec b. Cabernet saugvinjon

Figure 1. Typical symptoms of leafroll virus on red grapevine variety

Table 1. List of investigated Macedonian grapevine cultivars and infection rate (%)

Locality	Region	Years of investigation	No. of analyzed samples	No. of infected samples	Infection rate (%)
Stip	Three cesmi / Ezovo	2008-2010	18	10	62.5
	Krividol	2008-2012	14	12	85.7
	Kavaklija	2008-2013	25	12	48
	DolniBalvan / Batanje	2013	7	3	42
Kocani	Starilozja	2008-2009	21	17	80.9
Karaorman	Balabanci	2008-2010	6	3	50
Argulica	Tupanec	2010	20	16	80
Sarcievo	Sarcievo	2008-2013	49	26	53
Sveti Nikole	Erdjelija	2009	18	8	44.4
Amzabegovo / Pesirovo	Amzabegovo / Pesirovo	2012-2013	23	20	86.9
Pesirovo	Pesirovo	2012-2013	23	12	52.1
Crniliste	Crniliste	2012-2013	5	2	40
Ovce pole	Private field	2012-2013	2	0	0
Veles	Sopot	2008, 2010, 2012	16	4	25
Tikves grape production area	Kavadarci, s. Cemersko	2011	19	15	78.9
	Kavadarci, Krnjevo	2011-2013	26	12	46.1
	Kavadarci, Raec	2013	9	3	33.3
	DemirKapija	2011-2013	9	2	22.2
	Negotino, IloVilarov	2011	9	5	55.5
	Negotino, s. Lepovo	2011	6	4	66.6
Kumanovo		2010-2013	9	1	11.1
Valandovo	Josifovo	2009-2012	18	12	66.9
Gevgelija	Avlaki	2011-2012	7	2	28.5
Skopje	Skovin	2011-2012	20	14	70
Bitola		2011	7	0	0

Table 2. Viruses' status recorded by ELISA-test for GLRaV -1, -2, -3 and -7 on different grapevine variety and RT-PCR results

Locality	Region	Variety	Laboratory analyzes					
			GLRaV 1+3	GLRaV 1	GLRaV 2	GLRaV 3	GLRaV 7	RT-PCR
Stip	Three cesmi/ Ezovo	Vranec	+	-	-	+	-	+ (-3)
	Krividol	Vranec	/	-	-	-	-	nt+
	Kavaklija	Black burgundec	+	+	-	+	-	(-1, -3)
	DolniBalvan / Batanje	Vranec	+	+	-	+	-	nt
Kocani	Starilozja	Pinot noir	/	+	-	-	-	nt
Karaorman	Balabanci	Vranec	/	-	-	+	-	nt
Argulica	Tupanec	Vranec	/	-	-	-	-	nt
Sarcievo	Sarcievo	Vranec	+	+	-	+	-	nt
SvetiNikole	Erdjelija	Italian riesling	-	+	-	+	-	nt
Amzabegovo/Pesirovo	Amzabegovo/Pesirovo	Vranec	/	+	-	-	-	nt
Pesirovo	Pesirovo	Vranec	+	+	-	+	-	nt
Crniliste	Crniliste	Vranec	+	-	-	+	-	nt
Ovce pole	Private field	Pinot noir	+	+	-	+	-	nt
Veles	Sopot	Cabernet sauvignon	+	-	-	+	-	nt

Tikves grape production area	Kavadarci,	Pinot noir	+	+	-	-	-	nt
	s. Cemersko	Kratosija	+	+	-	-	-	nt
	Kavadarci, Krnjevo	Vranec	+	-	-	+	-	+ (-3)
	Kavadarci, Raec	Vranec	+	+	-	-	-	nt
	DemirKapija	Vranec	+	+	-	-	-	nt
	Negotino, IloVilarov	Vranec	+	-	-	+	-	nt
	Negotino, s. Lepovo	Vranec	+	+	-	+	-	nt
Kumanovo		Vranec	+	-	-	+	-	nt
Valandovo	Josifovo	Vranec	+	+	-	+	-	+ (-1, -3)
Gevgelija	Avlaki	Frankovka	+	-	-	+	-	+ (-3)
Skopje	Skovin	Vranec	+	+	-	+	-	nt
Bitola		Italian riesling	-	-	-	-	-	nt

Presence of virus: - = negative, + = positive, nt – not tested.

Table 3. Primer pair for reverse transcription-polymerase reaction (RT-PCR) amplification

Virus	Primer	Length (bases)	Sequence (5'-3')	Position	Amplified size (bp)
GLRaV-1	GLRaV1-M3	22	TCTTTACCAACCCCGAGATGAA	7245-7266	232
	GLRaV1-M4	22	GTGTCTGGTGACGTGCTAAACG	7455-7476	
GLRaV-2	GLRaV2-CP1	20	GGTGATAACCGACGCCTCTA	6745-6764	543
	GLRaV2-CP2	20	CCTAGCTGACGCAGATTGCT	7268-7287	
GLRaV-3	GLRaV3-M3	22	TACGTTAAGGACGGGACACAGG	13383-13404	336
	GLRaV3-N2	20	TGCGGCATTAATCTTCATTG	13699-13718	

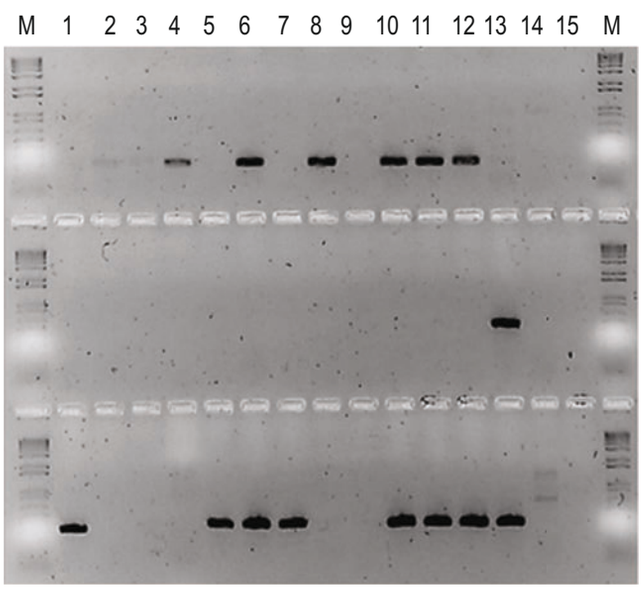

Figure. 2 Agarose gel electrophoresisshowing results of RT-PCR detectionusing primer pair for GLRaV -1, -2 and -3. Total RNA from ten representative grapevine petioles was used at lines 1-10. M-marker (1 Kb Plus DNA Ladder, Invitrogen), 11, 12, 13 positive control (positive grapevine sample from Milano's lab), 14 healthy plant (extracted from healthy grapevine) 15 negative control

viruses from leafroll complex in different levels and high levels of their infections. GLRaV-1 and GLRaV-3 are such dominance group of leafroll viruses in all investigated regions in the Republic of Macedonia. The virus status was also similar to those determined in other countries of the Mediterranean region, such as Croatia (Voncina et al., 2011), in most vine-growing regions in Italy (Savino et al., 2001), Cyprus, Malta, Greece (Digiaro et al., 2000) and also present in Serbia (Sivcev et al., 2011) from where most grapevine cultivars and propagative material come.

Conclusion

In all investigated grape growing regions, we have leafroll viral complex mostly present in wine grapevines, but the damage is on a low level and still not economically very important. Based on the results shown in this paper, we confirm the presentce of GLRaV-1 and GLRaV-3 and absence of GLRaV-2 and GLRaV-7 in all investigated regions. This is the first report on the presence of leafroll viral complex which is very important for wine and plant material producers in order to develop a better balanced strategy for plant health management of mother plants. In this survey we provide clear evidence that appropriate sanitation procedures will be necessary in the future for many Macedonian grapevine regions in order to provide healthy plants and virus-free planting material.

Acknowledgements

Field surveys, sample collection and DAS-ELISA test were performed at Goce Delcev University in Stip, Republic of Macedonia. RNA extraction and molecular characterization were carried out during Dr. Kostadinovska's stay at the Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, University of Milan, Italy within the Student mobility for studies (SMS) programme (Student Academic Year 2012-2013) of the ERASMUS Grant Agreement HEI.

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