# OCCURRENCE OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS COMPLEX IN THE REPUBLIC OF MACEDONIA

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## INTRODUCTION

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 economical importance, it is critical to determine which species of GLRaV are predominant in each region in Macedonia where this disease occurs (**Tab. 1**).

Fig. 1 Typical symptoms of leafroll virus on red grapevine variety a. Vranec b. Cabernet sauvignion

Fig. 2 Typical symptoms of GlRaV on local variety Stanusina

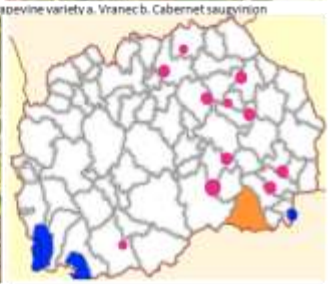

Fig. 3 Macedonian map with mark investigation regions

**Table 1.** List of investigated Macedonian grapevine cultivars: region, locality, number of analyzed samples and number of wines which were according to results of ELISA-test infected with GLRaV -1 and GLRaV -3

| Locality | Region | Years of investigation | No. of analyzed samples | No. of infected samples | Infection rate (%) |
|---|---|---|---|---|---|
| Stip | Three cesni | 2008 | 2 | 0 | 0 |
| | Ezovo | 2008-2010 | 16 | 10 | 62.5 |
| | Krividol | 2008-2012 | 14 | 12 | 85.7 |
| | Novo selo | 2009 | 1 | 0 | 0 |
| | Kavakija | 2008-2013 | 25 | 12 | 48 |
| | Dolni Balvan | 2013 | 3 | 1 | 33.3 |
| | Batanje | 2013 | 4 | 2 | 50 |
| Kocani | Stari Icoja | 2008-2009 | 21 | 17 | 80.9 |
| Kanaorman | Balabanci | 2008-2010 | 6 | 3 | 50 |
| Argulica | Tupanec | 2010 | 20 | 16 | 80 |
| Sarclevo | Sarclevo | 2008-2013 | 49 | 26 | 53 |
| Sveti Nikole | Erdjelija | 2009 | 18 | 8 | 44.4 |
| Anzabegovo/Pesirovo | Anzabegovo/Pesirovo | 2012-2013 | 23 | 20 | 86.9 |
| Pesirovo | Pesirovo | 2012-2013 | 23 | 12 | 52.1 |
| Creiliste | Creiliste | 2012-2013 | 5 | 2 | 40 |
| Ovce pole | Private field | 2012-2013 | 2 | 0 | 0 |
| Veles | Sopot | 2008, 2010, 2012 | 16 | 4 | 25 |
| | Kavadarci, s. Cemersko | 2011 | 19 | 15 | 78.9 |
| Tikves grape production area | Kavadarci, Krnjevo | 2011-2013 | 26 | 12 | 46.1 |
| | Kavadarci, Raec | 2013 | 9 | 3 | 33.3 |
| | Demir Kapija | 2011-2013 | 9 | 2 | 22.2 |
| | Negotino, Ilo Vilarov | 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 | Skopin | 2011-2012 | 20 | 14 | 70 |
| Bitola | / | 2011 | 7 | 0 | 0 |

## CONCLUSION

In almost all infected plants, the symptoms were expressed as leaf reddening, slightly downward leaf rolling and remarkable difference in rootstock and scion diameter. 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.

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.

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## MATERIALS AND METHODS

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) (**Fig. 1&2**) were detected in all marked vineyard regions in the Republic of Macedonia (**Fig.3**).

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 (**Fig. 4**).

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 previously (Rowhani, 1992). The total number of 387 grapevine symptomatic samples from 17 regions including 27 localities, were surveyed from 2008 to 2013 (**Tab. 1**).

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 (10mM) 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 5x 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 25 ºC for 5 min, 50 ºC for 60min and 70ºC for 15 min. RT was used in 25 µl PCR reaction containing 14.3 µl water, 2 µl $MgCl_2$ (25mM), 5 µl 5xbuffer, 0.5 µl dNTPs, 0.5 µl each of forward and reverse primers and 0.2 µl Taq DNA Polymerase.

## RESULTS AND DISCUSSION

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. 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 (**Fig. 6**).

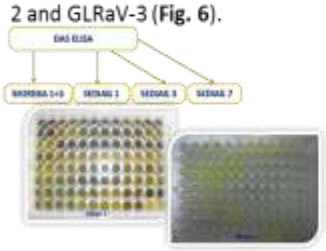


**Fig. 4** DAS ELISA lab test

**Fig. 5** Transport of virus on test plant *Nicotiana bentamiana*

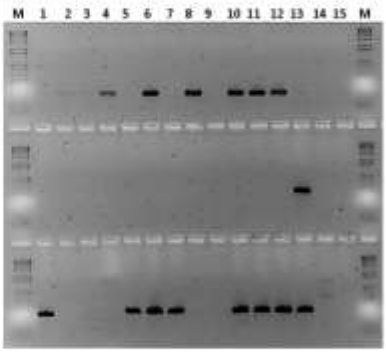


**Fig. 6** Agarose gel electrophoresis showing results of RT-PCR detection using primer pair for GLRaV -1, -2 and -3. Total RNA from ten representative grapevine petioles was used at lines 1-10. M-marker (1 Kb Plus DNA Ladder, Invitrogen), 11, 12, 13 positive control, 14 healty plant (extracted from healthy grapevine) 15 negative control

## REFERENCES

Rowhani A., 1992. Use of F (ab') 2 antibody fragment in ELISA for detection of grapevine viruses. American Journal of Enology and Viticulture **43**: 38-40.

MacKenzie D.J., McLean M. A., Mukerji S., Green M. 1997. Improved RNA Extraction from Woody Plants for the Detection of Viral Pathogens by Reverse Transcription-Polymerase Chain Reaction. Plant Disease/Vol. 81 No. 2: 222-226.