Original scientific paper
Оригиналан научни рад
UDC: 634.1/.8-295
DOI: 10.7251/AGREN1602143D

University of Banjaluka, Faculty of Agriculture
Agro-knowledge Journal

Sanitary Status of the Grapevine Germplasm Collection in Republic of Srpska

Duška Delić[1], Biljana Lolić[1], Gordana Đurić[1, 2], Tatjana Jovanović-Cvetković[1]

[1]*Faculty of Agriculture, University of Banjaluka, Republic of Srpska, BiH*
[2]*Genetic Resources Institute, University of Banjaluka, BiH*

Abstract

In July 2015, 179 grapevine plants belonging to 16 grapevine autochthonous cultivars were assessed for sanitary status using DAS ELISA test for the presence of: *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus* 1 (GLRaV-1), *Grapevine leafroll-associated virus* 2 (GLRaV-2) and *Grapevine leafroll-associated virus* 3 (GLRaV-3). Furthermore, survey for the phytoplasma presence and laboratory analyses using nested-PCR/RFLP assay was conducted at the beginning of September 2015 on grapevine cultivars which were not positive in DAS ELISA test for the presence of the four viruses. Out of 179 tested plants with DAS ELISA test, 146 (81%) were positive for the presence of at least one virus. The most widespread viruses were GFLaV- 1 and GFLaV- 3 with approximately 80 % of grapevines infected. Nested–PCR/RFLP assay showed that out of 33 tested samples 2 were positive for the presence of phytoplasmas from 16SrXII group. Sanitation of infected grapevine cultivars is needed in near future.

Key words: *Vitis vinifera* L., Grapevine Fanleaf Virus, Grapevine Leafroll-associated Virus, phytoplasma

Introduction

So far, 62 different viruses have been identified on grapevines (Martelli, 2015) from which one third are associated with complex-named diseases such as: infectious degeneration and decline, leafroll and rugose wood. Generally, the virus infection on grapevine can negatively influence the yield, sugar content and acidity of the must, berry color, resistance to biotic and abiotic stress, length of growing cycle etc. Moreover virus infection is one of the possible causes of intravarietal morphological variability (Walter and Martelli, 1996; Mannini and Credi, 2000). Although frequently on virus-infected vines the characteristic symptoms can be observed, there are also many cases of latent infections without many symptoms. Therefore much attention has been paid to the sanitary status of plant material.

In Bosnia and Herzegovina (BiH) neither systematic clonal selection, nor sanitary selection have ever been done. On the other hand there is an enlarged interest of producers and consumers for BiH autochthonous cultivars. Planting of healthy plant material is a base for high quality yield. For that reason our main objective was to improve the quality and sanitary status of BiH autochthonous cultivars starting with screening for the most important grapevine viruses and phytoplasmas in germplasm collection in Trebinje. The grapevine germplasm collection was established in the period from 2009 to 2013 through programme on plant genetic resources of the Republic of Srpska. Genetic Resources Institute of University of Banja Luka is the owner of this collection. The collection is placed in Trebinje (south-east part of the country) in "Petropavlov" monastery and it is constituted of 179 trees belonging to 16 grapevine autochthonous cultivars, among which the most famous are: Žilavka, Bena, Blatina, Kadarun, Surac, Rezaklija, Radovača, Plavka, Dobrogoština, Meginovka, Žlozder and Krkošija.

The main objective of this work is to find out grapevine cultivars in the collection free of the main viruses and phytoplasmas. The virus and phytoplasma free grapevines will be then placed under protective infrastructure with insect proof net and monitored until establishment of the virus and phytoplasma free mother stock.

Material and Methods

Evaluation of the virus presence

At the beginning of July, grapevine collection was surveyed for the presence of virus symptoms and leaves with petioles were sampled from each plant (179) for laboratory analyses. Viruses were detected using ELISA.

Commercial antisera (BIOREBA, Switzerland) against *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associatedvirus 1* (GLRaV-1), *Grapevine leafroll-associatedvirus 2* (GLRaV-2) and *Grapevine leafroll-associated virus3* (GLRaV-3) (EU directive 2005/43/EC) were used in DAS-ELISA method according to instructions of manufacturer. For all testing, the coating antibodies and conjugate antibodies were incubated for 4 h at 30 °C, while samples and controls were incubated overnight at 4°C. Results were read after adding the substrate (*p*-nitrophenyl-phosphate in 10 % diethanolamine, pH 9.8) to the wells. The incubation time was 30 to 60 min. The presence or the absence of virus was determined by comparing absorbance at 405/492 nm of the samples with that of the threshold value. Absorbance values greater or lower than the threshold were considered, respectively, as positive or negative results. The threshold was determined as two times the mean absorbance value of the negative control.

Evaluation of the phytoplasma presence

At the end of August 2015 all grapevine trees that showed to be negative in ELISA test were additionally examined and sampled for laboratory analyses for phytoplasma presence. Leaf midribs were used for extraction of total DNAs using DNeasy Plant mini kit (QIAGEN) following protocol described in Green et al. (1999).

Nested PCR/RFLP analyses with phytoplasma universal primers were used for the phytoplasma identification. All reactions were performed 20 µL volume using P1/P7 (Deng and Hiruki 1991; Schneider et al., 1995) and R2/F2n primers in nested PCR (Gundersen and Lee, 1996) (600 nM final concentration). PCR was performed using *Taq* polymerase (*Sigma*-Aldrich) and a program consisting of initial denaturation at 94°C for 1 min and 30s followed by 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min repeated for 34 cycles. The protocol terminated with a final extension step at 72°C for 10 min. Nested-PCR products were visualized in by electrophoreses in 1% agarose gel.

All nested-PCR products, which showed positive reactions for phytoplasma infection, were submitted to the RFLP analyses with *Mse*I and *Taq* restriction enzymes. RFLP digestions were ran and visualized in 3% agarose gel electrophoreses.

Results and Discussion

Results evaluation of the virus presence

During field survey for the virus presence the most frequent symptoms observed on grapevines in collections were exhibited virus symptoms such as

mosaic, chlorosis, leaf bubbling, leaf deformations, vein clearing, leaf yellowing or reddening and leaf rolling (Fig. 1a, b, c, d, e) .

Results obtained performing DAS ELISA test indicate a rather high level of grapevine virus infection in the grapevine germplasm collection, where out of 179 tested grapevine plants 146 (82%) were virus infected. The highest level of infection was with grapevine viruses that cause leafroll disease (GLRaV-1 and GLRaV-3), which generally seem to be the most represented viruses in grapevine (Gugerli, 2003; Maixner, 2005; Martelli, 2016). GLRaV-3 prevailed where 46% (83/179) of tested grapevines were positive for the virus presence. These data tally with the notion that GLRaV-3 is more common in the Mediterranean (Ahmed et al., 2004; Cabaleiro and Segura, 2006) and GLRaV-1 in the northern viticultural regions of the world (Credi and Giunchedi, 1996; Kominek et al., 2003). GLRaV-2 was found only in 3 out of 179 (1.7%) grapevines. However infection with GLRaV-1 was found also in high percentage 82/179 (45%).

The principal means of dissemination of GLRaV-1 and GLRaV-3 in some Mediterranean and overseas countries is through infected plant material with several insect vectors from families *Pseudococcidae* and *Coccidae* which are quite ubiquitous (Cabaleiro and Segura, 1997; Krüger et al., 2006; Almeida et al., 2013). On the other hand the low percentage of infected plants with GLRaV-2 can be due to the fact that for this virus there is no insect vector and it is only transmitted through plant propagation material (Meng et al., 2005). Obtained results were not unexpected because causal agents of leafroll disease complex have been laboratory ascertained on several autochthones cultivars in Herzegovina region (Delić et al., 2005; Karačić et al., 2014).

The incidence of nepoviruses GFLV was significantly lower since out of 179 tested grapevines, 16 (9%) were positive. It is worth mentioning that soil analyses showed that there were no vector nematodes in the collection.

Multiple infections with two or more viruses were also common. The most common combination was infection with GLRaV-1 and GLRaV-3, which is the case in 12 % of all grapevines tested (Table 1). Also, there were plants infected with three viruses. Similar situation with the virus infection was also observed during evaluation of the sanitary status of Croatian autohtones grapevine in Dalmatia (Karoglan Kontić et al., 2009).

All 33 plants were found to be negative for the presence of four tested viruses with DAS ELISA test. Generally Žilavka, Kadarun and Radovača were the grapevines with the highest level of infection with the tested viruses.

Tab. 1. Incidence of grapevine cultivars infection with different viruses and virus combinations (total tree number)
Учесталост заразе сорти винове лозе са различитим вирусима и њивовим комбинацијама (укупан број садница)

Cultivar *Copma*	grapevines infected with / *винова лоза заражена са*										
	GLRaV-1	GLRaV-2	GLRaV-3	GFLV	GLRaV-1 + GLRaV-2	GLRaV-1 + GLRaV-3	GLRaV-2 + GLRaV-3	GLRaV- 1 + GFLV	GLRaV-3 + GFLV	GLRaV-1 + GLRaV-3 + GFLV	GLRaV-2 + GLRaV-3 + GFLV
Žilavka	11/22	-	12/22	-	-	7/22	-	-	-	-	-
Bena	9/16	-	11/16	-	-	9/16	-	-	-	-	-
Kadarun	10/12	-	-	8/12	-	-	-	6/12	-	-	-
Blatina	-	2/15	10/15	1/15	-	-	1/15	-	-	-	1/15
Plavka	3/15	-	9/15	1/15	-	-	-	-	1/15	-	-
Drenak	1/2	-	2/2	-	-	1/2	-	-	-	-	-
Surac	-	-	2/25	4/25	-	-	-	-	-	2/25	-
Crni prošip	-	-	4/4	-	-	-	-	-	-	-	-
Rezaklija	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Radovača	15/15	1/15	-	-	1/15	-	-	-	-	-	-
Alikant buše	1/7	-	6/7	-	-	1/7	-	-	-	-	-
Trnjak	3/7	-	4/7	-	-	2/7	-	-	-	-	-
Dobrogoština	2/5	-	5/5	-	-	2/5	-	-	-	-	-
Meginovka	-	-	8/11	-	-	-	-	-	-	-	-
Krkošija	-	-	10/12	2/12	-	-	-	-	-	-	1/12
Žlozder	6/7	-	-	-	-	-	-	-	-	-	-
Total	82 / 179	3 / 179	83 / 179	16 / 179	1 / 179	22 / 179	1 / 179	6 / 179	1 / 179	2 / 179	2 / 179

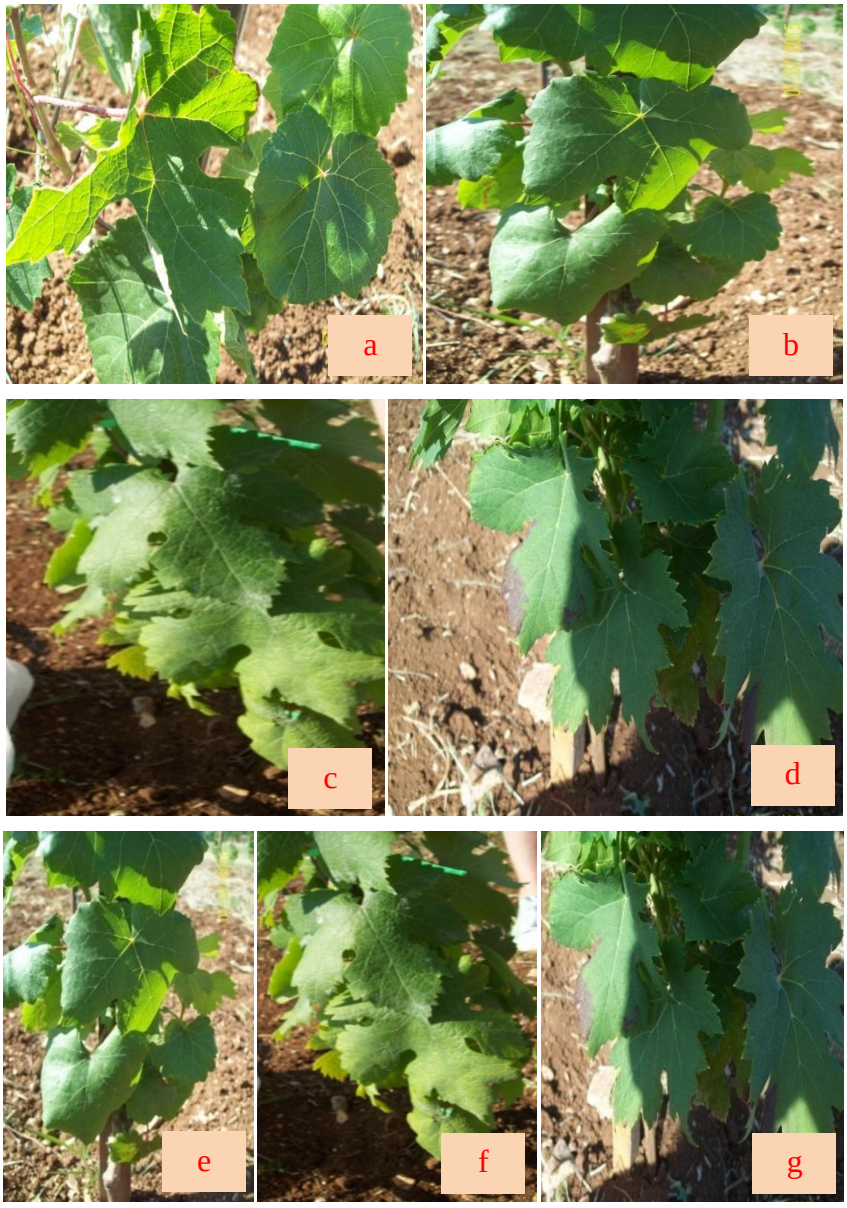

Fig. 1. Virus infection-like symptoms observed in grapevine germplasm collection:
Симптоми налик вирусној зарази примећени у колекцији гермплазме винове лозе:

a) mosaic (*мозаичност*) b) vein clearing (*прозрачност лисног нерва*),
c) leaf bubling (*мехуравост листа*), d) leaf deformations (*деформације листа*),
e) leaf rolling (*увртање листа*), f) yellowing (*жутило*), g) leaf reddening (*црвенило*).

Results evaluation of the phytoplasma presence

During the survey for the phytoplasma presence, plants that were negative for infection with four viruses in DAS ELISA test were additionally examined for the presence of phytoplasma symptoms. On some black grapevine varieties symptoms such as leafrolling and partial vein reddening were noticed (Fig. 2).

Fig. 2. Phytoplasma-like symptoms observed
Примећени симптоми налик зарази фитоплазмама

Nested-PCR assays showed that out of 33 tested grapevines from the collection of two plants (Kadarun and Bena cultivars) were positive for phytoplasma infection. RFLP analyses showed that phytoplasma from 16SrXII group was the causal agent. So far phytoplasmas from 16SrXII group were the only phytoplasmas found to cause grapevine yellowing diseases in Bosnia and Herzegovina (Delić et al., 2011a,b).

In autumn 2015, 31 grapevine trees belonging to 11 autochtones cultivars which were free from tested viruses and phytoplasmas were restored to the Institute for the Genetic Resources in Banjaluka under *insect-proof protective* structures. For that purpose plants were replanted in 50 L containers in substrate tested and found free of the presence of nematodes of the *Xiphinema* genus. In spring 2016, these plants were registered for certification program as future mother stocks.

Conclusion

In this survey cultivars with different population size and economical importance have been investigated.

Nevertheless, the results show that enough healthy grapevines can be found as basis for the further clonal selection procedure. Moreover, all ecotypes of Rezaklija cv. were found to be free of the tested viruses and phytoplasmas so it could also be an interesting clone for the breeding studies. Considering our findings, the situation is very serious, thus it is necessary to undertake sanitation combining thermotherapy and tissue culture of the cultivars and monitor them under necessary infrastructure for conservation of virus and phytoplasma free mother stocks.

References

Ahmed, H. M. H., Digiaro, M. and Martelli, G.P. (2004). Viruses and virus diseases of grapevine in Egypt. *Bulletin OEPP/EPPO, 34*, 395-398.

Almeida, R.P.P., Daane, K., Bell, V., Blaisdell, K., Cooper, M., Herrbach, E. and Pietersen, G. (2013). Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology, 4*, 94. doi: 10.3389/fmicb.2013. 00094.

Cabaleiro, C. and Segura, A. (2006). Temporal analysis of grapevine leafroll associated virus 3 epidemic. *European Journal of Plant Pathology*, *114*(4), 441 – 446.

Credi, R. and Giunchedi, L. (1996). Grapevine leafroll-associated and grapevine virus A in selected *Vitis vinifera* cultivars. *Plant Pathology, 45*(6), 1110 -1116.

Delić D., Contaldo N., Paltrinieri S., Lolić B., Đurić Z., Hrnčić S. and Bertaccini A. (2011a). Grapevine yellows in Bosnia and Herzegovina: surveys to identify phytoplasmas in grapevine, weeds and insect vectors. *Bulletin of Insectology, 64*(supplement), 245-246.

Delić D., Lolić B., Karačić A. (2011b). Screening for phytoplasma presence in West Herzegovina vineyards. *Phytopathogenic Mollicutes, 1*(2), 87-90.

Delić D., Jovanović-Cvetković, T., Đurić, G. (2007). Prisustvo i rasprostranjenost Grapevine leafroll-associated virus-1 i 3 u Bosni i Hercegovini. *Pesticidi i Fitomedicina, 22*(1), 45-50.

Deng, S. and Hiruki, D. (1991). Amplification of 16S rRNA genes from culturable and nonculturable mollicute. *Journal of Microbiological Methods, 14*(1), 53–61.

Green, MJ., Thompson, D.A. and Mackenzie, D.J. (1999). Easy and efficient DNA extraction from woody plants for the detection of phytoplasmas by polymerase chain reaction. *Plant Diseases, 83*(5), 482-485.

Gundersen, D.E., Lee, I.M. (1996). Ultrasensitive detection of phytoplasmas by nested PCR assays using two universal primer pairs. *Phytopathologia Mediterranea, 35*(3), 144–151.

Gugerli, P. (2003). Grapevine leafroll and related viruses. In *Proceedings 14th ICVG Conference, Locorotondo/Bari, Italy, September, 12th-17th*. Retreived from: http://icvg.org/general-assembly/

Karačić, A., Đermić, E.,Vončina, D. and Ivanković, M. (2014). *Pojava virusa iz kompleksa uvijenosti lista vinove loze (GLRaV) na autohtonim kultivarima u Hercegovini*. Paper presented at the „58. Seminar biljne zaštite", 11.-14. veljače, Opatija. Hrvatska.

Karagolan Kontić, J., Preiner, D., Šimon, S., Zdunić, G., Pohulja, D. and Maletić, E. (2009). Sanitary Status of Croatian Native Grapevine Varieties. *Agriculturae Conspectus Scientificus, 74*(2), 99-103.

Kominek, P. and Holleinova, V. (2003). Evaluation of sanitary status of grapevines in Czech Republic. *Plant Soil Environment 49*(2), 63 – 66.

Krüger, K., Saccaggi, D. and Douglas, N. (2006). Grapevine leafroll-asociated virus 3-vector interactions: transmission by the mealybugs *Planococcus ficus* and *Pseudococcus longispinus* (Hemiptera: Pseudococcidae). *Extended Abstracts 15th Meeting of ICVG, Stellenbosch, South Africa,* 130-131.

Martelli, G.P. (2015). Grapevine Virology: A historical account with an eye to the studies of the last 60 years or so. In Ertunç, F., *Proceedings of the 18th Conference of the International Council for the Study of Virus and Virus-Like Diseases of the Grapevine (ICVG), Ankara, Turkey, September 7, 11, 2015* (p 16-21). Ancara University, Faculty of Agriculture.

Maixner, M. (2005). *Risks posed by the spread and dissemination of grapevine pathogens and their vectors*. Paper presented at the International symposium – Introduction and Spread of Invasive Species, 9 – 11 June, Berlin, Germany. Retreived from http://dpg.phytomedizin.org/file admin/alte_Webseiten/Invasive_Symposium/articles/articles.

Mannini, F. and Credi, R. (2000). Appraisal of agronomic and enological modifications in the performances of grapevine clones after virus-eradication. In *Extended abstracts 13th ICVG Conference, Adelaide, Australia, March, 12-17* (pp. 151-154).

Meng, B., Li, C., Goszczynski, D.E. and Gonsalves, D. (2005). Genome sequences and structures of two biologically distinct strains of grapevine leafrolla ssociated virus 2 and sequence analysis. *Virus Genes, 31*(1), 31-41.

Walter, B., Martelli, G. P. (1996). Selection clonale de la vigne: Selection sanitaire et selection pomologique. Effets des viruses sur la culture de la vigne et ses produits. *Bull. OIV, 69*(789-790), 945-971.

Санитарни статус колекције винове лозе у Републици Српској

Душка Делић[1], Биљана Лолић[1], Гордана Ђурић[1, 2], Татјана Јовановић-Цветковић[1]

[1] Пољопривредни факултет, Универзитет у Бањој Луци, Република Српска, БиХ
[2] Институт за генетичке ресурсе, Универзитет у Бањој Луци, Република Српска, БиХ

Сажетак

Почетком јула 2015. године, 179 чокота који припадају 16 аутохтони култивара винове лозе су анализирани користећи DAS ELISA тест на присуство вируса инфективне дегенерације винове лозе (*Grapevine fanleaf virus*, GFLV) и удружених вируса увијености лишћа винове лозе (*Grapevine leafroll-associated virus* 1,2,3; GLRaV-1,2,3). Такође почетком септембра 2015. године, надзор здравственог стања култивара који нису били позитивни на вирусе у DAS ELISA тесту је извршен и на присуство фитоплазми гдје је за лабораторијске анализе коришћена комбинована метода nested-PCR/RFLP. Лабораторијске анализе DAS ELISA тестом показале су да од 179 тестираних чокота, 146 (81%) су били позитивни на најмање један вирус. Најзаступљенији вируси били су GFLaV- 1 и GFLaV-3 у око 80 % заражених чокота. Nested–PCR/RFLP анализа показала је да од 33 тестирана чокота 2 су била позитивна на присуство фитоплазми из 16SrXII рибозомалне групе. У току је конзервација чокота који су били негативни на присутсво тетсираних патогена као и санитација заражених.

Кључне ријечи: *Vitis vinifera* L., вирус инфективне дегенерације винове лозе, удружени вирус увијености лишћа винове лозе, фитоплазма

Duška Delić
E-mail address: duska.delic@agrofabl.com

Received: July 12, 2016
Accepted: November 29, 2016