

Sanitary status of three Croatian native grapevine varieties (*Vitis vinifera* L.) from Dalmatia region included in clonal selection

Sanitarni status tri hrvatske autohtone sorte vinove loze (*Vitis vinifera* L.) s područja Dalmacije uključene u postupak klonske selekcije

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ABSTRACT

The research of sanitary status and virus presence was conducted on three native Croatian grapevine varieties (Kujudžuša bijela, Zlatica vrgorska and Trnjak crni) that are grown in Dalmatia region. The investigation was done on 492 vines from 31 vineyards. Vines were tested by ELISA (Enzyme-Linked Immunosorbent Assay) on four main economic viruses: arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), grapevine leafroll-associated virus 1 and 3 (GLRaV-1, GLRaV-3) as a first step in clonal selection of these rare native grapevine varieties. Altogether, 40 vines were free of all tested viruses. The research confirms high rate of virus infection and eroded sanitary status of all investigated varieties. The above indicates necessity of implementation clonal and sanitary selection programme.

Keywords: native grapevine varieties, ELISA, grapevine viruses, Dalmatia

SAŽETAK

Istraživanje sanitarnog statusa provedeno je na tri autohtone hrvatske sorte vinove loze (Kujudžuša bijela, Zlatica vrgorska i Trnjak crni) koje se uzgajaju u Dalmaciji. Istraživanjem je obuhvaćeno ukupno 492 trsa iz 31 vinograda. Uzorci su podvrgnuti serološkom testu ELISA (*Enzyme-Linked Immuno-Sorbent Assay*) za analizu na prisustvo četiri gospodarski najvažnija virusa: virus mozaika lista vinove loze (ArMV), virus lepezastog lista vinove loze (GFLV), virus uvijenosti lista tip 1 i tip 3 (GLRaV-1, GLRaV-3). Od ukupnog broja prikupljenih uzoraka, njih 40 je bilo potpuno slobodno od prisutnosti istraživanih virusa. Ovo istraživanje potvrđuje visok udio virusom zaraženih trsova te loš sanitarni status istraživanih sorata. Sve navedeno ukazuje na nužnost provedbe klonske i sanitarne selekcije.

Ključne riječi: autohtone sorte vinove loze, ELISA, virusi vinove loze, Dalmacija

INTRODUCTION

Viticulture in Croatia has great and rich tradition dating back to ancient times (Jelaska and Briza, 1967). During pre-phyloxera time (beginning of 20th century) just in the costal part of Croatia, Dalmatia, 400 native grapevine varieties were grown over more than 90 000 ha of vineyards (Bulic, 1949). Afterwards, with the advent of diseases and pest (*Plasmopara viticola*, *Uncinula necator* and *Phylloxera vastatrix*) and due to other economic, demographic, and political reasons came a forceful decline of vineyard areas and loss of many native grapevine varieties. Today in Croatia there is 19 000 ha of vineyards (PAAFRD, 2019) and no less than 125 native grapevine varieties (Maletić et al., 2015a). Native grapevine varieties play major role in wine production even today and especially in the Costal wine region (Dalmatia). In the last two decades, large efforts were made in preservation and revitalization of native grapevine varieties in Croatia (Pejić and Maletić, 2010; Maletić et al., 2015b; Maletić et al., 2016). One of the main problems in the above-mentioned process is a lack of certified planting material. Despite the enormous efforts, as of yet, only 12 varieties are involved in process of clonal selection (Šikuten et al., 2018). Clonal selection is one of the finest tools for preservation, control virus diseases and targeted selection within a certain gene pool (Roby et al., 2014). First step in clonal selection is a mass positive and sanitary selection. Up to now, more than 70 different viruses have been detected among grapevine species (Martelli, 2014). Among them, grapevine leafroll disease (GLD) and infectious degeneration, are especially responsible for crop loss, reducing grape quality and reducing longevity of vines (Rayapati et al., 2015; Andret-Link et al., 2004). In the last couple of decades, there were several studies of sanitary status of Croatian native grapevine varieties (Poljuha et al., 2004; Karoglan Kontić et al., 2009a, 2009b; Vončina et al., 2010, Vončina et al., 2011a; Vončina et al., 2019). All the studies show high rates of virus infected vines in all winegrowing regions of Croatia and especially in the costal winegrowing region. Positive mass and sanitary selection were conducted on three native grapevine varieties Zlatarica vrgorska, Trnjak and Kujundžuša during 2018 and 2019. During

selection process, potential mother vines are selected with the purpose of finding virus free vines among them. This survey was done as a first step in the clonal selection of these rare native grapevine varieties with a goal of producing certified clone material, free from four main economically harmful viruses: arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), grapevine leafroll-associated virus 1 and 3 (GLRaV-1, GLRaV-3).

MATERIAL AND METHODS

Positive mass selection and collection of samples

The samples were collected from 26 commercial vineyards located in two winegrowing areas, Vrgorac and Imotski (Dalmatia). Samples were taken from vines that are included in mass positive clonal selection, and the age of the vines varied between 50 and 60 years. During the mass positive selection, evaluation of agronomic traits and visual evaluation of sanitary status was done. Throughout autumn 2018, samples from varieties Zlatarica vrgorska and Trnjak crni were collected in winegrowing area Vrgorac. Samples from varieties Trnjak crni and Kujundžuša bijela were collected in wine growing area Imotski during autumn 2019. Each sample was represented by at least four wooden basal cuttings of vine shoot. After labelling, samples were stored at 4 °C and tested within 15 days. The number of samples per each variety was different and comparable to the economic importance of the variety. Kujundžuša bijela had the greatest number of samples- 235 from 15 locations, Trnjak crni 160 from 16 locations and Zlatarica vrgorska 97 samples from 10 locations.

Serological assay

Vines were tested using ELISA (Enzyme-Linked Immunosorbent Assay) for the presence of four viruses: arabis mosaic virus, grapevine fanleaf virus, grapevine leafroll-associated virus 1 and 3 using commercial kits from Agritest (Valenzano, Italy) following manufacturers instructions and isolation of potential antigen as described by Vončina et al. (2019).

RESULTS AND DISCUSSION

In September 2018 and 2019, from Imotski and Vrgorac vineyards, visual observations were made for the most important economic and ampelographic characteristics of Kujundžuša bijela, Zlatarica vrgorska and Trnjak crni (mass positive selection). The presence of four economically important viruses was evaluated on selected vines by ELISA, including two nepoviruses (ArMV, GFLV), and two closteroviruses (GLRaV-1 and GLRaV-3). Although no visible symptoms were observed, the results of the ELISA showed that all four viruses were present in all tested varieties (Table 1). Furthermore, the results showed mixed infections, ranging from two up to four viruses infecting one vine and were detected on all sampling locations. The highest infection incidence in mixed infections had the combination of two viruses. Small percentage of infected vines were infected with three viruses, while only one vine was infected with four viruses. The most common combination was with GLRaV-1 and GLRaV-3. The dominance was expressed by the GLRaV-1 which is not so common for Dalmatinska zagora (whole Dalmatian wine growing region) and is also contrary to Vončina et al. (2019) findings. The highest infection rates showed Kujundžuša bijela, in which more than 86% of tested vines were infected with one or more viruses. Moreover, only in this variety mixed infection with four viruses was recorded. Among tested vines, only 13.19% showed to be virus free of tested viruses (virus free). Similar situation is with Zlatarica vrgorska, where 16.49% of vines were free from tested viruses. The best

sanitary status considering virus infection was confirmed in cultivar Trnjak crni, where 30% were free from all four viruses.

In Table 2 the detailed infection incidence by sampling location and type of virus for variety Kujundžuša bijela are presented. When visual observations of phenotypic characteristics were conducted, Kujundžuša bijela seemed to have the largest number of healthy vines. However, ELISA showed different result, as mentioned above. The possible explanation for this discrepancy could be the timing of visual observations. In both years the observations were conducted during September at the coastal region, when high temperatures still occur. Hence, the virus symptoms could be masked or less expressed. In tested vines the highest infection incidence was recorded for GLRaV-1 virus, comprising 64.26% of all infected vines, followed by GFLV, with 54.89%. GLRaV-3 was recorded in 27.23% of infected vines. The data presented are not so common for the coastal region and are contrary to the data published by Vončina et al. (2019). Their data showed that the most common virus in Dalmatia region is GLRaV-3 (79.6%), followed by GVA (61.4%) and GLRaV-1 (40.8%). Furthermore, GFLV infection rate was 19.6%, which is considerably lower compared to the data in Table 2. Also, our results show presence of single infections with ArMV virus, while Vončina et al. (2019) detected ArMV only in mixed infections. This situation could be the result of infected planting material derived from the limited number of mother plants used to produce planting material but not tested for the presence of viruses.

Table 1. Virus infection incidence determined by ELISA on three Croatian native grapevines

Variety	Number of tested samples	Virus free samples (No./%)	Infected samples with 1 virus (No./%)	Infected samples with 2 viruses (No./%)	Infected samples with 3 viruses (No./%)	Infected samples with 4 viruses (No./%)
Kujundžuša bijela	235	31 13.19%	77 32.77%	106 45.11%	20 8.51%	1 0.43%
Zlatarica vrgorska	97	16 16.49%	46 47.42%	29 43.28%	6 6.19%	0
Trnjak crni	160	48 30%	63 39.38%	44 27.50%	5 3.13%	0

No.-number

Table 2. Virus infection incidence determined by ELISA in variety Kujundžuša bijela

Location	Number of tested samples	Virus free samples (No.)	Infected samples (No.)	ArMV (No.)	GFLV (No.)	GLRaV - 1 (No.)	GLRaV - 3 (No.)
Vučja Draga	22	6	16	0	2	9	9
Bušanje	50	1	49	4	47	42	4
Maršići	3	1	2	0	0	2	2
Blato	17	8	9	1	8	2	0
Vinjani Gornji (A. A.)	2	0	2	0	1	1	2
Pešine njive	14	0	14	0	2	9	10
Vinjani Gornji (M. P.)	6	2	4	0	2	3	0
Glavina Donja	7	0	7	0	5	6	7
Hršćevani	19	2	17	0	6	10	9
Drum	26	0	26	0	24	23	4
Zmijavci	20	2	18	1	6	12	8
Slivno	5	0	5	1	2	5	0
Dubrava	18	3	15	1	12	11	1
Poljica (Gudelji)	10	0	10	0	9	9	4
Poljica	16	4	12	1	3	7	4
TOTAL (No./%)	235	29 12.34 %	206 87.66 %	9 3.83 %	129 54.89 %	151 64.26 %	64 27.23 %

No.-number

Furthermore, the additional spread of ArMV and GFLV at the same sites/sampling locations could be mediated by *Xiphinema index* and *Xiphinema diversicaudatum*, nematodes known to be their vectors.

From the data shown in Table 3, Zlatica vrgorska showed high infection rates for GLRaV-3 (59.79%) and GLRaV-1 (54.64%). Among tested varieties, this is the only variety to confirm findings by Karoglan Kontić et al (2009) and Vončina et al. (2019), the most common virus for the Mediterranean viticultural region is GLRaV-3. The lowest virus incidence was recorded for GFLV and ArMV, 5.15% and 6.19%, respectively.

Among the three varieties tested, Trnjak crni had slightly better sanitary status, with 30% of vines free from tested viruses. The virus incidence in Trnjak crni is similar to Zlatica vrgorska, with two main viruses

again GLRaV-1(47.5%) and GLRaV-3 (36.88%). However, compared to Zlatica vrgorska, the incidence of GFLV in Trnjak crni is much higher (18.13%). Again, the ArMV was detected in only two vines on two sampling locations. In the area of Vrgorac vineyards (Majići, Vlaka, Kozica, Raščane, Jaložina, ZavoJane) not a single Trnjak crni was infected with ArMV. A possible explanation could be in the absence of the vectors, nematodes *Xiphinema index*.

The results of virus-screening obtained in this study indicate a high level of virus infection of grapevine in Dalmatinska zagora, part of the Dalmatian viticultural area. This was found for all three varieties studied, with slightly better results for Trnjak crni. These results confirm the problematic health status of native Croatian varieties, especially Dalmatian varieties.

Table 3. Virus infection incidence determined by ELISA in variety Zlatarica vrgorska

Location	Number of tested samples	Virus free samples (No.)	Infected samples (No.)	ArMV (No.)	GFLV (No.)	GLRaV - 1 (No.)	GLRaV - 3 (No.)
Dražević	16	6	10	0	2	7	1
Rupa	25	8	17	0	1	14	8
Umčani	3	0	3	0	0	3	0
Barva	11	0	11	0	0	8	11
Strmenjak	22	0	22	0	0	6	20
Topolac	14	1	13	5	0	11	13
Vrgorac	1	0	1	1	0	1	1
Vlaka	1	1	0	0	0	0	0
Kozica	2	0	2	0	1	2	2
Ravča	2	0	2	0	1	1	2
TOTAL (No./%)	97	16 16,49 %	81 83,51 %	6 6,19 %	5 5,15 %	53 54,64 %	58 59,79 %

No.-number

Table 4. Virus infection incidence determined by ELISA in variety Trnjak crni

Location	Number of tested samples	Virus free samples (No.)	Infected samples (No.)	ArMV (No.)	GFLV (No.)	GLRaV - 1 (No.)	GLRaV - 3 (No.)
Vučja Draga	53	23	30	0	7	11	19
Vinjani Gornji (S. Đ.)	33	8	25	0	4	20	2
Seline	19	0	19	0	2	19	19
Vinjani Gornji (A. A.)	10	0	10	1	2	5	6
Slivno	7	2	5	0	1	4	1
Vinjani Gornji (M. P.)	5	5	0	0	0	0	0
Bušanje	2	0	2	1	1	2	0
Runovići	2	0	2	0	2	0	2
Zmijavci	1	0	1	0	1	1	0
Vinjani Gornji (J.P.)	1	1	0	0	0	0	0
Majići	3	0	3	0	0	3	3
Vlaka	3	0	3	0	1	3	3
Kozica	17	8	9	0	5	6	4
Raščane	1	0	1	0	1	1	0
Jaložina	1	0	1	0	1	0	0
Zavojane	2	1	1	0	1	1	0
TOTAL (No./%)	160	48 30 %	112 70 %	2 1,25 %	29 18,13 %	76 47,5 %	59 36,88 %

No.-number

Karoglan Kontić et al. (2009) confirmed that the main grapevine viruses in Croatia are those that cause grapevine leafroll-associated diseases (GLRaV-1 and GLRaV-3). They found that 86% of tested Dalmatian vines were positive for GLRaV-3, while GLRaV-1 prevailed in the continental regions. The incidence of GFLV and ArMV in whole Croatia was significantly lower. These findings later confirmed Vončina et al. (2017), that detected 91.7% vines infected with GLRaV-3 in the Croatian coastal region. Moreover, the infection with GLRaV-1 (39.6%) and GFLV (33.3%) was also determined. Additionally, Karačić (2015) at the Herzegovina area tested six most important native cultivars (Žilavka, Blatina, Trnjak, Bena, Dobrogostina, Krkošija), and confirmed the prevalence of GLRaV-3, detected in 66.52% of the tested samples, compared to GLRaV-1 found in 22% of the samples. The lowest number of vines was infected with GFLV and ArMV.

The already mentioned and the latest research by Vončina et al. (2019), who tested the presence of viruses in 14 Croatian native varieties from 51 vineyards on 1116 vines in Dalmatia. Observing the four most economically important viruses, the most common infection was with the GLRaV-3 with the average incidence of 79.6% (888 vines), while GLRaV-1 was considerably lower, with 40.8% infected samples (455 vines). Besides single infected vines, a significant number of those infected with different combinations of two, three or four viruses was found.

Furthermore, evaluation of the incidence of virus infections was conducted by Vončina et al. (2011b) in two Croatian grapevine collection vineyards: National collection Jazbina (Zagreb) and Risika (Krk). Out of 95 tested vines per collection site, only 10.5% (10 vines) in Jazbina and 7.4% (7 vines) in Risika collection vineyard, were found to be virus free. Remaining was infected by one (or more) of the nine tested viruses and mixed infection was common in both collections. Out of four analysed viruses in this research, following results were obtained: GLRaV-3 was dominant in both collections, 78.9% in Jazbina and 76.8% at Risika collection vineyard.

In the Jazbina collection vineyard, GLRaV-3 was followed by GLRaV-1 (29.5%), GFLV (17.9%) and ArMV (12.6%). The second most represented virus in Risika collection vineyard was GFLV (42.1%), followed by ArMV (23.2%) and GLRaV-1 (11.6%). This research showed a deviation from the norms for all viruses in 2011, so there were certainly expectations for change in geographical division of viruses in the future. The possible explanation may lie in different population of virus vectors. According to Masten Milek (2009), scale insects (vectors of grapevine leafroll-associated virus) are widely spread throughout Croatia. Genus *Pulvinaria* consists of *Pulvinaria floccifera*, *Pulvinaria hydrangeae* and *Pulvinaria vitis* as the most important vine pest from this genus.

Comparing the results of mentioned research, GLRaV-3 prevailed in the Mediterranean, while GLRaV-1 was dominant in the northern viticultural regions. The results presented in this research show that only Zlatarica Vrgorska had a high level of GLRaV-3 infection (59.79%), unlike other two varieties where GLRaV-1 virus dominated.

CONCLUSION

This research showed a very high infection rate with the four viruses (ArMV, GFLV, GLRaV-1, GLRaV-2) in the investigated Croatian native grapevine varieties (Kujudžuša bijela, Zlatarica vrgorska and Trnjak crni). Current sanitary status and nursery production with the lack of certified plant material contribute to spreading of the viruses.

High rates of virus infection were found in almost all Croatian native grapevine varieties grown in coastal part (Dalmatia) of state. This sanitary status represents a real threat to the genetic variability and breeding value. Accordingly, to the findings, it is necessary to continue with the preservation and further clonal selection of the native grapevine varieties.

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