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14–15 March 2024

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PREFACE

"From the mouth of my immortal teacher Pasteur, I heard these words:
It is true that science is international, but every scientist must be a man who in his scientific work is warmed by love for the people from which he sprang and to whom he owes all his strength".

Prof. Dr. Milan Jovanović Batut (1847-1940)"
The first dean of the Faculty of Medicine in Belgrade

Agricultural science and agriculture as a profession monitor and study changes occurring in this area, point out problems in agricultural practice, and find solutions. The Faculty of Agronomy in Čačak, in addition to educating students, 29y traditionally organizes the Symposium on Biotechnology (SYMBIOTECH) every year. The main goal is to acquaint the wider scientific and professional public with the results of the latest scientific research, and bring together domestic and foreign scientists in the fields of primary agricultural production, food processing, and environmental protection. We work tirelessly in pursuit of excellence.

At the 2nd International Symposium on Biotechnology, a total of 80 papers were presented in the 7 sections: Field, Vegetable and Forage Crops, Pomology and Viticulture, Livestock Production, Plant Protection, Food Safety and the Environment, Food Technology, Nutritionism, and Applied Chemistry.

We owe great gratitude to the **Ministry of Science, Technological Development and Innovation of the Republic of Serbia** and the **City of Čačak** for their traditional financial support and patronage of SYMBIOTECH24. We thank companies, entrepreneurs, stakeholders and all long-time friends of the Faculty of Agriculture for their material and organizational support.

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APPLICATION OF MULTIPLEX RT-PCR FOR GRAPEVINE VIRUSES DETECTION

Bojana Vasilijević[1], Vera Katanić[1], Sanja Živković[2], Tanja Vasić[2], Stefan Kovačević[1], Darko Jevremović[1]

Abstract: Grapevine, a significant fruit crop globally, is a host of various viruses that negatively affect yield, plant vigor, and fruit quality. Multiplex polymerase chain reaction (mPCR) offers the ability to detect numerous viruses simultaneously. Our study aimed to evaluate the effectiveness of mRT-PCR for the detection of nine grapevine viruses in Serbia, including: grapevine fanleaf virus, grapevine leafroll-associated viruses 1, 2, and 3, grapevine rupestris stem pitting associated virus, grapevine virus A, grapevine virus B, grapevine fleck virus, and arabis mosaic virus. This study confirms mRT-PCR as an efficient method for simultaneous detection of multiple grapevine viruses.

Keywords: grapevine, viruses, detection, mRT-PCR

Introduction

Grapevine (*Vitis vinifera* L.) is considered one of the most important fruit crops worldwide. Due to its high impact on the socioeconomy, great attention is focused on the cultivation and protection of this culture. Nevertheless, a grapevine is known to host a large number of different viruses that reduce yield and plant vigor, decrease fruit quality, and shorten vine longevity (Djenane et al., 2021). So far, more than 80 viruses have been discovered to infect grapevines. Leaf degeneration, grapevine leafroll, rugose wood complex, and fleck disease are the four main disorders among viruses of worldwide economic importance (Basso et al., 2017; Martelli, 2017; Vu et al., 2023).

Grapevine fanleaf virus (GFLV) is the main causative agent of grapevine fanleaf disease. GFLV causes many symptoms including leaf deformation, yellowing, mosaicking, vein banding, abnormal branching, and shortened internodes. Also, it causes considerable crop losses, reduces fruit quality, and shortens the longevity of grapevines in the vineyard. Yield losses can reach 77% in the most severe cases. It is

[1]Fruit Research Institute, KraljaPetra I 9, 32102 Čačak, Serbia (bvasilijevic@institut-cacak.org)
[2]University of Niš, Faculty of Agriculture, Kosančićeva 4, 37000 Kruševac, Serbia

transmitted specifically by the ectoparasitic nematode *Xiphinema index*, and belongs to the genus *Nepovirus* (Andret-Link et al., 2004; Djenane et al., 2021).

A group of viruses known as grapevine leafroll-associated viruses (GLRaVs), with at least 12 serologically distinct variants identified, named GLRaV-1 to GLRaV-12 are associated with leafroll disease. Ten of these viruses belong to the *Ampelovirus* genus, GLRaV-2 and GLRaV-7 belong to the *Closterovirus* and *Velarivirus* genus, respectively. The most widespread leafroll viruses are GLRaV-1 and GLRaV-3, followed by GLRaV-2 (Gambino, 2015; Martelli, 2017). Symptomatic vines infected with GLRaV-3 showed less vigorous growth and lower fruit yields compared to healthy ones. These viruses are primarily spread through vegetative propagation and grafting. The transportation of planting and propagation materials facilitates the long-distance movement of GLRaVs (Alabi et al., 2016).

Grapevine rupestris stem pitting associated virus (GRSPaV) is a member of the genus *Foveavirus* and is linked with Rupestris stem pitting disease. A close association between the presence of GRSPaV and symptoms of vein necrosis has been recently observed (Gambino and Gribaudo, 2006). GRSPaV is perhaps the most prevalent grapevine virus, and it can occur as distinct variants, each showing different symptoms on various *Vitis* spp (Meng and Rowhani, 2017). The presence of GRSPaV together with other viruses causes severe damage to infected grapevine plants very often. So, it's indicated that the presence grapevine virus A (GVA), may be required for rugose wood symptoms to occur. The virus is grapevine-specific and spreads through vegetative propagation, grafting, and possibly pollen and seed. However, no biological vectors have been reported (Stankovic et al., 2023).

The most widespread virus within the fleck complex is grapevine fleck virus (GFkV), which is the type species of the genus *Maculavirus*. Although latent in *V. vinifera*, GFkV induces specific foliar symptoms in the indicator *V. rupestris*, appearing as translucent spots. Leaves with intense flecking become wrinkled, twisted, and may curl upward. In contrast to the other viruses reported only in specific geographical areas, GFkV is ubiquitous. All these viruses are primarily spread through infected propagating material (Martelli, 2017).

Multiplex polymerase chain reaction (mPCR) is the simultaneous detection of multiple targets (e.g. viruses) in a single reaction using different primer pairs of each target. This method, which expands on the practical use of PCR, can result in significant time and labor savings in the laboratory without compromising the usefulness of the experiment. Multiplex PCR has been developed for the detection of multiple viruses in different plant species, including grapevine, cucurbits, wheat,

potato, and blueberry (Gambino and Gribaudo, 2006; Kwon et al., 2014; Kumar et al., 2017; Lee et al., 2023; Deb et al., 2023).

Multiple infections in grapevines are frequent, and the use of one reaction for the detection of several viruses is of great importance to facilitate the analysis. The aim of our study was to evaluate the multiplex reverse transcription-polymerase chain reaction (mRT-PCR) for simultaneous detection of nine viruses infecting grapevines in Serbia.

Materials and methods

The material for this study was selected from the collection of grapevine plants maintained at the greenhouse of the Fruit Research Institute, Čačak. The collection was formed in 2020 by rooting hardwood cuttings collected in grape orchards in Rasina district, Serbia. Cuttings were collected from plants showing various symptoms on leaves that could be ascribed to viral infections and from asymptomatic plants. Leaf samples for the analysis were collected from two-year-old plants and stored at -20°C until analysis. For this study, a total of 13 samples were selected and analyzed (Table 1).

Table 1. The list of tested samples used in this study

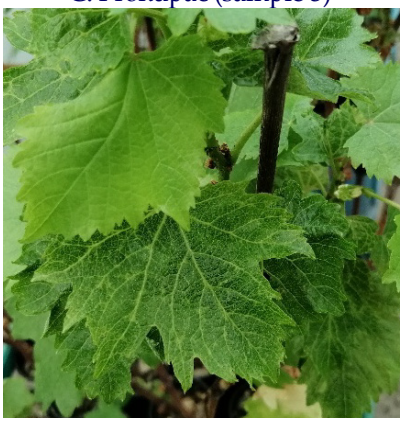
Sample number	Cultivar	Locality
1	Smederevka	Trmčare
2	Prokupac	Trmčare
3	Prokupac	Kobilje
4	Prokupac	Kobilje
5	Prokupac	Trmčare
6	Rajnski Rizling	Trmčare
7	Tamjanika	Trmčare
8	Smederevka	Trmčare
9	Prokupac	Trmčare
10	Prokupac	Trmčare
11	Prokupac	Mala Kruševica
12	Grakče	Padež
13	Prokupac	Donja Zleginja

A. Smederevka (sample 1)

B. Prokupac (sample 2)

C. Prokupac (sample 3)

D. Rajnski rizling (sample 6)



E. Prokupac (sample 11)

F. Prokupac (sample 13)

Figures 1A–1F. Symptoms associated with virus infections on grapevine cultivars

Total nucleic acids (TNA) were extracted from frozen leaf petioles using a 2% CTAB buffer, following the protocol of Li et al. (2008) with minor modifications as described by Jevremović et al (2019). The first-strand cDNAs were synthesized by reverse transcription (RT) reactions using Maxima Reverse Transcriptase (Thermo Fisher Scientific, USA) in accordance with the manufacturer's protocol. The obtained cDNAs were used as templates in multiplex polymerase chain reaction with nine virus-specific primer pairs (Table 2). The mPCR conditions were as follows: initial denaturation at 94°C for 4 min; followed by 35 cycles at 94°C for 30 s, 50°C for 1 min and 72°C for 90 s; with a final extension at 72°C for 10 min. mRT-PCR reactions were conducted in TPersonal thermocycler (Biometra, Germany) and PCR products were analyzed on 5% polyacrylamide gel stained with silver-nitrate.

Table 2. Specification of primers used for virus detection

Virus	Primers	Primes sequences 5'-3'	Product size (bp)	Reference
GFLV	Forward	ATGCTGGATATCGTGACCCTGT	118	Gambino and Gribaudo (2006)
	Reverse	GAAGGTATGCCTGCTTCAGTGG		
GRSPaV	Forward	GGGTGGGATGTAGTAACTTTTGA	155	
	Reverse	GCAAGTGAAATGAAAGCATCACT		
GFkV	Forward	TGACCAGCCTGCTGTCTCTA	179	
	Reverse	TGGACAGGGAGGTGTAGGAG		
GLRaV-1	Forward	TCTTTACCAACCCCGAGATGAA	232	
	Reverse	GTGTCTGGTGACGTGCTAAACG		
GVA	Forward	GAGGTAGATATAGTAGGACCTA	272	
	Reverse	TCGAACATAACCTGTGGCTC		
GLRaV-3	Forward	TACGTTAAGGACGGGACACAGG	336	
	Reverse	TGCGGCATTAATCTTCATTG		
ArMV	Forward	TGACAACATGGTATGAAGCACA	402	
	Reverse	TATAGGGCCTTTCATCACGAAT		
GVB	Forward	GTGCTAAGAACGTCTTCACAGC	460	
	Reverse	ATCAGCAAACACGCTTGAACCG		
GLRaV-2	Forward	GGTGATAACCGACGCCTCTA	543	
	Reverse	CCTAGCTGACGCAGATTGCT		

Results and discussion

PCR analysis revealed virus presence in all 13 tested samples (Table 3). PCR products of the expected size were obtained from tested grapevines, and no

amplification products were observed in the negative control (sterile water). Positive reactions were obtained with positive controls of tested viruses (Figure not shown). Seven out of nine tested viruses were detected in analyzed samples: GFLV, GRSPaV, GFkV, GLRaV1, GLRaV3, ArMV and GVA. The most detected virus was GRSPaV that was confirmed in all 13 samples, followed by GLRaV-1 (12), GFLV (9), GLRaV-3 (8), GFkV (4), GVB (2), and ArMV (1). The presence of GVA and GlRaV-2 was not confirmed in tested samples.

Table 3. Results of mRT-PCR detection in grapevine samples

No	**virus**								
	GFLV	GRSPaV	GFkV	GLRaV1	GVA	GLRaV3	ArMV	GVB	GLRaV2
1	+*	+	+	+	-**	+	-	+	-
2	-	+	-	-	-	-	-	-	-
3	-	+	-	+	-	-	-	-	-
4	-	+	-	+	-	+	-	-	-
5	+	+	+	+	-	+	-	-	-
6	+	+	-	+	-	+	+	+	-
7	-	+	-	+	-	-	-	-	-
8	+	+	-	+	-	-	-	-	-
9	+	+	-	+	-	+	-	-	-
10	+	+	-	+	-	+	-	-	-
11	+	+	+	+	-	+	-	-	-
12	+	+	-	+	-	-	-	-	-
13	+	+	+	+	-	+	-	-	-

*Positive reaction, **Negative reaction

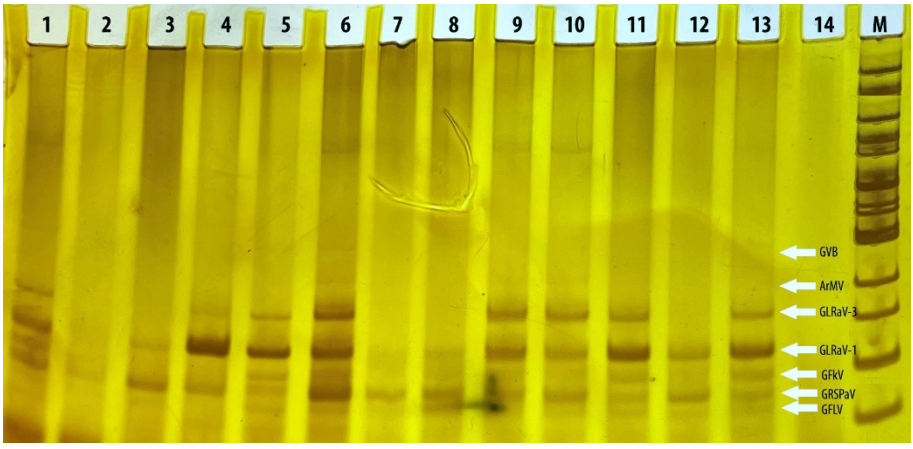


Figure 2. mRT-PCR detection of grapevine viruses (lines 1–13: analysed samples, line 14: negative control; line M: 100 bp DNA Ladder (Solis BioDyne, Estonia)

Lanes 1–13 (Table 2) show bands of different intensities for the desired PCR products around the expected size. In general, stronger bands indicate relatively higher virus concentration in tested sample. As presented in figure 2, in all tested samples, except sample 2, mixed infections were detected.

Several research groups reported simultaneous detection of grapevine viruses. Nassuth et al. (2000) published mRT-PCR procedure for the detection of five grapevine viruses: GVA, GVB, GLRaV-3, ArMV and GRSPaV. Gambino and Gribaudo (2006) used a multiplex procedure to detect the nine most important and widespread viruses of grapevine. A large number of samples were tested using ELISA, single-PCR and mRT-PCR. Based on the obtained results, the authors recommended the use of sensitive and reliable mRT-PCR for analysis in order to reduce costs and save time. López-Fabuel et al. (2013) developed a real-time RT-PCR multiplex detection method for the detection of five major grapevine viruses (GFLV, ArMV, GLRaV-1, GLRaV-3, and GFkV). Later on, the authors upgraded the method, including four viruses: GVA, GVB, GRSPaV, and GLRaV-2. This approach improved detection by broadening the range of targeted viruses. Bruisson et al. (2017) successfully identified the same set of nine grapevine viruses by TaqMan® RT-qPCR.

In previous research, Gambino (2015) demonstrated that the detection limits of multiplex RT-PCR were lower than those of single RT-PCR (primer sets undergoing separate testing in sRT-PCR before multiplex analysis). The reason is that, in the multiplex assay, the mix of primers competes for all templates instead of just one. Furthermore, compared to other advanced methods like real-time RT-PCR, the multiplex protocol did not show greater sensitivity. However, the level of sensitivity is considered reliable enough for the proposed purpose. It offers an effective and quick diagnostic process that's reliable, affordable, and easy to use, even for labs without much expertise or expensive equipment. In our study, the performance of mRT-PCR was reliable for routine diagnostics, allowing quick and multiple detection of viruses infecting grapevines. The method is recommended for rapid screening of the material. For certification purposes, to produce nuclear stock and to analyze higher categories of planting material, a combination of different methods is recommended (biological, serological, and molecular).

So far, eleven viruses infecting grapevines have been identified in Serbia: GVA, GVB, GLRaV-1, -2, -3, GFkV, ArMV, GFLV, raspberry bushy dwarf virus (RBDV), GPGV (grapevine Pinot gris virus), and GRSPaV (Sivčev et al., 2011; Jevremović and Paunović 2011; Mandić, 2018; Živković et al., 2022; Stanković et al., 2023). In all these studies, serological (ELISA) and molecular RT-PCR analyses were applied. In our study we presented the results of the application of mRT-PCR for the simultaneous amplification of several RNA plant viruses. This technique developed by Gambino and Gribaudo (2006) was found to be effective for detecting Serbian isolates of viruses infecting grapevines. Based on the results, this protocol can be recommended for the analysis of grapevine in Serbia, which allows rapid and simultaneous detection of multiple viruses.

Conclusion

The findings of our investigation confirmed the efficiency and suitability of multiplex RT-PCR for the simultaneous detection of multiple viruses in grapevine samples. The method was successfully applied for the detection of Serbian isolates of grapevine viruses, both in single and multiple infections.

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