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Full Length Article

A Survey for Two Major Grapevine Viruses in Georgian Vineyards, Caucasus Region

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Abstract

This study represents the distribution of *Grapevine fleck* and *G. fanleaf* viruses in the vineyards of the Kakheti region, the number one winemaking area in the republic of Georgia and highlights the benefit of the timely identification of phytopathogens using reliable laboratory assays. The main goal of the proposed study was to identify grapevine viruses in two prominent cultivars: *Rkatsiteli* and *Saperavi*. A survey was conducted in the 2020–2021 growing seasons. In total 600 grapevine samples were collected from 30 vineyards and analyzed using Double-antibody sandwich enzyme-linked immunosorbent assay (Das-ELISA) and One-Step RT-PCR analysis. Out of 123 positive samples 112 tested positive for *G. fleck* virus and 11- for *G. fanleaf* virus respectively based on Das-ELISA assay. Mix infection was demonstrated in 2 tested *Saperavi* cultivars. *G. fleck* virus was prevalent and both viruses were predominantly distributed in the *Saperavi* cultivar. Randomly selected 28 positive and 20 negative samples were further confirmed using a One-Step RT-PCR assay that revealed the effectiveness of Das-ELISA. A comprehensive diagnosis is important for controlling grapevine viruses and helps develop regulatory measures further to establish improved strategies for using virus-free planting materials.

Keywords: *Vitis vinifera* L.; Grapevine viruses; Viral disease; Serological assay; Molecular assay

Introduction

Grapevine fanleaf virus (GFLV) and *Grapevine fleck* virus (GFkV) are one of the most widely distributed and economically significant phytopathogens affecting wine grapes (*Vitis vinifera* L.) (Bahder *et al.* 2013; Martelli 2017). GFLV is a soil-borne viral pathogen of grapevines responsible for fanleaf degeneration (Bovey *et al.* 1990; Martelli and Savino 1990; Martelli 2014; Zherdev *et al.* 2018). The disease occurs worldwide in most temperate grapevine distributed regions (Andret-Link *et al.* 2004; Maliogka *et al.* 2015) and is transmitted *via* ectoparasitic nematode *Xiphinema index* with the propagating material (Martelli and Savino 1990; Villate *et al.* 2008). Symptoms caused by GFLV in grapevines may differ in patterns and severity and may include distorted, asymmetrical leaves, chlorotic mottling, and yellow mosaic (Martelli and Sovino 1990). GFkV is associated with the fleck disease complex with the ability of non-mechanical transmission (Bahder *et al.* 2013). GFkV is only found in the phloem and is transmitted through propagation and grafting (Sabanadzovic *et al.* 2001; Kanuya *et al.* 2012; Poojari *et al.* 2016; Zherdev *et al.* 2018). There are no reported vectors for GFkV. Disease caused by GFkV has been linked with reduced growth and low quality of wood for propagation. Foliage fleck symptoms including clearing of the veinlets and mosaic patterns with distorted leaves can be observed during GFkV infection (Fajardo *et al.* 2012; Martelli 2014). Georgia is considered the birthplace of wine as studies revealed evidence of cultivating grapevines during the early Neolithic Period from Georgia's country (McGovern *et al.* 2017). Nowadays Georgia is home to about 500 varieties of indigenous grapes that make up one-sixth of the world's total grapevine varieties. The country has up to 50,000 hectares of grapes, consisting of 75% white grapes and 25%-red respectively including endangered vines only found in Georgia. Georgia's number one winemaking area is the Kakheti region situated in the east part of the country comprising nearly 80 grape varieties, including the two most prominent – *Rkatsiteli* (white) and *Saperavi* (red) cultivars (Wines Georgia 2021). The main goal of this study was to survey vineyards in the Kakheti region to detect the presence of GFkV and GFLV in two main *Rkatsiteli* and *Saperavi* cultivars. Therefore, a better evaluation regarding the distribution of grapevine viruses in eastern Georgia and the assessment of the impact with the economic

consequences to the winegrape industry can be done. Correspondingly, as a wine-producing area, it's reasonable to have information regarding the dissemination of grapevine viruses in eastern Georgia to take all the necessary measures for the subsequent elimination of the grapevine viruses.

Materials and Methods

Survey and sampling

To investigate the status of virus infection in wine grape vineyards in the Kakheti region, 600 grapevine samples (including 300 *Rkatsiteli* and 300 *Saperavi* cultivars) from a total of 30 different vineyards (20 samples for each vineyard) were collected from June to October 2020–2021. As the distribution of grapevine viruses can be uneven in grapevine tissue according to the season and tissue source (Martelli and Sovino 1990; Constable and Randoni 2011) mature basal leaves with petioles and veins were collected and placed in sealed plastic bags for further transportation. A survey was conducted based on typical symptoms as well as randomly as grapevines can be completely symptomless. Collected samples were stored at 4°C maximum of 2–3 days for serological analysis and up to 1 week for further molecular analyses.

Serological analysis

Das-ELISA (BIOREBA AG, Reinach, Switzerland) was performed for the detection of GFkV and GFLV. Samples of grapevines fitted in extraction plastic bags (BIOREBA) were homogenized in the special extraction buffer "Grapevine" (Tris base 200 m*M*, NaCl 137 m*M*, PVP K25 2% (w/v), PEG 6000 1% (w/v), NaN3 0.02% (w/v), Tween 20 0.05% (w/v); pH 8.2) at a ratio of 1:10 (w/v). The test reaction was performed on 96 well plates according to manufacturers' instructions. Appropriate $OD_{405/492}$ values were obtained after the run of the samples into the Elisa Reader Optic Ivymen® system 2100-C.

RNA extraction

RNA was extracted from randomly selected 28 positive and 20 negative plant samples previously tested by Das-ELISA. Plant RNA Purification kit (OxGEn molecular solutions® Georgia) was used to isolate RNA from the tested samples. Following the recommendations preparing the lysate, binding, washing, and the final elution steps were done as it is described in the manufacturers' instructions. Extracted RNA concentration was assessed with a Qubit® RNA HS Assay Kit using a Qubit 3.0 fluorometer (Thermofisher Scientific) following the manufacturers' instructions.

One-step RT-PCR analysis

SuperScriptTM IV One-Step RT-PCR System (Invitrogen by Thermo Fisher Scientific) was used to perform revers-transcription (RT) and PCR steps continuously in one tube. The reaction mix was prepared as per the manufacturer's instructions. The assay was performed in a 50 μL mixture containing 25 μL 2×Platinum SuperFi RT-PCR Master Mix, 0.5 μL SuperScript IV RT Mix, 2.5 μL of each primer, up to 50 ng/μL template RNA and an appropriate volume of nuclease-free water to fill up the reaction mix up to 50 μL. Corresponding primers (Table 1) were used at final concentrations of 10 μM. The reaction mix was placed in the thermal cycler (SymplyAmp Applied biosystems) preprogrammed as follows: 1 cycle of RT at 50˚C for 10 min and RT inactivation/initial denaturation at 98˚C for 2 min, followed by 40 cycles of amplification at 98˚C for 10 s, 61˚C (GFLV), 70˚C (GFkV) for 10 s and 72˚C for 30 s and a final extension at 72˚C for 5 min. The appropriate annealing temperature for the used primers was defined using the Tm calculator (Thermo Fisher). Pre-stained 1% Agarose gel (UltraPureTM Agarose; Invitrogen) with ethidium bromide (Invitrogen) was prepared using TAE buffer (20 m*M* sodium acetate, 1 m*M* EDTA pH 8.0, 40 m*M* Tris base) in order to analyze obtained PCR amplicons by agarose gel electrophoresis. Visualization of PCR amplicons was done in the SMART 5 VWR gel documentation system. RT-PCR run consisted of samples without RNA, molecular grade water, and the negative sample obtained from Das-ELISA assay as negative controls.

Results

As a result, 600 grapevine samples from 30 vineyards covering different villages of the Kakheti region were tested for the presence of GFkV and GFLV. Notably, no symptoms of the viral infection were observed in the case of GFLV positive, while some symptoms were observed only in a few GFkV positive grapevine samples including leaf yellowing and mosaic pattern (Fig. 1).123 (20.5%) samples resulted as positive by Das-ELISA for both surveyed viruses among them 74 for *Saperavi* cultivar and 49 for *Rkatsiteli* respectively. 112 samples (18.7%) tested positive for GFkV with an infection rate from 0.0 to 50% and only 11 samples (1.8%) tested positive for GFLV where the infection rate varied from 0.0 to 15% in both surveyed grapevine cultivars. The study showed that GFkV was predominantly distributed in *Saperavi* cultivar consisting of 65 samples out of 112 Das-ELISA positives while in *Rkatsiteli* cultivar GFkV was found in 47 samples respectively. Regarding GFLV out of 11 positive 2 samples were seen in *Rkatsiteli* cultivar whereas the rest 9 samples were found in *Saperavi* cultivar (Table 2). Mix infections were demonstrated only in *Saperavi* cultivar where both viral agents were detected in 2 tested samples. The percentages of positive samples in the two surveyed cultivars are shown in Fig. 2. Twenty-eight positive samples obtained from Das-ELISA assay including 22 GFkV positive samples from 12 *Rkatsiteli* and 10

Table 1: The primers used in One-Step RT-PCR to amplify the RNA-dependent RNA polymerase (RDRP) and coat protein (CP) genes. Viruses included GFkV and GFLV F (forward) and R (reverse) primers

Virus	Primer's ID	Fragment length (nt)	Gene	Sequence (5'-3')	Reference
GFLV	GFLV-V1/F	322	CP gene	ACCGGATTGACGTGGGTGAT	Sànchez *et al*. (1991)
	GFLV-C1/R			CCAAAGTTGGTTTCCCAAGA	
GFkV	GFkV-585/F	533	RdRP gene	CTCAGCCTCCACCTTGCCCCGT	Naidu and Mekuria (2010)
	GFkV-1117/R			CAATTTGGCTGGGCGAGAAGTACA	

Table 2: Vineyards surveyed for GFkV and GFLV viruses in the Kakheti region. Samples tested by Das-ELISA and One-Step RT-PCR are presented

Regions	Cultivar	No. of vineyards surveyed	GFkV			GFLV		
			No. of ELISA positive samples/tested samples	Infection rate (%)	No. of PCR positive samples/tested samples	No. of ELISA positive samples/tested samples	Infection rate (%)	No. of PCR positive samples/tested samples
Ikalto	Rkatsiteli	1	8/20	40	1/1	0/20	0	0
Koghoto	Rkatsiteli	1	3/20	15	1/1	0/20	0	0
Alvani	Rkatsiteli	1	8/20	40	1/1	0/20	0	0
Napareuli	Rkatsiteli	1	5/20	25	1/1	0/20	0	0
Kvareli	Rkatsiteli	1	6/20	30	1/1	0/20	0	0
Telavi	Saperavi	5	19/100	19	3/3	3/100	3	1/1
Alvani	Saperavi	1	10/20	50	1/1	1/20	5	1/1
Pshaveli	Saperavi	1	8/20	40	1/1	0/20	0	0
Napareuli	Saperavi	1	5/20	25	1/1	0/20	0	0
Akhmeta	Rkatsiteli	1	0/20	0	0	0/20	0	0
Akhshani	Rkatsiteli	1	1/20	5	1/1	0/20	0	0
Ruispiri	Rkatsiteli	1	2/20	10	1/1	0/20	0	0
Gulgula	Rkatsiteli	1	1/20	5	1/1	1/20	5	1/1
Telavi	Rkatsiteli	1	0/20	0	0	0/20	0	0
Tsinandali	Saperavi	1	0/20	0	0	0/20	0	0
Shakriani	Saperavi	2	0/40	0	0	0/40	0	0
Akhmeta	Saperavi	1	6/20	30	1/1	2/20	10	1/1
Akura	Rkatsiteli	1	6/20	30	1/1	0/20	0	0
Vanta	Rkatsiteli	1	2/20	10	1/1	0/20	0	0
Tsinandali	Rkatsiteli	1	3/20	15	1/1	1/20	5	1/1
Busheti	Rkatsiteli	1	0/20	0	0	0/20	0	0
Kisiskhevi	Rkatsiteli	1	2/20	10	1/1	0/20	0	0
Nasamkhrali	Saperavi	1	1/20	5	1/1	3/20	15	1/1
Shalauri	Saperavi	1	10/20	50	1/1	0/20	0	0
Kondoli	Saperavi	1	6/20	30	1/1	0/20	0	0

Saperavi vineyards (one sample per vineyard) and six GFLV positive samples from 2 *Rkatsiteli* and 4 *Saperavi* vineyards were randomly selected and confirmed to be positive for GFkV and GFLV by One-Step RT-PCR using GFkV and GFLV specific primers (Table 2). Furthermore, randomly selected twenty Das-ELISA negative samples were also analyzed by One-Step RT-PCR and tested negative for both viruses. Agarose gel electrophoresis ascertained a single DNA fragment with appropriate size while negative controls remained without any amplification (Fig. 3).

Discussion

The prevalence of GFkV could be explained by using infected plant materials as there is no vector revealed for this virus. GFLV had a limited distribution compared to the GFkV as also described in previous studies (Basso *et al.* 2017; Porotikova *et al.* 2021). The reason that GFLV incidence is low could be the absence of occurrence of its vector (nematode: *Xiphinema index*), grapevine cultivated soils in vineyards that may not encourage the survival of the nematode, also it could be associated with the limited existence of GFLV in propagated grapevine materials that are used to establish new vineyards. Overall analysis showed that within two tested viral species, GFkV was prevalent and both viruses were predominantly distributed in *Saperavi* cultivar while the opposite trend was seen for the *Rkatsiteli* cultivar where lower prevalence for both viral agents was detected. Regardless of those symptoms observed only in a few GFkV positive grapevine samples including leaf yellowing and mosaic pattern it cannot be said that they were the result of GFkV infection as symptomless *V. vinifera* is common unless the virus infects *Vitis rupestris cv.'St. George'* (Fajardo *et al.* 2012) cultivar. Although there are reported studies on the distribution of grapevine viruses such as *GLRaV-1*, *GLRaV-3* and *GVA* in Georgian vineyards (Megrelishvili *et al.* 2016), the aforementioned symptoms cannot be assigned even to those viral pathogens until applying of molecular techniques for further confirmation is done. The occurrence of both etiological viral agents was

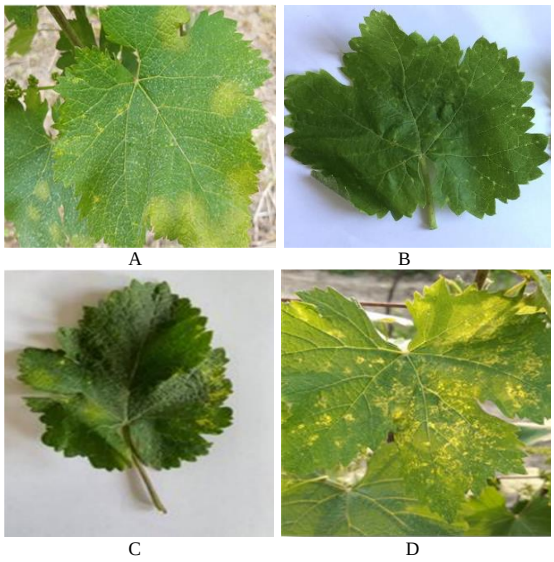

Fig. 1: (**A**), (**B**) and (**C**) grapevine samples from GFkV positive *Saperavi* cultivars; (**D**) grapevine sample from GFkV positive *Rkatsiteli* cultivar

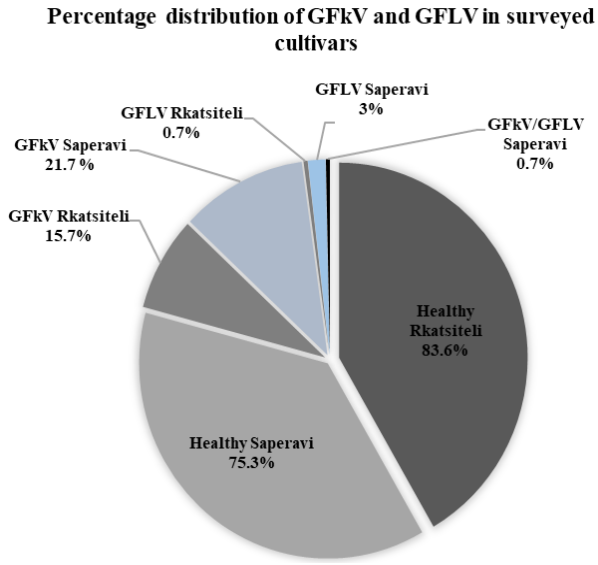

Fig. 2: Distribution of grapevine viruses in two main cultivars: *Rkatsiteli* and *Saperavi* (Percentage of plants positive and negative for both viruses by Das-ELISA assay are presented)

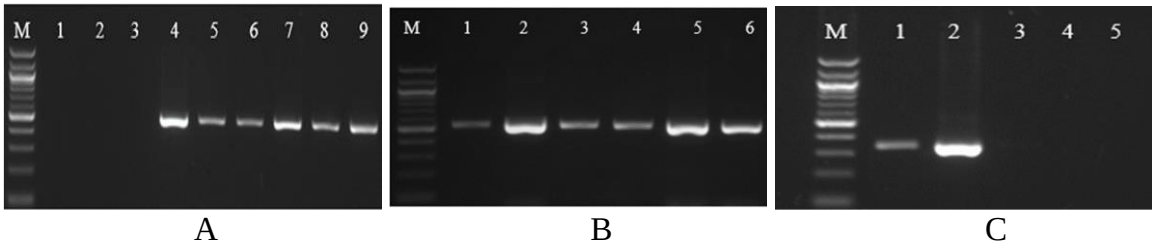

Fig. 3: Amplification of the expected 533 nt. (**A**; **B**) DNA fragments with GFkV-specific primer pair GFkV-585/F-1117/R and 322 nt (**C**) DNA fragments with GFLV-specific primer pair GFLV-V1/F- C1/R resulted from One-Step RT-PCR; M: 100 bp DNA ladder; (**A**) Lanes: 1-3 Negative controls; Lanes 4–9 GFkV positive samples in *Saperavi*; (**B**) Lanes: 1-6 GFkV positive samples in *Rkatsiteli*; (**C**) Lane: 1- GFLV positive sample in *Rkatsiteli*; Lane: 2- GFLV positive sample in *Saperavi*; Lanes: 3-5 Negative controls

established using serological and molecular methods. Obtained results showed that the extracted RNAs and the synthesized cDNA templates were free of extra contaminants, resulting in a high level of RNA and DNA preparations produced for gene expression level assays by One-Step RT-PCR indicating high sensitivity of molecular-based approaches as well as reliability and effectiveness of Das-ELISA assay. The benefit of Das-ELISA was to avoid false negatives in case of weakly positive samples with an antigen concentration near the detection limit and therefore allowed taking full advantage of the sensitivity of the Das-ELISA test.

Conclusion

The study has revealed the presence of grapevine viruses in surveyed vineyards in the east part of Georgia. Within the tested grapevines, most of the GFkV positive and all of the GFLV positive samples were symptomless. Thus, it is of high importance to apply appropriate, reliable and sensitive diagnostic assays for timely detection of the viral agents distributed in the vineyards. Das-ELISA can be employed in routine diagnostic tests for large-scale screening as it has been shown to be a consistent and effective tool for testing samples. Laboratory diagnostic assay such as the PCR technique should be applied to validate and confirm the results obtained from the serological method. The presence of single and mixed virus infection underscores the need for further investigation of the vineyards for the presence of other economically significant grapevine viruses. The health status of grapevines in vineyards should receive appropriate attention to prevent the dissemination of viral etiological agents, improve regulatory measures and establish management of using "clean" planting materials from laboratory-tested vines. A comprehensive diagnosis is important for the effective control of grapevine viruses and further for the control

of certified propagation material with virus-free status as it is the only trustworthy and credible strategy to control and prevent the spread of the diseases in viticulture.

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Author Contributions

All authors contributed equally to this work.

Conflict of Interest

All authors declare that they have no competing interests and that all co-authors have agreed to have seen and approved the manuscript for submission

Data Availability

The data are available with the authors

Ethics Approval

Authors declare that the study does not involve research neither on the human nor on live animal studies

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