

Current advances in understanding the *Grapevine Pinot gris virus*

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ABSTRACT

Grapevine virus diseases are widely spread in all viticultural areas of the world and they are considered detrimental, their presence is the most limiting factor of plants performances and vineyard life lasting. A large number of viruses have been identified in grapevine. From them, reported for the first time in 2012, *Grapevine Pinot gris virus* (GPGV) is spread all over the world. This study brings new knowledge about the virus distribution in plants throughout one year, the organ and period of sampling, for a reliable diagnostic. Additionally, the influence of high temperature on the physiological processes was investigated. Plants belonging to the 'Negru mare' genotype, natural infected with GPGV and *Grapevine fleck virus* (GFkV), maintained in a greenhouse, constituted the biological material. ELISA analyses of different grapevine organs and tissues (apex, leaf blade, petiole, young shoot, tendril, inflorescence, cane) located along the length of the shoot, carried out on different phenophases (before flowering, after flowering, veraison, dormancy) revealed that the conductive tissue was the most appropriate for diagnosis in the active growth period when the air temperature did not exceed 30°C. The measurement of the leaf temperature in two moments of the day (morning and afternoon), when the air temperature records increasing values revealed that at high temperatures (above 37°C), as a result of the intensification of the transpiration processes, the temperature at the leaf level does not increase progressively with the air temperature. Measurements of stomatal opening in two periods of the day in infected and virus-free grapevines showed that GPGV+GFkV complex infection caused sometimes a significant increase in stomatal opening both in the morning and in the afternoon, which could be a deficiency in the management of water and gas exchanges in plants.

Keywords: *Vitis*, GPGV, GFkV, ELISA, virus distribution, stomata, leaf temperature

INTRODUCTION

Grapevine viral diseases are widely spread in all viticultural areas of the world and they are considered detrimental, their presence is the most limiting factor of plant's performances and vineyard life lasting (Martelli and Boudon-Padieu, 2006; Giribaldi *et al.*, 2011; Mannini and Digiario, 2017). Until now, 86 viruses belonging to different families have been reported to infect grapevines (Fuchs, 2020). Viral infection produces physiological and structural alterations, affecting: photosynthesis, gas exchange, chlorophyll content, carbon translocation, phloem transport, and cellular metabolism (González *et al.*, 1997; Bentamini *et al.*, 2004; Endeshaw *et al.*, 2014; Martínez-Lüscher, 2019; Kappagantu *et al.*, 2020).

Grapevine (*Vitis spp.*), a woody species, is a model plant for the study of plant-virus interactions in both controlled and field conditions (Perrone *et al.*, 2017), but the evaluation of the effects of a virus disease is not easy because a grapevine plant can be infected by numerous viral entities at a time. Also, the losses produced by virus infection depend on the plant age, environmental and growing conditions, grapevine cultivars and rootstock (Golino *et al.*, 2003; Mannini, 2003; Pesqueira *et al.*, 2012). Thus, it should not be forgotten that field-grown plants are simultaneously subjected to different abiotic and biotic stress factors (Suzuki *et al.*, 2014). Belonging to *Trichovirus* genus in the *Betaflexiviridae* family, *Grapevine Pinot gris virus* (GPGV) has been reported in the last few years in the major grapevine growing regions of the world - USA, China, Canada, Europe, Australia (Saldareli *et al.*, 2017). Having symptomatic and asymptomatic strains, it is known to occur in both red and white wine grape cultivars, as well as in table grapes or rootstock, most of the time in combination with other viral infections. This paper aims to supplement the knowledge on GPGV infection with new data on the distribution of the virus in the plant, in order to establish a correct diagnosis (the period of sampling and the type of tissue), as well as its influence on the development of physiological processes in the plant, under the conditions of global warming.

MATERIALS AND METHODS

The studies were carried out on three grapevine plants belonging to *V. vinifera* L. 'Negru mare' genotype, maintained in vegetation pots in the greenhouse, identified as being infected with the viral complex GPGV and *Grapevine fleck virus* (GFkV).

The viral distribution in the plant was monitored during the vegetation phenophases (before flowering, after flowering, veraison, dormancy) by analyzing different organs (apex, leaf blade, petiole, young shoot, tendril, inflorescence, cane) collected from three shoots/plant, from three zones of the shoot (base, middle, top), by Enzyme linked-immunosorbent assay (ELISA) method (Clark and Adams, 1977), using commercial reagents (Bioreba, Switzerland). ELISA results were presented as optical density (OD) read on spectrophotometer, with a double filter, at 405/492 nm. All values above the cut-off (x3 OD mean of negative control) calculated for each test series, were positive considered.

The air temperature in the greenhouse was monitored throughout the vegetation period using a digital thermometer (Huger Electronics GmbH, Germany). The influence of abiotic factors represented by temperature, and biotic factors represented by a viral infection on the development of physiological processes in grapevines, in the context of plant adaptation to global warming, was studied by biometric measurements of the stomatal ostiole correlated with the temperatures measured at the leaf level (registered with the device FLIRONE PRO, Estonia) and of the environment. In 2021 two days of July, in the morning and the afternoon, the temperatures were recorded in three areas of each leaf (top, lower lobes), located in the middle zone of the shoot of each plant under study. The imprints were taken from the three zones of each leaf (Gokbayrak *et al.*, 2008) and analyzed with an OLYMPUS BX 41 (Germany) microscope, equipped with a digital camera. From each zone/imprint, 5 fields were analyzed, each field measuring the opening of the ostium of 10 stomata, using the Quick PHOTO MICRO 2.2 photo-documentation system. In the graphical representation, values are mean $\pm$ sd based on 150 measurements/leaf.

The control was represented by three grapevine plants of 'Negru Mare' genotype, GPGV+GFkV-free, regenerated by *in vitro* chemotherapy from infected plants, maintained under the same conditions. Statistical significance of differences between virus-infected plants compared with the control (healthy) was analyzed by SPSS 10 for Windows, taking $P < 0.05$ as significant according to one-way ANOVA.

RESULTS AND DISCUSSIONS

Since the development of laboratory methods for viral diagnostic, it has been studied, depending on each virus, which is the most reliable organ/tissue to analyze as well as the period suitable for sampling: Walter and Etienne (1987), Bovey *et al.* (1980), Fiore *et al.* (2009) for *Grapevine fanleaf virus* (GFLV); Monis and Bestwick (1996) for *Grapevine leafroll-associated virus type 1, 2, 3* (GLRaV-1, 2, 3); Buciumeanu *et al.* (2008) for *Grapevine virus A* (GVA). As a rule, ELISA diagnostic kits recommend the tissue type and optimal sampling period. GPGV being one of the last grapevine virus that can be identified by ELISA, the insert kit states: analysis of leaf samples collected from the same plant from the base, middle and apex shoot demonstrated that the best tissue for diagnosis is represented by young leaves located at the top and middle of the plant, collected in the spring.

At the same time, phloem tissue scraped from canes during the dormant period remains a good tissue for diagnosis.

In the present study, samples collected in April 2021, from various organs of a plant previously diagnosed with the GPGV + GFkV complex led to positive ELISA results only from the shoot placed at the base of the plant for GPGV, while GFkV was identified in all analyzed organs (Table 1). The plant from which the samples were collected had four internodes and showed symptoms of GPGV (deformations of the leaves, leaf mottling) at that time.

Table 1. Optical density values at 405/492 nm in samples collected from various grapevine organs belonging to the 'Negru mare' genotype, for the diagnosis of GPGV and GFkV, in the phenophase before flowering (April 2021). Values marked in red are ELISA positive. Cutt-off values were 0.123 for GPGV and 0.114 for GFkV

Virus	Apex	Basal leaf	Apical leaf	Basal shoot	Apical shoot	Inflorescence
GPGV	0.093	0.042	0.046	0.128	0.073	0.098
GFkV	0.171	1.112	0.851	3.409	0.266	0.330

In the phenophase of active growth of the plant, after flowering, when the shoots reached the size of 8-20 internodes (June 2021), the petiole of the leaves located at any level of the shoot was ELISA positive for GPGV (except 3 cases out of 23 tests), while only the leaf blade at the top, but in a rather small proportion (3 out of 9 tests), registered a positive result. Also, all samples of tendril were positive for GPGV (Table 2). Regarding the diagnosis for GFkV, with two exceptions, all types of organs analyzed led to positive results, confirming viral infection (Table 3).

Table 2. Optical density values at 405/492 nm in samples collected from various grapevine organs of three plants belonging to 'Negru mare' genotype, for the diagnosis of GPGV, in the phenophase of berries developing (June 2021). Values marked in red are ELISA positive. Cutt-off values were 0.105 and 0.108 (two series of analyses)

Plant	Shoot	Type of organ								Inter-nodes (No.)
		Basal leaf		Middle leaf		Apical leaf		Apex	Tendril	
		Blade	Petiole	Blade	Petiole	Blade	Petiole			
1	1	0.038	0.165	0.037	1.218	0.075	-	0.118	0.299	20
	2	0.037	0.097	0.042	0.726	0.043	0.072	0.093	0.197	18
	3	0.039	0.236	0.046	0.605	0.056	1.428	0.069	1.104	8
2	1	0.042	0.366	0.043	0.241	0.112	-	0.113	0.261	19
	2	0.034	0.156	0.041	0.504	0.431	1.507	0.067	0.351	12
	3	0.044	0.284	0.042	0.597	0.095	0.869	-	0.691	12
3	1	0.040	0.071	0.040	1.020	0.764	-	-	3.297	12
	2	0.040	0.529	0.040	1.054	0.052	1.727	-	2.648	13
	3	0.043	0.112	0.042	0.363	0.102	-	-	2.833	13

The veraison period is not reliable for the GPGV diagnosis because, with four exceptions (a petiole sample of a top leaf, 2 apices, and 2 tendrils), all analyzed organs were ELISA negative (Table 4). At the same time, the same samples tested for GFkV were positive (Table 5).

Table 3. Optical density values at 405/492 nm in samples collected from various grapevine organs of three plants belonging to the 'Negru mare' genotype, for the diagnosis of GFkV, in the phenophase of berries developing (June 2021). Values marked in red are ELISA positive. Cutt-off values were 0.105 and 0.096 (two series of analyses)

Plant	Shoot	Type of organ								Inter-nodes (No.)
		Basal leaf		Middle leaf		Apical leaf		Apex	Tendrill	
		Blade	Petiole	Blade	Petiole	Blade	Petiole			
1	1	2.093	0.075	3.418	3.471	0.444	-	0.777	3.523	20
	2	2.525	2.556	0.450	2.748	1.099	0.307	0.856	0.279	18
	3	2.046	1.133	0.280	1.957	0.802	0.385	0.101	1.510	8
2	1	2.290	1.392	0.128	1.202	0.740	-	0.508	2.702	19
	2	2.086	1.398	2.097	0.035	1.105	0.651	0.313	1.245	12
	3	2.828	1.190	3.067	2.142	1.876	0.997	-	1.829	12
3	1	3.532	2.884	2.688	1.954	1.930	-	-	1.656	12
	2	3.522	2.582	2.141	2.058	2.752	1.761	-	2.046	13
	3	3.542	2.774	1.588	1.676	2.390	-	-	1.892	13

Table 4. Optical density values at 405/492 nm in samples collected from various grapevine organs of three plants belonging to the 'Negru mare' genotype, for the diagnosis of GPGV, in the veraison phenophase (August 2021). Values marked in red are ELISA positive. Cutt-off values were 0.102 and 0.204 (two series of analyses)

Plant	Shoot	Type of organ									
		Basal leaf		Middle leaf		Apical leaf		Median leaf of lateral shoot		Apex	Tendrill
		Blade	Petiole	Blade	Petiole	Blade	Petiole	Blade	Petiole		
1	1	0.042	0.044	0.040	0.043	0.041	0.043	0.055	0.043	-	0.055
	2	0.042	0.042	0.042	0.042	0.041	0.05	0.034	0.041	-	-
	3	0.041	0.042	0.041	0.041	0.043	0.041	0.033	0.045	-	-
2	1	0.041	0.041	0.041	0.042	0.041	0.041	0.037	0.042	-	-
	2	0.043	0.042	0.040	0.042	0.042	0.061	0.033	0.078	-	-
	3	0.046	0.042	0.040	0.051	0.093	0.111	0.077	0.117	-	-
3	1	0.074	0.072	0.068	0.077	0.071	0.259	0.066	0.073	1.357	0.269
	2	0.079	0.079	0.112	0.076	0.08	0.086	0.082	0.109	-	-
	3	0.090	0.088	0.087	0.087	0.084	-	0.08	0.093	0.721	0.426

Regarding the use of phloem tissue as material for virus detection in grapevine, while for GFkV all samples collected along the length of the cane were positive, in the case of GPGV in only five out of nine tests viral infection was confirmed (Table 6). In all tables, the OD 405/492 nm values give information on viral concentration in different organs or tissues of the plant.

Studies on the efficiency of viral diagnosis by ELISA for the most important and damaging grapevine viruses revealed the period of sampling as a limiting factor in connection with the temperatures recorded at the time of sampling, without mentioning which should be the temperature threshold for a reliable diagnostic. GFLV was consistently identified in the leaves of the shoot tip throughout the growing season (June–October), with viral

concentration decreasing in the middle and lower leaves at the end of the season (Bovey *et al.*, 1980). In the case of GLRaV-3, from April to November, the basal leaf blades and petioles were the most suitable tissues for diagnosis (de Sousa, 1997). For GPGV, the insert kit does not recommend testing from mature leaves during the summer and at the end of the growing season.

Table 5. Optical density values at 405/492 nm in samples collected from various grapevine organs of three plants belonging to the 'Negru mare' genotype, for the diagnosis of GFkV, in the veraison phenophase (August 2021). Values marked in red are ELISA positive. Cutt-off values were 0.114, 0.204 and 0.171 (three series of analyses)

Plant	Shoot	Type of organ									
		Basal leaf		Middle leaf		Apical leaf		Median leaf of lateral shoot		Apex	Tendril
		Blade	Petiole	Blade	Petiole	Blade	Petiole	Blade	Petiole		
1	1	1.491	1.593	1.652	2.837	1.445	0.367	2.276	2.119	-	2.637
	2	1.628	1.741	1.135	1.924	1.373	1.030	1.989	1.945	-	-
	3	1.160	1.702	2.919	1.532	0.499	0.473	0.161	3.244	-	-
2	1	1.573	2.186	2.416	1.251	1.720	1.506	0.680	1.900	-	-
	2	2.586	3.180	2.254	1.574	1.525	1.924	3.058	3.308	-	-
	3	2.057	1.862	1.897	2.288	3.556	3.584	3.556	3.559	-	-
3	1	3.586	3.576	3.526	3.528	1.131	1.357	3.552	3.421	1.064	3.370
	2	3.559	3.566	3.574	3.574	3.552	3.531	3.001	2.850	-	-
	3	2.328	1.321	2.019	2.170	0.574	-	3.447	2.942	-	1.306

Table 6. Optical density values at 405/492 nm in samples collected in the dormant period (December 2021) along the length of the cane, on three plants belonging to the 'Negru mare' genotype, for the diagnosis of GPGV and GFkV. Values marked in red are ELISA positive. Cutt-off values were 0.336 for GPGV and 0.171 for GFkV

Virus	Plant 1			Plant 2			Plant 3		
	Inter-node 1	Inter-node 8	Inter-node 15	Inter-node	Inter-node 8	Inter-node 15	Inter-node 1	Inter-node 8	Inter-node 15
GPGV	0.924	0.458	0.279	0.315	0.245	0.211	1.116	0.349	0.770
GFkV	2.683	2.494	3.236	3.549	3.506	3.127	3.540	3.543	3.533

During the study, the temperature range in the greenhouse around sampling (8-14 April 2021) was between 37.4 and 42.4°C, when only a single young shoot sample at the base of the plant was positive. The phenophase after flowering, with thermal amplitudes between 26.9 and 29.6°C, was the one in which the maximum efficiency of GPGV diagnosis was recorded in all tested organs. Although the temperatures decreased at the end of August (15.2-19.4°C thermal range), the efficiency of GPGV diagnosis is not improved.

Therefore, the efficiency of GPGV detection can be influenced by high temperatures, over 40°C during sampling, but most likely, the most correct diagnosis is the ones resulting by testing the plant organs having conductive tissues (petiole, young shoot, tendril, cane).

In the context of global warming, the influence of high air temperature on grapevine plants was evaluated by comparing the air temperature in the greenhouse at two times of the day, two different days, with the temperatures recorded in different zones of the leaf. On the morning of the first day, at an air temperature of 26.4°C, the leaf temperature was around 25.8°C both in the upper lobe and in the basal lobes of the leaf. Similarly, in the next set of measurements, at an air temperature of 29.6°C, the leaf temperature was around 29°C. When midday air temperatures increased to 37°C, differences from leaf temperatures began to

increase, probably as a result of increased transpiration. The figures show that there are no differences in the temperature values recorded on different zones of the leaf (Figure 1, 2).

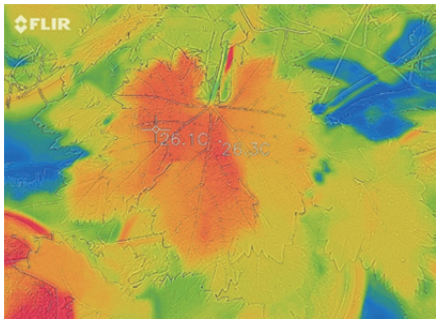


Figure 1. The temperature recorded in the morning at the base of a leaf from the middle of the shoot, using the FLIR device

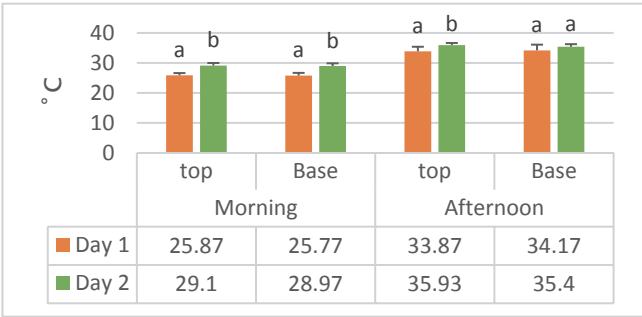


Figure 2. Temperature of the leaf. Values are averages, bars represent the standard deviation of the mean, letters represent the significance of differences of averages of temperature recorded in day 1 compared to day 2, at P < 0.05,

The photosynthesis and transpiration are two physiological processes regulated by the functionality of stomata. Also, stomata opening represents the gateway for the bacteria or fungi. The influence of viral infection on the stomatal function has been poor studied. Incipient studies showed that *Tobacco mosaic virus* caused a significant decrease in stomatal index, stomatal density, and transpiration rate in tobacco plants (Murray *et al.*, 2016). In the present study, GPGV+GFkV complex viral infection induced an increase in stomatal opening both in the morning and in the afternoon, with significant differences as compared to the control in the first set of measurements (day 1) in the afternoon and the second measurement (day 2) in the morning (Figure 3, 4). The ability of some pathogens (bacteria, fungi) to modify the stomatal behavior in host plants has been studied before (Melloto *et al.*, 2006, Allègre *et al.*, 2007). Research regarding the influence of GFLV on some characteristics of the stomata showed that the viral infection causes an increase in length, a decrease in diameter, as well as a decrease in stomata density, especially in symptomatic leaves (Guță and Buciumeanu, 2011).

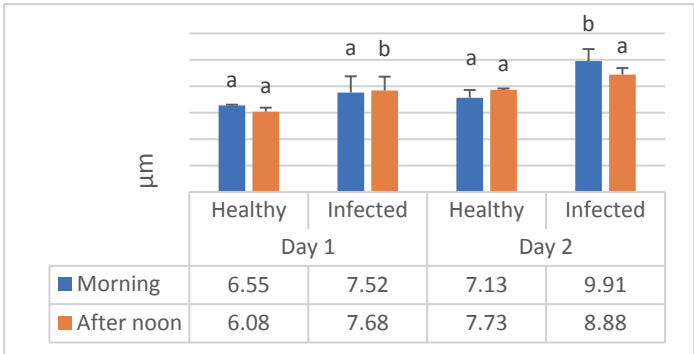


Figure 3. Stomatal opening in GPGV+GFkV-infected grapevine compared to healthy plants. Values are means, bars represent standard deviations, letters represent the significance of differences at P < 0.05

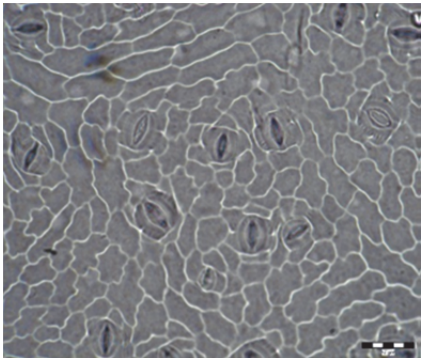


Figure 4. Microscopic capture (x400) of stomata in GPGV+GFkV-infected grapevine, Negru mare

CONCLUSIONS

Most often, the grapevine is subjected in nature to the simultaneous action of several viral infections. Therefore, it is difficult to evaluate the influence of only one virus without associating it with the climate conditions, age of the vineyard, the agricultural technique applied, and the area of culture.

Grapevine Pinot Gris virus is among the last identified and described grapevine viruses that have spread rapidly in vineyards worldwide, whose pathogen-plant interaction is in the process of elucidation. The availability of the ELISA diagnostic kit for *Grapevine Pinot Gris virus* allowed the processing of a large number of samples collected from different organs of the plant, during the phenophases of vegetation. The results revealed that organs with conducting tissues (petiole, tendril, young shoot, cane) seem to be the most reliable samples for viral diagnosis. The reliability of the diagnosis cannot be based on only one analysis. The temperature at the level of the leaf is close to the air temperature, but when the temperatures reach high values, the transpiration and implicitly the temperature in the plant is regulated through the stomata opening mechanisms. The presence of viral infection can affect this mechanism leading to effects on plant development.

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