*Guță, I.C., Buciumeanu, E.C., Ciripan, L.M. (2023): ELISA diagnosis of Grapevine Pinot gris virus. Agriculture and Forestry, 69 (3): 35-44. doi:10.17707/AgricultForest.69.3.03*

DOI: 10.17707/AgricultForest.69.3.03

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# ELISA DIAGNOSIS OF GRAPEVINE PINOT GRIS VIRUS

## SUMMARY

The grapevine production capacity and the products quality depend largely on the health of the cultivated plants. Of the pathogens affecting the grapevine culture, viruses cause significant damages. For this reason, it is extremely important to use diagnostic methods to identify the pathogens and to choose the correct method of plant protection. A reliable method for the routine diagnosis of many economically important grapevine viruses is Enzyme -Linked Immunosorbent Assay (ELISA), a number of kits allowing the detection of different grapevine viral pathogens being commercially available. The increased incidence of *Grapevine Pinot gris virus* (GPGV) in plantations around the world and the availability of an ELISA kit, made it possible to identify this pathogen in Romania as well. The *in-house* validation of GPGV kit was necessary. The results showed that the most reliable type of tissue for analysis is the leaf petiole in the beginning of the growing season and the phloem tissue in the dormancy period. As the ELISA response decreased over the time both for aliquots of positive control of kit and positive plant storage samples, using the plant control is necessary, but carefully for the type of plant tissue and period of sampling.

**Keywords:** GPGV, validation, cut-off, repeatability, reproducibility, robustness

## INTRODUCTION

Historically, visual diagnosis was the first and most used approach to diagnosing grapevine viral diseases, but without identifying the pathogen. Leaf reddening and curling, for instance, can be caused by the *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 2* (GLRaV-2) and *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine red blotch-associated virus* (GRBaV) or *Grapevine flavescence dorée* (FD) phytoplasma presence, *flavescence dorée* phytoplasma infection, or the Gra*pevine red blotch-associated virus* (GRBaV). Moreover, the same virus can cause differing

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Notes: The authors declare that they have no conflicts of interest. Authorship Form signed online.
*Recieved:12/04/2023* *Accepted:10/07/2023*

symptoms in different grapevine varieties. For example, leaf yellowing in white cultivars or reddening in red genotypes can be caused by leafroll-associated viruses. Sometimes, the virus infections have no symptoms. A GLRaV-3 infection in white varieties results in leaf blade yellowing, whereas in red-skinned varieties it causes leaf reddening. Finally, in some cases, a viral infection can be asymptomatic. This is why it is rather difficult to correctly detect and identify a virus on the basis of visual symptoms only, without the use of laboratory testing (Zherdev *et al.*, 2018).

ELISA (enzyme-linked immunosorbent assay) is one of the grapevine viral pathogen detection techniques based on coupling of two reactions of high specificity: antigen (represented by viral protein) – antibody reaction and enzyme – substrate reaction, through a compound called conjugate (Clark and Adams 1977). Currently, a number of ELISA kits allowing for the detection of different grapevine viral pathogens are commercially available.

In ELISA diagnosis, the green parts of the plant (foliar limb, petiole) and the phloem tissue scraping from cane are usually tested. Depending the virus, the season, the development phenophase, the pathogen may be unevenly distributed in different part of the plant. This is the reason why in the diagnostic kit are additional recommendations regarding the sampling period and the tissue types preferred for testing.

The evolution of grapevine viral disease diagnosis like next-generation sequencing method, allows the identification of new pathogens assimilated to characteristic symptoms.

By Illumina high throughput sequencing method, field symptoms of chlorotic mottling and leaf deformation observed since 2003 on the Pinot gris cv., in Italy, have been attributed to a new virus, named Grapevine Pinot gris virus (GPGV), related with trichoviruses (Giampetruzzi *et al.*, 2012). At the meeting of virologists in the frame of the 17$^{th}$ ICVG Congress was announced the discovery of four new grapevine viruses, including GPGV (Martelli, 2012). According to EPPO, updated 21 February 2022, the virus is widespread, and it has been reported in 58 countries on five continents (Demian *et al.*, 2022).

ELISA has been used for the GPGV detection in vineyards and in the certification program both in field-grown and greenhouse-grown grapevines (Tarquini *et al*., 2018; Bertazzon *et al*, 2021).

In Romania, the study about the incidence of GPGV in germplasm collection highlighted that out of 95 samples collected from plants presenting specific symptoms to GPGV infection, 60 were confirmed as infected. On the other hand, out of 75 samples from asymptomatic grapevines, 22 were ELISA positive (Guță and Buciumeanu 2021).

Since a part of our activity is the grapevine viruses monitoring, the reliable use of the ELISA diagnostic method was also studied for *Arabis mosaic virus+Grapevine fanleaf virus* (ArMV+GFLV), GLRaV-1+3 and *Grapevine fleck virus* (GFkV) (Guță and Buciumeanu, 2010, 2011, 2012). Because GPGV is a new virus entered into the study, the aim of this work was to verify the safety of GPGV diagnosis by ELISA reagents.

## MATERIAL AND METHODS

ELISA is performed in the grapevine virology laboratory belonging to National Research and Development Institute for Biotechnology in Horticulture Ştefăneşti, Argeş, România, who analyzes the most damaging and widespread grapevine viruses: ArMV+GFLV, GLRaV-1+3, GFkV and *Grapevine virus A* (GVA), both for routine diagnosis and research purposes, using commercial reagents BIOREBA, Switzerland.

Specific equipment (incubator, plate washer, spectrophotometer) and certified NUNC F-96 maxisorp microtiter plates were used.

The results presented in this work were obtained during GPGV testing, over three years, with three diagnostic kits, named A, B, C. The working instructions as recommended by the manufacturer were followed.

Positive and negative control are intended to verify the ELISA performance. Each kit contains one positive control (lyophilized extract of infected plants) and one negative control (lyophilized extract of healthy plants); the expected values of ELISA readings are indicated in the datasheet. For several determinations, the controls are used as aliquots, stored at -20°C. The maintenance in time of the quality of the controls was studied.

The reproducibility intra-determination and the repeatability inter-determination is assessed on positive samples (plant infected extracts) with different optical density (OD) readings, positive and negative controls, stored as aliquots at -20°C. Three repetitions of each extract have been performed for the repeatability. In this case, standard deviation of the mean must be smaller than cut-off. The reproducibility was assessed on aliquots of samples from a grapevine infected plant tested several times.

Because the laboratory has a collection of virus infected grapevine plants for the purpose of use as positive controls, three GPGV infected plants were analyzed during several vegetation phenophases. Three types of plant tissue (limb, petiole, cane) have been used.

The laboratory analyzes a large number of samples both for research purposes and for the maintenance of the collection of viticultural germplasm. Therefore it is necessary sometimes to rationalize the reagents by using them at half volume. In this paper is studied the ELISA method robustness for 100 and 200 µL/well work volume.

The minimum limit of virus detection is the cut-off value. The cut-off value was calculated for each ELISA plate individually, being as three times the mean value of negative control (all values above this cut-off were regarded as positive). Reading were performed with dual filter 405/492 nm.

## RESULTS AND DISCUSSION

Viral diseases cause grapevines modifications affecting the quantity, quality of production and longevity of the plantation. Early identification of the infection makes it possible to take measures to limit the damage of virus infection. Diagnostic methods have evolved significantly in recent times, and allow a reliable diagnosis. It is of critical importance to reduce test costs and duration in order to provide wide-scale diagnosis that can be included in comprehensive plant protection plans.

Because in the diagnostic process, positive and negative control are intended to verify the ELISA performance, it is necessary to use every time the aliquots stored at -20°C, even if their OD readings may gradually decrease over time, as indicated in datasheet.

Our study showed a strong variation up and down of positive control after 60 min readings, to all kits, A, B, C, both for 100 (kit A and B) or 200 µL/well (kit C) analysis volume. In all tests OD indicated positive signal, but after eight month (kit A), five month (kit B), two month (kit C), the value was closely of the cut-off (Figure 1, 2, 3). Unexpected, using the aliquot from kit A on the validation of kit B, OD reading increased, although 12 months had passed since solubilization (Fig. 1, point 12). The positive control of the kit C (currently in use) had a similar behaviour, even if 200 µL/well volume was used (Figure 3).

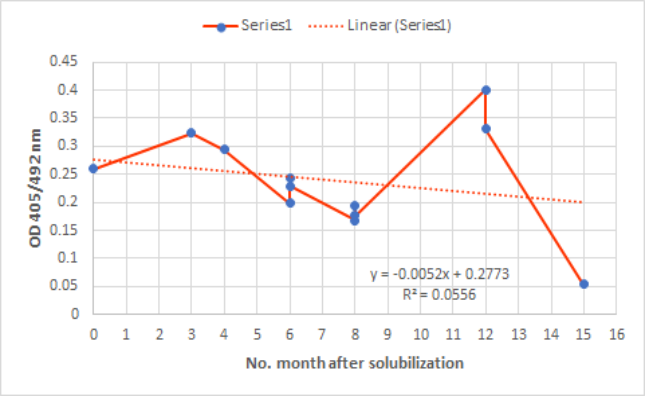


Figure 1. Evolution of ELISA optical density (OD) readings at 405/492 nm of the positive control from Kit A (series 1), used in different tests as aliquots, after solubilisation, for the working volume of 100 µL/well

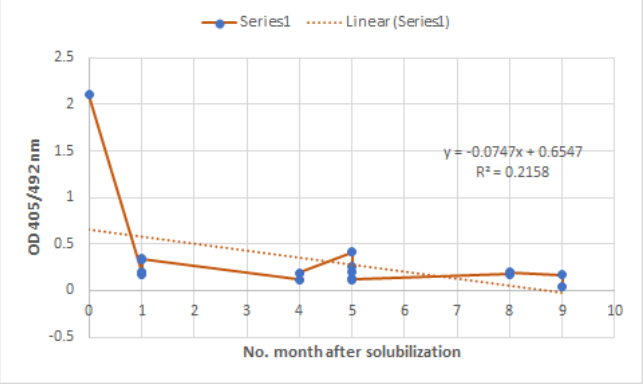


Figure 2. Evolution of ELISA optical density (OD) readings at 405/492 nm of the positive control from Kit B (series 1), used in different tests as aliquots, after solubilisation, for the working volume of 100 µL/well

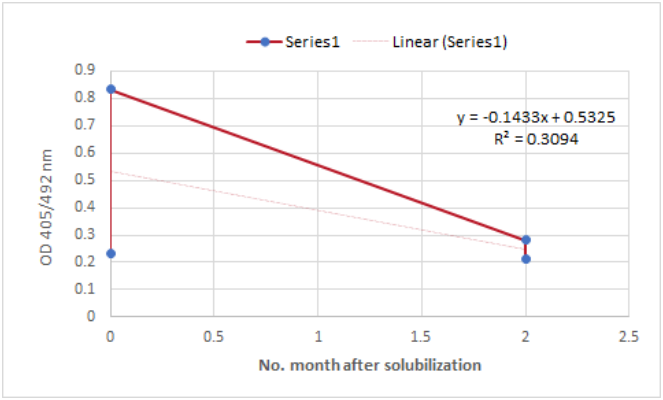


Figure 3. Evolution of some ELISA optical density (OD) readings at 405/492 nm of the positive control from Kit C, used in different tests as aliquots, after solubilisation, for the working volume of 200 µL/well

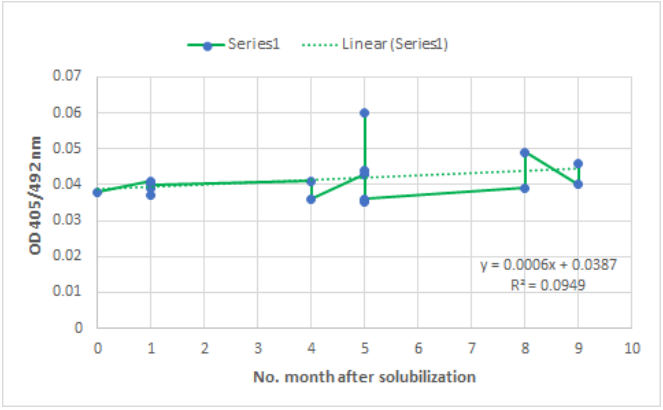


Figure 4. Evolution of ELISA optical density (OD) readings at 405/492 nm of the negative control from Kit B (series 1), used in different tests as aliquots, after solubilisation, for the working volume of 100 µL/well

Also, OD readings at 60 min of negative controls (lyophilized extracts from healthy grapevine plants) have not decreased linearly over time (Figure 4). The study of repeatability of the OD readings of different two samples, positive and negative control showed the precision intra-determination of diagnostic kit (Table 1).

On the other hand, repeated testing of different aliquots samples, both foliar limb or phloem tissue have highlighted decrease over time of 60 min OD readings. In some cases, after several month, the ELISA response was negative (Table 2 and 3).

The robustness of ELISA diagnostic kit for GPGV identification was investigated too, on the validation of kit C comparatively with kit B. OD at 60

min. reading of positive control C after reconstitution was much smaller than extinction values recommended in data sheet (>1.400) with kit C components, while the results were negative with kit B (still in the operating period).

Surprisingly, a positive control A had a positive response 22 months after reconstitution. A positive phloem tissue sample had a negative response 10 months after extraction (Table 4).

Table 1 Repeatability intra-determination: ELISA readings (OD 405/492 nm) for three repetitions of two phloemic tissue samples, positive kit control A and negative kit control A (100 µL working volume)

| Repetition | ELISA readings (OD 405/492 nm) | | | |
|---|---|---|---|---|
| | Sample 1 | Sample 2 | Positive kit control | Negative kit control |
| 1 | 0.522 | 0.879 | 0.260 | 0.054 |
| 2 | 0.510 | 0.886 | 0.254 | 0.046 |
| 3 | 0.512 | 0.815 | 0.256 | 0.047 |
| *Mean ± std* | *0.514 ± 0.006* | *0.860 ±0.039* | *0.256 ± 0.003* | *0.049 ± 0.004* |

*cut-off = 0.147*

Table 2 Reproducibility inter-determination: ELISA readings (OD 405/492 nm) for some repetition of positive samples (Kit A; 100 µL working volume)

| Repetition | Sample 1* | Sample 2* | Sample 3** | Sample 4** | Sample 5* |
|---|---|---|---|---|---|
| 1 | 0.709 | 0.527 | 0.514 | 0.860 | 0.308 |
| 2 | 0.479 | 0.533 | 0.151 | 0.149 | 0.294 |
| 3 | - | - | - | 0.158 | 0.174 |

** foliar limb*

*** phloem tissue sample*

Table 3 Repetability inter-determination: ELISA OD results (OD 405/492 nm) for some repetition of positive samples (Kit B; 100 µL working volume)

| Repetition | Sample 1 | Sample2 | Sample 3 | Sample 4 | Sample 5 |
|---|---|---|---|---|---|
| 1 | 1.384 | 0.138 | 0.514* | 0.860* | 0.722 |
| 2 | 0.515 | 0.079** | 0.082** | 0.096** | 0.098** |
| 3 | 0.134 | 0.054** | - | - | - |

** OD from the first diagnosis, Kit A, phloemic tissue sample, 16 month ago*

*** negative result of the test*

Technical sheet recommends GPGV diagnosis from young leaves at the top, or middle of the plant, sampled in the spring. The results obtained in ELISA tend to indicate that there is a heterogeneous distribution of the virus from one leaf level to another and that the sampling period is important to consider in the spring (Guide d'information sur l'émergence du virus du Pinot gris et sa propagation, 2019). To verify the ELISA performance, the manufacturer suggests also the use of the plant control.

Table 4 Validation of ELISA Kit C

| Sample | Kit B | | Kit C | |
|---|---|---|---|---|
| | 100 µL/well cut off = 0.117 | 200 µL well cut off = 0.201 | 100 µL/well cut off = 0.114 | 200 µL well cut off = 0.204 |
| Positive control C at solubilization | 0.073 | 0.073 | 0.311* | 0.232* |
| Positive control B – aliquot after 10 months from solubilization | 0.037 | 0.069 | 0.096 | 0.146 |
| Pozitive control A - aliquot after 22 months from solubilization | - | 0.063 | 0.127* | 0.222* |
| Positive floemic tissue (OD = 0.136, ten months before) | 0.037 | 0.067 | 0.053 | 0.089 |
| Positive floemic tissue (OD = 0.860, ten months before) | - | - | - | 0.166 |
| Positive floemic tissue (OD = 0.514, ten months before) | - | - | 0.090 | - |

** the positive responses*

In our study, in April, all the samples taken from the leaves (limb and petiole) at the base or the top of the shoot, were negative. Thermal amplitude (42°C average) and the maximum temperatures (43°C average) reached in the greenhouse during the sample period, may be the cause of these results.

At the beginning of June, after flowering, petiole samples were positive, while the limb readings showed negative results, along the length of the shoots (Figure 5).

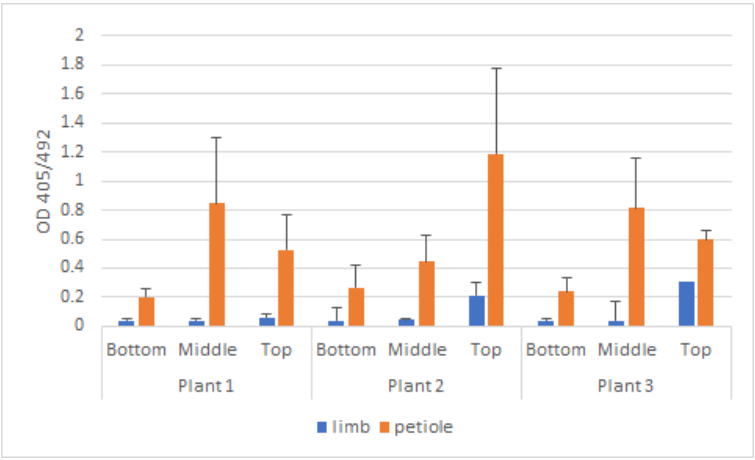


Figure 5 ELISA diagnosis of GPGV from leaves (limb and petiole) collected from the bottom, middle and top of the shoots of three infected plants. Values are average from three repetition (3 shoots/plant), bars represent the std.

In this phenophase of vegetation, although the maximum temperatures recorded were around 42°C, the thermal amplitude decreased to 27°C in average. In this condition, the petiole as conductive tissue was the best material to sample for a correct result.

As expected, the analysis of the phloemic tissue, along the length of the canes, during the dormancy period confirmed the GPGV infection of plants.
Grapevine (*Vitis vinifera* L.) is an important crop who is affected over of 80 distinct number of viruses (Martelli, 2017), some of them with important economic impact, requiring early identification to limit damages. That is why it is necessary to constantly develop new diagnostic methods with high detection sensitivity at an affordable cost price.

ELISA is a robust method with a high detection sensitivity, 1 to 10 ng virus/ml. Eight from fourteen specific grapevine viruses, among which the GPGV, have the possibility of identification through ELISA method.

Diagnostic methods based on nucleic acids amplification are more sensitive but requires expensive equipment and reagents.

There were only few studies that comparatively investigated the serological and molecular methods of grapevine virus diagnosis. When using phloem tissue as a biological material for analysis, some researchers indicated an equal reliability of ELISA and RT-PCR (Ling *et al*., 2001; Fiore *et al*., 2009). Chen *et al.* (2003) indicated that RT-PCR was more reliable for the detection of GLRaV-3 than ELISA while other studies showed that ELISA may be more sensitive than RT-PCR for the detection of GLRaV-1 and -3 (Cohen *et al.*, 2003). ELISA identification of GLRaV-1, -2 and -3, GVA, GFkV and GFLV from leaf petioles was more sensitive than RT-PCR as compared to the use of leaf blade and phloem tissue during the summer and early fall (Fiore *et al*., 2009).

The possibility of the simultaneous detection of a large number of viruses by one assay using DNA chips is essential for the diagnosis of grapevine infections. Since mixed infections with several pathogens at a time is an often-seen in vineyards, this feature of the DNA chip technique is a significant advantage not only for research purposes, but for further commercialization.

## CONCLUSIONS

The results obtained on GPGV diagnosis by ELISA using BIOREBA commercial kit, showed that the most reliable type of tissue for analysis is the leaf petiole at the beginning of the growing season and the phloemic tissue in the dormancy period.

ELISA response decreased over the time both for kit positive aliquots control and positive plant storage samples.

Using the plant control is necessary, but carefully for the type and time of sampling. Compliance with work volume/well as manufacturer recommended, eliminated any doubts on the results obtained.

The presence in the GPGV detection kit of several controls, without dividing the solubilized control into small volumes (aliquots), would increase the diagnosis reliability.

ELISA remains a reliable method for the routine diagnosis of many economically important grapevine viruses. Although virus titre shows seasonal fluctuations and the viruses may be unevenly distributed in grapevines, particularly for recent infection, ELISA provides reliable diagnosis if samples are collected at the optimal time in the specified grapevine tissue. ELISA results should be supplemented by molecular tests in critical situations, since some viral strains may be not detected by one or other type of tests.

## ACKNOWLEDGEMENTS

This work was supported by a grant of the Romanian Ministry of Research, Innovation and Digitization, NUCLEUS program No. 42N/2019, Project number 01 01 Investigations on the presence of a new virus *Grapevine Pinot Gris virus* in local viticultural material: diagnosis and eradication techniques.

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