THE DISTRIBUTION OF THE *FLECK VIRUS* IN NATURALLY INFECTED VINE

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Abstract. WALTER & ETIENNE (1987) studied the conditions for the detection of GFLV and ArMV by ELISA in grape tissues at different times of the year. According to their results, the leaves, roots and peeled wood (sawdust) are the best sources of virus. In our research we have highlighted the fact that the fleck virus of the vine can be detected safely, according to the instructions of the reagent kit manufacturer, from young plant material (leaves located at the top of young shoots) in the vegetation stages, except during very hot periods of the year, or at rest, from the phloemic tissue of one-year-old strings. The results of the study led to the conclusion that GFkV can be safely diagnosed using as biological material the phloemic tissue taken from one-year-old vine shoots, at rest, and in vegetation, the analyses on the foliar limbo of the leaf led to the expected results compared to the petiole.

Keywords: virus, ELISA, vine.

Rezumat. Distribuția *virusului fleck* la vița-de-vie infectată natural. WALTER & ETIENNE (1987) au studiat condiţiile pentru detectarea GFLV şi ArMV prin ELISA în ţesuturile viţei-de-vie în diferite perioade ale anului. Conform rezultatelor lor, frunzele, rădăcinile şi lemnul decojit (rumeguşul) sunt cele mai bune surse de virus. În cercetările noastre am evidenţiat faptul că virusul fleck al viţei-de-vie poate fi detectat în siguranţă, conform indicaţiilor producătorului kitului de reactivi, din material vegetal tânăr (frunze situate în vârful lăstarilor tineri) în etapele de vegetaţie, exceptând perioadele foarte călduroase ale anului, sau în repaus, din ţesutul floemic al coardelor de un an. Rezultatele studiului au dus la concluzia că diagnosticarea GFkV se poate face în siguranţă utilizând ca material biologic ţesutul floemic prelevat de la coarde de un an, în perioada de repaus, iar în vegetaţie, analizele din limbul foliar al frunzei au condus la rezultatele așteptate comparativ cu peţiolul.

Cuvinte cheie: virus, ELISA, vița-de-vie.

INTRODUCTION

The fleck complex consists of several viruses and diseases: *Grapevine fleck virus* (GFkV), *Grapevine asteroid mosaic-associated virus* (GAMaV), *Grapevine rupestris vein feathering virus* (GRVFV), *Grapevine redglobe virus* (GRGV), *Grapevine rupestris necrosis*, which cause latent or semi-latent infections in *Vitis vinifera* L. and most American *Vitis* hybrid rootstock species (MATELLI & BOUDON-PADIEU, 2006). The fleck complex influences the vigor, rooting capacity of rootstocks, grafting (UYEMOTO et al., 2009).

The disease is latent in European vine species and most American rootstocks. The symptoms are expressed on *Vitis rupestris* and consist in the clarification of the third and fourth nerves, producing localized, translucent spots. The leaves with intense spots are embossed, bent and twisted towards the top. Fleck is a ubiquitous graft-borne disease reported in all wine-growing countries of the world (BOVEY & MARTELLY, 1992; MARTELLI, 1993). Numerous observations and experiments highlight the effects of the presence of the GFkV infection, alone or in combination with other viruses and viral diseases. The presence of GFkV decreases rhizogenesis and grafting; the effects depend on the virulence of the viral isolate. GFkV alone or in the presence of leaf twisting induces a reduction in grafting and affects the vigor of plants in nurseries.

GFkV infection associated with nerve necrosis and nerve mosaic decreased the amount of wood cut in rootstocks 420A and Kober 5BB by 52 and 37%, respectively, and did not affect growth in Teleki 5A. Simultaneous infection with GFkV and leaf twisting causes the ajinashika disease in the *Koshu* Japanese grape variety, characterized by a reduction in the amount of sugar in the fruit (WALTER & MARTELLI, 1996). In other studies, the concentration of the virus was in most cases higher in the leaves from the tip of the shoot in calves infected with GFLV (BOVEY et al., 1980), ArMV & RRV (RÜDEL et al., 1983) or the chromate mosaic virus - GCMV (LEHOCZKY et al., 1984).

Following field experiments, it is considered that, in general, vines on their own roots are more tolerant to viral infections (GARAU et al., 1997). Studies conducted at National Research Development Institute for Biotechnologies in Horticulture Stefănesti-Arges (INCDBH) followed the phytosanitary condition of viticultural propagation material from different areas of the country, from the point of view of the presence of viral infections. Among the analysed viruses, the highest spread (7.84%) was registered in the case of GFkV (BUCIUMEANU & GUŢĂ, 2007).

TRIOLO & MATERAZZI (1987) showed that marbling decreases rhizogenesis and grafting to *V. rupestris*, depending on the infectious viral isolate. Also, differences were observed regarding the rooting capacity and vegetative multiplication in vitro depending on the phytosanitary condition of the vine (WALTER, 1988; BARBA et al., 1993; GONZÁLES et al., 1995). In addition to the negative effect on rhizogenesis, the influence of GFkV infection on plant size is demonstrated, with severe strains of the virus inducing varying degrees of grapevine dwarfism (BOVEY & MARTELLI, 1992).

GFkV infection is characterized by a severe change in mitochondria in structures called “multivaculate bodies”, while GAMaV induces peripheral vesiculation of the cytoplasm. Disrupted organs are considered viral replication sites (BOVEY & MARTELLI, 1992).

The vector is not known for any of the viruses of the fleck viral complex. Observations from Italy, South Africa and Japan suggest the natural spread of GFkV in the field; A similar behaviour was reported in Greece for a disease thought to be a mosaic asteroid but now identified as grapevine rupestris vein feathering. The primary spread of these and other viruses of the complex is through the infected propagating material. GFkV transmission by coccyx has been reported but has no epidemiological significance. GFkV is not transmitted by seed (GONZÁLES et al., 1995).

Indexing on *Vitis rupestris* allows, with a reasonable level of confidence, the distinction of different viruses of the complex based on the different reaction of the indicator. Polyclonal and monoclonal antibodies to GFkV were obtained. Thus, ELISA is commonly used for routine tests for GFkV detection (CLARK & ADAMS, 1977; WALTER & CORNUET, 1993) but cannot be used for all members of the complex because antisera are not available. Virus-specific primers were pooled in a single RT-PCR multiplex assay for GFkV, GRGV, GAMaV, and GRVFV. Due to the latency of symptoms, health selection in European varieties and most American hybrid rootstocks is uncertain. GFkV can be removed by heat therapy, meristem or apex culture. Recently, alternative methods have been developed such as in vitro chemotherapy and electrotherapy with very good results, up to 100% eradication of the virus (GUŢĂ et al., 2014).

Our research has shown that GFkV can be safely diagnosed using as biological material the phloemic tissue taken from vines for a year, at rest. This study also showed that, in the plant, the virus multiplies and migrates in all organs, at rest; during the advancement in vegetation, a viral plant is fully infected, the identification of the virus being dependent on the viral concentration and sensitivity of the method.

MATERIALS AND METHODS

Biological material. The distribution of the fleck virus has been studied on naturally infected vine plants belonging to the *Pinot noir* genotype, which are in the collection of the breeding laboratory of INCDBH Ștefănești-Argeș. The tests were performed during the entire vegetative cycle of the vine, both during the rest period and during three stages of active growth of the plant. During the resting period, samples were taken from both the buds and the phloemic tissue of the merits of the strings. After budding, during the growth period of the shoots, the tests were performed in three stages: May when the plant shoot was 53 cm, June - 70 cm and July - 120 cm. The samples consisted of leaves (leaf limbo and petiole) located at the base, middle and top of the shoot, arranged on both the main shoot and the offsprings, as well as the growing tips of the shoots (intensely regenerative apexes).

Three GFkV-infected vines belonging to the *Pinot noir* genotype were available in the laboratory's collection of viral plants. Each plant was driven with three growth shoots, so the results were expressed as averages on repetitions.

Detection and dosing of fleck virus in different areas of the vine plant was performed using serological ELISA analysis. The method has a high sensitivity of detection (1 - 10 ng virus / ml) and can detect viruses of different morphologies, in purified preparations or unexplained extracts, from infected herbaceous hosts or culture plants. The peculiarity of this method consists in the coupling of two highly specific reactions: the antigen reaction (represented by the viral protein) - antibody and the enzyme reaction - substrate, through a compound called conjugate. This compound is obtained by labelling the antibodies with an enzyme. Most often, for plant viruses, the used enzyme is alkaline phosphatase, with the corresponding substrate, para-nitrophenylphosphate and the antibodies are specific to the concerned virus.

The principle of the ELISA technique is as follows: the virus from the test sample is selectively captured and immobilized by the specific antibody, absorbed on a solid surface (96-well polystyrene microtiter plate); the captured virus reacts with the next specific antibody to which the enzyme (conjugate) has been bound; after washing, the complex complexed with the virus is detected colorimetrically, by adding the appropriate enzymatic substrate; substrate hydrolysis gives a bright yellow coloration, whose intensity can be measured spectrophotometrically at 405 nm (DO405nm) (CLARK & ADAMS, 1977) (Fig. 1).

For the safety of the work, each microplate must contain at least one positive control and for the separation of the positive samples from the negative ones a threshold is established, as being twice the average of the negative control values. The necessary reagents consisted of standardized test kits, produced by Bioreba - Switzerland. The diagnosis of fleck virus was made by DAS-ELISA which uses antibodies that are bound to the surface of the microplate to capture antigens. The presence of antigens is detected using specific antibodies coupled with alkaline phosphatase (conjugate). Finally, it is added to the enzyme substrate (pNPP); by its hydrolysis a bright yellow compound detectable at 405 nm is obtained when the antigen is present. The serological method based on a colour reaction allows a visual presentation of the results, the positive samples being yellow compared to the negative ones (Fig. 2), but for the safe evaluation of the viral concentration it is necessary to read the samples with a spectrophotometer. In the standard procedure, the absorbance (OD 405nm) is measured after 1 h and 2 h from the deposition of the substrate. For a good interpretation of the results it is recommended to read OD 405 nm at 30 min, 1 h and 2 h from the deposition of the substrate. Readings later than 3-4 hours provide better identification.

The interpretation of the result can be better achieved by calculating a detection threshold. This is obtained as twice the average of the OD 405 nm values corresponding to the negative control. A sample is considered infected if the OD is higher than this threshold; the sample can be considered “doubtful” if its OD value is close to that of the threshold. The validation of the laboratory results is done according to the OD values obtained for NC, PC and extraction buffer, measured after 1 h of incubation of the substrate at 37 °C. The values are specified in the kit insert

and are different depending on the kit; these are obtained after subtracting the average OD values of the substrate from the values read for NC, PC and extraction buffer. Being a qualitative method, the uncertainty is not estimated.

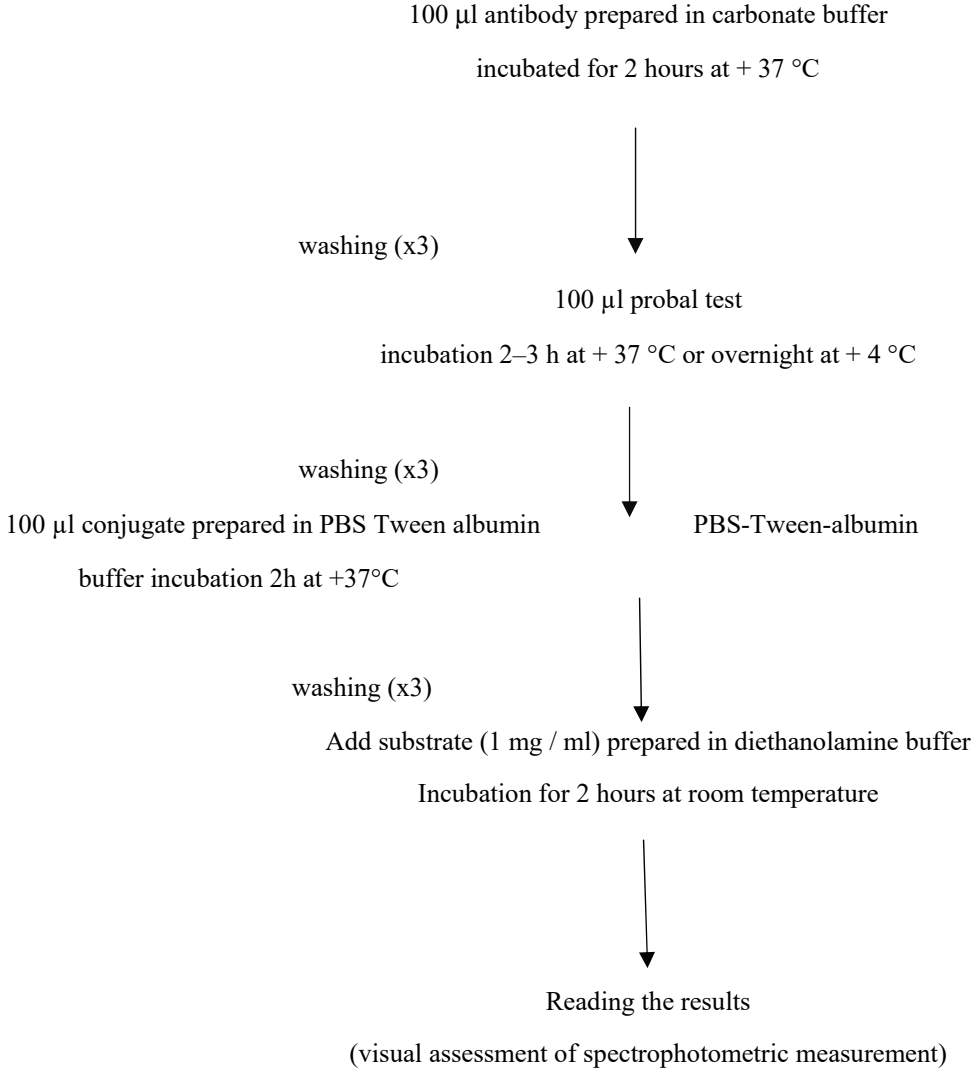

Figure 1. The standard procedure for using the ELISA technique.
(Clark, M.F., Adams, A.N., 1977. Characteristics of the microplate method of the enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen.Virol. 34: 475-483).

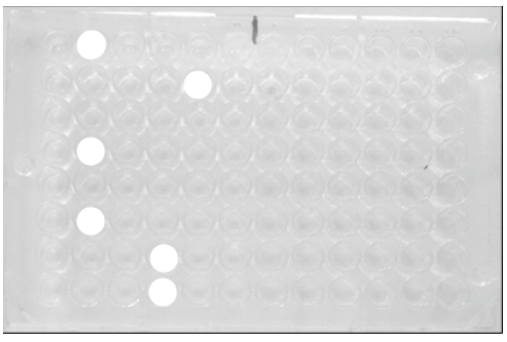

Figure 2. ELISA plate where the positive samples can be visually highlighted (white colour) (original).

RESULTS AND DISCUSSIONS

Assessment of viral concentration at rest. During the vegetative dormancy of the vines, the GFkV concentration was studied over the length of the one-year strings using as phloem material the phloemic tissue taken after bark removal. The used shoots had 10 internodes, numbered from base to tip. The ELISA revealed an

approximately equal viral concentration distributed over the length of one-year strings with a significant decrease towards their peak (Table 1).

Table 1. The concentration of GFkV virus on the length of the vine strings belonging to the Pinot noir genotype.

Position on shoots	DO 405 nm (cutoff = 0,249)
1	0,908
2	0,972
3	0,974
4	0,938
5	0,944
6	0,791
7	0,888
8	0,888
9	0,870
10	0,859

* values are averages per repetition

Assessment of viral concentration at rest. At budding, the recorded values were lower compared to the rest period in both the internode and the bud complex of the winter eye. This may be due to the circulation of raw and processed sap with the onset of physiological processes in the plant, knowing that the virus migrates into the plant through the conducting vessels (Fig. 3).

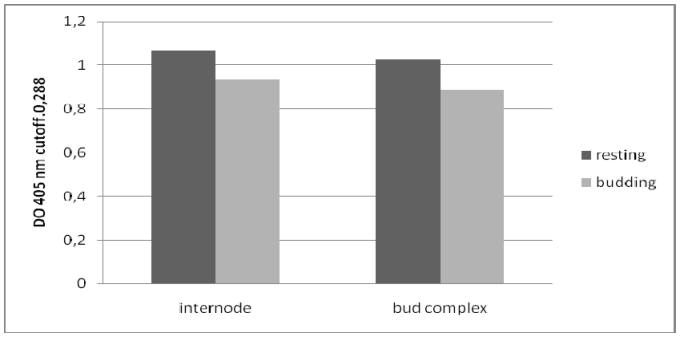

Figure 3. GFkV concentration in the cord internode and bud complex of the winter eye, resting and budding.

Dosage of GFkV during the growth period. The first test during the vegetation period was performed in early May, when the shoots of the studied plants were between 50-75 cm and about 10 leaves, respectively internodes. The leaves located at the base and middle of the shoot (internode 5) were harvested, from which samples of petiole and leaf limbo were taken, and from the top the intensely regenerative tip was harvested. ELISA results were positive except for the petioles of the leaves located in the middle of the shoots. The values of viral concentration were higher in the leaf blade compared to the petiole. The highest absorbance readings were recorded at the leaves at the base of the shoots and at the intensely regenerative apex (Table 2).

Table 2. GFkV concentration in different areas of the vine at the beginning of the growing season.

Position on shoots	Vegetative organ	Viral concentration DO 405 nm (cutoff = 0,501)	Test result
Base	Petiole	1,120	+
	Leaf blade	1,176	+
Middle	Petiole	0,484	-
	Leaf blade	0,879	+
Top	Intense regenerative apex	0,962	+

* values are averages per repetition

In July, when the shoots reached increases of about 20 internodes, the third set of samples was collected, from nodes 8, 11 and 20. The results revealed again this time higher readings of the absorbance in the leaf limbo in while in the petiole the viral concentration decreases below the cutoff level, towards the top of the shoot. Also, apexes located on children starting from the base to the top of the main shoot were analysed. The viral concentration decreased in the apexes collected from children located at the top of the main shoot (Table 3).

Vine testing for GFkV infection during the warmer periods of the year. The detection kit used to identify the fleck virus on the vines recommends avoiding the analyses during the warm periods of the year. Samples were collected at temperatures that reached 45 °C in the greenhouse during the day. Leaves (petiole and limbo) located at different levels of the main shoot were analysed. The results were positive, which confirmed the presence of viral infection, detectable even in the mentioned conditions (Table 4).

Table 3. Evaluation of GFkV infection at different levels along the length of the vine plant shoot.

Position on shoots	Vegetative organ	Viral concentration DO 405 nm (cutoff = 0,273)	Test result
node 8	Petiole	0,587	+
	Leaf blade	1,082	+
node 11	Petiole	0,250	-
	Leaf blade	0,606	+
node 20	Petiole	0,096	-
	Leaf blade	0,086	-
node 3	Young shoot apex	0,398	+
node 7	Young shoot apex	0,092	-
node 14	Young shoot apex	0,216	-

* values are averages per repetition

Table 4. Diagnosis of GFkV in plant length in plants of vines in conditions of high temperatures.

Position on shoots	Vegetative organ	Viral concentration DO 405 nm (cutoff = 0,243)	Test result
Base	Petiole	0,612	+
	Leaf blade	0,756	+
Middle	Petiole	0,590	+
	Leaf blade	0,687	+
Top	Petiole	0,576	+
	Leaf blade	0,712	+
Top	Intense regenerative apex	0,320	+

* values are averages per repetition

CONCLUSIONS

The results of the study led to the conclusion that GFkV can be safely analysed using as biological material the phloemic tissue taken from the vines for one year, at rest, and in vegetation, the analyses of the leaf blade of the leaf led to the expected results compared to the petiole. This can be explained by the existence of a viral concentration below the detection limit of the method.

The working protocol indicated by the reagent kit recommends the use as detection material during the vegetation period of young leaves located at the top of the shoots of the vine plant. The study identified the presence of viral infection in mature leaves located at the base of the plant. Moreover, there have been several cases when tests performed on apical leaves have been ELISA negative. In terms of test period, the present study showed that the infection with the fleck virus of the vine can be detected even in hot periods of the year, the virus being present in leaves located at all levels of plant shoots, from the base to the top.

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