USEFULNESS OF THE GRAPEVINE VIRUS-INFECTED COLLECTION

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Abstract
In order to use the virus-infected material as reference in various studies, a grapevine virus collection was established at NRDIBH Ştefăneşti-Argeş. The vines are infected with 1-3 of the main specific viruses of this crop: fanleaf virus, leafroll associated virus serotypes 1+3, fleck virus and virus A. Different lots of plants belonging to the same cultivar are infected with different viruses. The own rooted or grafted potted plants are maintained in an insect-proof greenhouse. The main goals of the study of grapevine under the influence of virus infection had in view: symptoms, in vitro behaviour of virus infected grapevine, virus elimination, plant positive control in the diagnostic process. The symptoms produced by viral infection can affect the whole plant (systemic symptoms) or they are visible on certain parts of the plant (local symptoms). In vitro studies of virus infected grapevines comparatively with the healthy material aimed with the quantitative and qualitative aspects of the culture: multiplication and rooting rates, shoots elongation, abnormal cuttings and vitrification phenomena. Infected grapevine cultivars and clones were subjected to virus elimination through thermotherapy, chemotherapy or electrotherapy, combined with in vitro culture. The diagnosis of leafroll, fleck, vein necrosis and corky bark diseases have been done by in vitro micrografting, as rapid biological method of virus detection. Samples collected from infected vines were used as material testing for virus detection by ELISA in inter-laboratory comparisons and Iaboratory-performed validation.

Key words: Vitis, virus, reference material

1. INTRODUCTION

Viruses and phytoplasmas are widely distributed plant pathogens, and there is not effective cure for already infected plants in the field. They cause considerable economic losses and are therefore a major concern to worlwide phytosanitary agencies (Laimer et al., 2005).
All known grapevine pests include about 70 infections agents belonging viruses (58), viroids (5), phytoplasmas (8), xylematic bacteria transmitted by insects (1). This represents the largest number of intracellular pathogen agents found for a single plant. The diseases produced by them reduce the vigour and longevity of plants or the quality and quantity of production. The contaminated propagating material is the first responsible for spreading these diseases in the viticultural areas of the world. Consequently all efforts should be done for improvement of sanitary conditions and the protection of healthy clones (Martelli and Boudon-Padieu, 2006).
The presence of viral pathogens in grapevine tissue pose still questions on their interaction with the host and the sensitive and cost effective method for the detection and elimination of the major viruses of this crop.
The grapevine virus collections were established to serve as models for host-pathogen relationship and pathogen elimination experiments. Collections of grapevines are pools of reference and source materials or standard type cultivars and viruses, virus and virus-like diseases (Golino, 1992; Gugerli et al., 2009).
The purpose of this paper is to show how to use a grapevine virus collection, the necessity of establishing and maintaining such type of collections.

2. MATERIAL AND METHOD

In order to use the virus infected biologic material as reference (positive controls) in our studies, a grapevine virus-infected collection was established at the National Research & Development

Institute for Biotechnology in Horticulture Ştefăneşti-Argeş. Grapevine cultivars and clones identified as positive for virus infection in the frame of routine and research diagnostic activities were added to the grapevine virus collection, maintained in controlled conditions, in an insect-proof green-house (Figure 1).

Figure 1. Grapevine virus collection

The collection comprises more than 20 representative grapevine cultivars and clones, infected with 1-3 viruses or affected by virus-like diseases. Different lots of own-rooted or grafted plants belonging to the same cultivar are infected with different viruses or viral complexes (Table 1) .

Table 1. The grapevine virus-infected collection

Specification (Virus/virus disease/ virus-like disease)	Infected cvs (No)
(GFLV +ArMV)	5
GLRaV-1+3	6
(GFLV +ArMV) +GLRaV-1+3	1
GFkV	10
GVA	1
GLRaV-2	1
GFkV + GLRaV-1+3	3
GLRaV-1+3 + GVA	2
GFkV + GLRAV- 2 + GLRaV-1+3	1
GFkV + GVA+ GLRaV-1+3	1
GVA + GLRaV-1+3 + GLRaV-2	3
GLRaV 1+3 + Vein mosaic	1
Vein necrosis	1
Corky bark	1

The study of grapevine biological material in the presence of virus infection had in view the main dangerous and widespread viruses of this crop (fanleaf virus GFLV, arabis mosaic virus - ArMV, leafroll associated virus serotypes 1,2,3 - GLRaV-1,2,3, fleck virus - GFkV and virus A - GVA), and their presence in the native varieties/clones important in the strengthening of the Romanian viticulture patrimony.

The main directions of use of plants belonging to the grapevine virus-infected collection are the studies regarding: symptoms, *in vitro* behavior of virus infected grapevine, virus elimination, plant positive control in the diagnostic process.

3. RESULTS AND DISCUSSIONS

Symptoms

The symptoms are observable effects induced by the presence of the virus on the growing, development and metabolism of infected plant. Frequently, the symptoms in the field are the same, whether they are caused by one or more viruses. Thus, is not possible to identify the specific infections based on symptoms.

Infected grapevines from the collection show morpho-anatomical modifications and the presence of the viruses was confirmed by ELISA testing. The symptoms produced by viral infection can affect the whole plant (systemic symptoms) or they are visible on certain parts of the plant (local symptoms). They often consist of modification of the size (abnormal development) and aspect of the plant (mosaic, yellows, rings, necrosis).

Thus, fanleaf disease symptoms consisting of short internodes, double nodes, malformed shoots and yellow mosaic have been observed on infected grapevine cv. Italia and Fetească albă cvs. in colection, starting from early stages of vegetation. Also, low fruit quality have been registered in the presence of GFLV infection (Figure 2).

Figure 2. Yellow mosaic on grapevine cv. Fetească albă

Leafroll affected Fetească neagră variety showed reddish and downrolled leaves with narrow green bandes along the primary and secondary veins (Figure 3).

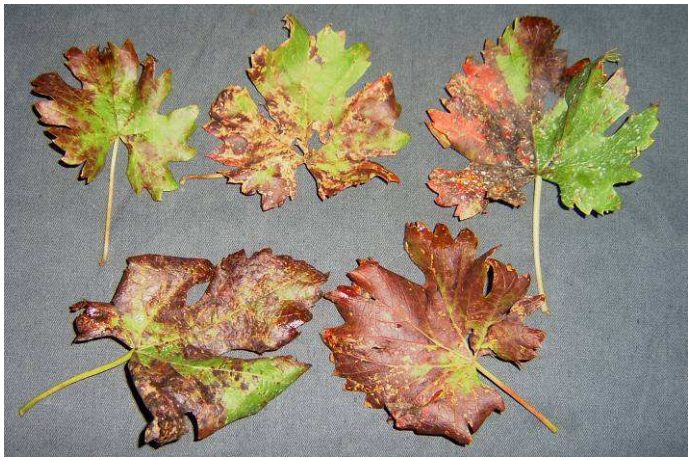

Figure 3. Leafroll symptoms on grapevine cv. Fetească neagră

In vitro behaviour of virus infected grapevine

The *in vitro* culture represents an easy tool for investigation the behaviour of the plants under the influence of the virus in uniformly conditions. *In vitro* studies of virus infected grapevines comparatively with the healthy material had in view quantitative and qualitative aspects of the culture: multiplication and rooting rates, shoots elongation, abnormal cuttings and vitrification phenomena. The non-uniformity of regenerative potential of different grapevine genotypes in the presence of virus infections and also a significant diminish of regenerative capabilities especially due the GLRaV-1+3 infection were registered. The quantity of GFLV infected material obtained by multiplication was apparently superior to the healthy one due the adventive buds and primordia to shoots elongation detriment (Figure 4). The quality of GFLV infected material was lower due the vitrification processes, abnormal cuttings and necrosis observed during the culture (Vişoiu et al., 2000a; Guță et al., 2009).

Figure 4. Virus disease symptoms on in vitro grapevine (a - fanleaf; b - leafroll)

Virus elimination

The infected cultivars and clones detected as positive for virus infection were subjected to virus elimination through heat treatment and/or *in vitro* meristem, apex, axillary bud culture by adapting the working protocols (especially the duration of the exposure) to the particularities of each virus (Buciumeanu and Vişoiu, 2000). During the *in vitro* culture and acclimatization phase, the persistence of the virus in regenerated plants was routinely checked. Thus, the efficiency of virus elimination by thermotherapy and/or *in vitro* culture was confirmed by ELISA tests in each step of the virus elimination technology (Buciumeanu and Vişoiu, 1996).

The healthy plants have been transferred into nuclear stock greenhouse for germplasm preservation under a severe regime for avoiding any virus infection (Vişoiu et al., 2000b).

New studies of the last years on virus elimination in grapevine deals with *in vitro* chemotherapy and electrotherapy applicable for many types of viruses at a time. Also, in the frame of virus eliminating studies, the phytotoxic effect of antiviral drugs ribavirin [(1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1H-1,2,4-triazole-3-carboxamide] and oseltamivir (3R,4R,5S)-4acetylamino-5-amino-3(1-ethylpropoxy-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate) used in controlled medium in various concentration and period of exposure was investigated (Guță et al., 2010).

The total elimination of GFLV and GFkV was achieved by chemotherapy using 40 mg/l and respectively 10 mg/l ribavirin, after 30 days of culture of grapevine apices on viricid medium; the ribavirin was ineffective in GLRaV 1+3 elimination. The treatment with 40 mg/l oseltamivir led to 71% elimination of GLRaV 1+3 after one subculture. Also, the elimination of GLRaV1+3 was 80-100% successful by electrotherapy in continuos electric field, at 10 V/cm – 10 min, folowed by the *in vitro* culture of apices collected from treated plant (Guță, 2010).

Electrotherapy with discontinuos current allowed to obtain 14-100% virus elimination in grapevine, at 10000 kHz – 10 min.
In order to obtain more efficient cleaning methodologies for initial material supplies, further investigations will allow the optimization of the factors of virus free plants regeneration under the influence of viricides and continuous/ alternative electric field. The effectiveness the virus detection and elimination methods with the aim to protect grapevine germplasm are in progress.
General objective of these cleaning methodologies are the scientific substantiation, the innovation and the development of new technologies for obtaining virus-free grapevine propagating material and a rise of technological competences by promotion of technologies and knowledge transfer in the agricultural field respecting the principle of long term development. Specific objective is to create efficient sanitation technologies: rapid, with low cost energy consume and a high rate of virus-free plants.

Plant positive control in the diagnostic process
ELISA (with DAS-, TAS- and DAS- biotin variants) is the most used method both for diagnosis and studies regarding the sampling strategy for different viruses (detection of the most reliable source of antigen and period of the year in which the analyze is performed).
The accuracy of ELISA results is proved by intra-laboratory validation of the method, by checking the suitability for the circumstances of use of each new reagent kit. The performance criteria as: repeatability, reproducibility, accuracy and robustness were explored. Validation was performed with reference material (positive and negative controls of the kit and samples taken from virus-infected grapevine belonging to the collection of the laboratory) (Guță and Buciumeanu, 2010). Also, healthy grapevine (virus-free) as negative control was used. Commercial ELISA reagents are purchased from BIOREBA, Switzerland.
The diagnosis of leafroll, fleck, vein necrosis and corky bark diseases have been done by a rapid biological method, *in vitro* micrografting (Buciumeanu et al., 2001). This method allows the detection of virus/virus-like disease in 2-3 months comparatively to the woody indexing procedure (1–3 years). Both laboratory and biological assays require internal standards (virus-infected plants). Grapevine virus-free indicators necessary for biological indexing procedures (wood grafting, green grafting, *in vitro* micrografting) are available in the germplasm collection (virus-free).

4. CONCLUSIONS
The grapevine virus collection is reference and source of material for diagnostic purposes for our own laboratory and also for similar others in the world.
Samples collected from infected vines were used as material testing for virus detection in inter-laboratory comparisons scheme and laboratory-performed validation.
The grapevine virus collection allowed the study of the behaviour of the plants in the presence of virus infections.
The infected plants in collection constitute demonstrative materials for grapevine growers and students in horticulture.

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