Continuity of grapevine virology at NRDIBH Ştefăneşti-Argeş in the service of a viable viticulture in Romania

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ELENA–COCUȚA BUCIUMEANU1, EMILIA VISOIU1, IONELA CÂTĂLINA GUȚĂ1, CARMEN FLORENTINA POPESCU1
1National Research and Development Institute for Biotechnology in Horticulture Ștefănești-Argeș, 37 București-Pitești Road, 117713-Ștefănești, România
Tel/Fax: 0248-266814; E-mail: ebuciumeanu@yahoo.com

Abstract

One of the most important missions of the National Research and Development Institute for Biotechnology in Horticulture Ștefănești-Argeș is to establish the initial grapevine plant-material collection and to preserve it in proper conditions. The infected cultivars and clones detected as positive for virus infection were subjected to virus elimination through heat treatment and/or in vitro culture by adapting the working protocols, especially the duration of the treatment, to the particularities of each virus. Starting from 1993, ELISA 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). In order to use the virus infected biologic material as reference (positive controls) in our studies, a grapevine virus infected collection was established. The behaviour of the infected grapevine material in comparison to the healthy one, both in vivo and in vitro conditions, have been investigated. The studies of cellular modifications in the presence of virus infection emphasized the usefulness of ultrastructural changes both for understanding the biological answer of the plants under stress conditions and as a valuable aid in virus diagnosis purposes.

Keywords: grapevine, germplasm collection, virus elimination, diagnosis, in vitro, survey

Introduction

Viticulture represents an important part of agriculture in România, both from the economic and social point of view. The total area under vines is about 189.7 thousands ha, of which 177.1 thousands wine grapes and 12.6 thousands table grapes. Being an intensive culture, the area of vineyard covers only 2% of the agricultural area. However, economically, viticulture covers 10% from the value of the agriculture production. In our country, viticulture represents the labor activity of about 33 000 families that is about one million people.

The quality of the grape depends on several influences and one of the most essential production factors is grapevine planting material. For this purpose, the European Community has formulated an uniform Community certification scheme which aims to ensure both type identity and health status. Romania as UE country, follows in the viticulture field the strengthening of the viticulture patrimony and the promotion in the new vineyards especially of Romanian grapevine varieties for competitive quality of wine in European space. With the aim of establishing of long lasting vineyards it is necessary to use healthy, virus-free planting material.

The improvement of native and foreign cultivars and clones by sanitary selection, detecting the viruses using advanced methods and developing the virus elimination techniques are essential for the efficiency of viticulture. Thus, the activity in grapevine virology field has been oriented to four main directions:
- Establishing and maintenance of the initial grapevine plant-material collection;
- The study of grapevine biological material in the presence of virus infection;
- Monitoring of grapevine viruses/virus diseases/virus-like diseases in vineyards;

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- Service activity.

Establishing and maintenance of the initial grapevine plant-material collection

All known grapevine pests include about 70 infections agents belonging to 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 (G.P. MARTELLI & E. BOUDON-PADIEU [1]).

One of the most important mission for National Research and Development Institute for Biotechnology in Horticulture Ștefănești-Arges (NRDIBH Ștefănești) is to establish the initial grapevine plant-material collection and to preserve it in proper conditions, as is required both by the European and national legislation.

The activity for sanitary selection and virus elimination in grapevine was carried out at the NRDIBH Ștefănești since 1988, according to the national planning for producing planting material and based on the certification scheme used in countries having a major contribution to European grape production (M. OȘLOBEANU & al. [2]). The program initiated for obtaining virus-free grapevine plants was constantly developed over the last years due to the increasing number of cultivars and clones needed to be available as healthy material. The plant material introduced within collection is produced by applying thermotherapy and/or tissue culture, and guaranteed for authenticity (trueness-to-type) and phytosanitary status.

According to the certification scheme followed by our laboratory, the testing for virus infection was carried out of the beginning by using herbaceous test plant and indexing on woody plant indicators. Since 1993, the research has registered progress in the study of viruses and viral diseases of grapevine after introducing the Enzyme –Linked Immunosorbent Assay (ELISA) method with commercial reagents, for the detection of the most economically important grapevine viruses: fanleaf virus + arabis mosaic virus (GFLV+ArMV); leafroll associated virus serotypes 1, 2, 3 (GLRaV-1,2,3); fleck virus (GFkV) and virus A (GVA) (E. BUCIUMEANU & al. [3]; I. TITA & E. BUCIUMEANU [4]).

Moreover, a virus-free grapevine collection of 224 grapevine cultivars and clones from which 95 are native cultivars, 41 (33 scions and 8 rootstocks) are autochthonous clones obtained from international cultivars was established, including plants which were tested for their authenticity and phytosanitary status, according to the European standards. This collection is used either as starting material for propagation and planting, or as plant material in studies concerning genetic variability of characters, adaptability to environment, and parental plants in breeding programs.

These healthy plants are preserved in greenhouse as germplasm collection, dedicated to initial grapevine plant material generation, under a severe regime for avoiding any virus infection (Table 1).

The virus-free grapevine germplasm collection provides the initial planting material for the establishment of new base planting vineyards. The setting up of new plantations with healthy planting material would lead to a rise in the technical-economic competitiveness by the efficaciousness of all obtaining and exploitation steps, from grafting to preserving the production characteristics.

The germplasm collection is annually checked for the most dangerous grapevine viruses (GFLV, GLRaV-1,2,3, GFkV, GVA), quarantine viruses (arabis mosaic virus - ArMV, raspberry ringspot virus - RRSV, strawberry latent ringspot virus - SLRV, tomato black
ringspot virus – TBRV, tobacco ringspot virus - TRSV, tomato ringspot virus – ToRSV peach rosette mosaic virus - PRV) and phytoplasmas (flavescence doré, bois noir).

Table 1. The grapevine virus-free collection

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Seedless table cultivars</td>
<td></td>
<td>3</td>
<td>-</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Seeded table cultivars</td>
<td></td>
<td>26</td>
<td>10</td>
<td>11</td>
<td>47</td>
</tr>
<tr>
<td>White, rose and flavoured wines</td>
<td></td>
<td>46</td>
<td>12</td>
<td>15</td>
<td>73</td>
</tr>
<tr>
<td>Red wines</td>
<td></td>
<td>11</td>
<td>11</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>Resistant cultivars</td>
<td></td>
<td>7</td>
<td>-</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>Rootstocks</td>
<td></td>
<td>2</td>
<td>8</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>95</td>
<td>41</td>
<td>88</td>
<td>224</td>
</tr>
</tbody>
</table>

The national grapevine collection is the result of research activities performed through national projects and was the subject of more than 35 papers published in national and international reviews, and presented to various scientific meetings and events.

**The study of grapevine biological material in the presence of virus infection**

The main goals of the study of grapevine biological material under the influence of virus infection had in view: comparative studies of healthy and virus infected plants, the virus diagnosis and elimination, virus purification, ultrastructural studies.

**Comparative studies of healthy and virus infected plants**

A virus contaminated plant decays after 4-5 years of culture, according to the virus/viruses type, of the genotype, environment conditions and of the agrotechnology used. The virus diseases reduce the grape yield by 80%, affecting at the same time the quality of fruit: the accumulation of carbohydrates, and the aspects (discoloration, short berries, millrandage), the grapes becoming unmarketable. The plantations using healthy material have a long term exploitation period at the specific quantitative and qualitative parameters and require small quantities of pesticides and maintenance activities.

The behaviour of the grapevine material under the influence of infection (with individual viruses and/or viral complexes), in comparison to the healthy one was done both **in vivo** and **in vitro** conditions.

**In vivo** studies of virus infected grapevines comparatively with the healthy plants had in view: plant growing and development (physiological analysis and biometric modifications), biochemical compounds, quality and quantity of the crop. Infected plants in the field which showed morpho-anatomical modifications and the presence of the viruses were confirmed by ELISA testing. Biochemical modifications were concerned with the determinations of the content of polyphenols, soluble glucides, chlorophyllous pigments in the leaves, juice acidity and sugar accumulation in grapes. The characteristics of the grapes were modified in the presence at virus infection (GRLaV-1+3, GFLV+ArMV, GFkV); low amounts of sugar in juice and higher acidity have been registered with virus infected grapes. Biochemical analysis helps to complete the range of investigations useful to assess a viral infection; as such modifications represent a general response of plants to a virus and also another pathogen or
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factor of stress, virus detection based on plant biochemical composition cannot be used as diagnosis method (I.C. GUȚĂ & al. [5, 6]).

The in vitro culture represents an easy tool for investigation the behaviour of the plants under the influence of the virus in uniform conditions. In vitro studies of virus infected grapevines compared 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 in different grapevine genotypes due to the presence of virus infections and also a significant diminishing of the 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 primordial in the detriment to shoots elongation. The quality of GFLV infected material was lower due the vitrification processes, abnormal cuttings and necrosis observed during the culture (E. VIȘOIU & al. [7]; E. VIȘOIU & E. BUCIUMEANU [8]; I.C. GUȚĂ & al. [9]).

Virus infected grapevine collection

In order to use the virus infected biologic material as reference (positive controls) in our studies, a grapevine virus infected collection of 17 cultivars was established. The grapevines are infected with 1-3 of the main specific viruses of this crop (Table 2). Different lots of plants belonging to the same cultivar are infected with different viruses.

Table 2. The grapevine virus infected collection

<table>
<thead>
<tr>
<th>Specification</th>
<th>Infected cvs (No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GFLV + ArMV)</td>
<td>5</td>
</tr>
<tr>
<td>GLRaV-1+3</td>
<td>6</td>
</tr>
<tr>
<td>(GFLV + ArMV) + GLRaV-1+3</td>
<td>1</td>
</tr>
<tr>
<td>GFkV</td>
<td>7</td>
</tr>
<tr>
<td>GVA</td>
<td>1</td>
</tr>
<tr>
<td>GLRaV-2</td>
<td>1</td>
</tr>
<tr>
<td>GFkV + GLRaV-1+3</td>
<td>2</td>
</tr>
<tr>
<td>GLRaV-1+3 + GVA</td>
<td>1</td>
</tr>
<tr>
<td>GFkV + GLRaV-1+3 + GLRaV-1+3</td>
<td>1</td>
</tr>
<tr>
<td>GFkV + GVA + GLRaV-1+3</td>
<td>1</td>
</tr>
<tr>
<td>GVA + GLRaV-1+3 + GLRaV-2</td>
<td>3</td>
</tr>
<tr>
<td>Vein mosaic</td>
<td>1</td>
</tr>
<tr>
<td>Vein necrosis</td>
<td>1</td>
</tr>
<tr>
<td>Corky bark</td>
<td>1</td>
</tr>
</tbody>
</table>

The own rooted and grafted potted plants are maintained in greenhouse provided with insectproof net.

Virus diagnosis and elimination

All 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 (E. BUCIUMEANU & E. VIȘOIU [10]). 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 (E. BUCIUMEANU & E. VIȘOIU [11]). Also, GFkV was eliminated by somatic embryogenesis (C.F. POPESCU & al. [12]).
The healthy plants have been transferred into nuclear stock greenhouse for germplasm preservation under a severe regime for avoiding any virus infection.

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 diagnosis of leafroll, fleck, vein necrosis and corky bark diseases have been done by a rapid biological method - *in vitro* micrografting (E. BUCIUMEANU & al. [13]; E. VIŞOIU & al. [14]; E. VIŞOIU & E. BUCIUMEANU [15]). This method allowed the detection of virus/virus-like disease in 2-3 months compared with the woody indexing procedure (1–3 years) (Table 3).

**Table 3. Viruses/virus and virus-like diseases detection at NRDIBH Ștefănești – Argeș**

<table>
<thead>
<tr>
<th>Viruses/virus and virus-like disease</th>
<th>Diagnosis method/ starting year</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFLV; GFLV (+ArMV*)</td>
<td>wood grafting/1989; ELISA/1993; sap inoculation</td>
</tr>
<tr>
<td>GLRaV-1; GLRaV-2; GLRaV-3</td>
<td>of herbaceous hosts/1994; <em>in vitro</em> micrografting/2002</td>
</tr>
<tr>
<td>GLRaV-1+3</td>
<td>wood grafting/1989; ELISA/1993</td>
</tr>
<tr>
<td>Leafroll disease/GLRaV-3</td>
<td>ELISA/ 1995</td>
</tr>
<tr>
<td>GLRaV-7</td>
<td><em>in vitro</em> micrografting /2002</td>
</tr>
<tr>
<td>GTA</td>
<td>wood grafting/1989; ELISA/ 2002</td>
</tr>
<tr>
<td>Corky bark</td>
<td>wood grafting/1989; <em>in vitro</em> micrografting /1997</td>
</tr>
<tr>
<td>Vein necrosis</td>
<td>wood grafting/1989; <em>in vitro</em> micrografting /1997</td>
</tr>
<tr>
<td>Vein mosaic</td>
<td>wood grafting/1989</td>
</tr>
<tr>
<td>TBRV*; SLRV*; RRSV*</td>
<td>ELISA/ 2002</td>
</tr>
</tbody>
</table>

*Common viruses (and also, phytoplasmas) for different crop plants are routinely analyzed by the quarantine laboratories.

Grapevine virus-free indicators necessary for biological indexing procedures (wood grafting, green grafting, *in vitro* micrografting) are available in the germplasm collection.

**Virus purification**

Virus particles were extracted from leaves of infected grapevines by fractionated polyethylene glycol 6000 (PEG 6000) precipitation and purified by deferential centrifugation. Purification routine procedures for closterovirus-like particle associated with leafroll disease (GLRaV-3) and also for isometric viruses (GFLV and GfKV) were described, and after various centrifugation steps were examined by ELISA. Thus, the loss of virus during the purification scheduled steps and the concentration of the preparations were estimated. The purified preparations were used in electronmicroscopical studies and for checking the *in vitro* virus resistance with antigen property (C. GRECU & al. [16]).

**Ultrastructural studies**

The cellular modifications induced in the presence of GFLV or GLRaV-3 infection were studied by electron microscopy of grapevine infected tissues. Ultrastructural analysis revealed unusual cytoplasmic accumulation of inclusion bodies with different origins (mitochondria, chloroplast, endoplasmic reticulum, cell membrane) and aggregates of virus particles, in function of the type of infection. These studies emphasized the usefulness of ultrastructural changes both for understanding the biological answer of the plants in stress conditions and as a valuable aid for virus diagnose purposes (A. BREZEANU & E. BUCIUMEANU [17]; E. BUCIUMEANU & A. BREZEANU [18]).

Improvement of activity

Our research group displayed a remarkable interest in the improving grapevine virus elimination techniques, chemotherapy and electrotherapy (I.C. GUȚĂ & al. [19]) compared with heat treatment and/or in vitro culture as classical methods. In order to obtain more efficient cleaning methodologies for initial material supplies, further investigations will allow the optimization of the factors involved in virus free plants regeneration under the influence of viricides and continuous/alternative electric field, respectively, applicable for many types of viruses at a time. 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.

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. Also, random amplification of polymorphic DNA (RAPD) technique was used to confirm the genetic stability (trueness-to-type) among virus free plants recovered by in vitro chemotherapy (I.C. GUTA & al. [20]).

The experiments had in view the most dangerous grapevine viruses (GFLV+ArMV, GLRaV 1+3, GFkV, GVA) and their presence in the native varieties/clones important in the strengthening of the Romanian viticulture patrimony.

The effectiveness the virus detection and elimination methods with the aim to protect grapevine germplasm are in progress. Some progress in RT-PCR application for detection of leafroll associated viruses GLRaV1+3 was registered (E.BUCIUMEANU & D. ISPAS [21]).

In the future, one of our research targets is the diversifying of the experiments by enlarging the number of intracellular pathogens (virus, viroid, bacteria, phytoplasma) and host plants taken into study.

Monitoring of grapevine virus/virus diseases/virus-like diseases in vineyards

The main activity to prevent the spreading of the grapevine virus diseases until now is the rigorous sanitary selection. The observations of phenotypical modifications induced in the field plants in the presence of virus infection, followed by virus diagnostic tests in the lab led, to the identification of infected material and prevented the spreading of the diseases. The Romanian research has already done progress in the study of viruses and viral diseases of grapevine after introducing the ELISA method, especially in the routine diagnosis. However, more research is needed to be done in order to further clarify the situation.

Virological analyses carried out until now revealed that Romanian grapevine varieties are infected with many viruses (D. BOSCIA & L. DEMARINIS [22]; B.N. MILKUS & al. [23]). One field survey for the main grapevine viruses detection on native varieties (Fetească neagră, Fetească regală, Tămăioasă românească, Victoria, Augusta) in own plantations of the institute have been performed. The results show that the majority of the samples collected showed at least the presence of one of the tested viruses (GFLV+ArMV, GLRaV 1+3, GLRaV-2, GFkV, GVA) (E. BUCIUMEANU &. al. [24]).

In the future, a survey of the most spread grapevine viruses/virus diseases/virus-like diseases of this crop all over the vineyards of the country is necessary.

Service activity

In order to obtain high quality grapevine planting material, service activity of the virology laboratory follows the Romanian legislation, in accordance with EU and EPPO
requirements. This activity consists of the virus diagnosis, maintenance and delivery of grapevine initial material.

**Virus/virus disease diagnosis**

The virology laboratory is working for the following customers: research and development units for viticulture and also for private grapevine growers.

The service activity aims with checking the phytosanitary status of grapevine from:
- planting material meant to own fields and to commercialization;
- the new clones or varieties obtained in breeding programmes;
- initial planting material;
- vineyards with mother plants meant to nursery.

Virological analyses of grapevine, serological (ELISA) and biological (wood grafting and *in vitro* micrografting) methods are accredited and validated. ELISA is the most efficient method for grapevine viruses detection: GFLV+ArMV, GLRaV-1+3, GLRaV-2, GFkV and GVA.

**Recognition of the virology lab competence**

The virology laboratory is accredited by RENAR - Romanian Accreditation Association in conformity with the requirements of standard SR EN ISO/CEI 17025:2005, for virological analyses of grapevine using the following methods: serological tests ELISA (DAS-, TAS- and DAS-biotin variants) and biological tests (indexing by wood grafting and *in vitro* micrografting). The quality of the grapevine virological tests, the working techniques and also the thrust of the customers in our results are improved by participation of the lab to intercomparative schemes.

**Maintenance of grapevine initial material**

Grapevine initial material of Romanian and international grapevine fruitful cultivars and rootstocks is maintained *ex situ* and *in vitro* conditions for medium and long term.

**Initial material delivery**

In order to establish grapevine basic mother plantations, in 2004-2009 period was delivered initial material (buds, cuttings, rooted plants) for 4 – 4.2 ha of scions and rootstocks to the viticultural research units. Traditional Romanian grapevine varieties and largely used rootstocks were predominant.

**References**


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