Grapes recovery from viruses during reproduction by biotechnological method

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Abstract. Grape regenerant plants obtained from apical meristems were assessed for the presence of the viruses using PCR and enzyme-linked immunosorbent assay (ELISA). The vast majority of the samples studied for the presence of the most common viruses (Grapevine Leafroll-Associated Virus – 1 (GLRaV-1); Grapevine yellow mosaic virus; Grapevine vein banding virus; Grapevine Leafroll Virus; Grapevine stem pitting virus) by ELISA showed negative results. Testing of regenerant plants of the Augustine variety clones showed that only one plant out of 80 plants showed a positive reaction to the presence of this virus. Analysis of 80 plants of the Moldova variety clones revealed a positive reaction to the yellow mosaic virus (Grapevine yellow mosaic virus) in 1 plant as well as in the original donor plant (Moldova variety). The rest of the regenerated plants (79 pcs.) were absolutely healthy. PCR analysis showed that the spectrum of fragments of the original genotypes of Moldova varieties (lanes 1–4) and Augustin (lanes 5–8) contained DNA fragments of 450 bps, corresponding to Candidatus Phytoplasma vitis Flavescence doree. In Bart variety (lanes 9–11), no pathogen was detected. Analysis of the spectrum of fragments of grape regenerant plants obtained from apical meristems (lanes 1–4) and Augustine (lanes 5–8) showed the absence of the pathogen Candidatus Phytoplasma vitis Flavescence doree.

1. Introduction

Modern standards for planting material require its recovery from viral and mycoplasma infections, parasitic nematodes, ticks and other pests, and diseases. It demands new effective methods of obtaining material free from pests and diseases as well as its accelerated reproduction to be entailed into the technological schemes for the recovery and reproduction of planting material [1, 2]. The development of highly efficient technologies for the production of healthy planting material for fruit and berry crops is becoming increasingly important due to the need to intensify horticulture [3, 4]. The successful development of biotechnological research including modern trends for the improvement of garden plants, their accelerated reproduction, obtaining new promising forms and plants storage is the basis for the sustainable horticulture development [5].

Harmfulness of viral diseases of fruit crops is determined by a number of reasons. First, viral diseases are latent at the initial stages of development (and many for a longer period). A decrease in yield, deterioration in the quality of a crop, slowdown in tree growth and even its death often occur for no apparent reason. The second factor in the high severity of viral diseases is their rapid spread while viral pathogens are transferred in a variety of ways: insect pests, pollen during flowering, mechanical
tools for reproduction and garden care (pruners, saws, knives). The third reason is the impossibility of using fast-acting chemical and biological means of control [6].

The strategy for controlling viral diseases includes the following aspects: culling the plantations infected with economically important viruses; using special recreation activities and producing healthy clones in a laboratory; compliance with organizational and agrotechnical measures, vector control; producing the varieties being immune and tolerant to viruses [7].

Methods of dry air thermotherapy, in vitro culture and chemotherapy are usually applied to heal plants from viruses. The best healing results are usually obtained by a combination of several methods. At the same time, on the one hand, an urgent task is to improve the known methods in relation to the biological characteristics of plants and the properties of specific viruses. On the other hand, it is important to search for and develop new highly effective ways to heal fruit and berry crops from viruses [8]. Consequently, the role of virology in modern nursery farming will increase every year due to the need to increase the release of healthy planting material and this trend should be taken into account when developing a strategy for horticulture development [9]. The most effective way is to improve and reproduce seedlings isolated apexes cultivation in combination with accelerated clonal micropropagation [10]. When testing the type of sterilizing agent, it was shown that the use of 12% hydrogen peroxide yield favourable results in clearing the meristems from infection and does not cause tissue necrosis, which increases the yield of material for further reproduction [11].

A technology aimed to obtain healthy clones of grapes based on the use of modern methods for diagnosing viral diseases and bacterial cancer and clonal micropropagation in culture in vitro has been proposed. Practical application of this technology enabled to obtain a collection of phytosanitary grape clones free from viral and bacterial infections [12]. Cobos R., Mateos R., Alvarez-Perez J., Olego M. et al. believe that vine stem fungal pathogens such as Diplodia seriata and Phaeomoniella chlamydospora can infect plants through wounds caused by cutting. They lead to vine trunk diseases and harvest decline [13].

2. Research methodology
The studies were carried out in the laboratory “Biotechnology of agricultural plants” of the Federal State Budgetary Educational Institution of Higher Education “Chechen State University” and at the Federal State Budgetary Scientific Institution “Chechen Research Institute of Agriculture”.

The research objects were grape varieties, various nutrient media and their components, growth regulators.

The research subjects were biotechnological techniques, methods, technology of in vitro grapes cultivation.

As a result of numerous experiments, the Murashige-Skoog growing medium was determined in various modifications in terms of the content of mineral and hormonal compositions that affect the rooting, growth and development of grape plants in vitro.

The initial plant material for cultivation was taken from intact grape plants grown at the experimental production site of the Chechen Research Institute of Agriculture.

Intensively growing green shoots of grape varieties Augustine, Moldova and Bart harvested from vegetating grape bushes were used as an initial explant in in vitro culture. The ripe vine was cut into two-, three-eyed stems 8–13 cm long, and placed for germination at a temperature of 25–30 °C. After 25–30 days green shoots appeared on the stems, and the apical shoots 2–3 cm long were cut off. The grape varieties Moldova, Augustin, Bart were selected for the research.

Method of enzyme-linked immunosorbent assay (ELISA). To assess regenerated plants of grapes and apple rootstocks, samples of plant material were formed according to the standard (STO VNIIKR 4.007–2011). The plants were visually inspected at the stage of adaptation and completion of growing. At least 10 plants were taken from each experimental sample of planting material. In the case of the external signs of the disease, the field was examined in more detail. The material was stored at a temperature of +40 °C, interleaved with filter paper in plastic bags with holes for aeration. The presence of Grapevine Leafroll-Associated Virus – 1 (GLRaV-1) in grape varieties Augustin, Moldova
and Bart was determined by ELISA. The enzyme immunoassay was carried out using ELISA kits for determining viral pathogens in plants Nano Diagnostics: Grapevine Leafroll-Associated Virus – 1 (GLRaV-1) – TAS ELISA / Coating Antibody (OOO “Laboratory Diagnostics”, Moscow) in accordance with the manufacturer’s instructions. The results of the analyzes were recorded on a Bio-Rad 680 (680XR) microplate photometer at a wavelength of 405 nm (immunogram 1).

**PCR analysis technique.** Young leaves of grapes and apple rootstocks were used as a basic material. Fresh leaves were used directly for DNA isolation, or stored at – 800 °C until being used. When taking samples in greenhouse conditions, part of them (replicates) were placed in silica gel and dried in it (1 g/100 g of raw weight). The DNA yield was calculated per unit of raw and/or dry weight of the material. To prepare the plant homogenate, the material was preliminarily cut into small pieces using a scalpel, then ground in a porcelain mortar using a porcelain pestle. Approximately 100–150 mql of the material was taken and transferred into Eppendorf tubes (1.5 ml), where they were intensively homogenized in 400ml of LB extraction buffer using a Poter homogenizer. It was pressed tightly against the tube walls and the homogenization time was minimized as much as possible (until the extraction buffer was intensely stained green). Then the samples were incubated in a thermostat during 40 minutes at 650 °C.

The specificity of primer systems was studied using some types of gram-positive and gram-negative bacteria, as well as samples of apple and grape plants uninfected with phytoplasmosis pathogens. One of the existing methods of PCR diagnostics were applied “in real time” to identify Candidatus Phytoplasma vitis Flavescence doree being the causative agent of grapes golden yellowing, a quarantine pest for the territory of Russia and the North Caucasus, and Candidatus Phytoplasma solani Bois noir, the causative agent of grape bark blackening.

### 3. Research results

Grape regenerant plants obtained from apical meristems were assessed for the presence of viruses and other pathogenic microorganisms using PCR and enzyme-linked immunosorbent assay (ELISA), providing highly sensitive immune diagnostics of plant material for the presence of phytopathogens in a short time frame. Shoot leaves were used as plant samples.

**ELISA.** The vast majority of the samples studied for the presence of the most common viruses (Grapevine Leafroll-Associated Virus – 1 (GLRaV-1); Grapevine yellow mosaic virus; Grapevine vein banding; virus; Grapevine Leafroll Virus; Grapevine stemm pitting) by ELISA showed negative results. (Table 1).

The parent plant of Augustine variety showed positive result in 1 of the five viruses tested being the (Grapevine Leafroll Virus). Testing of regenerant plants of its clones (AG-56-06; AG-56-12-1; AG-56-23-3; AG-56-31-1) showed that only one plant (AG-56 -06) out of 80 plants had a positive reaction with regards to the presence of the indicated virus. Analysis of 80 plants of clones of the Moldova variety (MO-126; MO-127; MO-128; MO-129) revealed a positive reaction to the yellow mosaic virus (Grapevine yellow mosaic virus) in 1 plant (MO-126) as well as in the original donor plants (varieties Moldova). The rest of the regenerated plants (79 pcs.) were absolutely healthy.

The content of infected plants in the total number of analyzed plants was 1.25 %. Identified infected plants (clones) of grapes were seized and destroyed. Plants that showed no viruses as a result of testing were assigned the category of virus-free basic clones. Thus, the use of meristem apexes enabled to rehabilitate the starting material of varieties Augustine and Moldova from (Grapevine yellow mosaic virus) and (Grapevine Leafroll Virus) in vitro.
Table 1. Results of testing experimental samples of planting material of grape varieties Augustin, Moldova and Bart for the presence of the most common viruses using ELISA

| Sample         | Grapevine Leafroll-Associated Virus – 1 (GLRaV-1) | Grapevine yellow mosaic virus | Grapevine vein banding virus | Grapevine Leafroll Virus | Grapevine stemm pitting |
|----------------|--------------------------------------------------|-------------------------------|-------------------------------|--------------------------|--------------------------|
| Augustine      | 1 2 3 4                                          | 1 2 3 4                       | 1 2 3 4                      | 1 2 3 4                  | 1 2 3 4                  |
| original (control) | - - - -                                            | - - - -                      | - - - -                      | - - - -                  | - - - -                  |
| AG-56-06       |                                                  | -                             | -                             | -                        | -                        |
| AG-56-12-1     |                                                  | -                             | -                             | -                        | -                        |
| AG-56-23-3     |                                                  | -                             | -                             | -                        | -                        |
| AG-56-31-1     |                                                  | -                             | -                             | -                        | -                        |
| Moldova        |                                                  | -                             | +                             | -                        | -                        |
| original (control) | - - - -                                            | -                             | +                             | -                        | -                        |
| MO-126         |                                                  | -                             | -                             | -                        | -                        |
| MO-127         |                                                  | -                             | +                             | -                        | -                        |
| MO-128         |                                                  | -                             | -                             | -                        | -                        |
| MO-129         |                                                  | -                             | -                             | -                        | -                        |
| Bart           |                                                  | -                             | -                             | -                        | -                        |
| original (control) | - - - -                                            | -                             | -                             | -                        | -                        |
| B-12           |                                                  | -                             | -                             | -                        | -                        |
| B-26           |                                                  | -                             | -                             | -                        | -                        |
| B-89           |                                                  | -                             | -                             | -                        | -                        |

Note: 1 – donor plants (original); 2 – Plants in vitro (shoots); 3 – plants after adaptation (regenerants); 4 – plants in the greenhouse (planting material)

PCR. The regenerant plants of grapes varieties Moldova, Augustin and Bart were assessed for the presence of quarantine phytoplasmosis for the territory of Russia and the North Caucasus being the pathogens of golden yellowing of grapes (Candidatus Phytoplasma vitis Flavescence doree, FD) and blackening of the bark (Candidatus Phytoplasma BN Bois noir) were analyzed using PCR.

The analysis was carried out by real-time PCR (RT-PCR) using species-specific primers selected for the 6SrRNA gene zone to identify the phytoplasmosis pathogens of the grapes FD and BN. The amplification of DNA isolated from grape plants and target phytoplasmas was performed in the PCR mode with electrophoretic detection of the amplification results. Results of RT-PCR of FD tuf primers and FD-FAM probe with phytoplasmic DNA samples. After conducting PCR on the tuf gene zone, DNA fragments of the desired size of 450 base pairs (bps) were detected.

As a result of PCR analysis, it was found that the spectrum of fragments of the original genotypes of varieties Moldova (lanes 1–4) and Augustin (lanes 5–8) contained DNA fragments of 450 bps, corresponding to Candidatus Phytoplasma vitis Flavescence doree. In Bart variety (lanes 9–11), no pathogen was detected. Analysis of the spectrum of fragments of vine regenerant plants obtained from apical meristems (lanes 1–4: clones MO-126; MO-127; MO-128; MO-129) and Augustin (lanes 5–8: clones AN 56-02; AG 56-12-1; AG 56-23-3; AG 56-31-1) showed the absence of the pathogen Candidatus Phytoplasma vitis Flavescence doree.

Thus, grape regenerant plants obtained from apical meristems in contrast to the original varieties Moldova and Augustin no longer had fragments corresponding to the pathogen Candidatus
Phytoplasma vitis Flavescence doree in their spectrum, which confirms the release of the original plant material from the pathogen passing through the culture meristem apexes in vitro.

Grapevine Fan Leaf Virus (GFLV) and Grapevine Fleck Virus (GFkV) viruses in grape plants were detected by PCR in combination with ELISA (Immunocapture PCR – with immune capture PCR). The analysis was carried out using a kit of reagents for diagnostics of plant pathogens Nano Diagnostics in the Immunocapture RT-PCR format: Grapevine Fan Leaf Virus (GFLV) – Immunocapture RT-PCR Kits / ACDcap Immunocapture Kit for RT-PCR and Grapevine Fleck Virus (GFkV) – Immunocapture RT -PCR Kits / ACDamp Immunocapture RT-PCR Kit (OOO “Laboratory Diagnostics”, Moscow) in accordance with the manufacturer’s instructions.

As a result of PCR analysis viruses Grapevine Fan Leaf Virus (GFLV), Grapevine Fleck Virus (GFkV), Grapevine Leafroll-Associated Virus – 1 (GLRaV-1) were not detected in samples of regenerated grape plants from Bart variety (clones B-12; B-26; B-89), Augustin variety (clones AN 56-02; AG 56-12-1; AG 56-23-3; AG 56-31-1); Moldova variety (clones MO-126; MO-127; MO-128; MO-129). The absence of bands at the level of the positive control for regenerated plants confirms that viruses were not detected in the samples studied.

As part of research 1,347 grape regenerant plants were tested by means of PCR analysis and ELISA for the presence of the most common phytoplasmosis and viruses. The test results confirmed that they had no internal infection. These plants were assigned the category of “virus-free” basic clones (Table 2).

Table 2. List of “virus-free” basic grape clones with confirmed health status by PCR and ELISA methods

| Original variety | Regenerant plants / clones | List of pathogenic microorganisms | Number of plants, pcs. |
|------------------|---------------------------|----------------------------------|------------------------|
| Augustine        | AG-56-03                  |                                  | 12                     |
| Augustine        | AG-56-12-1                |                                  | 26                     |
| Augustine        | AG-56-23-3                |                                  | 22                     |
| Augustine        | AG-56-31-1                |                                  | 22                     |
| Augustine        | AG-56-03                  |                                  | 22                     |
| Augustine        | AG-56-11                  |                                  | 86                     |
| Augustine        | AG-56-22-3                | GFLV                             | 63                     |
| Augustine        | AG-56-36-1                | GFkV                             | 18                     |
| Moldova          | MO-126                    | GLRaV-1                          | 74                     |
| Moldova          | MO-127                    | Gymv                             | 26                     |
| Moldova          | MO-128                    | Gvbv                             | 44                     |
| Moldova          | MO-133                    | GLV                              | 51                     |
| Moldova          | MO-126                    | CPv                              | 11                     |
| Moldova          | MO-141                    | Arabis mosaic virus              | 11                     |
| Moldova          | MO-148                    |                                  | 86                     |
| Moldova          | MO-151                    | Flavescence doree ine            | 45                     |
| Moldova          | MO-152                    | stemm pitting                    | 22                     |
| Moldova          | MO-153                    | CPhytoplasma solani              | 22                     |
| Moldova          | MO-154                    | Bois noir                        | 34                     |
| Moldova          | MO-155                    |                                  | 21                     |
| Moldova          | Б-12                      |                                  | 22                     |
| Moldova          | Б-26                      |                                  | 28                     |
| Moldova          | Б-89                      |                                  | 28                     |
| Moldova          | Б-111                     |                                  | 25                     |
| Bart             | В-113                     |                                  | 23                     |
| Bart             | В-125                     |                                  | 63                     |
| Bart             | В-126                     |                                  | 99                     |
| Bart             | В-261                     |                                  | 86                     |
| Bart             | В-315                     |                                  | 25                     |
| **Totally**      | **29**                    |                                  | **1117**               |
4. Conclusion

Part of infected plants in the total number of analyzed plants was 1.25%. Plants that showed no viruses on the test were categorized as “virus-free basic clones”. The use of meristem apexes enabled to rehabilitate the initial material of Augustine and Moldova varieties from Grapevine yellow mosaic virus and Grapevine Leafroll Virus in vitro. Thus, using ELISA and PCR analysis, it was confirmed that a healthy grape planting material was obtained from viruses and other pathogenic microorganisms using clonal micropropagation.

References

[1] Batukaev A A, Magomadov A S, Malykh G P and Batukaev M S 2014 Improving the technology of growing grape seedlings and increasing the productivity of grape plantations Bulletin of the Chechen State University 1 223–227

[2] Batukaev A A, Mukailov M D, Batukaev M S, Minkina T and Sushkova S Use of growth regulators in grapes grinding by in vitro method 18th International Multidisciplinary Scientific GeoConference SGEM-2018 (Vol 18 Issue 6.2) (Albena, Bulgaria 30 June – 9 July) pp 783–790

[3] Batukaev A A, Palaeva D O, Batukaev M S and Sobralieva E A 2018 In vitro reproduction and ex vitro adaptation of complex resistant grape varieties Advances in Engineering Research 151 895–899

[4] Bozhidai T N, Kastritskaya M S, Kukharchik N V, Meskhidze A M and Metriveli M V 2016 Regenerative capacity of blueberries at the stage of introduction into culture in vitro Biotechnology in fruit growing Proceedings of an international scientific conference (Samokhvalovichi) pp 105–107

[5] Kulikov I M, Upadyshev M T and Vysotsky V A 2016 Achievements and promising directions of biotechnological research in the FSBSU “All-Russian Institute of Selection and Technology of Horticulture and Nursery” Biotechnology in fruit growing Materials of an international scientific conference (Samokhvalovichi) pp 26–28

[6] Krasinskaya T A 2004 Micropropagation of various forms of Cerasus Mill. In vitro Fruit growing: scientific works Institute of Fruit Growing of the National Academy of Sciences of Belarus 16 26–31

[7] Upadyshev M T, Kulikov I M, Petrova A D, Metlitskaya K V and Donetskikh V I 2018 Modern methods of healing fruit and berry crops from harmful viruses (Moscow: FGBNU VSTISP) 160 p

[8] Upadyshev M T et al. 2014 Improvement of horticultural crops from viruses using environmentally friendly methods Fruit and berry growing in Russia 40(1) 329–333

[9] Kornatsky S A 2001 The most significant elements of industrial technology of clonal micropropagation of tree cultures In Industrial production of healthy planting material for fruit crops (pp 103–104) (Moscow)

[10] Vysotsky V A 1983 Culture of isolated tissues and organs of fruit plants: health improvement and microclonal reproduction Agricultural biology 7 42–48

[11] Tashmatova L V and Mantseva O V 2016 Features of the development of blackberries with different forms of growth in culture in vitro Biotechnology in fruit growing Materials of the international scientific conference (Samokhvalovichi) pp 85–88

[12] Khaustov E I, Prolanyuk E R, Sultanova O D and Bondarchuk V V 2016 Biotechnology of obtaining healthy clones of grapes Biotechnology in fruit growing Materials of the international scientific conference (Samokhvalovichi) pp 113–115

[13] Cobos R, Mateos R, Álvarez-Pérez J, Olego M, Sevillano S, González-García S, Garzón-Jimeno E and Coque J 2015 Effectiveness of natural antifungal compounds in controlling infection by grapevine trunk disease pathogens through pruning wounds Applied and Environmental Microbiology 81(18) 6474–6483