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Chemotherapy of apple shoots *in vitro* as method of viruses eradication

Abstract. Studies carried out in Kazakhstan and abroad have shown the widespread prevalence of viral, bacterial and fungal diseases on apple commercial varieties and clonal rootstocks. Current paper presents the results on the viruses eradication from *in vitro* shoots of varieties and clonal rootstocks of apple (*Malus domestica* Borkh.) using chemotherapy and obtaining healthy super-elite planting stocks. Ribavirin at concentrations of 75 and 100 mg/L caused severe *in vitro* shoot necrosis. Three subcultures on Murashige-Skoog medium with 50 mg/L ribavirin was efficient for elimination of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), and *Apple mosaic virus* (ApMV) from *in vitro* shots of *Malus* varieties and clonal rootstocks. A virus-free *in vitro* collection (42 accessions) was established, which was used for create a cryobank of shoot tips at -196°C and to obtain virus-free planting stocks. The percentage of *in vitro* shoots rooting ranged from 50% to 90%. The survival rate of *in vitro* shoots rooted in the soil substrate is more than 90%.

Key words: apple, chemotherapy, *in vitro* culture, ribavirin, virus-free planting stocks

Introduction

The apple (*Malus* sp.) is popular because the high nutritional, therapeutic and prophylactic properties as well as a high potential productivity and adaptive ability to growing conditions. The protection of plants from pests and diseases, including viruses ensure high yields of fruit crops. Studies carried out in Kazakhstan and abroad have shown the widespread prevalence of viral, bacterial and fungal diseases on apple commercial varieties and clonal rootstocks. The causative agents of these diseases are very harmful: they cause disruption of physiological processes, suppress growth, reduce generative productivity, resistance to stressful environmental factors, rooting and the yield in mother plantations, increase the susceptibility of plants to stress [1-3].

Four viruses such as *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), and *Apple mosaic virus* (ApMV) have been identified in apple orchards in Kazakhstan [3; 4]. Dolgikh et al. previously reported seven viruses of the apple in Kazakhstan pointing to the most harmful are: ACLSV, ASPV, ASGV [5]. ACLSV is the most widespread virus, which affects 75% of infected apple plants. In addition, there have been reported mix-infection in varieties and rootstocks [3].

The specificity of the ecology of viruses complicates the fight against them due to the obligatory nature of intracellular parasitism. Their propagation is closely related to the metabolism of the host plant cell, and this is the main obstacle to the direct suppression of the pathogens vital activity of plant viral diseases. It is challenging to distinguish healthy plants from infected ones. The detection and elimination of viruses and subsequent micropropagation of virus-free plants is the basis for the production of high-quality planting material. These have been methods of virus eradication from shoot tips and *in vitro* shoots such as thermotherapy, cryotherapy, chemotherapy, as well as a combination of these methods [6-10].

Over the past 50 years, world science has made significant progress in the study of viral diseases of apple, a prerequisite for which was the development of highly sensitive serological and molecular diagnostic methods and biotechnological methods of treatment.

The method of cryotherapy for apple and combined cryo- and chemotherapy methods for potatoes were previously successfully applied in the

Institute of Plant Biology and Biotechnology (IPBB) (Laboratory of Germplasm Cryopreservation) [3; 11]. However, apple cryotherapy did not demonstrate a 100% result in virus elimination, part of the collection remained infected with viruses. Therefore, in order to achieve higher rates, it was decided to use chemotherapy of *in vitro* plants. The technique consists in the use of chemical compounds (antiviral drugs) in the composition of the nutrient medium. These drugs inhibit the multiplication of viruses. In this regard, a large number of different substances were tested on model objects (tobacco mosaic virus, potato viruses) both in Kazakhstan and abroad, of which synthetic analogues of purine and pyrimidine bases (in particular, 2-thiouracil and 8-azaguanine), ribonuclease enzymes and some others substances that disrupt the metabolism of nucleic acids of an infected plant and are included in the genomic RNA of viral particles, reducing their specific infectivity. Many authors have successfully used ribavirin (1-beta-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide), a synthetic nucleoside, a broad-spectrum antiviral drug [7-8; 11-12].

Ribavirin does not affect the synthesis of RNA in plant cells and inhibits only the synthesis of viral RNA. Nevertheless, it has a depressing effect on plant growth with an increase in its concentration, which varies from 20 to 100 mg/L [7, 8]. The highest efficiency of ribavirin was shown to suppress potato viruses [7; 9; 11]. For apple, chemotherapy was carried out by F. Paprštein et al. As a result, 76% of the shoots recovered from the ASGV, ACLSV, ASPV viruses [8]. However, these authors carried out the experiment only for one variety Fragrance, while the literature provides data that the effectiveness of virus recovery depends on the characteristics of various genotypes. As a result, studies carried out on single varieties or in a limited number cannot be reliable and applicable to large collections, there is no guarantee that similar results will be obtained for other varieties [6; 7; 9-11].

The aim of this work was to assess the effectiveness of chemotherapy for elimination of the viruses from the infected apple varieties and rootstocks *in vitro*.

Materials and methods

Study design. The experiments were performed in five replications, n = 20. The work was carried out with the apple *in vitro* collection of the Germplasm Cryopreservation Laboratory of the IPBB. For the viruses testing, 77 *Malus* accessions were used, of which 61 were *Malus domestica* Borkh. (varieties and clonal rootstocks) and 16 accessions – *Malus sieversii* (Ledeb.) M. Roem. The collection was obtained in the period from 2002 to 2020 from field plants provided by the Kazakh Research Institute of Fruit Growing and Viticulture, "Liza" Farm, "Suzdaleva" Farm, "Integration-Turgen" LLP, wild forms were collected from the Main Botanical Garden (MBG) and from various areas of the Ile-Alatau National Park [13].

Plants were propagated *in vitro* on Murashige-Skoog (MS) medium [14] containing 30 g/L sucrose, 0.5 mg/L 6-benzylaminopurine (BAP), 0.01 mg/L indolylbutyric acid (IBA), 4.0 g/L Plant TC agar (Phytotechnology Laboratories®, Lenexa, KS), 1.25 g/L Gelrite™ (Phytotechnology Laboratories®), pH 5.7. All cultures were grown in glass culture vessels (237 mL) (Phytotechnology Laboratories®) in a growth room at 24°C±1°C with 16 h photoperiod of white fluorescent lights at 40 μM m^{-2} s^{-1} (standard growth room conditions) with two types of OPPLE tubular fluorescent lamps: YK 21RR 16/G 21W 6500K RGB and YK 21RL 16/G 21W 4000K RGB supplied by ElectroComplex in Corporation, Almaty, Republic of Kazakhstan (http://elcor.kz) [13]. Plants were subcultured i*n vitro* every 3-4 weeks.

Treatment. For chemotherapy, virus infected apple accessions were used, varieties: Aport Alexander (ACLSV, ApMV, ASPV), Gold Rush (ACLSV, ASPV), Red Free (ACLSV), Landsberger Renette (ACLSV, ASPV), Suislepper (ACLSV, ASPV), and clonal rootstock: B 7-35 (ASGV). *In vitro* shoots, were cultivated (standard growth room conditions) in 237 ml culture vessels (5 pieces each) on MS medium containing 30 g/L sucrose, 0.5 mg/L BAP, 0.01 mg/L IBA, 4.0 g/L Plant TC agar, 1.25 g/L Gelrite™, pH 5.7 with the addition of the 50, 75 or 100 mg/l ribavirin (F. Hoffman-La Roche Ltd. Switzerland, manufactured by Pantheon Inc., Canada). The duration of cultivation (1 passage) was 45 days, the number of passages: from 1 to 3.

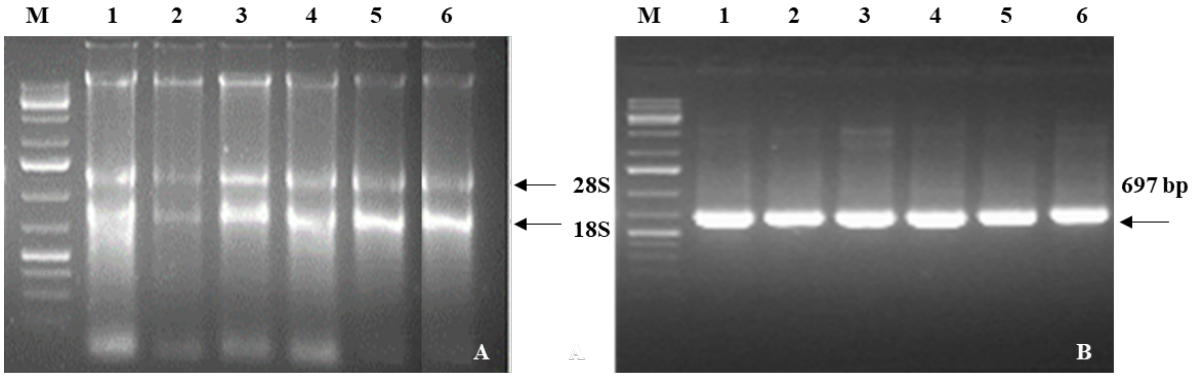
Assessment. After chemotherapy, detection was performed for 4 apple viruses: ACLSV, ASGV, ASPV, ApMV, according to the method described by Gasik et al. [17], with modifications [3].

Four pairs of specific oligonucleotides were constructed by multiple alignment of known nucleotide sequences of various isolates taken from the database of National Center for Biotechnology Information (NCBI) using the program BioEdit [18]. According to the results of the comparative analysis, the most conserved regions of the genomes ACLSV, ASGV, ASPV, APMV were identified, for which the primers were designed (Table 1). The designed primers were synthesized by Mycrosynth (Switzerland).

Table 1 – A set of oligonucleotides for the simultaneous testing of four apple viruses

Virus	N	Sequence of nucleotides	PCR product size (nucleotide pairs)
ASPV	201803	ATGTCTGGAACCTCATGCTGCAA	370
	201804	TTGGGATCAACTTTACTAAAAAGCATAA	
ACLSV	201805	TTCATGGAAAGACAGGGGCAA	677
	201806	AAGTCTACAGGCTATTTATTATAAGTCTAA	
ASGV	201801	GAAGACGTGCTTCAACTAGC	579
	201802	TTTTAGACCAGTGGCAAAGT	
ApMV	201807	AGGGTCCTGAGCAGTCGAGA	264
	201808	GTTTGGAGGGGCTTCCCACT	

The quality of the isolated RNA was determined by the separation of two ribosomal RNAs (28S and 18S) on an electrophoretogram (presented on Figure 1A). The samples were also checked for the quality of the isolated RNA using the control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (presented on Figure 1B). Verification was carried out using specific primers for GAPDH [19].



A – Total RNA isolated from the leaves; B – RT-PCR products GAPDH

Figure 1 – Electrophoresis in 2% agarose gel for apple 1-6 samples (*M. domestica*); Lane 1 – Aport Alexander; Lane 2 – Gold Rush; Lane 3 – Red Free; Lane 4 – Landsberger Renette; Lane 5 – Suislepper; Lane 6 – B 7-35; M – molecular marker (GeneRuler 1kb)

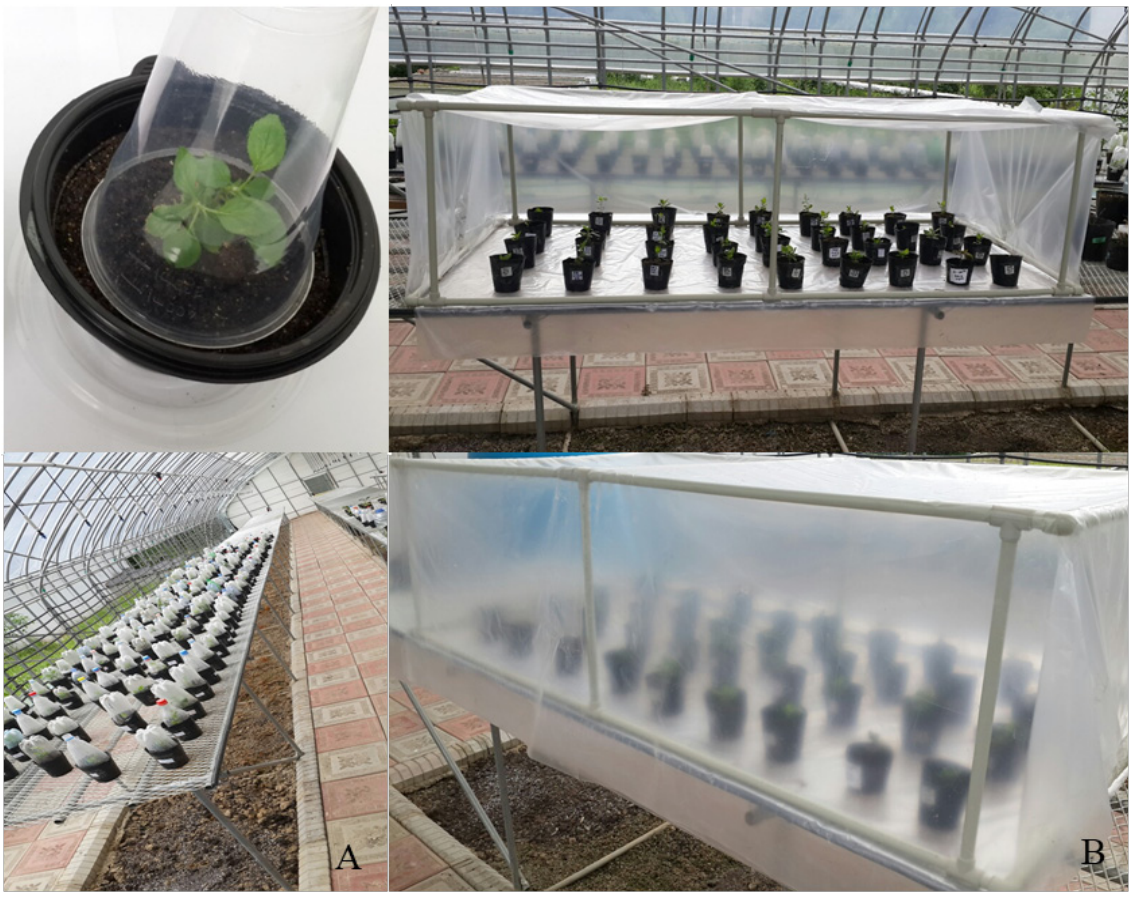
Reverse transcription and polymerase chain reaction (PCR) were performed using reagents from Fermentas, following the manufacturer's recommendations. To obtain cDNA of viruses, a reverse primer was used, reverse complementary to the desired region of RNA; for the PCR reaction, a second reverse primer was used, thereby increasing the specificity to viruses. PCR products were analyzed after electrophoresis (voltage 8 V/cm) in 2% agarose (Phytotechnology Laboratories®) gel containing 0.5 µl/ml ethidium bromide (Sigma-Aldrich) in 1x TAE buffer [3].

For *in vitro* rooting of virus-free aseptic plants in 237 ml culture vessels, a nutrient medium was used: ½ MS, 0.25 mg/L IBA, 4.0 g/L Plant TC agar, 1.25 g/L Gelrite™, pH 5.7 [20]. Rooting plants were grown in standard growth room conditions.

A sterile substrate was used as a soil: a mixture of black earth ("Ahty", https://almaty.flagma.kz/342682/), peat (Kekkila DSM 1 W EXTRA, Finland), perlite (Union Perlite, Almaty, RK https://perlite.kz/) in a percentage ratio: 50:40:10. Plants with roots (Root length – 3.5-8.0 cm) were transferred in a soil substrate in polyethylene containers (250 ml)

("Ahty"). The containers were closed with plastic transparent cap (presented on Figure 2A) or placed in a mini-greenhouse (presented on Figure 2B) to retain moisture necessary for adaptation of the shoots.

Procedures. During the first week, the adaptation of planting stocks to the soil substrate was carried out in standard growth room conditions. Then they were transferred to a greenhouse (temperature from 18°C to 24°C, humidity 40% without additional lighting. A week later, the plastic cap was removed for 10-15 minutes or the polyethylene of the mini-greenhouse was opened for ventilate the plants; on the following days, the duration of ventilation was increased to 8 hours. Duration of adaptation was from 3 to 6 weeks. For subsequent adaptation, the plastic caps were removed or the container with the planting stocks was transferred from the mini-greenhouse to the greenhouse.



A – Apple planting stocks in a soil substrate under transparent plastic caps;
B – Apple planting stocks in a mini-greenhouse

Figure 2 – Adaptation of planting stocks to the soil substrate and greenhouse conditions

Data analysis. Statistical analysis of the experimental data was conducted according to standard procedures described in the manual by Lakin 1990 and the SYSTAT Version 12.0 software package (Systat Software Inc., San Jose, CA. All data presented as percentages ± standard error, were subjected to arcsine transformation before the statistical analysis. ANOVA using SAS, version 9.2, for windows (SAS Institute Inc. Cary, NC) was applied to analyze the data [15-16].

Results and discussion

The chemotherapy experiments of virus-infected in vitro apple plants revealed that 75 or 100 mg/L ribavirin in the nutrient medium has a detrimental effect on plants for some accessions. A high percentage of plant necrosis *in vitro* was noted (Table 2). The chemotherapy with 50 mg/L ribavirin demonstrated only 5% of the shoots necrosis (Aport Alexander and B 7-35) (Table 3).

After one subculture on ribavirin, viruses were not detected in clonal rootstock B 7-35. Following two subcultures on ribavirin Gold Rush, Red Free and Suislepper were virus-free. For the varieties Aport Alexander and Landsberger Renette, it took three subcultures on ribavirin to be completely free from the viruses detected before chemotherapy. This study demonstrates that three subcultures on 50 mg/L ribavirin resulted 100% shoots free of ACLSV, ApMV, ASPV, and ASGV.

Table 2 – Shoot necrosis on Murashige-Skoog medium with various concentrations of ribavirin

Accession name and viruses in original sample	% shoot necrosis on MS medium with 100 mg/L ribavirin	% shoot necrosis on MS medium with 75 mg/L ribavirin	% shoot necrosis on MS medium with 50 mg/L ribavirin
Aport Alexander (ACLSV+ ASPV+ApMV)	100	100	5.0
Gold Rush (ACLSV+ASPV)	-	25.0	0
Red Free (ACLSV)	55.0	10.0	0
Landsberger Renette (ACLSV+ASPV)	40.0	10.0	0
Suislepper (ACLSV+ASPV)	10.0	15.0	0
B 7-35 (ASGV)	-	100	5.0
Mean ± st. dev.	51.25±37.50[a]	43.33±44.23[a]	1.67±2.58[b]
- The experiment was not carried out Means indicated by different letters differ significantly at $p \leq 0.05$			

Table 3 – Results of detection of four viruses (ACLSV, ASGV, ASPV, ApMV) in apple accessions using RT-PCR after chemotherapy, ribavirin concentration 50 mg/L

Accession name and viruses in original sample	After one subculture on MS medium with 50 mg/L ribavirin	After two subcultures on MS medium with 50 mg/L ribavirin	After three subcultures on MS medium with 50 mg/L ribavirin
Aport Alexander (ACLSV+ ASPV+ApMV)	17 – ACLSV+ASPV (85%) 3 – virus-free (15%)	1 – shoot necrosis (5%) 15 – ASPV (75%) 4 – virus-free (20%)	19 – virus-free (100%)
Gold Rush (ACLSV+ASPV)	15 – ASPV (75%) 5 – virus-free (25%)	20 – virus-free (100%)	-
Red Free (ACLSV)	10 – ACLSV (50%) 10 – virus-free (50%)	20 – virus-free (100%)	-
Landsberger Renette (ACLSV+ASPV)	16 – ASPV (80%) 4 – virus-free (20%)	7 – ASPV (35%) 13 – virus-free (65%)	20 – virus-free (100%)
Suislepper (ACLSV+ASPV)	6 – ASPV (30%) 14 – virus-free (70%)	20 – virus-free (100%)	-
B 7-35 (ASGV)	1 – shoot necrosis (5%) 19 – virus-free (95%)	-	-
Mean ± st. dev. Virus-free shoots	45.83±31.85[a]	88.0±16.81[b]	100.0±0[b]
- The experiment was not carried out			

After chemotherapy the virus-free shoots were micropropagated on a MS medium without ribavirin. After three subcultures of 4 four weeks each, a repeated test for the viruses was carried out and no viruses were detected. Accordingly, regardless of the amount of viruses in the original accession, three subcultures on ribavirin at a concentration of 50 mg/L is sufficient to obtain virus-free plants. The efficient protocol of chemotherapy for virus eradication from *Malus* varieties and clonal rootstocks *in vitro* shots was developed.

A virus-free *in vitro* collection of 54 varieties and 7 clonal rootstocks was established, which was later used to create a cryobank and obtain healthy

planting stocks (presented on Figure 3A, 3B). The percentage of *in vitro* rooting ranged from 50% to 90%. The survival rate of planting stocks in the greenhouse is more than 90% [20]. Thus, we carried out a full cycle of obtaining healthy apple planting stocks, from *in vitro* initiation to transfer of *in vitro* plants into the soil substrate (presented on Figure 3C).

In the future, the collection of aseptic in vitro plants and the obtained planting stocks will be used for scientific purposes, to create a cryobank, as well as to obtain super-elite planting stocks that can be involved in the breeding process in nursery and farm enterprises to improve existing and create new varieties and clonal rootstocks, as well as for the international exchange of genetic resources.

A – Virus-free *in vitro* shoots of the Golden Delicious variety; B – Virus-free *in vitro* apple collection

Figure 3 – Production of virus-free apple planting stocks

Conclusion

Apple, one of the most popular fruit crops in the world. The widespread spread of viruses on apple crops, their toxic effects cause concern throughout the world. Therefore, early diagnosis of viral diseases and the possibility of plant material therapy are relevant. The developed method of treatment for viruses (chemotherapy) allows to obtain 100% of in vitro material to heal, and is an effective solution to the existing problem.

The obtained developments can solve the problem of providing the Kazakhstan market with high-quality domestic apple planting material. Promotion of the sale of high-quality planting stocks will increase yields and improve the quality of fruit products, and in turn provide the country's population of apples with high taste and marketability, not damaged by pests and diseases. High-quality, competitive products can be sold not only domestically, but also abroad, which will increase the rating of Kazakhstan in the world market.

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