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APPLE CULTIVARS AND ROOTSTOCKS ASSAY FOR THE IDENTIFICATION OF DIVERSE VIRUSES AND HEALTHY GENOTYPES FOR BREEDING

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SUMMARY

The prevalence of harmful viruses, *viz.*, apple stem grooving virus (ASGV), apple stem pitting virus (ASPV), apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV), and tomato ringspot virus (ToRSV) in apple tree plantations in the Ryazan, Yaroslavl, and Moscow regions, Russian Federation, based on genotype features, planting type (industrial, collection, and repository), and tree age was studied during 2018–2021. The prevalence of harmful and latent viruses ranged from 49.6% to 53.8% in apple cultivars and from 8.3% to 100% in apple clonal rootstocks. The most common virus in the Moscow and Yaroslavl regions was ACLSV (34.7% and 53.8%) and that in the Ryazan region was ApMV (33.3%). Relative to that in young apple trees, the virus influence in older tree tissues was higher by 20% to 43% depending on virus type. Monoviral infection prevailed (59% of all trees were infected with one virus) in the studied apple tree cultivars. Of the cultivars, 25% were infected by a complex of two viruses (ASPV + ACLSV), 10% were infected by three viruses, and 6% were infected by four viruses. The highest prevalence of latent viruses was observed in old Russian cultivars (53.5%) and selections from old foreign cultivars (57.2%). Virus occurrence was slightly lower (51.6%) in new Russian cultivars than in other cultivars. Columnar apple tree cultivars had the lowest virus occurrence (30%) and were found to be more tolerant than other genotypes. The highest virus incidence was recorded in industrial orchards (63.4%), followed by that recorded in collection (20.8%) and repository (18.3%) plantations. By using ELISA, the apple genotypes that were free from harmful viruses were identified as source plants, i.e., 183 plants from 18 apple tree cultivars and 131 plants from clonal rootstocks. Results indicated that virus-free apple germplasm is highly effective for the successful implementation of breeding and genetic improvement.

Keywords: *Malus domestica* Borkh., viruses, cultivars, rootstock, diagnostics, ELISA

Key findings: Using virus-free apple germplasm is entirely necessary for the successful implementation of breeding and genetic improvement.

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INTRODUCTION

In the present era, the occurrence of unhealthy planting material of fruit crops and the prevalence and harmfulness of viruses have increased significantly as a result of the enhanced anthropogenic load on natural cenoses and agrocenoses. Harmful latent viruses include apple stem grooving virus (ASGV), apple stem pitting virus (ASPV), apple

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chlorotic leaf spot virus (ACLSV), and apple mosaic virus (ApMV). Latent pome crop viruses are mainly spread by infected planting material (Hadidi *et al*., 2011; Katwal *et al*., 2016; Megrelishvili *et al*., 2017).

Latent viruses are widespread in seed crop plantations worldwide. In Korea, 47.6% of apple trees are infected with three different viruses, i.e., ACLSV, ASGV, and ASPV, but not by ApMV (Cho, 2015; Cho *et al*., 2016). Other studies reported a high prevalence of latent viruses (97.3%) in apple trees in Korea (Lee *et al*., 2020). In India, a survey of 17 apple plantations revealed that the frequencies of ASGV, ASPV, ACLSV, and ApMV range from 5.4% to 92% (Katwal *et al*., 2016). In 13 provinces in China, 65% to 80% of apple trees are infected with viruses, with infection by a complex of two or more viruses being highly prevalent (Ji *et al*., 2013). The overall prevalence of dormant viruses in apple trees in Georgia is 23% (Megrelishvili *et al*., 2017), whereas that in Tunisia is 80% (Mahfoudhi *et al*., 2013). In the Russian Federation, the prevalence of viruses in apple trees from the end of the 20th century to the second decade of the 21st century ranged from 42% to 50% (Prikhodko and Magomedov, 2011; Upadyshev *et al*., 2014; Metlitskaya *et al*., 2018).

Harmful viruses result in apple yield losses of 7% to 48% (Clever and Stehr, 1996). ACLSV in combination with other viruses reduce apple yields by 10% to 30% (Cieniewicz and Fuchs, 2016; Fuchs, 2016). ASGV, ASPV, and tomato ringspot virus (ToRSV) cause necrosis and phloem and xylem dysfunction in the grafting zone that results in reduced growth, leaf chlorosis, and eventually decreased productivity. ApMV infections in susceptible cultivars cause yield reductions of up to 50% (Fuchs, 2016).

Reducing viral diseases in horticultural crops is possible only through the wide introduction and use of virus-free planting material obtained from specialized scientific centers for breeding. Regular monitoring is entirely needed to control the spread of viruses and the development of possible epiphytotic diseases (Kulikov and Upadyshev, 2015; Kulikov *et al*., 2018). The national standard of the Russian Federation GOST R 53135-2008 states that the planting material of pome crops must be free from viruses, i.e., ASGV, ASPV, ACLSV, and ApMV.

A virus-free collection of apple trees is crucial for breeding purposes (Keshavarz and Hajnajari, 2021). Many countries have planned programs and the exchange of resistant plant genetic resources to reduce the spread of viruses domestically (Barba *et al*., 2015; Motyleva *et al*., 2021). In the majority of the countries, the gene banks of fruit and small fruit crops are stocked with pest- and disease-free planting materials that have been screened for varietal purity, productivity, and genetic stability (FAO, 2014; Borisova *et al*., 2018). In German fruit crop gene banks, 75% of apple cultivars are identified as matching varietal types (Höfer *et al*., 2019).

Gene banks should be preserved *in situ* (botanical gardens and collections in the field) and *ex situ* (under artificial conditions; greenhouses; and laboratories, including cryopreservation and *in vitro*) in consideration of the Global Plant Conservation Strategy (Jackson and Kennedy, 2009; Ren *et al*., 2019). In the United States, the gene pool conservation system includes more than 20 gene banks with approximately 540 000 plant and seed samples (Bretting *et al*., 2011). The German Federal gene pool contains more than 151 000 *ex situ* samples (Oppermann *et al*., 2015). In the Russian Federation, the largest collection of plant genetic resources, which comprises 320 000 samples (1.2 million storage units), is in the Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (https://www.gov.spb.ru/static/writable/ckeditor/uploads/2021/03/25/32/1272). The *in situ* conservation of genetic resources is considered preferable given the possibility of species development under changing environmental conditions.

The objectives of the present study are to determine the prevalence of the harmful viruses ASGV, ASPV, ACLSV, ApMV, and ToRSV in apple cultivars and rootstocks depending on the region, varietal characteristics, planting type, and age and to identify and isolate virus-free genotypes for subsequent use in future breeding efforts.

MATERIALS AND METHODS

Experimental conditions

This study was carried out during 2018–2021 at the Laboratory of Virology, Federal Horticultural Research Center for Breeding, Agrotechnology, and Nursery, Moscow. The samples for the diagnosis of ASGV, ASPV, ACLSV, ApMV, and ToRSV were acquired from industrial apple plantations in the Moscow, Ryazan, and Yaroslavl regions. All three regions have a temperate continental climate. Ryazan (Ryazan 54°37' north latitude, 39°41' east longitude) is in south Moscow, and

Yaroslavl (Yaroslavl 57°52' north latitude, 39°12' east longitude) is located north of Moscow (Moscow 55°45' north latitude, 37°36' east longitude). In Ryazan, the average annual temperature is +4.6 °C, the average precipitation is 450–550 mm, and the vegetation period is 230–250 days. In Yaroslavl, the average annual air temperature is −5.0 °C, the average precipitation is 500–600 mm, and the vegetation period 165–170 days. In the Moscow region, the average annual air temperature is +3.5 °C, the average rainfall is 420–640 mm, and the vegetation period is 170 days.

Data recorded

The virus infection index of leaf samples was determined. The infection index was calculated as the ratio of sample extinction to the extinction of the negative control, i.e., 1.0 to 1.59: no virus; 1.6 to 1.99: probable infection; and 2.0 and higher: reliable infection. A total of 319 apple trees were tested, and 1920 virus tests were performed.

Chemicals

The ELISA for ASGV, ACLSV, ApMV, and ToRSV was performed by using diagnostic kits containing immunoglobulins, conjugates, and positive and negative controls from Loewe, Germany. Diagnostic kits were also used in the ELISA for ASPV and were obtained from Bioreba AG, Switzerland. All other chemical substances chosen for the analysis were of analytical quality (minimal purity 99%) and were bought from Sigma–Aldrich, USA, and Panreac Applichem, Spain.

Sample preparation

Leaf samples were selected from four sides of the apple tree crown, from the middle part of the crown, and from the base of the shoot. Three leaves were taken from each side of a twig, and a composite sample was prepared. Leaf samples were homogenized in sample (conjugate) buffer containing PBS buffer (pH 7.4) with the addition of 2% polyvinylpyrrolidone and 0.2% bovine serum albumin. The samples were homogenized at the ratio of 200 mg of leaves per 3 ml of sample buffer. After homogenization, the samples were centrifuged on a Beckmann J2-21 M/E centrifuge (UK) at 2500 rpm for 20 min at +4 °C. The supernatant was plated in Costar (USA) 96-well microplates at the amount of 200 µl per well in two replicates.

ELISA

Sandwich ELISA was performed in accordance with the method described by Clark and Adams (1977). The coating buffer (pH 9.6) for immunoglobulins contained 1.59 g l^{-1} Na_2CO_3, 2.93 g l^{-1} $NaHCO_3$, and 0.2 g l^{-1} NaN_3 in distilled water. The coating buffer was applied to the microplates and incubated in a refrigerator at +4 °C for 16 h. The microplates were washed three times with PBS wash buffer (pH 7.4). After washing, the samples were applied to the microplates and incubated at +4 °C for 16 h. After incubation, the microplates were washed four times with PBS wash buffer (pH 7.4). The conjugate was diluted at the rate of 1:200 in conjugate buffer. The conjugate was applied to the microplates and incubated in a TS-1/80 SPU thermostat (Russia) at +37 °C for 4 h. The microplates were washed three times with PBS wash buffer (pH 7.4). Then, 1 mg ml^{-1} 4-nitrophenylphosphate was added to the substrate buffer (97 ml l^{-1} diethanolamine, 0.2 g l^{-1} NaN_3 distilled water, pH 9.8). The substrate buffer was applied to the microplates at +20 °C in the dark. After 2 h, the results of the analysis were recorded on a Stat Fax 2100 (USA) plate photometer at the wavelength of 405 nm.

Statistical analysis

For the comparative evaluation of the apple trees of different ages (8–10 and more than 20 years old), samples were taken from six apple cultivars (Lobo, Melba, Spartan, Antonovka, Papirovka, and Bogatyr') with five trees of each age. The results were expressed as the mean values of the infestation index with standard deviation. MS Excel (Microsoft Excel, v. 2016) software was used for statistical analysis.

RESULTS

The prevalence of harmful latent viruses in apple tree stands in the three surveyed regions of the Russian Federation varied from 49.6% to 53.8% (Figure 1). The total prevalence rates of the viruses in the apple trees in all of the three surveyed regions did not differ significantly. However, the prevalence of individual viruses in different areas exhibited certain features. The most common virus in the Moscow and Yaroslavl regions was ACLSV (34.7% and 53.8%, respectively) and that in Ryazan region was ApMV (33.3%). This difference may be connected to the planting of different apple cultivars in various regions. In

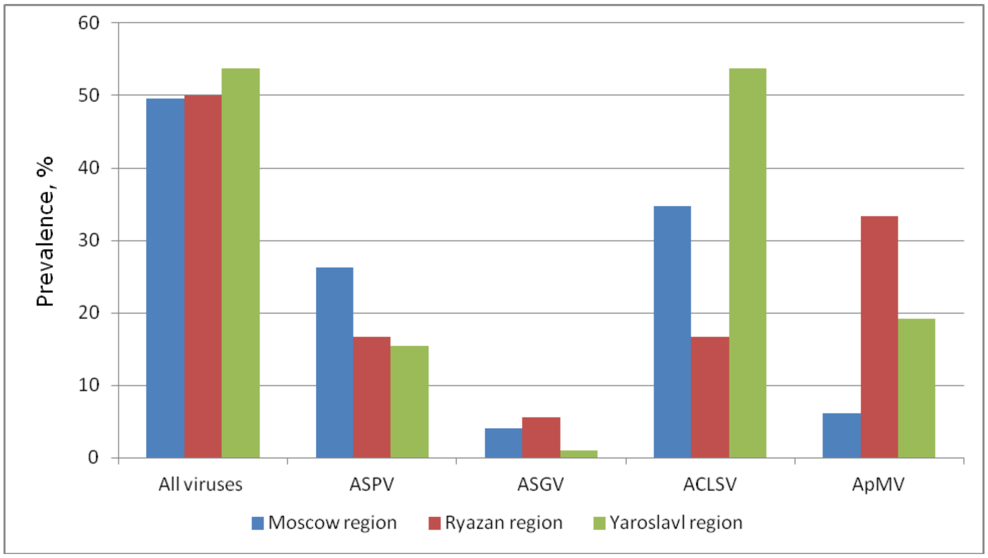

Figure 1. Prevalence of latent viruses in apple tree plantations in the Moscow, Ryazan, and Yaroslavl regions of the Russian Federation.

Table 1. Virus infection indexes of apple trees depending on age and virus type.

Age of trees (years)	ASPV	ASGV	ACLSV	ApMV
8–10	1.2 ± 0.08	1.0 ± 0.06	1.4 ± 0.11	1.5 ± 0.10
20 or more	1.5 ± 0.09	1.2 ± 0.07	2.0 ± 0.15	1.5 ± 0.12

Mean of 5 determination ± standard deviation

the Yaroslavl and Ryazan regions, old Russian cultivars (‘Antonovka’ and ‘Papirovka’) dominated, whereas new and old Russian cultivars and foreign breeding material were dominant in the Moscow region. ASPV had higher prevalence rates (15.5%–16.2%) than ASGV (1.0%–5.6%).

An examination of industrial apple tree stands of different ages revealed that the infection indexes of the studied viruses tended to increase as the ages of the trees increased (Table 1). The indexes of ASPV, ASGV, and ACLSV infection in 20-year old apple trees were 25%, 20%, and 43% higher than those in 8–10-year old trees. Monoviral infection prevailed in apple tree cultivars. Specifically, of all trees, 59% were infected by one virus; 25% were infected by a complex of two viruses (ASPV + ACLSV); and 10% and 6% were infected by three and four viruses, respectively.

The analysis of virus prevalence in apple tree cultivars of different origins revealed that old Russian cultivars (53.5%) and old cultivars of foreign selection (57.2%) had the highest prevalence of viruses (Figure 2). The virus incidence in new Russian cultivars was also rather high (51.6%) but was relatively low in columnar apple tree cultivars (30%). The highest frequencies of ASPV were found in old Russian cultivars (34.8%) and old cultivars obtained from foreign selections (40.2%). The other viruses were characterized by lower frequencies, i.e., ACLSV by 15% and 16.3% and ApMV by 10.9% and 12.8% (Table 2). ASGV was more often diagnosed in old Russian cultivars (17%) than in other cultivars.

The most infected (43%–100%) apple cultivars were ‘Antonovka’, ‘Orlovskoe polesye’, and ‘Sinap Orlovskii,’ which were mainly affected by ASPV. The virus prevalence in apple tree clonal rootstocks varied from 8.3% to 100% (Table 3). The semidwarf clonal rootstock 62-396 showed the highest infection prevalence. Specifically, 69.7% of the trees of this rootstock were infected by ACLSV. The medium-growing clonal rootstock 54-118 was mainly infected by ACLSV and was characterized by the low occurrence of other viruses. The strong-growing clonal rootstock 57-490 was characterized by the low occurrence of viruses.

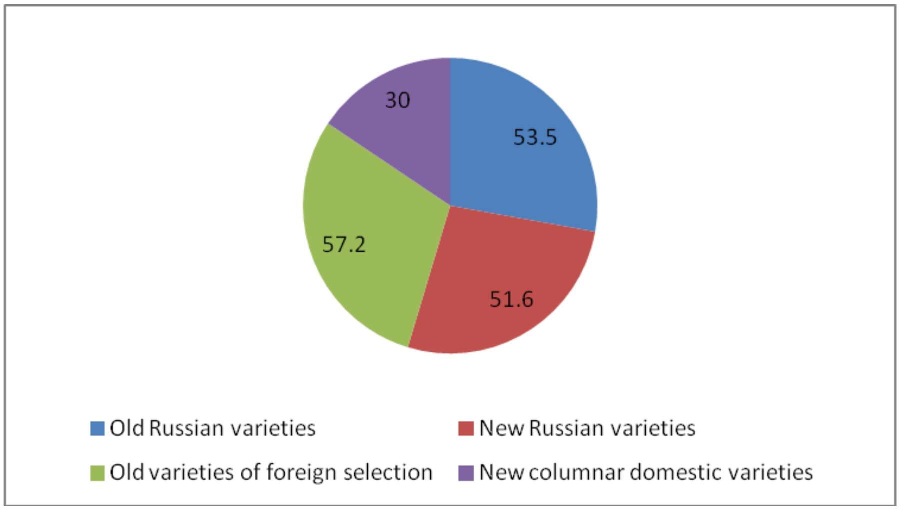

Figure 2. Prevalence of latent viruses (%) in apple cultivars in accordance with breeding affiliation (2018–2021).

Table 2. Prevalence of latent viruses in apple cultivars depending on breeding affiliation (2018–2021).

Breeding affiliation of cultivars	ASPV (%)	ASGV (%)	ACLSV (%)	ApMV (%)
Old Russian cultivars (Antonovka, Papirovka, Sinap Orlovsky, Orlik, Konfetnoe)	34.8	17.0	15.0	10.9
New Russian cultivars (Orlovskoye Polesie, Svezhest, Imrus, Podarok Grafskomu, Marat Busurin, Mayak Zagorya, Legenda)	26.4	8.6	12.4	2.8
Old cultivars of foreign selection (Lobo, Melba, Spartan, and Mantet)	40.2	6.3	16.3	12.8
New Russian column-shaped cultivars (Valyuta, Triumph, Ostankino)	20.0	6.7	6.7	0.0

Table 3. Virus prevalence in apple tree clonal rootstocks (Moscow region).

Clonal rootstocks	General prevalence of viruses (%)	ASPV (%)	ASGV (%)	ACLSV (%)	ApMV (%)
54-118	27.6	7.1	9.4	18.2	3.7
57-490	3.8	3.8	0.0	3.8	3.8
62-396	69.7	0.0	8.3	69.7	0

In apple trees in the Moscow region, the prevalence of harmful latent viruses varied from 42.6% to 63.4% depending on plantation type (Table 4). The highest prevalence of viruses was found in industrial apple tree orchards (63.4%). Virus prevalence was lower by 18.3% and 20.8%, respectively, in repository and collection plantations. In all plantations, the most common virus was ACLSV, which ranged from 42.3% to 58.5% in prevalence. ASPV had a relatively high prevalence in industrial orchards likely due to their apple cultivar composition. ASGV and ApMV exhibited low prevalence, and ToRSV was not found in quarantined samples. Similar to the present study, a previous study did not detect ToRSV in 98 samples obtained from 48 apple cultivars grown under Iranian conditions (Keshavarz and Hajnajari, 2021). Of the apple trees, 48.5%, 48.5%, and 3% were infected by one, two, and three viruses, respectively.

All of the studied apple cultivars and clonal rootstocks had virus-free plants (Table 5). The fruits of the apple cultivars ‘Valuta’, ‘Konfetnoe’, ‘Mantet’, ‘Marat Busurin,’ ‘Podarok Grafskomu,’ and ‘Svezhest’ were free from

Table 4. Virus prevalence in apple trees in different plantations in the Moscow region (2020–2021)**.**

Plantation type	Number of test samples	General prevalence of viruses (%)	ASPV (%)	ASGV (%)	ACLSV (%)	ApMV (%)	ToRSV (%)
Industrial garden	41	63.4	29.3	0	58.5	4.9	0
Repository	71	45.1	9.9	0	42.3	0	0
Collection plantations	47	42.6	0.0	4.3	42.6	0	0
Total	159	52.2	12.6	1.9	48.4	1.3	0

Table 5. Yields of virus-free apple tree cultivars and clonal rootstocks.

Apple cultivars and clonal rootstocks	Total number of tested plants (units)	Number of virus-free plants (units)	Virus-free plant yield (%)
Cultivars			
Antonovka	24	4	16.7
Bolotovskoe	8	2	25.0
Chekhovskoe	26	19	73.1
Imrus	10	3	30.0
Kandil Orlovskij	9	2	22.2
Konfetnoe	21	18	85.7
Lobo	17	11	64.7
Mantet	15	14	93.3
Marat Busurin	25	22	88.0
Mayak Zagorya	27	7	25.9
Melba	20	13	65.0
Papirovka	14	7	50.0
Podarok Grafskomu	17	15	88.2
Rozhdestvenskoe	9	6	66.7
Svezhest	13	11	84.6
Spartan	27	14	51.9
Triumph	22	8	36.4
Valyuta	7	7	100
Total	311	183	58.8
Clonal rootstock			
54-118	98	71	72.4
57-490	52	50	96.2
62-396	33	10	30.3
Total	183	131	71.6

harmful viruses. The fruit yields of these cultivars exceeded 80%. The virus-free plants of the cultivars ‘Lobo’, ‘Melba’, ‘Papirovka’, ‘Rozhdestvenskoe’, ‘Spartan’, and ‘Chekhovskoe’ were characterized by an average fruit yield (50%–73%). However, the virus-free plants of the apple cultivars ‘Antonovka’, ‘Bolotovskoe’, ‘Imrus’, ‘Kandil Orlovskii,’ ‘Mayak Zagorya,’ and ‘Triumph’ had low fruit yields of 16%–36%.

The virus-free plants of the apple clonal rootstocks 54-118 and 57-490 were characterized by high fruit yield, whereas that of rootstock 62-396 had a low yield. A total of 183 virus-free plants of 18 apple tree cultivars and 131 plants of clonal rootstocks were identified and isolated for further use in future breeding programs (Figure 3).

DISCUSSION

The trend of apple virus spread observed in this work was generally the same as that found in other countries. Under Iranian conditions, 71.4%, 47.5%, and 18.5% of apple tree specimens are infected with ACLSV, ASPV, and ASGV, respectively (Keshavarz and Hajnajari, 2021). Therefore, ACLSV was the most common virus of Iranian apples. In India, ASPV, ACLSV, and ApMV have approximately equal prevalence rates of 17.2%, 16.8%, and 15.2%, respectively, with ASGV showing a slightly lower prevalence rate of 12% (Kumar *et al.*, 2012). In Georgia, the prevalence rates of ASGV, ASPV, and ACLSV are insignificant at 11%, 8.5%, and 3.3%, respectively, and those

Figure 3. Apple tree cultivars free from harmful viruses (left) and clonal rootstocks 54–118 (right) under greenhouse conditions.

of ApMV have not been reported (Megrelishvili *et al*., 2017).

At the end of the 20th century, ACLSV was more often detected in apple trees than other viruses; however, infection by ASPV, which is more harmful than other viruses, has increased in recent years, and ASGV has also been found in some apple plantations (Upadyshev *et al*., 2017; Metlitskaya *et al*., 2018). The above trend has also been observed In other apple-growing countries. In Tunisia, ASPV (46% of samples) is predominant among latent viruses in apple trees, followed by ACLSV (39%) and ApMV (4%) (Mahfoudhi *et al*., 2013).

In the present study, monoinfection by apple viruses was prevalent. Virus infection tends to increase with plant age due to the enhancement of virus accumulation in plant tissues. Similar trends of virus infection and spread were also reported in past studies (Prikhodko and Magomedov, 2011). Given the absence of the natural vectors of latent viruses, the virus-free status of industrial orchards and mother plantations could be maintained for a long time and even for more than 10 years by cultivating virus-free apples (Petrova *et al*., 2016).

Earlier researchers also reported a higher prevalence of monoinfection (64%) in the central region of the Russian Federation compared with that of infection with virus complexes (Prikhodko and Magomedov, 2011; Motyleva *et al*., 2021). ASPV + ACLSV is the most common virus complex (23%) observed under Tunisian conditions (Mahfoudhi *et al*., 2013), and the same trend was also reported in the present study. At the same time, in apple plantations in China, infection by a complex of four latent viruses has the highest prevalence (27%), and infection by a complex of two viruses (ASPV + ACLSV) has the lowest (0.6%) (Ji *et al*., 2013). Moreover, in Korea, 84.8% of apple tree specimens are infected with a complex of two or more viruses, with monoinfection accounting for 12.4% of tree infection (Lee *et al*., 2020). Differences in the structure and prevalence of virus infection in different countries are likely related to different varietal compositions, age, and other plantation parameters.

In the present study, the highest prevalence of latent viruses was found in old cultivars of the Russian Federation and selections of old foreign apple breeding material. In past studies, the highest virus infestation was also observed in apple tree cultivars of foreign selection (76.5%), followed by that in old and new cultivars of Russian local selection, of which 48.4% and 44.6% of the samples, respectively, were infected. Some other studies reported that the apple cultivars 'Antonovka', 'Mantet', 'Lobo', and 'Welsi' are the most commonly infected genotypes (Prikhodko and Magomedov, 2011).

Rootstocks and scions should be virus-free to obtain virus-free grafted apple plants. In the early stage, apple seedlings are virus-free because virus transmission is not established through apple seeds. However, clonal rootstocks should be tested for the presence of major harmful viruses.

CONCLUSIONS

The characteristics of harmful latent viruses in apple tree agrocenoses were established on the basis of location, age, and varietal composition. The prevalence of harmful latent viruses in apple cultivars ranged from 49.6% to 53.8% and that in apple clonal rootstocks ranged from 8.3% to 100%. The most common virus in the Moscow and Yaroslavl regions was ACLSV (34.7% and 53.8%) and that in the Ryazan region was ApMV (33.3%). Depending on virus type, virus concentration was 20%–43% higher in the tissues of older apple trees than in young apple trees. Of the studied apple tree cultivars, 59% were infected by one virus, 25% were infected by a complex of two viruses (ASPV + ACLSV), 10% were infected by three viruses, and 6% were infected by four viruses. The highest prevalence of latent viruses was found in old Russian cultivars (53.5%) and old cultivars of foreign breeding material (57.2%). The occurrence of viruses was slightly lower (51.6%) in new Russian apple cultivars than in other cultivars. The lowest occurrence of various viruses (30%) was reported in columnar apple tree cultivars. The highest prevalence of viruses was found in an industrial apple tree orchard (63.4%), whereas low percentages of 18.3% and 20.8% were found in repository and collection plantations, respectively. ASPV had a relatively high prevalence (29.3%) in industrial apple orchards. Candidate apple genotypes that were free from viruses as indicated by ELISA results were identified and isolated from source plants, i.e., 183 plants from 18 apple tree cultivars and 131 plants from clonal rootstocks.

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