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Molecular diagnostics of viruses in apple rootstocks using RT-qPCR

The development of intensive horticulture in Kazakhstan requires the use of virus-free planting material, particularly apple rootstocks (*Malus domestica*), free of viral and viroid infections. Latent forms of pathogens, which do not exhibit visible symptoms but are easily transmitted through vegetative propagation, are considered the most hazardous. The objective of this study was to evaluate the phytosanitary status of apple rootstocks used in nurseries in southern Kazakhstan by employing reverse transcription quantitative polymerase chain reaction (RT-qPCR). A total of 24 samples from field and laboratory collections were analyzed. RNA extraction was performed using the "PhytoSorb®" kit, and diagnostics were conducted on the Bio-Rad CFX96 platform using multiplex LETGEN test kits. Four pathogens were identified: *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple green crinkle associated virus* (AGCaV), and *Apple hammerhead viroid* (AHVd). The viruses ASPV and AGCaV were found to be the most prevalent, occurring in both field and laboratory samples. These findings confirm the widespread circulation of these pathogens within the apple rootstock propagation system. The results emphasize the need for the implementation of regular molecular diagnostics, sanitation programs, and certification measures aimed at preventing the spread of latent infections and ensuring the sustainable development of horticulture in the region.

Keywords: *Malus domestica*, rootstock, viruses, viroids, RT-qPCR diagnostics.

Introduction

Apple (*Malus*) is one of the leading fruit crops and is cultivated in many countries worldwide, including under the agroclimatic conditions of Kazakhstan, where it occupies a significant share in fruit and berry production. Due to the high consumer demand and nutritional value of its fruits, apple plays a key role in the horticultural industry of the country. However, the successful development of the sector largely depends on the use of healthy planting material, particularly high-quality and virus-free rootstocks [1].

The production of rootstocks and the development of nursery practices in Kazakhstan are accompanied by a number of challenges, the most significant of which is the spread of viral infections among planting material [2]. Apple is predominantly propagated vegetatively—through grafting, cutting, or micropropagation. While this method of propagation ensures the preservation of varietal characteristics, it also facilitates the transmission of viral diseases, many of which may remain latent for extended periods without exhibiting visible symptoms [3, 4].

Latent viruses of fruit crops pose a serious threat to both commercial orchards and nurseries. Diseases caused by these pathogens reduce yield, impair the commercial and organoleptic qualities of fruits, and disrupt the compatibility between rootstock and scion. The greatest danger lies in the fact that, in the absence of pronounced symptoms, infected mother plants continue to be used for propagation, thereby contributing to the further spread of the infection [5].

The most significant viruses affecting apple include *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), *Apple mosaic virus* (ApMV), *Apple green crinkle associated virus* (AGCaV), as well as the recently identified viruses ARWV-1, ARWV-2, ApNMV, and the viroid AHVd. The presence of these pathogens is particularly critical during micropropagation [6, 7].

To address these challenges, modern molecular biology techniques, particularly reverse transcription quantitative polymerase chain reaction (RT-qPCR), enable accurate and sensitive detection of viruses even at the early stages of infection.

In the context of Kazakhstan, where the establishment of intensive orchards is a pressing task, the use of virus-free rootstocks is of critical importance. In this regard, the relevance of diagnosing and monitoring viral infections in nursery material is increasing. The application of molecular genetic techniques, such as reverse transcription quantitative polymerase chain reaction (RT-qPCR), enables highly accurate detection of viral pathogens even in asymptomatic plants [8]. This, in turn, provides an opportunity to establish a healthy foundation for the development of modern orchards and to enhance the efficiency of fruit and berry production in the country. The implementation of such approaches opens prospects for creating highly productive orchards resistant to viral degradation and contributes to the sustainable development of the industry at the national level [9].

The present study is aimed at identifying viruses and viroids of apple rootstocks circulating within the nursery production system of Kazakhstan, which will provide a basis for the implementation of phytosanitary control measures. The objective of the research is to assess the phytosanitary status of apple rootstocks used in Kazakhstani nurseries and to determine the spectrum of viral and viroid pathogens through the application of reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Experimental

Objects of the Study. The study was conducted on 24 samples of apple rootstocks (*Malus domestica Borkh.*) collected from various sources, including a nursery farm in the Almaty region and the collection of basic plants at the Kazakh Research Institute of Fruit and Vegetable Growing. Sample collection was carried out during the vegetation season (March–September 2024-2025).

Plant Material Collection. Each sample represented a single tree, from which 15–20 fully developed leaves were collected in four directions (east, west, north, south). Field monitoring included a visual assessment of symptoms, while in vitro samples showed no visible signs of infection. Leaves were stored at +4 °C until RNA extraction. Sampling was carried out in accordance with EPPO standards [10]. All samples were tested for the presence of viruses according to EPPO standards, as well as for newly widespread viruses and viroids, including ASPV, ACLSV, ApMV, ASGV, ARWV1, ApNMV, AGCaV, and AHVd.

RNA Extraction. RNA was extracted from leaves using the PhytoSorb® kit (Syntol LLC) following a modified protocol by Mekuria et al. [11]. Leaf tissue (200 mg) was ground in liquid nitrogen and extracted with a buffer containing 4.4 % PVP-40 and 1 % sodium metabisulfite, followed by vortex mixing. The quality and concentration of RNA were determined using a NanoDrop 1000 spectrophotometer. Purity was assessed by the absorbance ratios at 280/260 and 260/230 nm (1.8–2.3). Samples were stored at −20 °C.

RT-qPCR. Detection of viruses (ACLSV, ASPV, ApMV, ASGV, ARWV-1, ARWV-2, ApNMV, AGCaV) and the viroid AHVd was carried out using RT-qPCR with LETGEN® (TaqMan®) kits and the Bio-Rad CFX96 platform. Each reaction was performed in triplicate. The use of certified reagents and equipment ensured high sensitivity and eliminated false-positive results.

Throughout the study, the highly sensitive Bio-Rad CFX96 system and certified TaqMan-based kits were employed, providing high analytical specificity and preventing false-positive outcomes (Table 1).

Table 1

Sampling sites and names of plant material used for virus diagnostics

Location of samples	№	Name of samples
Nursery Farm, **Enbekshikazakh district, Almaty region**	1	M-9
	2	M-7
	3	MM-106
	4	B-9
Collection of basic plants under protected conditions, Department of Biotechnology of Horticultural Crops, Kazakh Research Institute of Fruit and Vegetable Growing, Almaty	5	Б 16-20
	6	Б 7-35
	7	62-396
	8	APM-18
	9	Zhetysu 5

Continuation of Table 1

Location of samples	№	Name of samples
Collection of basic plants under protected conditions, Talgar district, Almaty region, Almalyk village, Talgar Research Facility, Kazakh Research Institute of Fruit and Vegetable Growing	10	APM-18
Field collection, Talgar district, Almaty region, Almalyk village, Talgar Research Facility, Kazakh Research Institute of Fruit and Vegetable Growing	11	Б-7-35
	12	Б-16-20
	13	6-4-8
	14	Zhetysu 3
	15	Zhetysu 4
	16	Zhetysu 5
	17	Zhetysu 6
	18	Zhetysu 7
	19	Б-7-35
	20	Б-16-20
	21	Zhetysu 2
	22	Б-7-35 (M)
	23	Б-16-20 (M)
	24	62-396 (M)

Results and Discussion

The study included field and laboratory examinations of 24 samples of apple rootstocks (*Malus domestica* Borkh.) collected from various sources: a nursery farm in the Almaty region, the collection of basic plants under protected conditions, and the field collection of the Kazakh Research Institute of Fruit and Vegetable Growing. The results are presented using the following symbols: "+" indicates that a virus or viroid was detected in the sample, while "–" indicates its absence (Table 2).

Table 2

Name, origin, and results of virus detection in the studied apple rootstocks

Location of samples	Name of samples	Name of viruses			
		ACLSV	***ASPV***	***AGCaV***	***AHVd***
Nursery Farm, Enbekshikazakh district, Almaty region	M-9	+	+	–	–
	M-7	–	–	–	+
	MM-106	–	–	–	–
	B-9	–	–	–	–
Collection of basic plants under protected conditions, Department of Biotechnology of Horticultural Crops, Kazakh Research Institute of Fruit and Vegetable Growing, Almaty	B 16-20	+	–	+	–
	B 7-35	–	–	+	–
	62-396	–	–	–	–
	APM-18	–	–	–	–
	Zhetysu 5	–	–	–	–
Collection of basic plants under protected conditions, Talgar district, Almaty region, Almalyk village, Talgar Research Facility, Kazakh Research Institute of Fruit and Vegetable Growing	APM-18	–	–	–	–

Continuation of Table 2

Location of samples	Name of samples	Name of viruses			
		ACLSV	*ASPV*	*AGCaV*	*AHVd*
Field collection, Talgar district, Almaty region, Almalyk village, Talgar Research Facility, Kazakh Research Institute of Fruit and Vegetable Growing	B-7-35	–	–	+	–
	B-16-20	–	–	+	–
	6-4-8	–	+	+	–
	Zhetysu 3	–	–	+	–
	Zhetysu 4	–	–	–	–
	Zhetysu 5	–	–	+	–
	Zhetysu 6	–	–	+	–
	Zhetysu 7	–	–	–	–
	B-7-35	+	+	+	–
	B-16-20	+	+	+	–
	Zhetysu 2	–	–	+	–
	B-7-35 (M)	+	+	–	–
	B-16-20 (M)	–	+	–	–
	62-396 (M)	–	–	+	–

Diagnostics performed using the RT-qPCR method revealed positive results for three viruses—ACLSV, ASPV, and AGCaV—as well as for the viroid AHVd (Table 2). The remaining viruses (ApMV, ASGV, ApNMV, ARWV-1, ARWV-2) were not detected in any of the analyzed samples (Fig. 1).

ACLSV was detected in five samples: M-9 (nursery farm), B-16-20 (collection of basic plants under protected conditions, field collection of the Kazakh Research Institute of Fruit and Vegetable Growing), as well as its clone B-16-20 (M), and B-7-35 (field plot of the Kazakh Research Institute of Fruit and Vegetable Growing) along with its clone B-7-35 (M). The amplification curves exhibited signal enhancement at early cycles (Ct < 30), confirming a high viral load and the reliable presence of ACLSV.

ASPV was detected in six samples: M-9, 6-4-8, B-7-35, B-16-20 (field site), as well as in clonal forms B-7-35 (M) and B-16-20 (M). This virus exhibited a broader distribution compared to ACLSV. The recorded Ct values ranged from 20 to 35, indicating the presence of both latent and active forms of infection.

AGCaV was the most frequently detected pathogen, identified in 12 samples. It was found in the rootstocks B-16-20, B-7-35, 6-4-8, as well as in cultivars from the Zhetysu collection (Zhetysu 2, 3, 5, 6) and in the samples 62-396 (M) and Zhetysu-5. Its occurrence in materials from various sources indicates its active circulation. Such prevalence highlights the ongoing circulation of AGCaV within the nursery propagation system.

The viroid AHVd was detected in only one sample (M-7) originating from the nursery farm. Signal amplification was observed after cycle 39, exceeding the recommended Ct threshold of ≤35. Nevertheless, the presence of AHVd in one of the rootstocks warrants additional monitoring, given its potential phytopathogenicity.

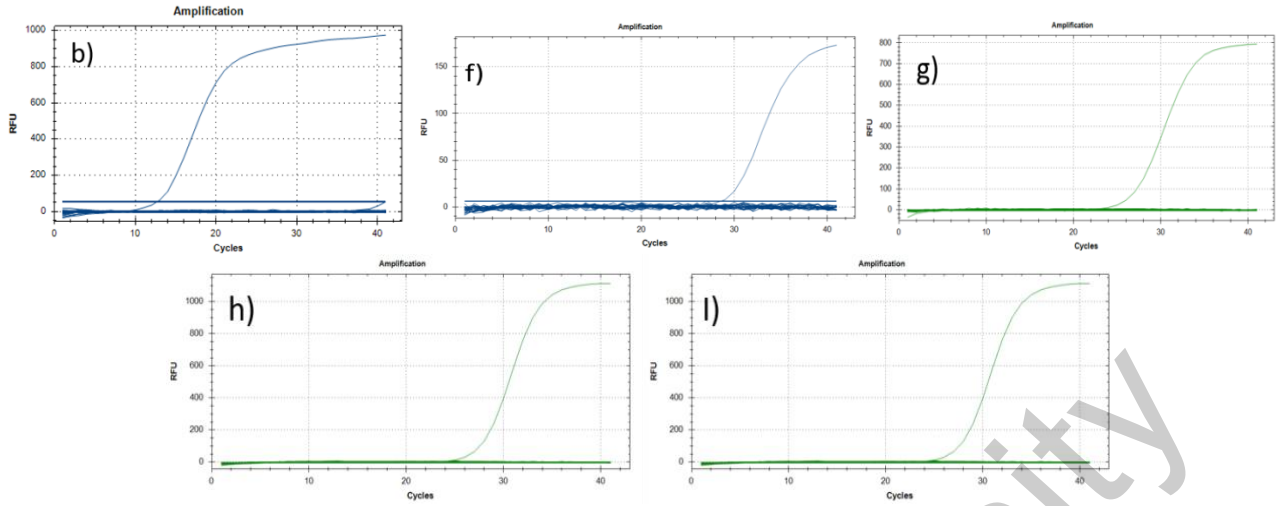

Figure 1. Negative amplification results obtained by RT-qPCR
b) ApMV; f) ASGV; g) ApNMV; h) ARWV-1; i) ARWV-2

This study was aimed at identifying viral pathogens in mother plant blocks and assessing the phytosanitary status of apple rootstocks. To date, field collections of pome crops in Kazakhstan have not undergone systematic virus testing, making the obtained results significant for developing strategies for sanitation and preventing the further spread of infectious agents in nursery production.

Monitoring, including visual inspection, molecular diagnostics [12], and selection of resistant genotypes [13], forms the basis of orchard protection. Since viral infections cannot be controlled with fungicides, the use of certified virus-free planting material remains the key preventive measure [14].

Particular attention should be given to AGCaV, which demonstrated the highest prevalence in both field and greenhouse samples. This virus is associated with fruit deformation, characterized by deep depressions, cracks, brown spots, and a reduction in tree productivity, in some cases leading to severe decline [15]. ASPV was also frequently detected, including asymptomatic carriage in clonal rootstocks. It induces pitting in the wood of certain cultivars (e.g., *Charden* and *Virginia Crab*), but often remains symptomless in commercial cultivars [16]. ACLSV negatively affects tree growth, yield, and fruit quality. In susceptible rootstocks (e.g., *Asami*), it triggers a hypersensitive reaction at the graft union, resulting in plant death—known as "topworking disease" [17]. Even in the absence of visible symptoms, infected plants exhibit impaired photosynthetic processes and reduced physiological potential. AHVd is considered pathogenic, as it has been associated with trunk cracking, necrosis, shoot weakening, and growth retardation across different continents [18]. Recently, AHVd was confirmed to be a true viroid capable of autonomous replication and inducing cellular disorders [19].

Our findings confirm that even latent infections caused by these pathogens can significantly affect the quality and viability of rootstocks. The detection of ACLSV, ASPV, AGCaV, and AHVd in symptomless samples underscores the necessity of routine RT-qPCR diagnostics and the implementation of sanitation programs for rootstock material [20, 21]. These results highlight the importance of an integrated approach to viral phytosanitary security. In particular, the application of *in vitro* elimination techniques, such as thermotherapy and shoot tip culture, can be recommended, as these methods have previously demonstrated their effectiveness in controlling PPV and ACLSV in stone fruit crops [22]. Therefore, the results of this study contribute to the development of a comprehensive strategy for virological control, integrating molecular, biotechnological, and breeding approaches to support sustainable nursery production in Kazakhstan.

Conclusions

The aim of this study was to assess the phytosanitary status of apple rootstocks used in nursery production in Kazakhstan and to identify the spectrum of viral and viroid pathogens. The results demonstrated the presence of both widespread and newly detected pathogens in collection mother blocks and nurseries. Furthermore, these findings emphasize the necessity of routine molecular diagnostics for the efficient detection and management of viruses, particularly within breeding and certification programs. Future research should focus on developing comprehensive strategies to control viruses and viroids within mother plant and nursery systems.

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Conflict of interest

The authors declare no conflict of interest.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Askarova M.A.** — Data curation, Writing — original draft, Writing — review & editing; **Yusupova Z.YA.** — Investigation, Methodology; **Madenova A.K.** — **Resources,** Validation; **Abdikerimova R.A.** — Formal analysis, Investigation, Methodology; **Kabylbekova B.Zh.** — Project administration, Supervision, Validation, Visualization.

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Алма телітушілеріндегі вирустық инфекцияларды RT-qPCR әдісімен анықтау

Қазақстанда интенсивті бақ шаруашылығын дамыту үшін, әсіресе вирус және вироид инфекцияларынан таза, сауықтырылған отырғызу материалын, соның ішінде алманың телітушілерін (*Malus domestica*) қолдану қажет. Ең қауіптілері — айқын белгілері жоқ, бірақ вегетативтік көбейту кезінде оңай таралатын латентті патогендер. Зерттеудің мақсаты — Қазақстанның оңтүстігінде тәлімбақтарда қолданылатын алма телітушілерінің фитосанитарлық жағдайын нақты уақыт режиміндегі кері транскрипциялы полимеразды тізбекті реакция (RT-qPCR) әдісімен бағалау. Талдау үшін далалық және зертханалық коллекциялардан 24 үлгі алынды. РНҚ бөліп алу «ФитоСорб®» жинағының көмегімен жүргізілді, ал диагностика Bio-Rad CFX96 платформасында LETGEN мультиплекстік тест-жүйелері арқылы орындалды. Нәтижесінде төрт патоген анықталды: *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple green crinkle associated virus* (AGCaV) және *Apple hammerhead viroid* (AHVd) вироиды. Ең жиі кездескен патогендер ASPV және AGCaV вирустары, олар әрі далалық, әрі зертханалық үлгілерде тіркелді. Алынған деректер алма телітушілерін көбейту жүйесінде осы патогендердің жоғары деңгейде таралғанын көрсетті. Зерттеу нәтижелері жасырын инфекциялардың таралуын болдырмау және аймақтағы бақ шаруашылығының тұрақты дамуын қамтамасыз ету үшін тұрақты молекулалық диагностика, сауықтыру және сертификаттау бағдарламаларын енгізудің маңыздылығын дәлелдейді.

Кілт сөздер: Malus domestica, телітуші, вирус, вироид, RT-qPCR анықтау

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Молекулярная диагностика вирусов на подвоях яблони методом RT-qPCR

Развитие интенсивного садоводства в Казахстане требует применения оздоровлённого посадочного материала, особенно подвоев яблони (*Malus domestica*), свободных от вирусных и вироидных инфекций. Наиболее опасными считаются латентные формы патогенов, не имеющие выраженных симптомов, но легко передающиеся при вегетативном размножении. Цель настоящего исследования заключалась в оценке фитосанитарного состояния подвоев яблони, используемых в питомниководстве юга

Казахстана, с применением метода полимеразной цепной реакции с обратной транскрипцией в реальном времени (RT-qPCR). Для анализа было отобрано 24 образца из полевых и лабораторных коллекций. Выделение РНК проводилось с использованием набора «ФитоСорб®», а диагностика — на платформе Bio-Rad CFX96 с использованием мультиплексных тест-систем LETGEN. В результате были выявлены четыре патогена: *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple green crinkle associated virus* (AGCaV) и вироид *Apple hammerhead viroid* (AHVd). Наиболее широкое распространение показали вирусы ASPV и AGCaV, встречающиеся как в полевых, так и в лабораторных образцах. Данные подтверждают высокую циркуляцию этих патогенов в системе размножения подвоев яблони. Результаты подчёркивают необходимость внедрения регулярной молекулярной диагностики, программ оздоровления и сертификации, направленных на предотвращение распространения скрытых инфекций и обеспечение устойчивого развития садоводства в регионе.

Ключевые слова: Malus domestica, подвой, вирусы, вироиды, RT-qPCR диагностика

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