Applying methods of replication and recovery of potato microplants (*Solanum tuberosum* L.) in seed production

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Abstract. Potatoes are strongly affected by pests and by pathogens of fungal, bacterial and viral nature. The most common and economically significant potato viruses are *Y* (PVY), *X* (PVX), *S* (PVS), *M* (PVM), and potato leaf twisting virus (PLRV). The development of a virus-free bio-resource collection *in vitro* is the basis for plant breeding development and transferring seed production to a healthier foundation. In this regard, the aim of this research was to apply methods of recovery and select optimal conditions for *in vitro* propagation of a collection of virus-free potato varieties. A collection of 22 healthy virus-free potato varieties was developed and kept *in vitro* in the FSBSI "FSC of Agricultural Biotechnology of the Far East named after A. K. Chaika". The recovery from viruses through joint use of tissue culture (apexes 2-4 mm) and chemotherapy (ribavirin) of the new potato variety Avgustin was carried out. The recovered test-tube plants, as well as the samples of six *in vitro* potato varieties that are in demand in plant breeding and seed production (Smak, Sante, Yantar, Zhukovsky ranny, Dachny, Adretta), were tested by enzyme immunoassay method (ELISA) for latent infection with viruses *Y* (PVY), *X* (PVX), *S* (PVS), *M* (PVM), and *L* (PLRV). The evaluation for Potato Spindle Tuber Viroid (PSTVd) was performed using PCR method. As a result of the study, no viral infections were detected in the recovered material and plants *in vitro*. The composition of nutrient medium for the microclonal propagation of potatoes that provides maximum value of the propagation rate is detected.

1 Introduction

Potato seed production on the “virus-free” basis is an important method of increasing yield and improving the quality of tubers [1]. A combination of field methods for selecting best clones and laboratory methods for seed material recovery using apical meristem culture, chemotherapy, and suppression of virus replication with chemicals is used to improve potatoes of various varieties that are valuable for a number of characteristics but are affected by viruses [2].

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About 40 viruses that infect potato were found in different countries and regions with different natural and climatic conditions. The *PLRV*, *PVY*, *PVX*, *PVS*, *PVM* and *PSTVd* viruses are among the most pathogenic ones. With the massive spread of these pathogens, crop losses can exceed 50%. Furthermore, the defeat of these viruses negatively affects the quality of tubers, as some of them may cause internal necrosis. Therefore, the most important practical tasks of original, elite and reproductive potato seed production require development and mastering of modern methods of virological control [3-7].

Highly stringent requirements are set for viruses Y (PVY) and L (PLRV), as for the most harmful, reducing potato yield by 50-80% [8]. For many regions, the tendency to decrease the quality of seed potatoes is particularly dangerous due to the spread and increasing harmfulness of various strains of Potato Virus Y, which causes severe symptoms of wrinkled and banding mosaics on plants [9, 10].

Potato Spindle Tuber Viroid (*PSTVd*) was found in different areas of the Far East Federal District (Amur Region, Primorsky, Kamchatsky, and Khabarovsk Krai) in 2006-2007. The isolates of *PSTVd* were found in varieties Adretta, Aksamit, Android, Zhukovsky ranny, Udacha, Veteran, Sante, PRI-12. Each of them had specific symptoms on tubers: spindle-like and pear shape, cracks, PINK EYES, etc. In two Far Eastern isolates, the nucleotide sequence of viroid RNA was detected [11].

While recovering potatoes, virological control is usually carried out using enzyme immunoassay (ELISA) and polymerase chain reaction (PCR). Both methods allow determining pathogens in samples. Besides, PCR identifies a species or a strain of an organism by a specific nucleotide sequence of its genome fragments. Potato Spindle Tuber Viroid (*PSTVd*) can only be identified using PCR [12].

Creating bio-resource collection *in vitro* is essential for plant breeding development and transferring seed production to a healthier basis. In this regard, the purpose of this work was to apply methods for recovery of varieties and find suitable conditions for *in vitro* reproduction of the collection of healthy virus-free potato varieties.

2 Materials and methods

The research was conducted at the FSBSI "FSC of Agricultural Biotechnology of the Far East named after A. K. Chaika" (Ussuriysk, Primorsky Krai).

The object of the research were varieties of the FSBSI "FSC of Agricultural Biotechnology of the Far East named after A. K. Chaika" (Dachny, Kazachok) and recovered virus-free potato microplants of foreign and domestic plant breeding, acquired from the Healthy Varieties Bank of the Lorch Potato Research Institute (Adretta, Bellaroz, Bryansky Delikates, Gala, Sante, Fioletovy, Fresco, Yubilyar). During the recovery of a new variety Avgustin was used [13]. For propagation and maintenance of virus-free test tube potato plants *in vitro*, the method of microclonal reproduction (microcutting) was applied [14]. During the Avgustin variety recovery process, the tissue culture method was combined with chemotherapy using an antiviral drug ribavirin. Apical (apexes) or axillary buds of etiolated and green tuber seedlings of 2–4 mm in size were introduced into the *in vitro* culture.

The nutrient medium for cultivation of potato plants was prepared according to the Murashige and Skoog Medium (MS0) recipe [15] with modified components of the composition. The variant I of the medium [16] consisted of (mg/l): casein hydrolysate – 50; mesoinositol – 80; thiamine – 0.5; pyridoxin – 0.5; ascorbic acid – 0.5; IAA (indole-3-acetic acid) – 1.0; kinetin – 0.2; indolebutyric acid – 0.2; sucrose – 20000. In the variant II of the medium [17] the content of casein hydrolysate (mg/l) was 40; mesoinositol – 0; thiamine – 0.2; pyridoxin – 0.1; ascorbic acid – 0.2; IIA – 1.0; kinetin – 0.04; indolebutyric acid – 0;

ferulic acid – 0.02; sucrose – 30000. The variant III of the medium had no phytohormones, other components were similar to the variant I.

The medium was sterilized at 0.9 atm for 20 minutes in a steam sterilizer GK-100-3. Instruments (tweezers and scalpels) were sterilized by dry heat in a dry heat cabinet FD 240 (Binder) for 2 hours at a temperature of 200 ° C. Microcloning of test tube potato plants was carried out under sterile conditions of a laminar box (BAVnp-01- "Laminar-S") - 1.5.

The materials and media were prepared according to the Butenko R.G. recommendations [18]. Tubes with potato microclones were cultured at 4.5-5.0 klk illumination, $22 \pm 30^\circ\text{C}$ temperature, 16 hour light day, and 60-70% air humidity.

The reproduction coefficient of micro-plants was determined on the 25th day of in vitro cultivation.

Test tube plants obtained through tissue culture were tested by ELISA for latent infection with viruses: Y (PVY), X (PVX), S (PVS), M (PVM), and L (PLRV), as well as for the presence of bacterial infection [GOST 33996-2016] in the testing laboratory for diagnostics of potato diseases of the "Federal Scientific Center of Agricultural Biotechnology of the Far East named after A. K. Chaika", authorized in the Rosselkhoztsentr Voluntary Certification System. Enzyme immunoassay was performed on tuber samples and plants in vitro [19]. The results were evaluated using an Infinite F50 photometer at a wavelength of 450 nm.

To assess latent infection with viruses (Y, X, S, M, L PSTVd), a PCR method was used. Total RNA was isolated from the green parts of plants by Andreeva G. N. et al. [20]. The RNA concentration was determined using a MaxLife fluorometer. PCR was performed in a 25-ml reaction mixture using kits of the "FITOSKRIN" series produced by "Syntol" to detect RNA of potato viruses by RT-PCR-RV in the QuantStudio 5 amplifier (Thermo Fisher Scientific). The results were interpreted based on the presence or the absence of an increase in the fluorescent signal for each sample, focusing on positive and negative reaction controls. To control the reaction and possible contamination, a blank sample containing a complete reaction mixture with distilled water added instead of RNA was used. As an external positive control, a K134 sample positive for the presence of the PVX virus was used by the ELISA method.

To prove the presence of significant differences between the average values, a single-factor analysis of variance followed by multiple comparisons of the Fisher averages (LSD method) was used.

3 Results and Discussion

In the FSBSI "FSC of Agricultural Biotechnology of the Far East named after A. K. Chaika", the production of original seed potatoes is carried out on a virus-free basis, using a collection of in vitro recovered virus-free potato varieties. 21 varieties are supported in vitro, including five of them bred by our institution: Yantar, Smak, Dachny, Kazachok, Avgustin. The varieties presented in the collection belong to different groups of ripeness (early, middle-early, and middle-late) and differ in biological and genetic parameters. During microclonal propagation in vitro, potato plants of different genotypes are characterized by different regenerative capacity. The morphogenetic potential of regenerating plants is directly dependent on the biology of a variety: early and middle-early potato varieties have a more active morphogenesis and increased regenerative ability compared to mid-season and late-ripening ones [21]. Therefore, when microclonal propagation of several varieties in vitro at once is carried out, there is a different varietal reaction of plants to the standard nutrient medium [22], which dictates the need to find the optimal composition of the medium components for each variety.

The main task in the production of virus-free potato plants is to increase the reproduction rate and accelerate plant growth after cuttings. During the microcutting process on three variants of nutrient media, a significant difference between the 10 varieties of potatoes taken in the experiment was revealed (Table 1). On average, the maximum values of propagation rates were obtained in micro-plants of the Sante (3.6) and Kazachok (3.5) varieties. The obtained propagation rate values show that the tested nutrient media differed at the 5% significance level. The variant I stood out among the studied media compositions. Its composition ensures high propagation rate for the majority of the studied varieties: Gala – 4.3, Fresco – 4.0, Sante – 4.3, while the average propagation rate in the experiment is 3.44. The average values of propagation rates on the medium variant II was 2.89, on the variant III – 2.51, while the average rate in the experiment – 2.9.

Table 1. Propagation rate of potato plants of different varieties depending on the nutrient medium compositions.

№	A factor	B factor - medium			$\bar{x} \pm S\bar{x}$
	Variety	Variant I	Variant II	Variant III	
1	Gala	4.3±0.3*	2.9±0.2	2.3±0.2	3.2±0.2
2	Bellaroza	2.6±0.2	2.8±0.1	3.0±0.2	2.5±0.1
3	Bryansky delikates	3.6±0.2	3.0±0.3	2.3±0.2	2.96±0.1
4	Fresco	4.0±0.3*	3.0±0.2	3.6±0.5	3.2±0.2
5	Sante	4.3±0.3*	3.5±0.3	3.0±0.2	3.6±0.2*
6	Adretta	3.4±0.2	2.7±0.3	3.4±0.3	3.2±0.2
7	Yubilyar	2.4±0.2	2.5±0.2	2.1±0.2	2.3±0.1
8	Dachny	3.0±0.2	2.5±0.2	2.3±0.3	2.6±0.1
9	Fioletovy	3.3±0.4	2.0±0	1.9±0.2	2.4±0.2
10	Kazachok	3.4±0.3	4.0±0.3*	3.1±0.2	3.5±0.2*
	$\bar{x} \pm S\bar{x}$	3.44**	2.89	2.51	2.9
LSD ₀₅	0.38	0.21			

* – the differences are significant at the 5% variety significance level (A factor).

** – the differences are significant at the 5% nutrient medium significance level (B factor).

The Kazachok variety is an exception – the medium variant II is preferable for its microclonal propagation.

The medium nutrient composition of the variants I and II ensured high growth and propagation of potato plants in vitro. Both variants contained phytohormones - IAA (indole-3-acetic acid) and kinetin. Variant III (without phytohormones) appeared to be not productive for the majority of the studied genotypes except Bellaroza and Adretta potato varieties, in which, upon cultivation on a medium of given composition, propagation rates are similar in value to indicators on other variants of media.

To clear the health of potato plants off viruses, cultures of apical meristems, chemo -, thermo -, cryo - and electrotherapy, as well as various combinations of these methods (complex or combined therapy) are used. Chemotherapy is based on the treatment of infected plants with substances with antiviral activity. Ribavirin, a synthetic analog of guanosine (1-β-D-ribofuranosyl-1H-1,2,4-triazol-3-carboxamide), with a prominent antiviral effect, is widely used to recover potatoes from viruses [23]. This drug was used in recovering a new potato variety Avgustin.

At the initial stage, it was important to determine the optimal conditions for sterilization of apical and axillary apices isolated from potato seedlings and to select an effective sterilizing agent [24, 25]. The use of chlorine-containing agent "Belizna" with sodium hypochlorite content of 5-14% provided a low yield of aseptic explants (26-30%). The

experiment showed that the most acceptable sterilizing agent for potatoes is 0.1% solution of diacid with the addition of TWEEN-80 (1-2 drops per 1 liter). Potato explants were immersed in it for 12 minutes, and then repeatedly washed with sterile distilled water. The sterilization efficiency using the diacid solution was 96.6% (Table 2).

Table 2. Efficiency of potato Explant sterilization during in vitro isolation.

Variant number	Planted in medium, pieces.	Infected, pieces.	Aseptic explants output, %
1	48	2	95.8
2	48	3	93.8
3	40	1	97.5
4	40	-	100
Total:	176	6	96.6

To recover the Augustin potato variety, buds (apexes) of 2-4 mm in size from tuberose seedlings grown in the dark (etiolated) and in natural light (green) were used.

After sterilization in 0.1% diocide solution, they were cultivated, depending on the variant of the experiment, either on a hormone-free MS medium, or on a MS medium with addition of ribavirin (20 mg/l). After 4 weeks of cultivating of isolates on nutrient media in the climacamera, the development of various morphological structures was observed: callus tissue, callus with roots, roots and green shoots (Table 3).

Table 3. Formation of morphological cultures during the cultivation of potato explants in vitro.

Variant number	Planted in medium pieces.	Callus		Callus with roots		Roots		Microshoots		Not developed	
		pieces	%	pieces	%	pieces	%	pieces	%	pieces	%
1	46	2	4.3	1	2.2	1	2.2	12	26.1	30	65.2
2	45	2	4.4	5	11.1	10	22.2	7	15.6	21	46.7
3	39	2	5.1	0	0	4	10.2	7	17.9	26	66.7
4	40	0	0	1	2.5	14	35.0	6	15.0	19	47.5
Total:	176	6	3.4	7	3.9	29	16.5	32	18.2	96	54.5

The largest number of shoots was formed on buds isolated from etiolated seedlings when cultivated on MS medium with the antiviral drug ribavirin - 26.1% of the total number of isolated in vitro. Regeneration of shoots from green apexes cultivated on a medium with ribavirin was observed only in 15.6 % of isolates. In variants where ribavirin was not added to a nutrient medium, the percentage of shoots formed from green apexes was 15.0, and from etiolated ones - 17.9. Microshoots obtained from the apexes of green sprouts of potato tubers were characterized by bigger length compared to the microshoots formed from the buds of etiolated sprouts.

The obtained shoots were propagated on the medium variant I. Samples for ELISA testing for the presence of viral and bacterial infections were taken from each microplant during microcutting, and the test showed no infection in the plants.

ELISA is a relatively simple and cheap method of identifying a wide range of infections, including viral infections. Viroids are an exception. Due to the lack of a protein coat, they cannot provide antigens for binding to antibodies and therefore cannot be detected by this method. Besides, among the disadvantages of ELISA method are a slightly

lower sensitivity in comparison to PCR and obtaining false positive and false negative results [26].

As a result of the study, the reaction flow was detected only in the positive control sample, while the reaction flow through the internal control channel of the reaction was detected in all samples. The obtained results indicate a normal course of the reaction in the studied samples and the absence of latent infection of the Potato Spindle Tuber Viroid. Tests for other viruses showed positive reactions for the viruses X, Y, S and M. Potato Virus Y appeared to be the most frequent and was found in almost all studied lines. In isolated cases, viruses S, X, M and L were detected. In all cases, they were accompanied by the PVY. It should be noted, that ELISA detected both X and Y viruses in K134 sample (Table 4).

Table 4. Detection of viral infections in recovered lines and varieties using ELISA and PCR methods.

Lines (Number of plants)	Viruses											
	PVX		PVY		PVS		PVA		PVM		PLRV	
	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR
T1k (3)	0	0	0	1	0	1	0	0	0	1	0	0
T2k (3)	0	0	0	1	0	0	0	0	0	0	0	0
T3r (3)	0	0	0	2	0	1	0	0	0	1	0	0
S8r (3)	0	1	0	3	0	0	0	0	0	0	0	0
S9k (3)	0	0	0	3	0	0	0	0	0	0	0	0
K134 (1)	1	1	0	1	0	0	0	0	0	0	0	0

The results indicate a lower sensitivity of ELISA compared to PCR. Other researchers had similar data [27, 28] when developing a PCR method for diagnosing viral infections and comparing methods. In the case of ELISA, false-positive results are possible due to cross-reactions, and false-negative results are possible with a low infectious load. At the same time, the applied PCR method with reverse transcriptase reaction and real-time detection using fluorescent probes can be considered a benchmark for specificity in detecting RNA of a specific infectious agent in a mixture of heterologous nucleic acids [29, 30]. The recovery efficiency was significantly different in studied lines. Thus, in the T1k line, one sample out of three had a complex infection of Y, S and M viruses, in the T2K line one sample was infected with PVY, and in the T3r line only one sample was free of infection. In the lines S8r and S9k no clean samples were found. All samples were infected with PVY, and the S8r sample was also infected with PVX.

It should be noted that the efficiency of recovery of planting material of potatoes is highly dependent on a number of factors. The most significant of them are the initial infectious load, its complexity and type of pathogen; explants used for introduction into in vitro culture, as well as the types of therapy used for recovery and their complexity. Potato Virus Y is the most common and malicious out of all viral pathogens. Also, it is well known that plants infected with PVY are the most difficult to recover. Using ribavirin, RNase and thermotherapy separately do not show positive results [31-34]. The situation is aggravated to a greater extent in the presence of infection with several viruses of the source material. Research conducted earlier has shown that the most effective integrated approach includes meristem culture, thermo -, chemo - and electro-therapy. However, using all approaches at the same time does not provide recovery of each sample [23]. Based on the

data obtained using the PCR, it became possible to reject the infected material and choose a healthy one for further replication. The yield of healthy plants as a result of the experiment was 33.3%.

4 Conclusion

Results on microclonal propagation of varieties in vitro on nutrient media with different composition were obtained. Variety-specific variants of nutrient media that provide the highest plant propagation ratio were selected. A new variety Avgustin was recovered using tissue culture (apexes 2–4 mm) with chemotherapy (ribavirin). Recovered tube plants of the Avgustin variety were tested for hidden infection with viruses: *Y (PVY)*, *X (PVX)*, *S (PVS)*, *M (PVM)*, *L (PLRV)* using PCR and ELISA methods. Using PCR method allowed identifying varieties with pathogens. The possibility of new varieties recovery from viruses on the basis of the FSBSI "FSC of Agricultural Biotechnology of the Far East named after A. K. Chaika" in order to provide healthy seeds for the region of the Far East was shown.

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