Diagnosing potato viruses using ELISA and RT-PCR method

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Abstract. The article contains the results of enzyme-linked immunosorbent assay and reverse transcription polymerase chain reaction for potato viral infections. Estimation of seed potato and vegetating potato plants infection degree with PVS, PVX and PVM viruses is provided as well as the extent of their dominance is revealed. The mixed infection of the examined samples of Dunyasha sort plant samples includes two viral components PVS+PVM which belong to the same Carlavirus group. PLRV and PVY viruses were not found in any of the samples which passed assay. Late Udovickiy sort showed the greatest viral reinfection resistance under the conditions of Kostanay province.
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Abbreviations: PVS - potato virus S, PVM - potato virus M, PVY - potato virus Y, PVX – potato virus X, PLRV – potato leafroll virus.

Introduction

Potato is one of the most important crops. In terms of food security as well as with population growing and starvation widening the UN General Assembly basing on scientists' opinion, aims to attract the world community's attention to the role of potato, believing that it is the potato that will solve the problem of mankind food security in the future and development of this industry has a very important strategic value. Over 3 billion inhabitants of the planet consume the potato as food crop and 150 countries grow it [1].

Annual world production of the potato exceeds 300 million tons. Kazakhstan cultivates an area of 160-170 hectares for the potato growing. The yield does not exceed 15 t/ha [2].

Currently the most successful crop protection measure is improvement of planting material by eliminating viruses from the crop tissue followed by cultivation and protection from re-infection [3]. In potato primary seed growing practice biotechnology methods are widely used to obtain improved source material including microclonal propagation allowing to get the seed material free from viral infections.

The yield of the seed potato freed from viruses exceeds the usual potato yield by 40-80% and sometimes by 2 times [4]. If seed potato originally contains at least some virus-infected tubers, the number of infected plants will steadily increase in each new vegetative reproduction even in the most favorable conditions and complete absence of infection carriers. Therefore the potato freed from viruses and mycoplasma both in explicit and latent form can be grown only if healthy (virus-free) source material is used for planting.

There are several methods to diagnose viral infections and identify their agents known: visual, serological, indicating, electronic and microscopic methods based on detection of changes in the chemical composition of virus-infected plants.

Currently, production of potato minitubers is most successfully developing in the Netherlands, Russia, Belarus, Ukraine and China. Seed growing with the use of minitubers became widespread in the EU [5-6].

Infection-free seed growing is currently the object for close cooperation of scientists from the Kazakh National Agrarian University, Kostanay Agricultural Research Institute, Kazakh Research Institute of Potato and Vegetable Growing.

Materials and methods

The objects of the study were the first filed generation sorts improved by apical meristem and regionalized in Kostanay province: middle-late sort Dunyasha and late sort Udovickiy.

The following surveys and observations were performed during vegetation:

The plant latent virus infection was tested using ELISA and RT-PCR to detect PVX, PVS, PVM, PLRV and PVY viruses.

In 2013 Kostanay Agricultural Research Institute conducted in vivo studies in the Tobol River floodplain.

The studies area climate is extremely continental with cold little snow winter and hot dry summer. Kostanay province climate is featured with prolonged cold spring, early fall of temperature in autumn and late summer rainfall. The end of May and most of June are the most dry when potato shoots. The plants have to spend fleeting soil and mother tuber

moisture reserves before rainfall starts. In terms of annual precipitation the year 2013 was more humid than the previous year 2012.

Potato growing techniques did not differ from the standard of the industry and corresponded to the zonal one. The potato was harvested manually using a continuous method. The total area of the plot was 120 m^2, discount area was 100 m^2.

Plant nutrition area was 70x30 cm. Variants were placed systematically with four-time repeatability.

Greenhouse minitubers of Dunyasha and Udovickiy sorts were planted manually in the hiller-precut furrows at all the selection garden plots. The furrows stretched from north to south as prevailing windroses are south-western. Planting time was the 2nd decade of May, the predecessor was wheat. The experiments were conducted with hard natural background without irrigation. The gardens were spatial isolated at a distance of 3 km from settlements and other potato fields.

The experimental area perimeter was surrounded with wheat plots as well as petunia and scented tobacco plots (biological traps) which isolated the potato from winged aphid species (virus carriers). The plot was constantly kept clean. Potato beetles and aphids were exterminated with 3 times spraying in the growing season using "Konfidor" and "Karate" preparations during the years of the studies.

50-100 leaves from each variant - depending on the plot size - were collected by a leaf from each plant (clone) as samples for enzyme-linked immunosorbent assay (ELISA).

Virus detection assays were carried out in the Institute of Molecular Biology and Biochemistry named after M.A. Aytkhozhin (Almaty).

The reverse transcription reaction and PCR were performed using Fermentas sets.

Reverse transcription reaction of the isolated RNA.

Transcription was carried out in two stages. The first stage involved preparing # 1 mixture of 11 µl containing 2.5 µg of RNA, 0.5 µl each 20 µM of primer mixture (ASense). The mixture was being heated for 5 min. at 75°C, then it was placed in ice.

The second stage involved preparing # 2 mixture of 19 µl containing 4 µl 5x buffer for the reverse transcription, 1.2 µl 25 mM of mixture of each of four dNTP, 1,2 µl of RNAsin, 0,1 µl of reverse transcriptase M-MuLV (200pc/µl) 9.2 µl of bidistilled water.

The mixture # 1 and # 2 were combined, transcription reaction was performed in the following temperature conditions:

Stage 1 - 42 °C - 1 h, 30 min. - 1 cycle,

Stage 2 - 70°C - 10 min. - 1 cycle [7-8].

The DNA amplification using the multiplex polymerase chain reaction (mPCR)

For the mPCR assay the following reaction mixture of 20 µl was prepared: 2µl 10x buffer for Taq-polymerase, 0,4 µl 25mM of dNTP mixture, 3,2 µl 25mM $MgCl_2$, 0,5 µl of BSA, 0,3 µl of mixture (Sense+ASense) of primers for each virus with concentration of 50 ng/µl, 0,3 µl of Taq-polymerase, 2-5 µl of the reverse transcription product. The reaction was carried out in the following temperature condition:

Stage 1 - 94°C 5 min. - 1 cycle,

Stage 2 - 94°C 30 s, 60°C 30 s, 72°C 1 min. - 30 cycles.

Stage 3 – 72°C 5 min. - 1 cycle [9-10].

Enzyme-linked immunosorbent assay.

The enzyme-linked immunosorbent assay was performed using BIOREBA (Switzerland) sets for diagnosis of plant viruses. All stages and calculation of the substances concentration were maintained according to the recommendations given in the sets instructions. The same company antibodies to the viral coat protein were used [11].

Results and discussion

In 2013 to test the presence of five virus variants in the potato early flowering phase 33 samples of the first field generation potato leaves of Dunyasha and Udovickiy sort were selected in late July.

Positive and negative controls attached to the brand sets were used for the virus diagnostics in all cases. The results are shown in Table 1.

Table 1. Results of ELISA for the potato samples in the early flowering phase at the clone test greenhouse (in vivo) of the Kostanay Agricultural Research Institute

	PVX	PVS	PVM	PVY	PLRV
K-	0,538	0,474	0,662	0,516	0,461
K+	3,351	1,501	1,643	0,948	3,496
Dunyasha sort					
0	0,433	0,463	2,547	0,462	0,491
1	0,427	3,316	1,831	0,441	0,479
2	0,427	3,383	2,174	0,452	0,460
3	0,428	3,271	1,803	0,433	0,469
4	0,434	3,210	1,960	0,440	0,468
5	0,445	3,188	2,131	0,454	0,456
6	0,427	3,149	2,212	0,445	0,472
7	0,433	3,108	1,810	0,429	0,461
8	0,422	2,637	1,592	0,450	0,472
9	0,412	0,457	1,682	0,421	0,442
10	0,408	2,157	1,562	0,428	0,443
11	0,411	2,309	1,413	0,428	0,430
12	0,409	2,113	1,421	0,432	0,428
13	0,409	2,295	1,194	0,430	0,445
14	0,405	2,296	1,278	0,416	0,448
15	0,415	2,377	1,499	0,424	0,441
16	0,410	2,514	1,166	0,423	0,451
17	0,506	2,420	1,611	0,428	0,457
18	0,690	2,449	1,578	0,435	0,453
19	0,545	2,559	1,663	0,438	0,454
20	0,546	2,516	1,538	0,509	0,432
21	0,578	2,550	1,341	0,572	0,454
22	0,562	2,565	1,733	0,589	0,464
23	0,566	2,503	1,776	0,541	0,459
24	0,424	2,579	1,787	0,450	0,472
25	0,418	2,402	1,828	0,479	0,449
Udovickiy sort					
1	0,420	0,427	0,459	0,437	0,431
2	0,415	0,426	0,453	0,436	0,448
3	0,410	0,418	0,475	0,435	0,451
4	0,414	0,421	0,462	0,430	0,436
5	0,406	0,418	2,046	0,427	0,439
6	0,414	0,424	2,152	0,429	0,436
7	0,406	0,427	2,276	0,427	0,444

Note. K – - negative control, K + - positive control. The figures show the meter readings measure at 405 nm.

Table 2 shows that in the early flowering phase, improved regionalized Dunyasha and Udovickiy sorts are virus-infected from 43% to 100%, infection composition varies.

Table 2. Prevalence of potato viral infections in the early flowering phase found using ELISA at the clone test greenhouse of the Kostanay Agricultural Research Institute

Sort	Total infected plants, %	Including virus-infected ones				
		PVX	PVS	PVM	PVS + PVM	PVS + PVM + PVX
Dunyasha	100	0	0	7.7	88.5	3.8
Udovickiy	43	0	0	43	0	0

PLRV and PVY viruses were not found in any of the samples which passed assay.

All samples of Dunyasha sort gave a positive response to the presence of PVM and PVS - 88,5%, which belong to the same *Carlavirus* group. Most of the plants were infected with these two viruses. Moreover, the meter indicates a higher content of PVS as compared to the amount of PVM in these plants. Two plants were infected with PVM only - 7,7%. One sample gave a positive response to the presence of PVX besides PVM and PVS - 3.8%. Positive signal for PVX virus infection is much weaker as compared to the signals for the presence of PVM and PVS. PVM was found at Udovickiy sort in the early flowering phase - 43 %.

After obtaining the assay results the infected plants of Dunyasha and Udovickiy sorts were rejected.

During potato mass flowering phase in early August 51 samples of the first field generation potato leaves of Dunyasha and Udovickiy sort were selected. The results are shown in Table 3.

PVX, PLRV and PVY were not found in any of the samples passed assay in the mass flowering phase (Table 4). Most of Dunyasha sort plants gave a positive response to the presence of PVM – 2,4% and PVS - 95,2%, which belong to the same *Carlavirus* group. Most of the plants were infected with these two viruses PVM+PVS-95%. Moreover, the presented results indicate a higher content of PVM virus as compared to the PVM titre in these plants.

The selected Udovickiy sort samples were not infected with viruses.

After obtaining the assay results the infected plants of Dunyasha sort were rejected.

In 2014 the tubers were checked for infection using ELISA and RT-PCR before planting into the soil. The presence of five virus variants was checked in 5 test-tube plants, including two variants of Dunyasha sort numbered 1, 2, and three variants of test-tube plants of Udovickiy sort numbered 3, 4, 5.

Total RNA preparations were isolated from the potato leaf samples of variant 1 and 2, 3, 4, 5 using trisol. The isolated preparations quality was analyzed in 1.2% agarose gel (the results are shown in Figure 1).

Table 3 Results of ELISA for the potato samples in the mass flowering phase at the clone test greenhouse (in vivo) of the Kostanay Agricultural Research Institute

	PVX	PVS	PVM	PVY	PLRV
1	2	3	4	5	6
K-	0,394 0,462	0,394 0,521	0,433 0,531	0,407 0,411	0,412 0,422
K+	2,364 2,261	1,049 0,946	0,666 0,669	0,677 0,646	2,743 2,699
Dunyasha sort					
1	2	3	4	5	6
1	0,408	2,226	2,704	0,423	0,430
2	0,398	1,708	2,961	0,413	0,420
3	0,394	1,924	2,560	0,425	0,410
4	0,399	2,009	2,729	0,415	0,424
5	0,394	2,153	2,771	0,420	0,424
6	0,405	0,411	2,620	0,411	0,408
7	0,468	1,674	2,757	0,414	0,418
8	0,404	1,658	2,808	0,417	0,411
9	0,405	2,318	2,879	0,401	0,405
10	0,392	1,492	2,299	0,432	0,413
11	0,395	1,352	2,063	0,415	0,417
12	0,392	1,724	2,396	0,414	0,413
13	0,389	1,674	2,334	0,420	0,414
14	0,395	1,813	2,461	0,409	0,423
15	0,388	1,809	2,414	0,418	0,412
16	0,400	1,423	2,412	0,395	0,421
17	0,405	1,604	3,386	0,431	0,439
18	0,403	0,407	0,567	0,418	0,426
19	0,401	1,578	3,086	0,424	0,425
20	0,406	2,177	3,201	0,410	0,423
21	0,411	2,276	3,361	0,421	0,436
22	0,404	1,981	3,518	0,420	0,422
23	0,401	2,342	3,321	0,406	0,431
24	0,449	2,111	3,145	0,419	0,428
25	0,429	2,140	2,919	0,412	0,421
26	0,401	2,566	3,284	0,416	0,422
27	0,412	2,224	3,035	0,428	0,461
28	0,401	2,268	2,733	0,421	0,415
29	0,393	2,246	2,558	0,417	0,416
30	0,393	1,592	2,575	0,416	0,403
31	0,395	1,720	2,664	0,443	0,411
32	0,401	2,214	2,937	0,429	0,418
33	0,397	2,386	2,631	0,422	0,409
34	0,400	1,927	3,071	0,426	0,407
35	0,400	2,132	2,939	0,412	0,409
36	0,397	2,297	2,839	0,420	0,423
37	0,396	1,710	2,997	0,422	0,424
38	0,413	2,056	2,690	0,405	0,431
39	0,399	2,401	3,502	0,430	0,421
40	0,396	2,180	3,502	0,431	0,417
41	0,398	1,995	3,502	0,417	0,428
42	0,403	2,524	3,502	0,415	0,412
Udovickiy sort					
1	0,403	0,412	0,516	0,428	0,424
2	0,392	0,413	0,500	0,426	0,417
3	0,399	0,397	0,526	0,430	0,434
4	0,403	0,403	0,555	0,420	0,414
5	0,402	0,410	0,525	0,416	0,422
6	0,400	0,403	0,492	0,429	0,415
7	0,402	0,408	0,488	0,426	0,422
8	0,401	0,408	0,515	0,418	0,439
9	0,400	0,412	0,500	0,397	0,404

Note. K – - negative control, K + - positive control. The figures show the meter readings measure at 405 nm.

Table 4. Prevalence of potato viral infections in the mass flowering phase found using ELISA at the clone test greenhouse of the Kostanay Agricultural Research Institute in 2013

Sort	Total infected plants, %	Including virus-infected ones				
		PVX	PVS	PVM	PVS + PVM	PVS + PVM + PVX
Dunyasha	97.6	0	0	2.4	95.2	0
Udovickiy	0	0	0	0	0	0

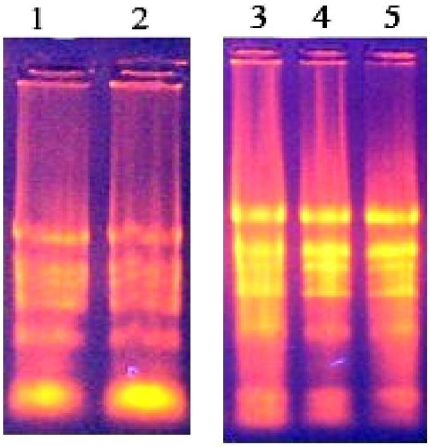

Fig. 1. The results of the isolated RNA preparations electrophoresis

See the reverse transcription (RT) reaction below. The RT reaction included 5 µg of RNA and using antisense primers (ASense) mixtures selected for each virus variant at a concentration of 20 µM (Table 5) [12].

Table 5. Sequence of the primers used to analyze RT+mPCR

Primer name	Position in the virus genomic RNA	Sequence of the primer	Primer length
PVX Sense	5664-5683	5' – tagcacaaca caggccacag – 3'	20
PVX ASense	6225-6205	5' – ggcagcattc atttcagctt c – 3'	21
PVS Sense	7543-7561	5' – tggcgaacac cgagcaaatg – 3'	20
PVS Asense	7707-7728	5'- atgatcgagt ccaagggcac tg - 3'	22
PLRV Sense	3653-3672	5' – cgcgctaaca gagttcagcc – 3'	20
PLRV ASense	3969-3988	5' – gcaatgggg tccaactcat – 3'	20
PVY Sense	8723-8742	5' – acgtccaaaa tgagaatgcc – 3'	20
PVY ASense	9183-9202	5' – tggtgttcgt gatgtgacct – 3'	20
PVM Sense	7242-7264	5' – gaaagctga aactgccaaa gatg - 3'	24
PVM ASense	7737-7762	5' – catctgcagt tatagcacct cttgg - 3'	25

Then using the DNA obtained multiplex PCR reaction (mPCR) involving five pairs of primers (concentration of 10 µM) for five virus variants was carried out.

Mainly for convenience of subsequent analyzes we prepared positive controls for each virus variant. Using the same method the viral RNA were isolated from the positive controls attached to BIOREBA ELISA sets. The pure RNA was then used in the RT reaction, and in mPCR similarly to the above stated with the only exception that the reaction included primer pairs specific for a particular virus variant. As a result, the DNA fragments of various sizes were produced which distinguished in mobility in 2.5% agarose gel (line K in Figure 2).

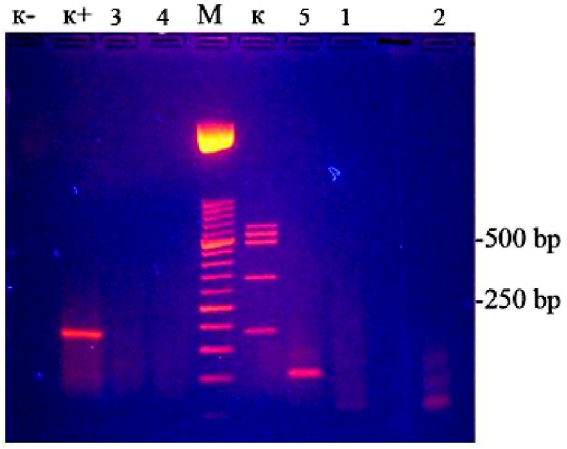

Fig. 2. The results of electrophoresis of the DNA fragments obtained during the assay of samples using the reverse transcription reaction and the multiplex PCR (K - positive control, M - marker 0,05 kb)

For PVX variant the fragment of 562 nucleotide pairs (np) was produced (topmost fragment in Figure 2, K line), for PVM variant - 520 np, PVY variant - 480 np, PLRV variant - 336 np, PVS variant - 187 np (the lowest fragment in Figure 2 in the same line). After measuring the concentration of the obtained PCR-products the fragments were mixed and further used as a positive control to determine the size of the viral fragments after the multiplex PCR. The DNA fragments obtained as a result of PCR with RNA control variant corresponded to the size data (K line in Figure 2). Figure 2 shows that none of the potato tested variants contains the fragment corresponding to a viral one. This evidences that these variants are free from the potato viruses analyzed [13].

Then using BIOREBA sets for diagnostic of plant viruses we carried out ELISA. The results are shown in Table 6. Positive and negative controls attached to the brand sets were used for the virus diagnostics in all cases.

Table 6. The results of the ELISA for the potato samples

	PVX	PVS	PVM	PVY	PLRV
K-	0.405	0.404	0.439	0.406	0.412
K+	2.066	0.985	1.058	0.948	3.549
Dunyasha sort					
1	0.417	1.823	3.449	0.422	0.438
2	0.422	2.348	3.545	0.434	0.433
Udovickiy sort					
3	0.401	0.418	0.549	0.428	0.417

Note. K-- negative control, K + - positive control. The figures show the meter readings measured at 405 nm.

According to the data above, after ELISA only one sample of Udovickiy sort was free from viral infection. PLRV and PVY viruses were not found in any of the samples which passed assay. Samples 1 and

2 show the presence of two viruses PVS and PVM, PVM titer is very high as compared to PVS.

Thus, ELISA and multiplex PCR data are contradictory for two samples 1 and 2. This can be explained as follows. The ELISA has a lower degree of reliability, since the virus presence was determined by the presence or absence of the coat protein (enzyme reaction with alkaline phosphatase which changes the solution color during the experiment, progresses only when the coat protein of each virus is present). As a rule the coding sequence of the one group virus coat proteins are more conservative than other proteins. A positive repose after reaction can be obtained even if the plant samples contain other related viruses of the same group: *Carlavirus* group which includes PVS and PVM or *Potyvirus* group which includes PVY [14-15]. Although the RT and associated PCR method is a multistage procedure, it differs by a greater sensitivity than ELISA since the primers involved in the reaction process are selected strictly in accordance with the nucleotide sequences of the viral genome. The RT and PCR primers were selected to conservative areas of the coat protein coding sequences for the diagnosis of PVM, PVS and PVY. Both these groups are very numerous and it is possible that the additional fragments we've found (see Figure 2) show the presence of other viruses.

Conclusions

PVX, PLRV and PVY were not found in any of the samples passed assay in the mass flowering phase.

The mixed infection in the examined samples of Dunyasha sort is represented by two components the most common of which were: in the early flowering phase PVS + PVM - 88.5% and in the mass flowering phase PVS + PVM - 95%.

In the early flowering phase Udovickiy sort samples were found to contain PVM - 43%, and in the mass flowering phase the samples were not infected.

Late Udovickiy sort showed the greatest viral reinfection resistance under the conditions of Kostanay province.

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