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DEVELOPMENT OF NEW qPCR-BASED IDENTIFICATION SYSTEMS FOR NON-QUARANTINE POTATO (*Solanum tuberosum* L.) PATHOGENS DISTRIBUTED IN THE TERRITORY OF RUSSIA

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Abstract

Russia is among the largest potato producers in the world. According to statistics for 2018, the sown area of potatoes amounted to 310.7 thousand ha, which is 3.5 % more than in 2017, and the gross harvest in the industrial sector is 7157 thousand tons. At the same time, potatoes are susceptible to infection by various plant pathogens of different taxonomic groups. In modern potato growing, the progressive spread of viral and bacterial diseases, which, in addition to reducing the yield, causes a catastrophic deterioration of tubers' quality, leads to a serious problem for commercial production. Not all dangerous pathogens belong to quarantine organisms in Russia. However, the need for their accurate and highly specific identification is not less than for quarantine organisms. Currently, common diagnostic methods in potato growing are indicator plants, serological and cytological tests. They are relatively reliable, but not always sensitive enough, time-consuming and their use requires highly qualified personnel. A modern alternative to these methods are diagnostic systems based on polymerase chain reaction (PCR), in particular, its real-time modification (quantitative PCR, qPCR). In the present study, for the first time in Russia, qPCR-based tests were developed for six non-quarantine pathogens — necrotic strains of potato virus Y (PVY^{N-NTN} and $PVY^{N:O}$), tobacco rattle virus (TRV), and pathogenic bacteria *Dickeya solani*, *D. dianthicola*, and *Pectobacterium atrosepticum*. Primers and fluorescent-labeled probes were designed based on nucleotide sequences deposited in the NCBI GenBank international database for the amplicon size not more than 500 bp. The specificity of the proposed systems was shown in tests with the genetic material of pathogens that infect potatoes, which are taxonomically close or occupy similar ecological niches, such as the ordinary strain PVY, potato mop-top virus, *Pectobacterium carotovorum*, and *D. zeae*. The quality of the proposed test systems was also evaluated using plant material, presumably infected with the analyzed pathogens. Nucleic acids were isolated using Proba-NK (for RNA) and Proba-GS (for DNA) reagents (AgroDiagnostica LLC, Russia). For the reverse transcription reaction, the RevertAid Premium First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used. PCR was carried out in the Tertsik thermocycler (DNA-technology, Russia), and quantitative PCR was performed in the DT-96 detection thermocycler (DNA-technology, Russia). To assess possible inhibition, an internal control sample (IC, a plasmid with a specific 560 bp insert) was added to the reaction mixture. Positive control samples (PCs) were cloned using the Quick-TA kit (Evrogen, Russia). Plasmid DNA concentration was determined (a NanoVue spectrophotometer, GE HealthCare, USA). Specific amplification products were sequenced (an ABI PRISM 3730 automated sequencer, Applied Biosystems, USA). Analytical sensitivity was evaluated by quantitative PCR, in which sequential 10-fold dilutions of plasmid DNA (PC in four independent replicates) in the range of 10^7 to 10^0 copies per reaction were used as templates. High sensitivity of the developed test systems, ranging from 10 to 500 copies of specific DNA per reaction, as well as high reproducibility (*Cv* 1.5-2.0

%) were shown. The maximum fluorescence increase for the developed hydrolyzed probes ranged from 1200 to 2000 units of background. The universality of the proposed amplification profiles can serve as the basis for adapting test systems to the multiplex PCR format. The obtained results indicate that these systems detect the analyzed pathogens with high specificity and sensitivity and can be used as part of phytosanitary control and routine detection of 6 non-quarantine pathogens in plants, planting material and food products.

Keywords: diagnostics, quantitative PCR, sensitivity, specificity, *Pectobacterium atrosepticum*, *Dickeya solani, Dickeya dianthicola*, necrotic strains of potato virus Y, PVY, tobacco rattle virus

Potato is among main crops with a global production of about 375 million tons. Diseases caused by plant pathogens of different nature belonging to different taxonomic groups become an important factor reducing the quantity and quality of potato yields. Annual losses caused by viroses make up 7% of the crop, by mycoses and bacterioses 14% [1, 2]. At present, more than 50 viral and bacterial pathogens of potato ~~diseases~~ are identified in regions with diverse climatic conditions [3, 4]. At the same time, only some of them are tested during the certification of seed potatoes in Russia, and there is no integrated monitoring system for viral and bacterial diseases of this crop [5, 6].

Modern potato growing is characterized by the progressive spread of viral pathogens, such as causative agents of necrotic spotting disease of tubers and rattle virus, as well as potato blackleg ~~bacteriosis~~. In addition to reducing the yield, these agents catastrophically deteriorate the standard quality of tubers, which becomes a serious problem for commercial production [7].

Potato tuber necrotic ringspot disease (PTNRD, the emerging of rings and arcs of dark brown color on tubers) is caused by necrotic strains of the virus Y (potato virus Y, $PVY^{N\text{-}NTN}$). At the same time, PTNRD-causing isolates that possess serological properties characteristic of the ordinary strain of potato virus Y (PVY^{O}), were identified. These strains isolated in Europe are assigned to the subgroup $PVY^{N\text{-}Wilga}$ necrotic strains (by the name of the variety on which the virus with similar properties was first detected) [8-10].

The group of PVY isolates with similar properties, discovered later in North America, was designated $PVY^{N:O}$ [11]. The PVY genome is a single-stranded RNA about 9700 nucleotides long. To date, the whole genome sequences and their polymorphism have been determined for all of the listed groups of isolates, and this fact greatly simplifies the study and development of diagnostic systems [12]. Potato stem-mottle disease (corky ringspot) which manifests itself as arcs, rings or flecks that form on or within tubers is caused by the tobacco rattle virus (TRV) which is spread primarily by stubby-root nematodes of the order *Dorylaimida* genus *Trichodorus* [13, 14].

According to the report from Laboratory of Phytoparasitology of the Center for Applied Hygiene and Epidemiology RAS which studies the distribution of *Trichodorus* nematodes, rattle virus is detected in 75% plant samples invaded by nematodes [15]. TRV refers to typical pathogens of natural focal diseases. Already in the first year that a healthy potato plant enters the infectious area, the tuber yield becomes unmarketable [16, 17]. The disease caused by the virus is described in many countries, and in the mid-1970s it was detected in the territory of the former USSR [16]. The virus is widely present in the Netherlands, Germany, Belgium, England, France, Poland, Sweden, Austria, Finland, the USA, Japan, the Baltic countries and Russia [18-20]. Identification of TRV is difficult, since a mixed infection is frequent [15, 17]. The TRV genome is a linear single-stranded RNA which consists of two parts, RNA-1 and RNA-2, 9000 to 11500 nucleotides long in total [21]. At present, a significant number of structures of the complete genomes of this virus have been determined for isolates of different geographical origin [22, 23].

The spread of potato blackleg in recent years has been noticeably pro-

gressing. A number of new pathogens causing watery rot of stems and soft rot of tubers have been identified [24]. Symptoms caused by *Dickeya* spp. bacteria on tubers and potato plants in many ways are similar to those of *Pectobacterium* spp. infection [25].

However, the *Dickeya* spp. can cause the disease when the amount of the infectious agent is small. *Dickeya* spp. can spread more easily over plant vascular tissues, are more aggressive, and at elevated temperatures, wet rot damage that they cause is stronger than in the case of *Pectobacterium atroseptica* infection. *Dickeya* spp. bacteria are typical in regions with a hot climate, however, due to local climate changes, these agents are recently observed in regions with a temperate climate, traditional for the production of potatoes. An important step in the study of these bacterial pathogens was the sequencing of their genome structures [26-28].

The main way to radically combat potato viral and bacterial diseases is to identify pathogens in the early stages of seed production, followed by culling the infected material. Classical diagnostic methods are reliable, but often do not have sufficient sensitivity, and also require highly qualified personnel and a significant time [29]. In this regard, new domestic PCR-based assays are needed to identify pathogens that degrade the quality of marketable potatoes and cause significant economic damage.

In the present paper, we report on the first Russian quantitative PCR-based systems for diagnostics and identification of six non-quarantine pathogens (necrotic strains of Y potato $PVY^{N\text{-}NTN}$ and $PVY^{N:O}$, tobacco rattle virus, pathogenic bacteria *Dickeya solani*, *Dickeya dianthicola*, *Pectobacterium atrosepticum*).

The purpose of the work is the development of original domestic systems for identifying non-quarantine potato pathogens based on the quantitative polymerase chain reaction, assessing the sensitivity and effectiveness of these systems, as well as the possibility of their use in the practice of plant pathology.

Materials and methods. PVY^{NTN}, $PVY^{N:O}$, TRV, *Pectobacterium atrosepticum*, *Dickeya solani*, *Dickeya dianthicola* were received from Lorch All-Russian Research Institute of Potato (VNIIKH) collection. In addition, plant pathogens taxonomically close or similar in symptoms on potato plants were used, i.e. PVY (potato virus Y, common strain), potato mop top virus (PMTV), *Pectobacterium carotovorum*, *Dickeya zeae*. Their isolates were collected during testing seed material from the Central and Southern Federal Districts of the Russian Federation. Monoclonal antibodies (Bioreba, Switzerland) were involved in tests. In addition, the proposed test systems were validated on the genetic material of 9 samples of potato tubers (*Solanum tuberosum* L., various varieties from Moscow Province) which were presumably infected by the analyzed pathogens.

Nucleic acids were extracted with Proba-NK kit (for RNA) and Proba-GS kit (for DNA) (AgroDiagnostica LLC, Russia) as per the manufacturer's protocol. For reverse transcription, the RevertAid Premium First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used.

The primers and fluorescently labeled probes were designed by aligning nucleotide sequences of a number of loci of detected organisms deposited in the NCBI database (GenBank, https://www.ncbi.nlm.nih.gov/genbank/). A ClustalW algorithm [30] was used for alignment. The selection and assessment of the physicochemical properties of oligonucleotides was performed with Oligo v. 6.71 software (https://www.oligo.net/oligo_updates.htm).

PCR protocols (Tertsik thermocycler, DNA-technology, Russia) were as follows: for primer pairs Y-NTN(F-R), Y-NW(F-R), TRV(F-R), Dsol(F-R) — 90 s at 93 °C; 20 s at 93 °C, 5 s at 64 °C, 5 s at 67 °C (5 cycles); 1 s at 93 °C, 5 s at 64 °C, 5 s at 67 °C (40 cycles); for primer pairs Patr(F-R), Ddi(F-

R) — 90 s at 93 °C; 20 s at 93 °C, 5 s at 64 °C, 5 s at 67 °C (5 cycles); 1 s at 93 °C, 5 s at 60 °C (40 cycles). qPCR was performed according to similar programs (a detecting thermocycler DT-96, DNA-Technology, Russia). The results were analyzed by a threshold method [31]. Each DNA sample was tested in triplicate. The composition of PCR buffer and the protocol of separation of amplification products by agarose gel electrophoresis were described in earlier papers [32, 33].

An internal control (IC), the pTZ57R/T plasmid with a specific 560 bp insert, was added to the reaction mixture to assess possible inhibition, so the reaction format was multiplex. Positive controls (PCs) were cloned using the Quick-TA kit (Evrogen, Russia) according to the manufacturer's protocol. The plasmid DNA concentration was measured by NanoVue spectrophotometer, GE Healthcare, USA). DNA samples were sequenced at Evrogen JSC (ABI PRISM BigDye Terminator v. 3.1 kit, Applied Biosystems, USA), followed by analysis of the reaction products (an ABI PRISM 3730 automatic sequencer, Applied Biosystems, USA).

Analytical sensitivity was evaluated by qPCR with sequential 10-fold dilutions of plasmid DNA as a template (PCs in four independent replicates) in the range from 10^7 copies to 10^0 (single copies) per reaction. A linear dependence of the threshold cycle on the number of copies of specific DNA in the reaction was plotted for each PC.

Standard deviations ±SD calculated from the averaged Cq value for a series of dilutions were used to assess reproducibility. The variability of Cq values was evaluated by testing each dilution thrice in triplicate. The coefficients of variation (*Cv*, %) were calculated.

Results. The analysis of the nucleotide sequences deposited in GenBank for the analyzed pathogens allows us to selected the following loci for the design of primers and probes: coat protein gene (GU980964, AF321554, AJ535662, GU550076, AJ315774) for PVY^{NTN}, *VPg* gene (EF638893, EF638901, EF638902-638892) for $PVY^{N:0}$, RNA-dependent RNA polymerase gene (JX267264-267270, MF918561-918567) for TRV, RNA polymerase σ-factor gene (*rpoD*, MH118541, LC275948-275957) for *D. dianthicola*. For the remaining pathogens, marker fragments of sequences of complete genomes were used, CP024956, CP009125, CP007744 for *P. atrosepticum*, CP024710, CP017453, CP016928 for *D. solani*. When designing primers, the corresponding loci of the target pathogens and closely related taxa were aligned. The desired temperature of primer annealing at least 60 °C was also accounted. The resultant four pairs of primers had a calculated annealing temperature of 64 °C, two pairs of 60 °C. The structure and properties of the designed primers, as well as the size of the amplification products are shown in Table 1.

1. Characterization of primers designed for identification of six non-quarantine potato (*Solanum tuberosum* L.) pathogens

Pathogen	Nucleotide sequence 5′→3′	T_o, °C	Amplicon, bp
PVY^{NTN}	Y-NTNF: TGAAACCAATCGTTGAGAAACA Y-NTNR: GTACTGATGCCACCGTCGT	60	290
$PVY^{N:0}$	Y-N0F: CAAGTCAAGCAGGAGGTTTG Y-N0R: CCCAGTCTGCCTTAGTTTA	60	350
TRV	TRVF: TTTCTTACATTCATGACTGGCT TRVR: TTGACCAACTCTCGCGGTAC	60	320
Pectobacterium atrosepticum	PatrF: CAGTAGGTTTGGGAGCAGCC PatrR: CCACTACCGATGATGCTCCC	64	280
Dickeya solani	DsolF: ATGTACTAATCAGACATGTTGCTT DsolR: TGTATCCTGATTAATTTGTGATCC	60	200
D.dianthicola	DdiF: TGTCCGATTTGATCACCGT DdiR: ATGCTGTTGTCATCATCGGAC	64	160

N o t e. PVY^{NTN} — potato virus Y (strain NTN), $PVY^{N:0}$ — potato virus Y (strain N:0), TRV — tobacco rattle virus; T_o — calculated temperature of primer annealing.

To assess the specificity of the developed primers, in addition to the DNA or cDNA of the analyzed pathogens, we used the genetic material of closely related or occupying similar ecological niches organisms, such as the PVY common strain, Potato mop-top virus (PMTV), bacteria *Pectobacterium carotovotum*, *Dickeya zeae*. Electrophoretic analysis of amplification products showed that all used primer pairs were strictly specific. In subsequent analysis we used qPCR format (Table 2).

2. Specificity of primers designed for identification of six non-quarantine potato (*Solanum tuberosum* L.) pathogens by qPCR method

Pathogen	Primer					
	Y-NTNF-R	Y-NOF-R	TRVF-R	PatrF-R	DsolF-R	DdiF-R
PVY^{NTN}	+ (29. 1)	–	–	–	–	–
$PVY^{N:0}$	–	–	–	–	–	–
PVY	–	–	–	–	–	–
TRV	–	–	+ (24. 1)	–	–	–
PMTV	–	–	–	–	–	–
Pectobacterium atrosepticum	–	–	–	+ (25. 3)	–	–
P. carotovorum	–	–	–	–	–	–
Dickeya solani	–	–	–	–	+ (27. 4)	–
D. dianthicola	–	–	–	–	–	+ (29. 3)
D. zeae	–	–	–	–	–	–

N o t e. PVY — potato virus Y (common strain), PVY^{NTN} — potato virus Y (strain NTN), $PVY^{N:0}$ — potato virus Y (strain N:0), TRV — tobacco rattle virus, PMTV — potato mop-top virus. The average values of threshold cycles per three replicates are given in parentheses; "+" is a positive result, "–" is a negative result.

3. Probes and analytical sensitivity of designed qPCR-based systems for identification of six non-quarantine potato (*Solanum tuberosum* L.) pathogens

Pathogen	Probe nucleotide sequence 5′→3′	T_o, °C	Sensitivity, DNA copies per reaction
PVY^{NTN}	(BHQ1)-TGCGGCCTTCAT(FAMdT)TGAATGTGCGC	82	1×10^1
$PVY^{N:0}$	(BHQ1)-GCAAGCCTTGGGCAG(FAMdT)AACACGACCA	81	1×10^2
TRV	(BHQ1)-GAACCGTGGCAGG(FAMdT)GAGAGGAGACAC	82	1×10^2
Pectobacterium atrosepticum	(BHQ1)-CGCGTCTTTTTT(FAMdT)GGGGTGTCGGCA	83	5×10^2
Dickeya solani	(BHQ1)-CGACGTGAAAATGTGA(FAMdT)GACTTCCATCC	82	5×10^1
D. dianthicola	(BHQ1)-TTCGTCTTCTTCGC(FAMdT)TTCGTCGTCGTCA	82	1×10^1

N o t e. PVY^{NTN} — potato virus Y (strain NTN), $PVY^{N:0}$ — potato virus Y (strain N:0), TRV — tobacco rattle virus; T_o — calculated temperature of probe annealing.

Another important feature for a diagnostic system is its analytical sensitivity. qPCR analyses with 10-fold dilutions of plasmids (PC) with known concentrations and copy numbers in the reaction mixture showed that the systems for PVY^{NTN} and *D. dianthicola* identification had the highest sensitivity (about 10 copies of the plasmid with the corresponding cloned DNA fragment per reaction), the system for *P. atrosepticum* identification was the least sensitive (500 copies per reaction) (Table 3).

The efficiency values for the developed test systems ranged from 91.2 to 98.0%. The values of standard deviations during testing of each of 10-fold dilutions in triplicate ranged from 0.09 to 0.53, and the *Cv* value did not exceed 2%. As an example, Figure 1 shows graphs of the threshold cycle vs. the number of plasmid copies with cloned DNA fragments of target objects (PC) per reaction, as well as graphs of fluorescence signal accumulation for PVY^{NTN} and *D. dianthicola* ~~test~~ systems as examples.

The quality of the developed test systems was also evaluated using potato tubers of different varieties presumably infected with the analyzed pathogens. In PCR, in addition to specific primers and probes, the IC (plasmid) with the corresponding pair of primers and probe were added to the reaction mixture. The presence of a band, corresponding to IC amplification product, was demonstrated on the electrophoregrams of all nine potato samples' cDNA/DNA analyzed by primer pairs to PVY^{NTN} and *D. dianthicola*. This indicates the absence of reaction

inhibition (Fig. 2). In turn, the absence of a specific band for negative control indicates the absence of contamination of the working area with specific amplicons. As follows from the electrophoregrams shown, PVY^{NTN} virus cDNA was revealed in samples No. 3 (cultivar Red Scarlett), No. 4 (cultivar Zhukovsky rannii), No. 7 (cultivar Favorit III), No. 8 (cultivar Pomdor), No. 9 (cultivar Floris), and *D. dianthicola* DNA in samples No. 2 (cultivar Red Scarlett), No. 3 (cultivar Red Scarlett), No. 6 (cultivar Romano), 8 (cultivar Pomdor), 9 (cultivar Floris).

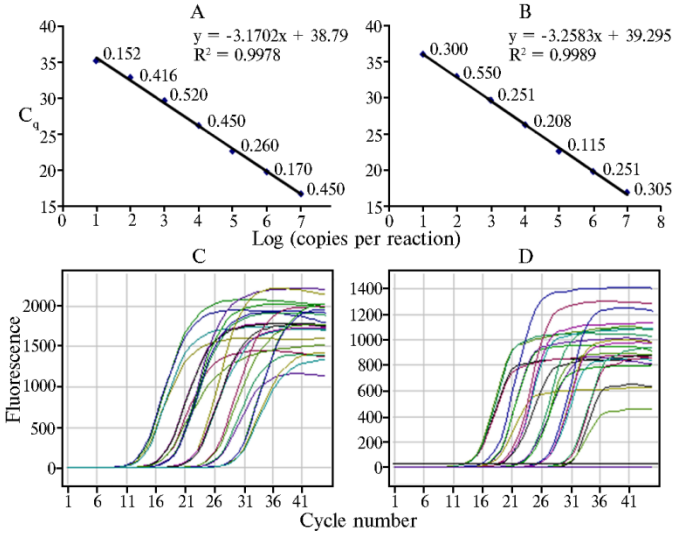

Fig. 1. Assessment of sensitivity and effectiveness of designed qPCR-based tests for identification of six non-quarantine potato (*Solanum tuberosum* L.) pathogens with 10-fold dilutions of specific positive controls: A and B — qPCR threshold cycle dependence on plasmid copy number per reaction (with the standard deviations for four replicates indicated next to each point), C and D — corresponding graphs of fluorescence accumulation. Sensitivity for PVY^{NTN} is 10 copies per reaction, 98.0% effectiveness (A), for *Dickeya dianthicola* 10 copies per reaction, effectiveness 96.7% (B).

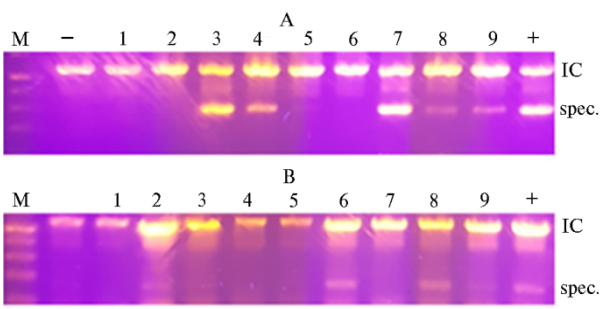

Fig. 2. Electrophoregrams of PCR amplification products (A — cDNA, Y-NTNF-R primer pair; B — DNA, DdiF-R primer pair) **in testing potato tubers presumably infected by non-quarantine pathogens.** IC — internal control, spec. — specific amplification product; 1, 2, 3 — Red Scarlett, 4 — Zhukovsky rannii, 5, 6 — Romano, M — molecular weight marker; "+" is a positive control sample, "–" is a negative control sample.

Note that several variants of systems for identification of the pathogens we examined in this paper have been currently described in special literature. Shvidchenko et al. [34] showed that the real-time PCR is significantly more specific than ELISA for detection of potato viruses, including PVY (the diagnostic sensitivity of ELISA relative to real time PCR is 68%). Also a number of multiplex PCR-based systems have been proposed, in particular for identification of the necrotic strains PVY$^{N:0}$ and PVY^{NTN} [35], as well as the main pectinolytic bacteria, including *P. atrosepticum* and *Dickeya* spp. (with minimum sensitivity of 0.01 ng/μl specific DNA) [36], however, these were not adapted to the qPCR format. Primers that specifically distinguish species of the genus *Dickeya* were described in 2013 [37] and successfully used to identify pathogens in vegetable crops in Northern Ireland [38]. For this test system a very high sensitivity, about 0.05 pg of DNA per reaction (about 10 copies of plasmid DNA), was shown.

Also, a number of systems of different sensitivity for detection of tobacco rattle virus have been described [39-41]. As per Holeva et al. [42], the sensitivity of the system was about 1 fg of plasmid DNA per reaction (approximately 10^2 copies).

Thus, the main characteristics of the proposed assays were close to those of the most effective foreign analogues or even exceeded them. The sensitivity of the systems ranged from 10 to 500 copies of specific DNA per reaction, and the amplification efficiency exceeded 90%. High reproducibility of the results is shown (*Cv* no more than 2%, standard deviations over dilutions no more than 0.53). The designed probes are shown to be high specific, and the internal controls are optimized. The quality of the test systems is confirmed by tests with plant material infected with the studied pathogens. The totality of the obtained results indicates that the developed ~~test~~ systems identify with high sensitivity and specificity six non-quarantine potato pathogens that cause significant economic damage. These systems can be important tools in monitoring plant pathogen infections of planting material, plants, and food products.

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