Matrix approach to the simultaneous detection of multiple potato pathogens by real-time PCR

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DNA/RNA microarrays, identification, micromatrix format, potato pathogens, rapid diagnostics, real-time PCR.

Abstract

Aim: Create a method for highly sensitive, selective, rapid and easy-to-use detection and identification of economically significant potato pathogens, including viruses, bacteria and oomycetes, be it single pathogen, or a range of various pathogens occurring simultaneously.

Methods and Results: Test-systems for real-time PCR, operating in the unified amplification regime, have been developed for \(Phytophthora infestans\), \(Pectobacterium atrosepticum\), \(Dickeya dianthicola\), \(Dickeya solani\), \(Ralstonia solanacearum\), \(Pectobacterium carotovorum\), \(Clavibacter michiganensis\) subsp. \(sedenonicus\), potato viruses \(Y\) (ordinary and necrotic forms as well as indiscriminative test system, detecting all forms), \(A\), \(X\), \(S\), \(M\), potato leaf roll virus, potato mop top virus and potato spindle tuber viroid. The test-systems (including polymerase and revertase) were immobilized and lyophilized in miniature microreactors (1-2 µl) on silicon DNA/RNA microarrays (micromatrices) to be used with a mobile AriaDNA™ amplifier.

Conclusions: Preloaded 30-reaction micromatrices having shelf life of 3 and 6 months (for RNA- and DNA-based pathogens, respectively) at room temperature with no special conditions were successfully tested on both reference and field samples in comparison with traditional ELISA and microbiological methods, showing perfect performance and sensitivity (1 pg).

Significance and Impact of the Study: The accurate, rapid and user-friendly diagnostic system in a micromatrix format may significantly contribute to pathogen screening and phytopathological studies.

Introduction

Successful development of agricultural industry is closely associated with the need to ensure effective protection of plants against a variety of bacterial, fungal and viral infections. Despite the fact that development of modern plant disease control methods provides significant increase in agricultural production, annual global crop losses caused by various pests and pathogens still reach 10–16% with additional 6–12% losses due to storage diseases (Chakraborty and Newton 2011). Moreover, pesticide use to control pests and diseases requires considerable financial investment reaching annual 40 billion US dollars worldwide (Pimentel and Burgess 2014).

Potato is one of the most important staple crops worldwide. The crop is crucial for welfare of billions of people; therefore, sustainable potato production is very important for global food security. Since vegetative propagation of potato predominates, plant pathogens, especially those of viral and bacterial origin, pose serious threats to potato industry. Among more than 40 viruses and viroids infecting cultivated potatoes, eight (potato leaf roll virus (PLRV), potato virus \(Y\) (PVY), potato virus \(X\) (PVX), potato virus \(A\) (PVA), potato virus \(S\) (PVS), potato virus \(M\) (PVM), potato mop-top virus (PMTV) and potato spindle tuber viroid (PSTVd)) are most economically significant in terms of their global distribution and effect on the crop yield. PVY and PLRV may cause
up to 80–90% of yield losses, while other above-mentioned viruses show yield losses within 10–40% (Ahmadvand et al. 2012; Palukaitis 2012). In addition, bacterial, oomycetal, and fungal pathogens cause about 14% of global potato losses (Oerke 2006). Among them, the most significant are Dickeya dianthiocola, Dickeya solani, Clavibacter michiganensis subsp. sepedonicus, Ralstonia solanacearum, Pectobacterium atrosepticum, Pectobacterium carotovorum subsp. carotovorum and oomycete Phytophthora infestans. Most of the above-listed potato viruses and bacteria are subjected to regulation in seed potato certification (UNECE 2014).

Growing globalization and international trade in seed and table potato, climatic changes leading to invasion of potato pathogens and pests to new areas, and emergence of pathogens resistant to modern pesticides have significantly increased the need of agricultural sector in tools for rapid, accurate and reliable diagnostics of potato diseases. These tools include the use of resistant cultivars, efficient pesticide application, and early, accurate, and reliable detection and identification of pathogens. The last one is particularly important for potato diseases of viral and bacterial nature, where disease-free seed material is the only effective way to restrict infection.

Classical diagnostic methods, such as a microscopic identification or pathogen cultivation are time-consuming and require highly qualified personnel. Currently, enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR)-based diagnostic methods are considered to be most effective (Ward et al. 2004).

Being relatively simple and inexpensive, immunoassay methods became popular quickly. The total number of ELISA tests used to detect agricultural pathogens worldwide reaches 10 million per year (Bonantz et al. 2005). Lateral-flow devices or test strips are also actively used, especially in field diagnostics, since they are technically undemanding, can be used by unqualified personnel, and provide result within just a few minutes. However, like any other enzyme-linked technique, this method works well for viral diseases and could be less sensitive in the case of bacterial and fungal pathogens (Sanati Nezhad 2014).

Conventional PCR methods provide high sensitivity and specificity of diagnostics, though they require precise thermocycling and contamination control during sampling, probe preparation, and analysis of PCR products, thus limiting applicability of the method without employing specially equipped laboratory facilities and highly qualified personnel. Nevertheless, PCR remains the most promising technology for in-field molecular diagnostics.

In recent years, a number of new amplification methods emerged, making it possible to overcome to some extent disadvantages of classic PCR. Application of technology based on isothermal DNA amplification (LAMP PCR) allows one to avoid the use of costly and bulky thermocyclers and significantly reduces reaction time. In addition, this technology increases efficiency and specificity of the reaction and facilitates interpretation of results. However, in most cases, LAMP PCR technology requires an expanded set of primers (up to 6) having certain amplicon length limitations, and also comprises hybridization step (Gill and Ghaemi 2008).

Use of FLASH (FLuorescent Amplification-based Specific Hybridization) PCR approach provides for fluorimetric registration of amplification in sealed reaction tubes which excludes sample contamination (Abramova et al. 2008; Ryazantsev and Zavriev 2009). However, no commercial kits for FLASH PCR are currently available, that could be used for simultaneous diagnostics of multiple pathogens.

Real-time PCR technique requires no analysis of PCR products upon completion of the reaction, thus providing for low risk of contamination and reducing time required for the analysis. The technique also enables simple quantification of target pathogen (Okubara et al. 2005). The method though has certain disadvantages, such as expensive equipment and reagents. Moreover, high sensitivity requires special attention to the conditions under which testing is being carried out, thus limiting its in-field use. The possibility of using different fluorescent dyes enables realization of multiplex PCR, thus reducing total time and cost of the analysis. At the same time, the number of pathogens tested is limited by the number of detection channels and multiple reactions running simultaneously in the same volume that imposes certain requirements on the primers used and increases the risk of nonspecific reactions (Mirmajlessi et al. 2015). In practice, the number of different pathogens detected by multiplex PCR does rarely exceed two.

Miniaturization and creation of microfluidic or stationary microchip systems has become next step in development of PCR techniques that allows PCR to be carried out in microreactors with the volume of only a few dozen picolitres (Nagai et al. 2001; Ahmad and Hashsham 2012). In addition to reducing cost of reagents, such tiny reaction volume provides faster and more accurate temperature control that reduces the total analysis time and improves effectiveness of the reaction.

To provide a wide-range application of microchip PCR systems for diagnostic purposes, such systems shall be easy to use, have low net cost of microchips and should not require highly qualified personnel. As a rule, microfluidic microchips do not meet these requirements, while stationary microchips with open microreactors are quite suitable as a basis for creating diagnostic systems (Slyadnev 2012).
Development of an optimized diagnostic systems based on stationary microchips has been also undertaken in Russia resulting in the AriaDNA® microchip amplifier (Navolozkii et al. 2010; Navolotskii et al. 2011) having certain advantages over similar systems. This system is based on the use of disposable microchip made of silicon or aluminium alloy and consisting of up to 48 microreactors/wells, and provides simultaneous multiplex PCR analysis in real-time mode (Fig. 1a). The microchip is highly thermal-conductive that reduces amplification time to 20–30 min. Special modification of the chip surface provides hydrophilic zone within the wells and hydrophobic zone beyond them, which ensures reliable retention of samples within the wells during thermocycling and prevents both cross-contamination and inhibition of the reaction (Fig. 1b). Fluorescent signal is registered for each well individually, so interference of signals from neighbouring wells is eliminated. In the case of employing reverse transcription PCR (RT-PCR), both reactions are sequentially performed in the same well. Another important advantage of the system is that all components of reaction mixture (including polymerase and reverse transcriptase) can be lyophilized in the wells that reduces time required for the analysis and simplifies the procedure – the operator shall only prepare DNA or RNA samples and add them into the wells under the layer of sealing mineral oil (Navolotskii et al. 2011).

Potential for the use of such microarrays (micromatrices) has been demonstrated earlier for express diagnostics of sexually transmitted diseases (Suvorova et al. 2012). The purpose of this study was the development of real-time PCR-based systems in a micromatrix format for express diagnostics of the most economically important potato pathogens, including PLRV, PMTV, PVX, PVA, PVS, PVM, PVY (PVYO and PVYN forms), PSTVd, P. atrosepticum, P. carotovorum subsp. carotovorum, D. dianthicola, D. solani, C. michiganensis subsp. sepedonicus, R. solanacearum and P. infestans.

Materials and methods

Phytopathogenic organisms

Strains of P. infestans MVK 118a-07, P. atrosepticum ECA-515, D. dianthicola ECH-103, D. solani D 2354 and R. solanacearum RS R-300 were obtained from the State Collection of Phytopathogenic Micro-organisms (ARRIP). For other plant pathogens, we used corresponding control materials from commercial ELISA or PCR diagnostic kits provided by Agdia Biofords, France (P. carotovorum, C. michiganensis subsp. sepedonicus, potato virus Y (general, PVY), necrotic form of potato virus Y (PVYN), and potato virus A); Lorkh All-Russian Research Institute of Potato Farming, Russia (PVYO, PVX, PVS, PVM, PLRV and PSTVd); and Loewe Biochemica GmbH, Germany (PMTV).

Oligonucleotide design

For each pathogen species, primers and fluorescent probes were designed using Oligo Primer Analysis software v. 6.0 (Molecular Biology Insights, Inc., Colorado Springs, CO, USA). Since amplification of test systems for RNA- or DNA-based pathogens shall occur simultaneously on the
same micromatrix (microarray), one of the key factors was the possibility to apply similar amplification parameters to provide efficient use of all primer pairs. Selectivity of the developed primers was tested using BLAST option of the NCBI database. Oligonucleotides developed for the target pathogens are listed in Table S1.

**Micromatrix (microarray) design**

In this study, two types of micromatrices were generated and tested (Fig. 2, Fig. S1):

i. **RT-PCR micromatrix ‘Potato pathogens. RNA’** for detection and identification of specific RNA sequences of potato viruses Y (PVY\(^\text{O}\) and PVY\(^\text{NTN}\)), X (PVX), A (PVA), S (PVS), M (PVM), leaf roll (PLRV), mop-top (PMTV), as well as potato spindle tuber viroid (PSTVd). The matrix design provided the possibility to simultaneously test two samples per matrix (Fig. 2a).

ii. **PCR micromatrix ‘Potato pathogens. DNA’** for simultaneous detection and identification of specific DNA sequences of *P. infestans*, *P. atrosepticum*, *P. carotovorum* subsp. *carotovorum*, *D. dianthicola*, *D. solani*, *C. michiganensis* subsp. *sepedonicus* and *R. solanacearum*. The matrix design provided the possibility to simultaneously test three samples per matrix (Fig. 2b).

In addition, due to high demand, test system for general PVY (detection and identification of potato virus Y independently of its form) was developed for the RT-PCR micromatrix ‘Potato pathogens. PVY’. The matrix design provided the possibility to simultaneously test 23 samples per matrix.

**DNA/RNA extraction**

DNA and RNA extraction was carried out using ‘AmpliSens\(^\circledR\)’ DNA-sorb-B DNA extraction kit’ and ‘RIBO-sorb’ kit for DNA/RNA extraction (The Research Central Institute of Epidemiology, Russia) according to the manufacturer’s recommendations. Final DNA concentration was determined at 260 nm using a SmartSpec Plus spectrophotometer (BioRad, Hercules, CA, USA). DNA samples were stored at \(-20^\circ\text{C}\) until use.

**Preparation of micromatrices and sample application**

Empty 30-cell Si/Al micromatrices were prepared according to the earlier described technology (Navolotskii et al. 2011). Reaction mixture of total 25 µl consisted of 0.6 µl of the dNTP mix (10 mmol l\(^{-1}\)), 2 µl of the oligonucleotide mix (5 pmol l\(^{-1}\) for each primer and 2.5 pmol l\(^{-1}\) for the probe), 0.9 µl of Taq-polymerase (5 units l\(^{-1}\); ‘Evrogen’, Moscow, Russia), and 19 µl of deionized water. For RT-PCR, 0.5 µl of MMLv-revertase (‘AmpliSens’, Russia) was added to reaction mixture. Stabilization and lyophilization of the PCR mix on micromatrix surface were performed by

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**Figure 2** Topology of PCR micromatrices designed for the diagnostics of (a) viral and (b) bacterial/oomycetal potato pathogens. PSTVd, potato spindle tuber viroid; PLRV, potato leafroll virus; PVX, potato virus X; PVA, potato virus A; PVS, potato virus S; PVM; potato virus M; PMTV, Potato mop-top virus; PVY\(^\text{O}\), potato virus Y; PVY\(^\text{NTN}\), potato virus Y\(^\text{NTN}\); CMS, Clavibacter michiganensis subsp. sepedonicus; Pi, *Phytophthora infestans*; Pa, *Pectobacterium atrosepticum*; Pcc, *Pectobacterium carotovorum* subsp. *carotovorum*; Ds, *Dickeya solani*; Dd, *Dickeya dianthicola*; Rs, *Ralstonia solanacearum*; IC, internal control; C+, positive control; C−, negative control.
After installing prepared micromatrix into holder cartridge, the whole reaction zone was covered with a sealing layer of mineral oil (620 μl) avoiding bubble formation. DNA or RNA samples were mixed with 10× PCR buffer (‘Evrogen’) at 1 : 9 ratio; the resulting DNA/RNA concentration of samples was 1 μg ml⁻¹. One microlitre of a sample or deionized water (negative control) was added into each well under the sealing oil layer according to the particular matrix topology.

Real-time PCR in micromatrix format

Real-time PCR was performed on two-beam AriaDNA Microchip Amplifier (Lumex-Marketing LLC, Saint-Petersburg, Russia). Thermal cycling conditions for DNA amplification were standard for this amplifier and included initial denaturation (94°C for 120 s) followed by 45 cycles of 94°C for 5 s and 60°C for 25 s. The total amplification time was 30 min. For RNA samples, the cycling additionally included preliminary reverse transcription stage (37°C for 20 min). Data acquisition was performed at the end of each 60°C step. Detection of fluorescence related to PSTVd, PVY°, PVS, PMTV, PVA, CMS, P. atrosepticum, P. carotovorum subsp. carotovorum and D. solani was followed using channel 1 (FAM). For PLRV, PVYNTN, PVX, P. infestans, D. diathicola, R. solanacearum, and internal control sample (IC), channel 2 (ROX) was used. Signal recording, calculation of threshold cycles (Cₜ) and analysis of the results were carried out automatically using AriaDNA software package (‘Lumex-Marketing’ LLC).

Real-time PCR in tube format

Real-time PCR in tube format (25 μl) was performed on LightCycler 480 II amplifier (Roche, Basel, Switzerland). Reaction mixture composition was the same as for the micromatrix format. Thermal cycling conditions for DNA amplification included initial denaturation (95°C for 10 min) followed by 45 cycles of 95°C for 15 s and 60°C for 5 s. The total amplification time was 30 min. For RNA samples, the cycling additionally included preliminary reverse transcription stage (37°C for 20 min). Data acquisition was performed at the end of each 60°C step. Detection of fluorescence related to PSTVd, PVY°, PVS, PMTV, PVA, CMS, P. atrosepticum, P. carotovorum subsp. carotovorum and D. solani was followed using channel 1 (FAM). For PLRV, PVYNTN, PVX, P. infestans, D. diathicola, R. solanacearum, and internal control sample (IC), channel 2 (ROX) was used. Signal recording, calculation of threshold cycles (Cₜ) and analysis of the results were carried out automatically using AriaDNA software package (‘Lumex-Marketing’ LLC).

Figure 3 Examples of the detection of various potato pathogens using the developed real-time PCR micromatrix systems. The thresholds are shown by horizontal lines. (a) Analysis of separate DNA samples isolated from Clavibacter michiganensis subsp. sepedonicus (CMS), Phytophthora infestans (Pi), and Pectobacterium atrosepticum (Pa); each sample was analysed in two wells of the same micromatrix. (b) Analysis of a mixed sample containing RNA from potato virus X (PVX), potato virus S (PVS), and potato virus A (PVA). (c) Detection of RNA of the necrotic form of potato virus Y (PVYNTN) on a micromatrix containing test systems for all RNA-based potato pathogens; signals from the wells containing test systems corresponding to other viruses do not exceed the threshold. (d) Detection of both ordinary (PVY°) and necrotic (PVYNTN) forms of potato virus Y (PVY) using the test system developed for general PVY. DNA/RNA concentration of samples is 1 μg ml⁻¹.

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for 60 s. For RNA samples, the cycling was performed after preliminary reverse transcription.

Detection limit determination

To determine detection limit of the developed test systems, serial dilutions of DNA or RNA of the target species were tested. The concentration range included 100, 10, 1 and 0.1 ng ml$^{-1}$ for DNA-based pathogens and 20,000, 1000, 100, 10, 1 and 0.1 ng ml$^{-1}$ for RNA-based pathogens. All dilutions were arranged in the same DNA or RNA micromatrix.

Specificity assessment

To evaluate specificity of the developed primer pairs, each of them was tested with potato DNA and samples of the whole set of DNA- or RNA-based pathogens included into the study. DNA or RNA samples were obtained from pure cultures of microorganisms, positive controls of the corresponding ELISA kits, or infection-free potato tuber as described above and adjusted to a final concentration of 1 µg ml$^{-1}$.

Shelf life estimation of the developed PCR matrices

The shelf life of the developed diagnostic micromatrices (microarrays) was estimated using plasmids containing DNA or cDNA sequences of some target potato pathogens (positive controls, Table S1). Plasmid samples were added to the corresponding microreactors of the tested matrices at concentration within the range of 1–300 ng ml$^{-1}$, and then PCR analysis was carried out. The analysis was made on fresh matrices and also after 3- and 6-month storage at room temperature in dry place without direct light exposure.

Field trials

Both developed micromatrices (‘Potato pathogens. RNA’ and ‘Potato pathogens. DNA’) were tested at the facilities of the ‘Rossel’khozsentr’ (Federal Budgetary Enterprise ‘Russian Agricultural Center’) branch located in Leningrad region (Russia) using some ‘live’ field samples, i.e., potato leaves and tubers collected from potato farms of the region and having symptoms of viral or bacterial infection. The number of plants sampled depended on their availability and varied from 3 to 5. For each sample analyzed, one tissue sample was taken from stolon (for DNA-based pathogens) and leaf, tuber sprout or apex bud (for RNA-based pathogens). The total number of samples per plant was 3–5. RNA/DNA extraction from plant tissues was carried out using AmpliSens® RIBO-sorb DNA/RNA extraction kit (Russian Central Institute of Epidemiology, Russia) according to the manufacturer’s recommendations. Results of the analysis were compared with those obtained by the use of commercial ELISA kits for detection of PVX, PVY, PVS, PVM, PVA and P. atrosepticum (Lorkh All-Russian Research Institute of Potato Farming, Russia) in accordance with the manufacturer’s recommendation.

Results

Diagnostic efficiency and specificity of developed test systems

Laboratory tests of the developed test systems were carried out using DNA and RNA samples of micro-organisms, test-systems for which were included in diagnostic matrices. The experiments showed their perfect efficiency: all nucleic acids searched for were detected and identified in samples containing DNA/RNA of a single pathogen, as well as in samples containing mixture of DNA/RNA of several pathogens (Fig. 3).

All test-systems were subjected to specificity/selectivity check at both development stage (using NCBI nucleotide-BLAST tool) and through laboratory testing. Each test-system was checked for possible cross-reactions with potato DNA and genomic DNA/RNA of potato pathogens used in the study. No cross-reactivity to nontarget species or false-positive results were observed (Tables 1 and 2, Fig. 3c).

Examination of all developed primers with potato DNA sample showed no amplification. In field studies, total DNA/RNA isolated from infected potato samples was used as matrix for amplification. Results obtained for pure bacterial cultures and field infected potato plants did not reveal significant differences, which meant no interference of potato DNA with the developed PCR-test-systems.

Reproducibility of the approach was examined by analysis of the same sample of each pathogen in 14× repetitions arranged on the same micromatrix. Examples of amplification curves for P. carotovorum subsp. carotovorum, P. infestans, PVS and PVYO are shown in Fig. 4. All test systems showed excellent reproducibility, and standard deviation of $C_t$ did not exceed 1-5%.

Detection limit determination

Examples of amplification curves obtained for serial dilutions of DNA- or RNA-based potato pathogens are shown in Fig. 5a,b. According to the results obtained, detection limit of the developed test systems was 1 ng ml$^{-1}$ for most of the pathogens; for general PVY it
was even lower (0.1 ng ml⁻¹). Therefore, detection limit of both diagnostic micromatrices for DNA- and RNA-based pathogens of potato was determined to be 1 ng ml⁻¹ that corresponds to ~1 pg of DNA/RNA (~10 copies) per reaction.

Sequential dilution of samples provided good linearity of the corresponding semilog graph, i.e., linear dynamic range of amplification was exhibited for the concentration range used. An example of standard regression curve built for general PVY is shown in Fig. 6.

Shelf life assessment of the developed PCR matrices

Fluorescence intensity of signals obtained from DNA- and RNA-based micromatrices stored for 6 and 3 months, respectively, remained at satisfactory level, and \( C_t \) values increased averagely by only 5%, which is quite acceptable for practical use (Fig. 7). In the case of longer storage, some part of micromatrices lost their efficiency, so the quality of analysis could not have been guaranteed.

Comparison of real-time PCR analysis in micromatrix and tube formats

To compare micromatrix and conventional tube formats of real-time PCR, samples of PVS and PSTVd were tested in parallel using AriaDNA® and LightCycler 480 II (Roche) amplifiers. The results showed that fluorescence curves, as well as starting cycles, were quite similar (Fig. S2). Therefore, tiny well volume (1.2 μl) of the matrices did not affect PCR efficiency as compared with the tube format (25 μl).

Field trials

Comparative diagnostic efficiency of the developed real-time PCR micromatrices with conventional ELISA is shown in Table 3. Examples of amplification curves are shown in Fig. S3. According to the obtained results, real-time PCR diagnostics in micromatrix format detected all viruses revealed by the compared ELISA kits. Moreover, in some samples we also detected low amount of PVY (ordinary and necrotic forms) and potato spindle tuber viroid. In addition, analysis of samples suspected for bacterial infection performed using ‘Potato pathogens. DNA’ micromatrix resulted in the identification of \( P. atrosepticum \) (samples 20, 21, 23) and \( P. carotovorum \) subsp. \( carotovorum \) (sample 22), confirmed by the parallel microscopic examination, while the ELISA assay demonstrated negative results. Within the framework of field trials, we also examined several ‘healthy’ potato samples with no disease signs, in which no infection was revealed by the ELISA method.
In all these cases, both micromatrices types showed negative results as well.

**Discussion**

Timely and precise diagnostics of plant diseases is one of the most important tools to minimize yield losses. It provides an adequate quality assessment of seeds and stored agricultural products, identification of quarantine objects in imported seed material, and timeliness and effectiveness of protective treatments. For end user of diagnostic test systems, these systems should fit the following criteria:

i. cost of analysis should not be too high;
ii. method of analysis should provide rapid results, high sensitivity and specificity, which is especially important for early diagnostics;

| Test system | Cms | Pi | Pa | Ds | Dd | Pcc | Rs |
|-------------|-----|----|----|----|----|-----|----|
| CMS         | 18.78 ± 2.14 | –   | –   | –   | –   | –   | –  |
| Pi          | –   | 22.06 ± 1.65 | –   | –   | –   | –   | –  |
| Pa          | –   | –   | 26.47 ± 3.23 | –   | –   | –   | –  |
| Ds          | –   | –   | –   | 24.96 ± 1.79 | –   | –   | –  |
| Dd          | –   | –   | –   | –   | 24.56 ± 2.70 | –   | –  |
| Pcc         | –   | –   | –   | –   | –   | 19.92 ± 1.67 | –  |
| Rs          | –   | –   | –   | –   | –   | –   | 28.93 ± 3.42 |

*Average C_{t} values (two wells) are listed in the table. No amplification was detected in blank cells. Designations: CMS, *Clavibacter michiganensis* subsp. *sepedonicus*; Pi, *Phytophthora infestans*; Pa, *Pectobacterium atrosepticum*; Pcc, *Pectobacterium carotovorum* subsp. *carotovorum*, Ds, *Dickeya solani*, Dd, *Dickeya dianthicola*, Rs, *Ralstonia solanacearum*.

Figure 4 Examples illustrating reproducibility of the approach: (a) *Pectobacterium carotovorum* subsp. *carotovorum*, C_{t} = 14.32 ± 0.15, (b) *Phytophthora infestans*, C_{t} = 14.89 ± 0.11, (c) PVS, C_{t} = 13.28 ± 0.05, and (d) PVYO, C_{t} = 15.97 ± 0.13. IC, internal control. DNA/RNA concentration of samples is 1 μg ml^{-1}. The number of repetitions for each sample was 14.
procedure of analysis should be quite simple and does not require highly qualified personnel and special facilities and conditions. Ideally, the equipment should be mobile enough to be used not only at stationary, but also at field laboratories; in addition, diagnostic kits should not require any special conditions of storage, transportation and use.

Possibility of simultaneous detection of a wide range of pathogens is also significant, since it saves user’s time and money. In addition, different regions differ in the level of expansion and harmfulness of plant pathogens that results in an outspoken demand for ‘geographically localized’ test systems with different sets of pathogens including those missing from standard commercial diagnostic kits.

Existing laboratory and field methods for plant pathogen diagnostics do not completely meet the above-mentioned requirements. Though a number of promising technologies for detection of a range of plant pathogens have been reported (Geister et al. 2004; Julich et al. 2011; Perdikaris et al. 2011; Dai et al. 2012; Koo et al. 2013; Papadakis et al. 2015; Safenkova et al. 2016), existing commercial diagnostic kits are mainly based on the ‘one pathogen – one test’ principle and, in some cases, have insufficient sensitivity or simplicity. Therefore, problem of development of user-friendly, inexpensive and highly productive diagnostic systems able to simultaneously analyze a large number of samples or to reveal a wide range of plant pathogens and, at the same time, suitable for in-field use still remains very relevant.

According to the results of this study, the proposed real-time PCR microarrays provide high sensitivity (detection limit is 1 ng ml$^{-1}$ and several DNA/RNA copies per a sample, with no cross-reactions observed in relation to nontarget pathogens) that corresponds to common commercial diagnostic systems. Excellent reproducibility and lack of cross-reactions in relation to other DNA- or

**Figure 5** Examples of the performance of the developed real-time PCR microchip system at different concentrations of (a) DNA from Pectobacterium atrosepticum and (b) RNA from PVY. Each sample was analyzed in two microreactors of the same micromatrix.

**Figure 6** Standard regression curve of a serial dilution of RNA from potato virus Y ($y = -3.32x + 20.24, R^2 = 0.995, E = 100.1\%$).
RNA-based pathogens confirmed high reliability of the approach. Tiny reaction volume reduces consumption of PCR reagents by 20 times as compared to classic tube format of PCR analysis, while amplification time makes only 33 min for 45 cycles that is at least twice more rapid than classic PCR. Important advantage is the special technology of preparation of disposable microarrays that significantly simplifies the procedure of analysis (operator shall only prepare DNA or RNA samples and apply them into the wells) and extends shelf life of the micromatrices under room temperature (3 and 6 months depending on the type of target pathogens). Though there were some other attempts to develop long-stored PCR chips or arrays (Rubina et al. 2004; Morrison et al. 2006; Kim et al. 2009), no one of them provided any commercialized results.

Therefore, the developed diagnostic micromatrices are extremely suitable for the early and accurate diagnostics of most important potato pathogens in field laboratories even by unskilled personnel. In addition, primer design based on the same ‘standard’ PCR conditions for all developed test systems makes it possible to produce diagnostic micromatrices with any possible combination of DNA- or RNA-based potato pathogens depending on the customer’s demand or region of use.

In spite of obvious advantages of the proposed approach, there are also some issues that should be addressed in future. First, plant material used for DNA/RNA extraction is heterogeneous and may include leaves, stems, undamaged tubers and rotten/multi-infected/damaged plant tissues. At the moment, no single optimal method exists that would provide for optimal DNA/RNA extraction from any type of a sample. Therefore, sampling and the choice of extraction method might lead to false results (mainly negative). Another issue is that interpretation of results may be difficult, especially in case of viral pathogens. First, we need to set \( C_t \) threshold level. According to our empiric data and basing upon considerations presented by Grosdidier et al. (2017), we consider it to be 35. Considering \( C_t \) slightly exceeding this value, one can never be sure whether it is false-positive result or just very low virus presence. The problem is to be addressed on case-by-case basis by getting greater statistics and comparison with result obtained by other methods on the same samples.

Field trials have demonstrated high sensitivity of the tested assays and their potential for in-field use and the express diagnostics of a wide range of potato pathogens even in the case of extremely low pathogen presence in plant tissues. Thus, the developed diagnostic systems may be used at the first stages of large-scale screening of seed
or stored potato for latent viral and bacterial infections, and also for testing plant material used for mass propagation in meristem cultures. In addition, real-time PCR/RT-PCR in micromatrix format has great potential for high-throughput phytopathological studies and monitoring of viral and bacterial potato diseases, so one of the next directions of our studies will be testing of our approaches in large-scale screening of potato for a wide range of infections. The first practical step has been already accomplished in collaboration with Rossel’khozsentr (Russian Agricultural Center) in large-scale screening of potato viral pathogens in potato seed material and plantings (Mal’ko et al. 2017).

This work represents a part of a large project intended to develop diagnostic PCR micromatrices for the detection of a wide range of potato pathogens including bacteria, fungi, viruses, viroids, phytoplasmas and nematodes. The final aim of this project is the development of a number of sensitive and efficient ready-to-use assays intended to cover most economically significant RNA- and DNA-based potato pathogens.

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### Conflict of Interest

No conflict of interests is declared.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Ready-to-use real-time PCR micromatrices for diagnostics of DNA-based (left) and RNA-based (right) pathogens of potato.

**Figure S2.** Comparison of efficiency of the developed diagnostic real-time PCR test-systems in the micromatrix and tube formats by the examples of potato spindle tuber viroid (PSTVd) and potato virus S (PVS). Left: fluorescence curves obtained on a Roche LightCycler 480 II amplifier (tube format, eight tubes per a sample). Right: fluorescence curves obtained on an AriaDNA® microchip amplifier (micromatrix format, 2 wells per a sample). DNA/RNA concentration of samples is 1 μg ml⁻¹.

**Figure S3.** Amplification of specific sequences of the field samples nos. (a) 2, (b) 6, (c) 16, and (d) 21 on the developed real-time PCR micromatrices (see Table 3). PVX, potato virus X; PVYO and PVYntn, ordinary and necrotic forms of potato virus Y; PA, *Pectobacterium atrosepticum*.

**Table S1.** List of primers and probes (PR) used in diagnostic PCR micromatrices for the detection of potato pathogens by a multiplex real-time PCR/RT-PCR.