Rapid and simple detection of two potato cyst nematode species by real-time multiplex PCR using preserved microarray-based test systems

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Summary. Accurate diagnostics of plant pathogens is very important to prevent the spread of infection and significant yield losses. This paper presents an easy, rapid and inexpensive system for the complex real-time multiplex PCR diagnostics of two potato cyst nematode species, *Globodera rostochiensis* and *G. pallida*, using disposable long-stored stationary PCR microarrays. Laboratory trials have demonstrated an excellent diagnostic efficiency and sensitivity of the developed test systems with the DNA detection limit equal to 1 and 10 pg for *G. rostochiensis* and *G. pallida*, respectively. The specificity of the developed primers was successfully confirmed using DNA samples of related nematode species and host plant. A small reaction volume (1.2 µl) and a special technology of lyophilisation and stabilisation of PCR reagents within microreactors significantly reduce the total time of amplification (~30 min) and the number of required manipulations. Ready-to-use microarrays with flexible architecture can be stored up to 6 months at room temperature without any loss of their detection efficiency. The developed test system has a good potential as a rapid field diagnostic method, especially in the case of a low pathogen concentration in plant tissues or soil.

Key words: *Globodera rostochiensis*, *Globodera pallida*, qPCR microarrays, plant disease diagnostics.

Potato is one of the most economically important crops in the Russian Federation. According to the FAOSTAT data, Russia took second place in the world potato production after China up to 2008, when it was overtaken by India (FAOSTAT, 2017). In recent years, the annual potato production in Russia is about 31-33.5 million tons; however, the average crop harvest is only 14.5-15 t ha^{-1} (Surinov *et al.*, 2015). This is significantly lower than in Europe and North America, where the average potato harvest exceeds 40 t ha^{-1} (FAOSTAT, 2017). Such a major gap is caused by difference in the applied potato growing technologies (including potato varieties used, frequency of application of fertilisers and pesticides, *etc*.) and also in poor seed quality resulting from the infection with various pathogens, including plant-parasitic nematodes.

Potato cyst nematodes (PCN), such as *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 and *G. pallida* Stone, 1973, are serious pests of potatoes, especially in the temperate zone, causing losses of about 9% of total potato production worldwide (Turner & Subbotin, 2013). Besides yield losses, infection with such pathogens is connected with a long-term survival of their cysts in the soil that results in the exclusion of the infected field from the potato production cycle for several years (Youssef, 2013). In Russia, PCN (*G. rostochiensis*) was first found in 1948 in the Kaliningrad region (infected area 491 ha); in 1982, this pest was observed already in 29 regions with the infected area equal to 11639 ha (Vasyutin & Tryakhov, 1999). To date, *G. rostochiensis* has become common in the European part of Russia, Southern Siberia and Far East, infecting almost 1.8 million ha in 61 regions of Russia (Isaev, 2014), while the estimated corresponding yield losses in Russia exceed one billion rubles or US$17.5 million (Simakov *et al.*, 2006). *Globodera pallida*, like some other plant pathogenic nematodes, is a quarantine organism, which is common in Europe but still has not been found in Russia (Limantseva *et al.*, 2014). Due to a high volume of seed potato imported mainly from The Netherlands, where this pathogen is regularly detected, a strict and constant quarantine control of seed material is required to

prevent the appearance of *G. pallida* in the territory of Russia.

Due to their damaging potential, PCN are subjected to quarantine regulations in many countries. Obviously, an accurate detection, identification and quantification of these species are necessary for their control in seed material and soil samples. Diagnostic methods based on morphology and morphometric characteristics of cysts are laborious and time-consuming, and include a comparison of samples with typical specimens stored in maintained collections, *i.e.*, requiring highly-qualified staff (Subbotin *et al.*, 2010). DNA-based methods represent more efficient tools for rapid, sensitive and reliable nematode identification, so the development of new efficient approaches for the molecular diagnostics of PCN represents a relevant task.

In recent years, a promising technology based on the use of disposable qPCR microarrays, characterised by a long-term ambient storage and flexible architecture, was developed and successfully tested for the express diagnostics of a range of urogenital infections of humans (Suvorova *et al.*, 2012) and potato viruses (Govorov *et al.*, 2015). The purpose of this study was the development of a similar diagnostic system providing a rapid and simple simultaneous identification of two potato cyst nematodes, *G. rostochiensis* and *G. pallida*.

MATERIALS AND METHODS

Biological materials. Cysts of the golden potato nematode, *Globodera rostochiensis* (strains nos M100206-M100207), were obtained from the State Collection of Phytopathogenic Microorganisms of the All-Russian Research Institute of Phytopathology (ARRIP). Cysts of the pale potato cyst nematode, *Globodera pallida* (strain no. M100113), were obtained from the Bari Unit of the Institute for Sustainable Plant Protection (Italy). The cysts of several PCN species of different geographical origin (*Heterodera avenae*, *H. filipjevi*, *H. latipons* (all from Turkey), *H. medicaginis* (Saratov Region, Russia), *H. schachtii* (Kiev Region, Ukraine), *H. cruciferae* (Moscow Region) and *H. glycines* (Far East, Russia)), used for the specificity assessment of test systems, were provided by the ARRIP Laboratory of Diagnostics of Phytopathogenic Microorganisms. In addition, cysts of two *G. rostochiensis* pathotypes (RO1 and RO2) obtained from Italy and two *G. rostochiensis* field samples of the RO1 pathotype collected from the infested fields of the Moscow and Lipetsk regions of Russia were used to validate the specificity and robustness of the developed assay. Potato tubers ('Red Scarlett') were obtained from the ARRIP Department of Potato and Vegetable Diseases.

Cyst collection and isolation. Cysts from soil samples were collected using a paper filter method. Aliquots of slightly dried infested soil (100-150 g) were placed in 1.5 l plastic glasses, with distilled water added to form a suspension, and mixed for 1 min using a glass rod. The suspension was then left for 10-15 s to provide the precipitation of heavy sand and soil particles. Water with suspended particles and cysts was poured through a 0.16-mm sieve, and fine particles were removed from the sieve by the washing with a water jet. The remained retentate was poured into funnels with filter paper (FT55-348, FILTRAK). After the completion of the filtering process, paper filters were placed under a binocular microscope (Carl Zeiss Stemi2000C) for a hand cyst collection (Pridannikov *et al.*, 2007). Collected cysts were stored at 4°C until use.

DNA extraction. DNA extraction from *G. rostochiensis* and *G. pallida* cysts was carried out using an AmpliSens® DNA-sorb B DNA extraction kit (Russian Central Institute of Epidemiology, Russia). One cyst of each nematode species was placed into a 1.5 ml Eppendorf tube containing a small amount of DNA extraction buffer and ground with a sterile pestle. The next steps of the DNA extraction were carried out according to the manufacturer's recommendations. DNA extraction from potato tubers was carried out using a DNeasy

Table 1. Primers designed for the qPCR-based diagnostics of *Globodera rostochiensis* and *G. pallida*.

Nematode species	Primers and probe sequences (5'–3')	Annealing temp., °C	Amplicon size, bp
Globodera rostochiensis	Forward: CGATGGTTGAACTGAACA Reverse: GCTAGCTGTACTCGGCCA FAM probe: [FAM]CGGGGCGTGTGAAGAGGC[BHQ1]	56 56 71	88
Globodera pallida	Forward: CGCGGATATGCTGACAT Reverse: GCTAGCTGTACTCGGCCA FAM probe: [FAM]AGACATGCCGCTGGGTACG[BHQ1]	59 56 67	124

Plant Mini Kit (Qiagen, USA) according to the manufacturer's recommendations. Final DNA concentration was determined at 260 nm using a SmartSpec Plus spectrophotometer (BioRad, USA). The obtained DNA samples were stored at −20°C until use.

Fig. 1. AriaDNA® real-time microarray amplifier with two qPCR microarrays and a holder (top) and a standard 30-well microarray in a holder (bottom).

Primer design. For each PCN species, three primer pairs and fluorescent probes were designed on the basis of all available ITS1 region sequences of these species from GenBank (https://www.ncbi.nlm. nihgov/genbank) using an Oligo Primer Analysis software v. 6.0 (Molecular Biology Insights, Inc., USA). Since the amplification of both test systems should occur simultaneously on the same microarray, one of the key factors was the possibility to apply similar amplification parameters to provide efficient use of both primer pairs. The specificity of the developed primers was tested using the BLAST option in the NCBI database. The developed primer pairs and probes were tested in a tube format using a DTlite real-time amplifier (DNA Technology LLC, Russia) with amplification conditions described below. The best set for each nematode species was selected (Table 1).

Microarray preparation and sample application. The empty 30-cell Si/Al microarrays (Fig. 1) were prepared according to the previously described technology (Navolotskii *et al.*, 2011). The preparation of preserved microarrays with lyophilised PCR mix was performed by GenBit LLC using a technology developed independently of this study. After the microarray installation into a holder cartridge, the reaction zone was accurately covered with a sealing layer of mineral oil (620 µl) avoiding formation of bubbles. One microliter of a sample DNA or deionized water (as a negative control) was added into a microreactor under the mineral oil layer. The negative control was included in each experiment.

DNA amplification and data analysis. The real-time qPCR assays were performed using an AriaDNA® microarray amplifier (Lumex Marketing LLC, Russia). Thermal cycling conditions for DNA amplification were standard for this amplifier and included an initial denaturation step at 94°C for 120 s followed by 45 cycles at 94°C for 5 s and 60°C for 25 s. The total amplification time was 30 min. Data acquisition was on the FAM (*G. rostochiensis* or *G. pallida* DNA) and ROX (internal control) channels at the end of each 60°C step. The AriaDNA® software (Lumex-Marketing LLC, Russia) was used to generate amplification curves for each reaction and to calculate the threshold cycle number (Ct); the baseline was set in an automatic mode.

Primer and probe specificity assessment. The specificity of the assays developed for *G. rostochiensis* and *G. pallida* was evaluated using cysts of several PCN species (*G. rostochiensis*, *H. avenae*, *H. filipjevi*, *H. latipons*, *H. medicaginis*, *H. schachtii*, *H. cruciferae* and *H. glycines*), and DNA of *G. pallida* and the host plant (*Solanum tuberosum* L., 'Red Scarlett'). Two different types of preserved 30-cell microarrays with applied Master mix for either *G. rostochiensis* or *G. pallida* diagnostics were prepared. For each microarray, sample DNA of each species (1 µg ml^{-1}) was added to a separate microreactor, and the signal intensity from each microreactor was registered during the amplification. For each test system, the experiment was arranged in three repetitions.

RESULTS

Detection range determination and regression curves. To determine the detection range of the system, seven serial dilutions of DNA extracted from *G. rostochiensis* cysts (120, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng μl^{-1}) and six serial dilutions of DNA extracted from *G. pallida* cysts (150, 10, 1, 0.1, 0.01, and 0.001 ng μl^{-1}) were tested. DNA of each species was tested in two repetitions for all dilutions; all dilutions were arranged in the same microarray.

Signal intensity curves for both PCN species are shown in Fig. 2. In the case of *G. rostochiensis*, only the lowest DNA content (0.0001 ng) provided a signal intensity that did not exceed the threshold level; all other dilutions were reliably detected. The threshold cycle (Ct) values for the six DNA dilutions varied from 19.25±0.27 (120 ng) to 35.03±0.27 (0.001 ng). In the case of *G. pallida*, no distinguishable signal intensity was observed for the DNA content of 0.001 ng, while other dilutions provided signal intensity exceeding the threshold level. The Ct values varied from 23.31±0.19 (150 ng) to 32.36±1.70 (0.01 ng). Therefore, the detection limit of the developed diagnostic systems was determined to be 0.001 ng (1 pg) for *G. rostochiensis* and 0.01 ng (10 pg) for *G. pallida* that corresponds to ~10 or ~100 DNA copies, respectively.

Standard regression lines were generated using the range of DNA concentrations from 120 to 0.001 ng μl^{-1} for *G. rostochiensis* and from 150 to 0.01 ng μl^{-1} for *G. pallida* (Fig. 3A & B). A linear dynamic range of amplification was exhibited for the concentration ranges used. The calculated regression equations were the following: y = –3.150x+29.125 (*G. rostochiensis*) and y = –2.457x+28.237 (*G. pallida*).

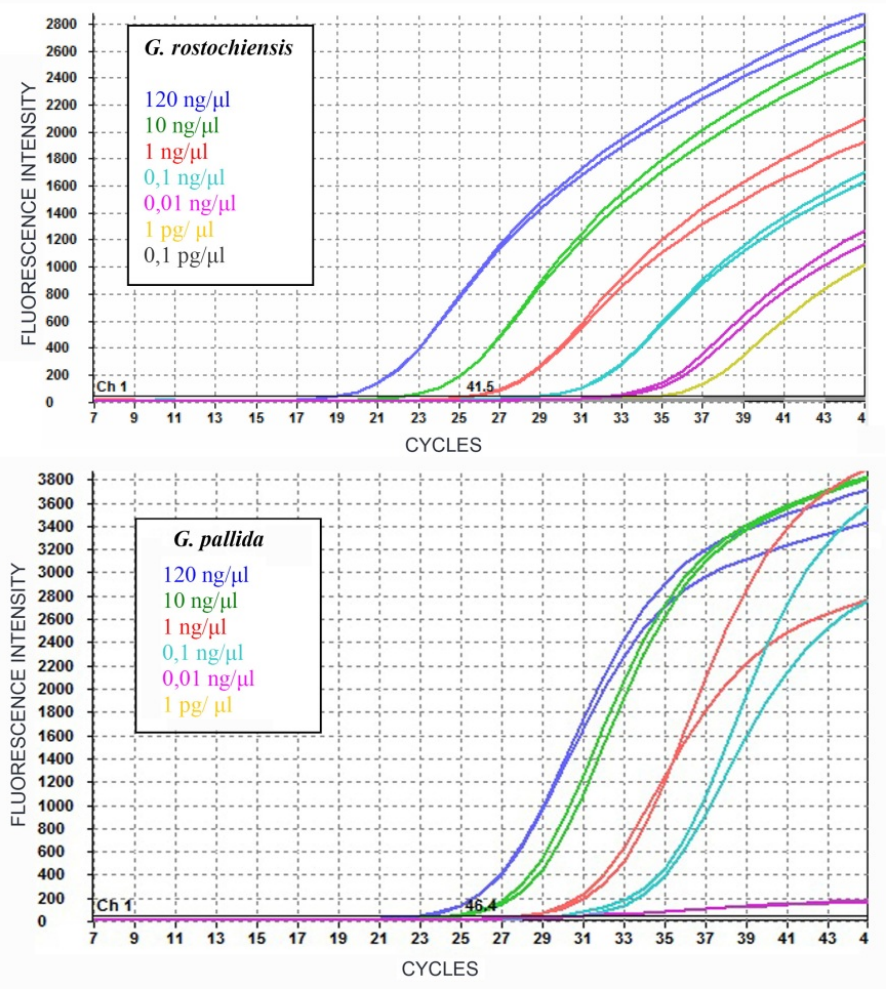

Fig. 2. Performance of the developed diagnostic systems for the serial dilution of DNA of *Globodera rostochiensis* (top) and *G. pallida* (bottom). The threshold level of detection (Ch1) is indicated by a grey line. Each concentration excepting the minimal ones is represented by two samples.

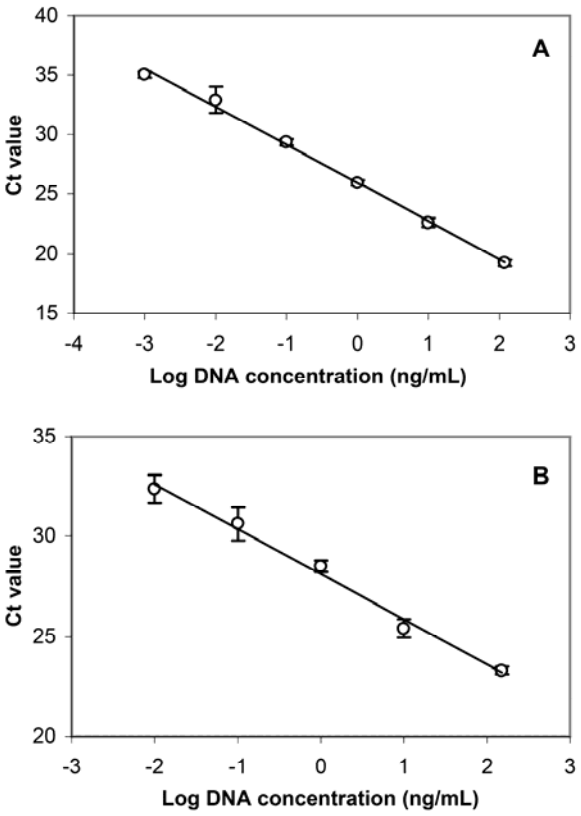

Fig. 3. Standard regression lines of a serial dilution of DNA from *Globodera rostochiensis* (A) and *G. pallida* (B). Threshold cycles (Ct) were plotted against the log of genomic DNA concentration. Regression equations and associated statistics are shown in the text.

The results of runs of the two serial dilutions of DNA of *G. rostochiensis* and *G. pallida* showed a high and moderately high efficiency (E = 96.3 and 78.4%, respectively) and a highly significant (R^2 = 0.991 and 0.982, respectively) negative correlation between the Ct value and the amount of nematode DNA over the range studied, with little variation between the replicated runs. These results demonstrate that amplifications were reproducible.

Primer and probe specificity assessment. The results of the specificity assessment of the developed test systems for two PCN species are shown in Fig. 4. In the case of *G. rostochiensis*, the corresponding test system successfully detected both RO1 and RO2 pathotypes. In both test systems, only DNA sample of a target pathogen provided sufficient signal intensity; signals from other species did not exceed the threshold level. The diagnostic specificity of the developed qPCR assays reached 100%, since there were no false positive results for DNA of other closely related pathogens and host plant.

Validation using field samples. Since *G. pallida* is a quarantine object and has not been found in Russia, there was no possibility to obtain any field samples of this pathogen. Therefore, only the assay for *G. rostochiensis* was tested using freshly collected samples from infested fields of the Moscow and Lipetsk regions. The results of the performed experiment are shown in Fig. 5. Both samples were successfully detected, confirming that the test system works satisfactorily with field samples.

Combination of the developed test systems on the same microarray. To evaluate the possibility of a simultaneous differential diagnostics of *G. rostochiensis* and *G. pallida*, both developed test systems were applied on the same qPCR microarray into different microreactors, and tested under standard amplification conditions of the AriaDNA® amplifier using DNA samples of both nematode species. The obtained results demonstrated an efficient diagnostics of both pathogens (Fig. 6).

DISCUSSION

An efficient molecular diagnostic systems intended for the detection of pathogenic microorganisms and suitable for the use in both field and stationary laboratories, should meet the following requirements. First, the cost of analysis should not be too high. Second, the applied approach should provide a rapid, sensitive and specific identification that is especially important for early diagnostics. Third, the procedure of analysis should be simple, *i.e.*, it should not require special premises and conditions and a highly-qualified personnel; ideally, the equipment should be mobile, and diagnostic kits should not require special storage and transportation conditions. Finally, the possibility of simultaneous detection of different pathogens saves time and money for the user.

In recent years a number of species-specific PCR primers based mainly on the polymorphisms in the internal transcribed spacer (ITS) and 18S regions of ribosomal DNA were successfully developed for the detection of *G. pallida*, *G. rostochiensis* and some other nematode species (Mulholland *et al.*, 1996; Bulman & Marshall, 1997; Fullaondo *et al.*, 1999; Zouhar *et al.*, 2000; Skantar *et al.*, 2007; Nega, 2014). Although being more advanced than the morphological diagnostics and providing high sensitivity and specificity, these methods still have some disadvantages, such as the risk of contamination of reaction mixture resulting in false positive or negative results, long duration of the analysis, and a complex process of the analysis and data interpretation.

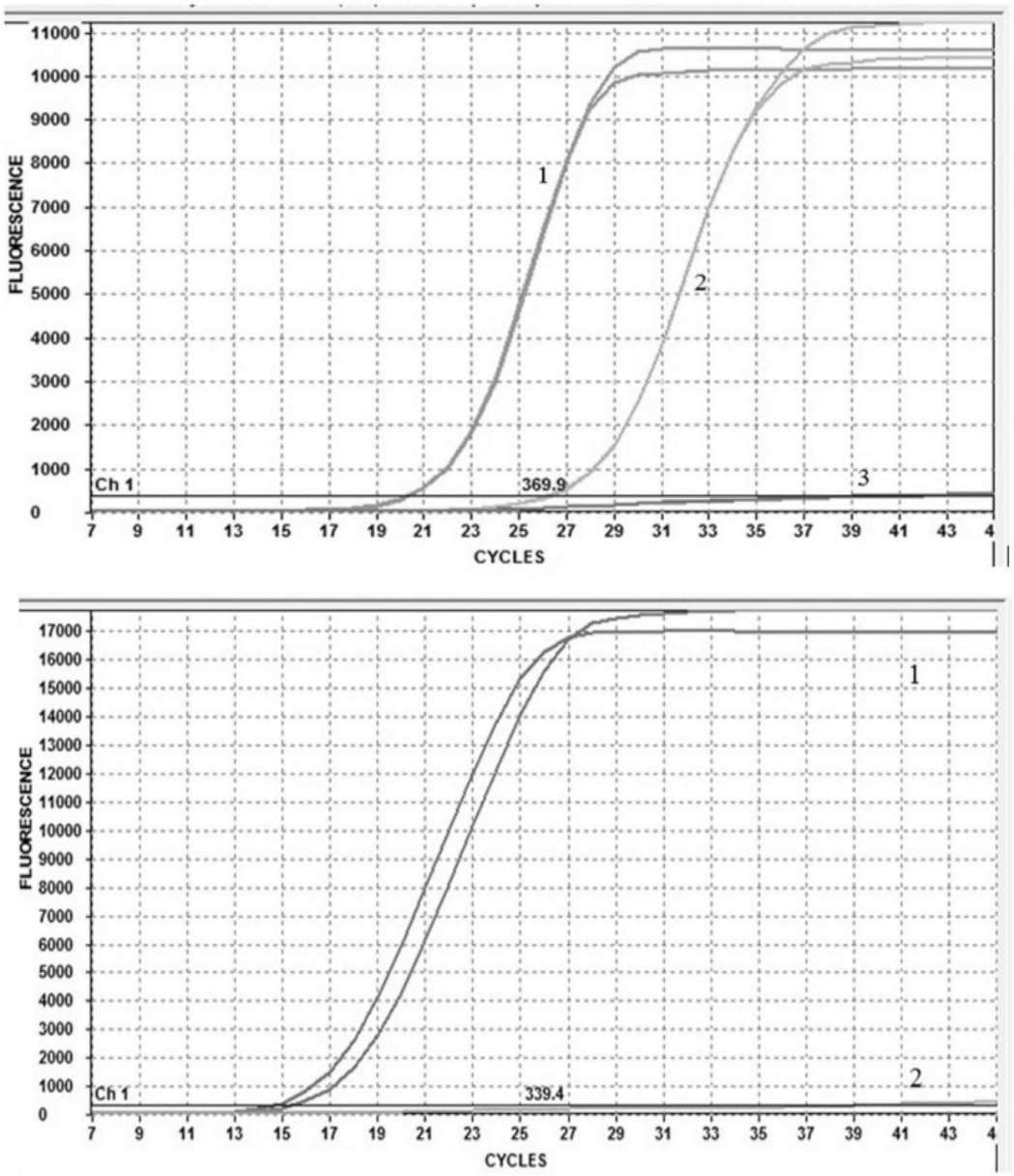

Fig. 4. (Top) Specificity assessment of the test system developed for the diagnostics of *Globodera rostochiensis*. 1 – *G. rostochiensis* RO1 (Ct = 20.37±0.37), 2 – *G. rostochiensis* RO2 (Ct = 26.34±0.23), 3 – DNA of host plant (*Solanum tuberosum*) and related PCN species *G. pallida*, *Heterodera avenae*, *H. filipjevi*, *H. latipons*, *H. medicaginis*, *H. schachtii*, *H. cruciferae* and *H. glycines* (all Ct values are below the threshold). (Bottom) Specificity assessment of the test system developed for the diagnostics of *G. pallida*. 1 – *G. pallida* (Ct = 15.25±2.32), 2 – DNA of host plant (*S. tuberosum*) and related PCN species *G. rostochiensis* (RO1 and RO2), *H. avenae*, *H. filipjevi*, *H. latipons*, *H. medicaginis*, *H. schachtii*, *H. cruciferae* and *H. glycines* (all Ct values are below the threshold). Ch1 indicates automatically calculated threshold level of detection.

The further development of PCR-based methods resulted in the appearance of a range of new approaches based on real-time PCR, FLASH (fluorescent amplification-based specific hybridisation) PCR, and multiplex PCR. These methods were also applied to PCN diagnostics (Madani *et al.*, 2005; Bačić *et al.*, 2008; Nowaczyk *et al.*, 2008; Ryazantsev *et al.*, 2009; Nakhla *et al.*, 2010). However, from the point of view of a final user, none of these methods is ideal. Real-time PCR provides the possibility to quantify the pathogen presence in a sample and does not require the stage

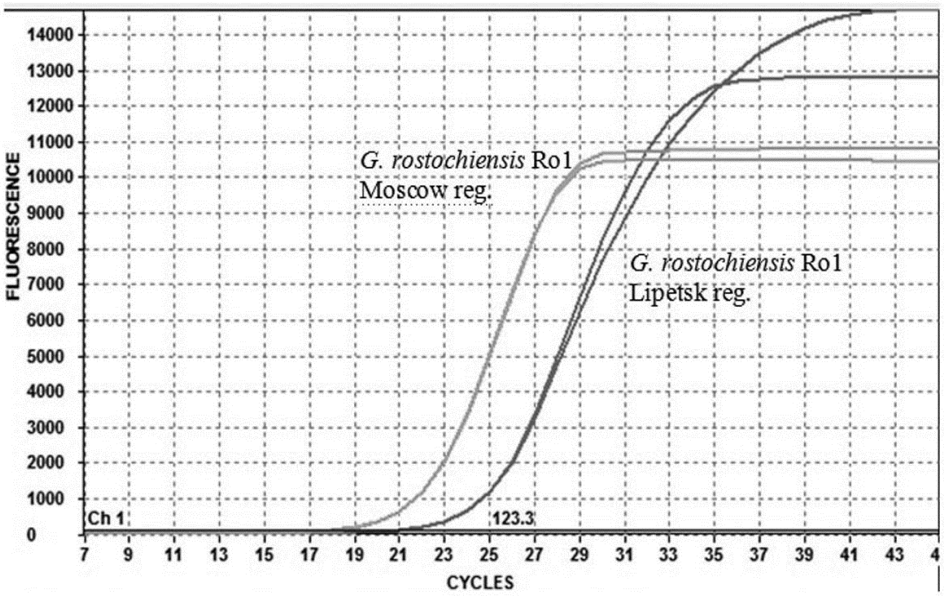

Fig. 5. Validation of the test system developed for the diagnostics of *Globodera rostochiensis* using fresh field samples collected in two regions of Russia. The final DNA concentration was ~1 µg ml^{-1}.

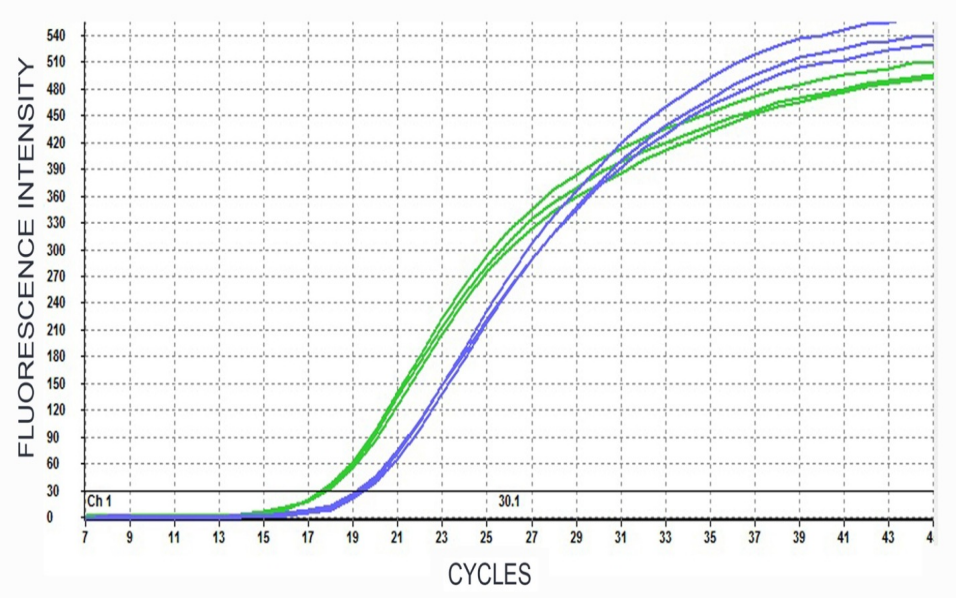

Fig. 6. Simultaneous diagnostics of *Globodera rostochiensis* (green lines) and *G. pallida* (blue lines) using a preserved qPCR microarray containing two different test systems. For each pathogen, three DNA samples (1 µg ml^{-1}) were added to individual microreactors containing the corresponding test system. The threshold level of detection (Ch1) is indicated by a grey line.

of detection of PCR products, thus reducing the total time of analysis to 2-3 h. Nevertheless, at the same time, this method requires expensive equipment, is based usually on the 'one test – one pathogen' principle, and is very sensitive to working conditions. FLASH PCR makes it possible to register the results of amplification without opening the test tubes. This reduces the risk of sample contamination, but it also requires specific equipment. Commercial kits for this diagnostic format are also based on the 'one test – one pathogen' principle. Multiplex PCR provides a

possibility of a simultaneous analysis of several pathogens in the same reaction mix that saves both time and costs. However, there is a high risk of nonspecific reactions in a complex PCR mix and there are some limitations on the number of primers used. Due to this fact, the number of pathogens that can be detected simultaneously is limited.

Thus, the existing methods of PCN diagnostics do not completely meet the above-mentioned requirements for ideal test systems. From this point of view, the proposed diagnostic system based on preserved qPCR stationary microarrays offers a number of advantages compared to the above-mentioned PCR-based methods. Use of microarrays provides a possibility of up to 30 or even more (depending on the microarray architecture) simultaneous independent PCR reactions. Small volume of microreactors and high heat conductivity of Si/Al microarrays reduces the total time of amplification to 20-30 min and the volume of required reagents that, in turn, reduces user's costs. Last but not least, a unique technology of immobilization of lyophilised components of PCR Master mix using special stabilising components provides an extremely long (up to 6 months) lifetime of preserved microarrays at a room temperature and significantly simplifies the procedure of analysis: a user should just prepare DNA samples and apply them into microreactors.

The performed study confirmed the possibility to use the proposed technology for the development of a diagnostic system for detection of two PCN species. The DNA detection limit for the developed assays was 1 pg for *G. rostochiensis* and 10 pg for *G. pallida*, which is comparable or even better than the sensitivity of other PCR-based methods for PCN detection (see, for example, Skantar *et al.*, 2007; Nowaczyk *et al.*, 2008; Nakla *et al.*, 2010; Christoforou *et al.*, 2014).

The necessity to develop original primers instead of using already validated assays, and the observed difference in the sensitivity and efficiency of two developed systems are explained by the fact that the microarray technology obviously requires the same amplification conditions for all wells. Therefore, the crucial condition for the development of primers and probes for different pathogens, which are planned to be combined on the same microarray, is that they should work under the same PCR conditions. This study represents a part of a large project intended to develop qPCR microarrays for the detection of a wide range of potato pathogens including bacteria, fungi, viruses, viroids, phytoplasmas and various nematodes. The final composition of such microarrays (excluding RNA-based pathogens, which require RT-PCR reaction and, therefore, different PCR conditions), may vary depending on the customer's demand or region of sales. Taking into account this fact, a decision has been made to use the same PCR conditions for all developed test systems, which would provide a possibility to produce diagnostic microarray systems with any combination of DNA-based potato pathogens. In this case, all developed primers should be suitable for the chosen 'standard' PCR conditions. This necessitates the development of novel compatible primers, whose sensitivity and efficiency may differ under these 'standard' PCR conditions. In the case of *G. pallida*, the developed test system provided the best possible sensitivity for the standard PCR conditions; although lower than that for *G. rostochiensis*, its sensitivity and efficiency still remained comparable with other existing assays (see above).

The specificity of the developed primers was successfully confirmed by the test with DNA and live material of related PCN species and the host plant. The test system for *G. rostochiensis* was also verified for both RO1 and RO2 pathotypes and for samples collected from two infected fields of different geographical locations. The possibility of a simultaneous detection of both pathogens using one microarray with a flexible architecture makes it possible to design preserved microarrays in accordance with the user's needs and may be useful in the case of soil infection with both pathogens, as was reported recently for Slovakia (Douda *et al.*, 2014). Therefore, taking into account the above-mentioned advantages of the qPCR microarray technology, the new test system for the diagnostics of two PCN species can be recommended for use by nematologists and plant pathologists. The further development of similar system for other plant-parasitic nematodes is planned.

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REFERENCES

BAČIĆ, J., STARE, B.G., SÍRCA, S. & UREK, G. 2008. Analyses of *Globodera rostochiensis* and *G. pallida* populations from Serbia by morphometrics and real-time PCR. *Russian Journal of Nematology* 16: 63-65.

BULMAN, S.R. & MARSHALL, J.W. 1997. Differentiation of Australian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New*

Zealand Journal of Crop Horticulture Science 25: 123-129.

CHRISTOFOROU, M., PANTELIDES, I. S., KANETIS, L., IOANNOU, N. & TSALTAS, D. 2014. Rapid detection and quantification of viable potato cyst nematodes using qPCR in combination with propidium monoazide. *Plant Pathology* 63: 1185-1192.

DOUDA, O., ZOUHAR, M., RENČO, M. & MAREK, M. 2014. Molecular and morphological exploration of a mixed population of two potato-parasiting nematode species, *Globodera rostochiensis* and *G. pallida*. *Helminthologia* 51: 3-6.

FAOSTAT. 2017. URL: http://www.fao.org /faostat/en/ #data/QC/visualize/ (accessed: April 23, 2017).

FULLAONDO, A., BARRENA, E., VIRIBAY, M., BARRENA, I., SALAZAR, A. & RITTER, E. 1999. Identification of potato cyst nematode species *Globodera rostochiensis* and *G. pallida* by PCR using specific primer combinations. *Nematology* 1: 157-163.

GOVOROV, D.N., ZHIVYKH, A.V., NOVOSELOV, E.S. & GOLIKOV, A.G. 2015. [Monitoring of bacterial and viral diseases of agricultural crops]. *Zashchita i Karantin Rastenii* 7: 35-37 (in Russian).

ISAEV, A.A. 2014. [*Reference Book on the Quarantine Phytosanitary State of the Russian Federation*]. Russia, Rosselkhoznadzor. 403 pp. (in Russian).

LIMANTSEVA, L., MIRONENKO, N., SHUVALOV, O., ANTONOVA, O., KHIUTTI, A., NOVIKOVA, L., AFANASENKO, O., SPOONER, D. & GAVRILENKO, T. 2014. Characterization of resistance to *Globodera rostochiensis* pathotype Ro1 in cultivated and wild potato species accessions from the Vavilov Institute of Plant Industry. *Plant Breeding* 133: 660-665.

MADANI, M., SUBBOTIN, S.A. & MOENS, M. 2005. Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using real-time PCR with SYBR green I dye. *Molecular and Cellular Probes* 19: 81-86.

MULHOLLAND, V., CARDE, L., O'DONNELL, K.J., FLEMING, C.C. & POWERS, T.O. 1996. Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. *BCPC Symposium Proceedings* 65: 247-252.

NAKHLA, M.K., OWENS, K.J., LI, W., WEI, G., SKANTAR, A.M. & LEVY, L. 2010. Multiplex real-time PCR assays for the identification of the potato cyst and tobacco cyst nematodes. *Plant Diseases* 94: 959-965.

NAVOLOTSKII, D.V., PERCHIK, A.V., MARK'YANOV, I.A., GANEEV, A.A. & SLYADNEV, M.N. 2011. Microchip analytic system for multiplex analysis by real-time polymerase chain reaction with reagents immobilized in microreactors. *Applied Biochemistry and Microbiology* 47: 221-227.

NEGA, A. 2014. Review on nematode molecular diagnostics: from bands to barcodes. *Journal of Biology, Agriculture and Healthcare* 4: 129-153.

NOWACZYK, K., DOBOSZ, R. KORNOBIS, S. & OBREPALSKA-STEPLOWSKA, A. 2008. TaqMan REAL-Time PCR-based approach for differentiation between *Globodera rostochiensis* (golden nematode) and *Globodera artemisiae* species. *Parasitology Research* 103: 577-581.

PRIDANNIKOV, M.V., PETELINA, G.G., PALCHUK, M.V., MASLER, E.P. & DZHAVAKHIYA, V.G. 2007. Influence of components of *Globodera rostochiensis* cysts on the *in vitro* hatch of second-stage juveniles. *Nematology* 9: 837-844.

RYAZANTSEV, D.YU., ABRAMOV, D.D. & ZAVRIEV, S.K. 2009. [Diagnostics of quarantine phytopathogens by the PCR method in the FLASH format]. *Sel'skokhozyaistvennaya Biologiya* 3: 114-117 (in Russian).

SIMAKOV, E.A., GLEZ, V.M., MANANKOV, V.V., ZHURAVLEV, A.A., ILYICHEVA, A.A. & PISARENKO, E.K. 2006. [*Methods of Assessment of Potato Cultivars for Their Resistance to Golden Nematode under Laboratory Conditions*]. Russia, Dostizheniya Nauki i Tekhniki APK. 19 pp. (in Russian).

SKANTAR, A.M., HANDOO, Z.A., CARTA, L.K. & CHITWOOD, D.J. 2007. Morphological and molecular identification of *Globodera pallida* associated with potato in Idaho. *Journal of Nematology* 39: 133-144.

SUBBOTIN, S.A., MUNDO OCAMPO, M. & BALDWIN, J.G. 2010. *Systematics of Cyst Nematodes (Nematoda: Heteroderinae). Nematology Monographs and Perspectives, 8 B*. The Netherlands, Brill. 512 pp.

SURINOV, A., BARANOV, E., BUGAKOVA, N., GELVANOVSKY, M., GOKHBERG, L., DIANOV, M., ELIZAROV, V., ZHITKOV, V., ZABELIN, V., IVANOV, YU., KEVESH, A., KOSAREV, A., LAIKAM, K., MASAKOVA, I., NESTEROV, V., OKSENOIT, G., RYBAK, O., RYABUSHKIN, B., TATARINOV, A. & KHOROSHILOV, A. 2015. [*Russia' 2015: Statistical Pocketbook*]. Russia, Rosstat. 62 pp. (in Russian).

SUVOROVA, O., PERCHIK, A., SLYADNEV, M., NAVOLOTSKII, D. & MUSHNIKOV, N. 2012. Rapid determination of sexually transmitted infections by real-time polymerase chain reaction using microchip analyzer. *Engineering* 5: 1-3.

TURNER, S.J. & SUBBOTIN, S.A. 2013. Cyst nematodes. In: *Plant Nematology* (R.N. Perry & M. Moens Eds). pp. 109-143. Wallingford, UK, CAB International.

VASYUTIN, A.S. & TRYAKHOV, A.N. 1999. [Occurrence of golden nematode in Russia]. In: *Abstracts of the 3^{d} International Nematology Symposium*. p. 92. St.-Petersburg, Russia, Zoological Institute RAS (in Russian).

YOUSSEF, M.M.A. 2013. Potato nematodes and their control measures: a review. *Archives of Phytopathology and Plant Protection* 46: 1371-1375.

ZOUHAR, M., RYSANEK, P. & KOCOVA, M. 2000. Detection and differentiation of the potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida* by PCR. *Plant Protection Science* 36: 81-84.

М.М. Никитин, Н.В. Стацюк, П.А. Французов, М.В. Приданников и А.Г. Голиков. Быстрая и эффективная диагностика двух видов цистообразующих нематод картофеля с использованием тест-систем на основе qPCR-матриц длительного хранения.

Резюме. Одним из важных средств для предотвращения распространения инфекции и существенных потерь урожая является своевременная и точная диагностика возбудителей болезней растений. В статье представлены результаты разработки тест-систем для комплексной, быстрой и эффективной диагностики двух видов картофельных цистообразующих нематод, *Globodera rostochiensis* и *G. pallida*, методом мультиплексного ПЦР в реальном времени с использованием одноразовых стационарных qPCR матриц длительного хранения. Результаты лабораторных испытаний показали высокую диагностическую эффективность и чувствительность разработанных тест-систем с пределом обнаружения на уровне 1 и 10 пг ДНК для *G. rostochiensis* и *G. pallida*, соответственно. Специфичность использованных в тест-системах праймеров была подтверждена с использованием образцов ДНК родственных видов нематод, а также растения-хозяина. Небольшой реакционный объем (1.2 мкл) и применение специальной технологии иммобилизации компонентов реакционной смеси в микрореакторах обеспечивают снижение расхода реагентов, существенное уменьшение общего времени анализа (в том числе времени амплификации до 30 мин) и упрощение процесса диагностики за счет сокращения количества необходимых манипуляций. Производимые в виде пресервов qPCR-матрицы могут храниться до 6 месяцев при комнатной температуре без потерь качества и эффективности диагностики. Разработанные тест-системы перспективны для применения в полевой экспресс-диагностике фитопатогенов, особенно в случае их низкой концентрации в почве или растительных тканях.