AGRICULTURAL BIOLOGY,
2020, V. 55, № 1, pp. 194-204
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 634.8:632.08:577.2

doi: 10.15389/agrobiology.2020.1.194eng
doi: 10.15389/agrobiology.2020.1.194rus

DEVELOPMENT OF REAL TIME PCR KIT FOR DIAGNOSTICS OF GRAPEVINE BLACK WOOD CAUSATIVE AGENT *Candidatus* Phytoplasma solani

S.A. BLINOVA[1], A.A. SHVARTSEV[1], S.V. SYKSIN[1], G.N. BONDARENKO[2], I.G. BASHKIROVA[2], S.M. GORISLAVETS[3], V.I. RISOVANNAYA[3], E.P. STRANISHEVSKAYA[3], V.A. VOLODIN[3], Ya.I. ALEKSEEV[1, 3, 4]

[1]*LLC Syntol*, 42, Timiryazevskaya ul., Moscow, 127434 Russia, e-mail Sofya.blinova@yandex.ru (✉ corresponding author), alexey.sva@yandex.ru, stason_16@inbox.ru, jalex@syntol.ru;
[2]*All-Russian Plant Quarantine Center,* 32, ul. Pogranichnaya, pos. Bykovo, Ramenskii Region, Moscow Province, 140150 Russia, e-mail reseachergm@mail.ru, bashkirovaid@mail.ru;
[3]*National Research Institute for Grape and Wine Magarach RAS,* 31, ul. Kirova, Yalta, Republic of Crimea, 298600 Russia, e-mail mgr.magarach@gmail.com, stranishevskayaelena@gmail.com, vitaliivolodin1988@gmail.com;
[4]*Institute for Analytical Instrumentation RAS*, 31-33, ul. Ivana Chernyh, St. Petersburg, 198095 Russia, e-mail jalex@syntol.ru

ORCID:
Blinova S.A. orcid.org/0000-0001-6782-8353
Shvartsev A.A. orcid.org/0000-0002-2786-9860
Syksin S.V. orcid.org/0000-0002-2753-3857
Bondarenko G.N. orcid.org/0000-0002-3826-1009
Bashkirova I.G. orcid.org/0000-0001-9014-4179
Gorislavets S.M. orcid.org/0000-0002-6749-8048
Risovannaya V.I. orcid.org/0000-0003-2208-798X
Stranishevskaya E.P. orcid.org/0000-0002-2840-5638
Volodin V.A. orcid.org/0000-000-2842-6092
Alekseev Ya.I. orcid.org/0000-0002-1696-7684

The authors declare no conflict of interests
Acknowledgements:
The studies were carried out as part of the VNNIIViV Magarach RAS State Task No. 0833-2015-0019.
Received October 16, 2019

Abstract

Today, phytoplasmas are causative agents of about three hundred different plant diseases. The greatest damage in European vineyards is due to two types of phytoplasmas, *Candidatus* Phytoplasma vitis Marzorati et al. 2006, the pathogen of flavescence dorée of grapevine, and *Candidatus* Phytoplasma solani Qualino et al., 2013, the causative agent of black wood of grapevine. Phytoplasma damage of vineyards can lead to crop losses of up to 25-30 %, and when infected up to 70 %, the vineyards should be completely uprooted. Symptoms of various phytoplasma diseases in grape are similar with each other and with viral and bacterial diseases that makes their visual differentiation to species impossible. The wide spread and high damage by phytoplasma diseases require deeper research of phytoplasma epidemiology and relevant molecular genetic diagnostic methods for monitoring phytopathogen in planting material. We have developed the first Russian kit for detection these pathogens by real-time polymerase chain reaction (Real-Time PCR) which allows effective identification of *Candidatus* Phytoplasma solani. A comparison of the developed kit with the recommended primers and probes for Real-Time PCR has shown a higher sensitivity and specificity as compared to existing diagnostic PCR systems. The goal of this work was to develop and test a kit for the detection of *Candidatus* Phytoplasma solani by the real-time polymerase chain reaction (qPCR). *Candidatus* Phytoplasma solani DNA samples and infected grape vines, roots and leaves of Chardonnay, Pinot noir and Bastardo Magarachsky varieties with visual signs of infection collected in the autumn of 2018 from the vineyards of the South Coast region of the Crimean peninsula were tested. Phytoplasma DNA was extracted as recommended by EPPO, with modifications, as well as with Cytosorb reagent kit (Syntol LLC, Russia). A pair of primers, SolaSeq_F 5′-AACTTAACCTTTTAACTAGGGC-3′ and SolaSeq_R 5′-CATCAAGGCATTTGCC-3′, was designed for *Candidatus* Phytoplasma solani DNA sequencing. To estimate the analytical sensitivity of the kit, a vector construct based on the Pal2T plasmid (Evrogen, Russia) was created with the insertion of the *Candidatus* Phytoplasma solani target 119 bp fragment of *SecY* gene. The sequence of *SecY* gene is conservative, unlike other genes recommended for diagnosis. The designed primers allow identification of all *Candidatus* Phytoplasma solani strains which sequences we found in the GenBank NCBI Nucleotide database on January 16, 2019. The developed kit was tested using various Real-Time PCR instruments. We have assessed the main characteristics of the kit, i.e. sensitivity, specificity, and reproducibility. Analytical sensitivity of the developed kit isn't less than 15 copies per PCR reaction. The analytical specificity was 100 % when tested with 37 closely related and accompanying microorgan-

isms, as well as four samples of grapes suspected to be infected by *Candidatus* Phytoplasma solani. There were no false-positive results in the analysis of other types of phytoplasmas and related microorganisms. Also, in analyzing target organism DNA samples, false-negative results were not found. The developed kit was tested on 194 samples of grapes suspected of being infected by *Candidatus* Phytoplasma solani. The specificity of *Candidatus* Phytoplasma solani detection was confirmed in all cases by DNA sequencing of positive samples. The developed kit allows rapid, accurate and high sensitive DNA identification of *Candidatus* Phytoplasma solani in plants at all stages of their vegetative development, including planting material, and can also be used for full-scale screening studies.

Keywords: phytoplasma, *Candidatus* Phytoplasma solani, grapes, real-time PCR, diagnostics, qPCR test kit, specificity, sensitivity, reproducibility, repeatability

In 1967, unicellular plant pathogens that were previously mistakenly assigned to the jaundice virus group due to the similarity of symptoms and the inability to culture on a nutrient medium [1-3] were classified as mycoplasma-like organisms due to morphological similarities and sensitivity to tetracycline antibiotics [4-6]. With the development of molecular biology methods based on the nucleotide sequencing ribosomal RNA genes [7, 8], these microorganisms were designated as a separate taxonomic group with the generic name *Candidatus* Phytoplasma [9, 10].

Phytoplasmas affect a wide range of plants, including grapes [11, 12]. Two types of phytoplasmas are most harmful for grapes, the grapevine yellow pathogen *Candidatus* Phytoplasma vitis Marzorati et al., 2006 (a quarantine object) [13, 16-18] and the black wood pathogen *Candidatus* Phytoplasma solani Qualino et al., 2013 [19-21]. The main host plant for the grapevine yellow pathogen *Candidatus* Phytoplasma vitis is a grape of the European-West Asian group, which includes almost all cultivated grape varieties. The yield loss of susceptible varieties can reach 100%, such plants die off 2-3 years after infection.

Phytoplasma *Candidatus* Phytoplasma solani also affects sugar corn, fruit crops and members of the *Solanaceae* family. Many wild-growing plants, in particular, stinging nettle and field bindweed, can be a reservoir for phytoplasmic infection [22, 23]. The distribution of phytoplasma in plants is not uniform. Phytoplasma is mainly localized in the phloem, so this part of the plant is preferable for the nucleic acid extraction and subsequent diagnosis. Some types of plants are tolerant to phytoplasma infection; therefore, when taking samples for analysis, attention should be paid to collecting diverse biomaterial (root fragments, vines, leaves) even without visual signs of infection.

Phytoplasmas lead to a yield decrease which varies from insignificant to almost complete loss [1, 12]. In grapes, the infection leads to yield losses of up to 25-30%, and at 70% infection the grape plants are completely uprooted. The prevalence of grape phytoplasmoses in the areas of cultivation can reach 70-80%, and the damage can exceed 40-80% [23].

According to EPPO (European and Mediterranean Plant Protection Organization, https://gd.eppo.int), as of November 2, 2019, *Candidatus* Phytoplasma solani is ubiquitous in Western and Eastern Europe, except for several countries in central and northern Europe [24]. Data to establish the really affected vineyards in Russia and other countries of the former USSR are insufficient [11]. For a comprehensive screening, it is necessary to develop a specific and easy-to-use kit for identification of *Candidatus* Phytoplasma solani.

For the first time in Russia, we developed a qPCR-based test system for detecting the *Candidatus* Phytoplasma solani and compared its quality with existing diagnostic systems for the main characteristics (specificity, sensitivity, reproducibility, repeatability).

Our goal was to develop and test a kit for detection of *Candidatus* Phytoplasma solani, the causal agent of the black wood of grapevine with real-time polymerase chain reaction (qPCR).

Materials and methods. *Candidatus* Phytoplasma solani DNA samples were received from the collection of the All-Russian Plant Quarantine Center (VNIIKR)-and contaminated grape material was provided by the Magarach All-Russian National Research Institute of Viticulture and Winemaking (VNNIIIV Magarach). Fragments of the vine, roots and leaves of Chardonnay, Pinot noir and Bastardo Magarachsky varieties with visual signs of *Candidatus* Phytoplasma infection were collected in the autumn of 2018 from the vineyards of the South Coast agroclimatic region of the Crimean Peninsula.

Samples for DNA extraction were ground manually and using a Precellys Evolution rotational homogenizer (Bertin Technologies, France). Phytoplasmic DNA was extracted according to EPPO recommendation [24] with modifications [25], and also with Cytosorb reagent kit (Syntol LLC, Russia) which provides cell lysis with a chaotropic agent guanidine hydrochloride followed by DNA sorption on silicon particles as described in patent application RU No. 2019111081/10 (021521).

Conventional PCR and qPCR were performed in the reaction buffer (3 mM $MgCl_2$, 0.25 mM dNTP, 2.5 units of SynTaq DNA polymerase with antibodies inhibiting enzyme activity) (Syntol LLC, Russia). The selected primers should meet the following criteria: G or C nucleotide at the 3′-end ("GC clamp"), the average annealing temperature 65 °C for primers, 2-5 °C higher for a probe. The presence or absence of secondary structures and the annealing temperature were checked via online Oligo Calc primer analysis software: Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html), ThermoFisher Multiple Primer Analyzer (https://www.thermo-fisher.com), Promega Biomath Calculators (5×PCR Buffer, 3 mM $MgCl_2$) (https://worldwide.promega.com/resources/tools/biomath/tm-calculator/). Fluorescent labels in qPCR were 6FAM and 6ROX dyes attached to the 5′-end of the probe. Fluorescence dampers were dyes RTQ1 and BHQ2 at the 3′-end of the probe. The concentration of primers and probes in the reaction mixture was 450 nM and 200 nM, respectively. qPCR was performed using four devices, ANK-32 (IAI RAS, Russia) [26], CFX-96 (Bio-Rad, USA), Rotor-Gene 6000 (QiaGen, USA), and DTprime 5 M1 (DNA Technology, Russia), and the following amplification protocol: 5 min at 95 °C; 15 s at 95 °C, 40 s at 60 °C (50 cycles). Results were deemed positive if the fluorescence signal exceeded the threshold which was set as a 10% difference between the moduli of the highest and lowest fluorescence).

A pair of primers SolaSeq_F 5′-AACTTAACCTTTTTAACTAGGGC-3′ and SolaSeq_R 5′-CATCAAGGCATTTGCC-3′ was designed for sequencing DNA of *Candidatus* Phytoplasma solani. Amplification program for conventional PCR was as follows: 5 min at 95 °C; 20 s at 95 °C, 20 s at 60 °C, 1 min at 72 °C (36 cycles); 5 min at 72 °C. DNA sequencing was performed on a Nanophor 05 instrument (Institute for Analytical Instrumentation RAS, Russia). The results were analyzed using DNA analysis software version 5.0.3.2 (Institute for Analytical Instrumentation RAS, Russia).

Bioinformatic analysis and data processing were performed using the software UGENE (UNIPRO, Russia) and AliView (NBIS, Department of Cell and Molecular Biology, Uppsala University, Sweden). The search for target DNA sequences was carried out in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) [27].

Target DNA in developing primers and probe was *SecY* gene with 119 bp specific fragment, positions from 513 to 632 bp (KU600099.1, NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) length) [27].

To determine the analytical sensitivity of the reagent kit, a vector con-

struct based on the Pal2T plasmid (Evrogen, Russia) was created with the insertion of the 119 bp target nucleotide sequence of *Candidatus* Phytoplasma solani. Ligation was carried out for 24 hours at 4 °C using T4 DNA ligase (ThermoFisher Scientific, USA). The transformation of *Escherichia coli* (Migula 1895) was done using heat shock method. The presence of the vector was checked by PCR colony method with standard M13 primers. Plasmid was isolated using a PlasGen kit (Syntol LLC, Russia). To increase the efficiency of PCR, the plasmid was cleaved with NotI restriction endonuclease (ThermoFisher Scientific, USA). The concentration of plasmid DNA was measured on a Quantus fluorimeter (Promega Corporation, USA) in triplicate. To determine the sensitivity of the developed kit, qPCR was run in 2-fold repetition with a series of 10-fold dilutions with a known plasmid concentration [28].

Efficiency of the kit was tested on 37 samples of closely relative and accompanying objects: *Vitis vinifera*, *Xanthomonas campestris*, *Cercospora beticola*, *Fusarium culmorum*, *Fusarium* sp., *Fusarium tricinum*, *Gibellina cerealis*, *Botrytis cinerea*, *Pleospora betae*, *Phomopsis helianthi*, *Pseudomonas fluorescens*, *Alternaria tenuissima, Aspergillus niger* (collection of Syntol LLC, Russia); *Candidatus* Phytoplasma vitis (20.9 Ct), *Candidatus* Phytoplasma rubi (18.9 Ct), *Candidatus* Phytoplasma pyri (21.4 Ct), *Candidatus* Phytoplasma mali (18.4 Ct), *Candidatus* Phytoplasma convolvuli (15.9 Ct), *Candidatus* Phytoplasma asteris (15.0 Ct), *Candidatus* Phytoplasma cirsii (33.5 Ct), *Candidatus* Phytoplasma taraxacum (14.0 Ct), *Candidatus* Liberibacter solanacearum (23.6 Ct), *Xanthomonas oryzae* 0227, *Cercospora kikuchii* (VNIIKR collection); *Xanthomonas euvesicatoria* DSM 19128, *Xanthomonas gardneri* DSM 19127, *Xanthomonas perforans* DSM 18975, *Xanthomonas vesicatoria* DSM 22252, *Xanthomonas translucens* pv. *translucens* DSM 18974 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany); *Xanthomonas phaseoli* CFBP 2534 (CIRM-CFBP, France); *Fusarium sambucinum* F139, *Fusarium graminearum* F877, *Fusarium verticillioides* F43, *Fusarium avenaceum* F623 (All-Russian collection of industrial microorganisms, State Research Institute of Genetics and Selection of Industrial Microorganisms of the National Research Center Kurchatov Institute, Moscow); *Fusarium oxysporum* F840, *Phytophthora cinnamomi* F3332, F3333, *Phytophthora cactorum* 985 (All-Russian collection of microorganisms, Skryabin Institute of Biochemistry and Physiology of Microorganisms (IBPM) RAS, Pushchino, Moscow Province).

In qPCR, grape, bacteria, and fungi DNA concentration was 50 ng per reaction. Threshold cycle of *Candidatus* Phytoplasma sp. was determined with UniRT primers [13]. For *Candidatus* Liberibacter solanacearum, primers recommended by EPPO [14] were used.

Results. Table 1 shows primers and probes published by E. Angelini with co-workers and C. Pelletier with co-workers and currently used [1, 11, 16, 29, 30], as well as the primers and probe we developed for qPCR (*BN*).

1. Primers and probes used to identify *Candidatus* Phytoplasma solani with their corresponding amplification protocols

Name, reference	Target	Amplification mode	Sequence
BNrt [16]	*16S rRNA*	5 min at 95 °C; 15 s at 95 °C, 40 s at 60 °C (50 cycles)	F 5′-GGTTAAGTCCCGCAACGAG-3′ R 5′-CCCACCTTCCTCCAATTATCA-3′ Pb 5′-(6FAM)AACCCTTGTTGTTAATTGCCATCATTAAG(RTQ1)-3′
mapBN [29]	*adk*	5 min at 95 °C; 15 s at 95 °C, 20 s at 50 °C, 20 s at 72 °C (50 cycles)	F 5′-ATTTGATGAAACACGCTGGATTAA-3′ R 5′-TCCCTGGAACAATAAAAGTYGCA-3′ Pb 5′-(6ROX)AAACCCACAAAATGC(BHQ2)-3′

Continued Table 1

BN (own data)	*SecY*	5 min at 95 °C; 15 s at 95 °C, 40 s at 60 °C (50 cycles)	F 5′-AATACCAGTACAATACGCTCGC-3′ R 5′-AAAGGTTGCATCAAGGCATTTGC-3′ Pb 5′-(6FAM)AACACTGCTGGAGTAATGCCTGTAATT(RTQ1)-3′

N o t e. F — forward primer, R — reverse primer, Pb — probe.

To compare the analytical sensitivity of recommended systems and that developed by us, qPCR was run with a series of 10-fold dilutions of *Candidatus* Phytoplasma solani DNA. Although the *BNrt* primers were not inferior in the threshold cycle to that we developed, a 1:100 dilution did not give a positive result, which indicates a lower sensitivity of *BNrt* compared to *BN*. The fluorescence signal of positive samples with *BN* and *BNrt* was 1,500 and 500 relative fluorescence units (RFU), respectively. In both cases, the concentration of primers in the reaction mixture was 450 nM and the probe concentration was 200 nM. The difference in threshold cycles of *mapBN* with *BNrt* and *BN* was +3.0-3.5 Ct. In addition to the lower sensitivity of the *mapBN* system, a 1:100 dilution showed a complete absence of fluorescence signal growth (Fig. 1).

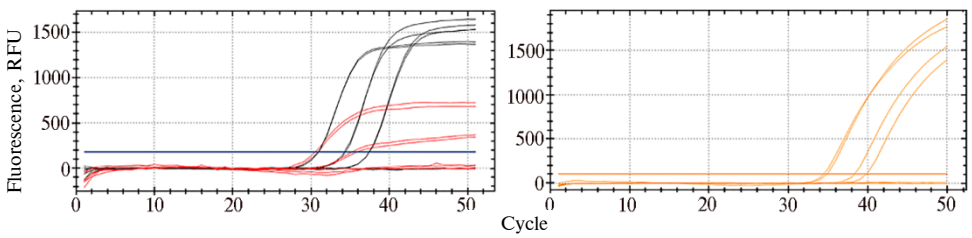

Fig. 1. Real-time polymerase chain reaction (qPCR) with a series of three 10-fold dilutions of *Candidatus* Phytoplasma solani DNA and different primers and probes: *BN* — black, *BNrt* — red, *mapBN* — orange (RFU — relative fluorescence units, a CFX-96 device, Bio-Rad, USA).

To estimate PCR specificity with *BNrt* primers, we used AliView software to align target fragments corresponding to primers and a probe for qPCR detection of *Candidatus* Phytoplasma solani. More than 100 DNA fragments were found in the NCBI database, 69 of which were of *Candidatus* Phytoplasma solani. Their identity with the target fragment for *BNrt* primers [16] ranged from 98 to 100%. Alignment showed a 100% coincidence of sequences of the primer and probe binding sites. This system, in addition to *Candidatus* Phytoplasma solani, also detected other 16SrXII phytoplasmas that cause witches' broom and potato purple top diseases [31, 32] on potatoes. In accordance with bioinformatic analysis, the *BNrt* system of primers and a probe for qPCR did not provide high specificity for the Candidatus Phytoplasma solani DNA detection.

The target gene for *mapBN* primers is *adk*. Sequence alignment showed a high specificity for attaching primers and a probe; however, only 10 sequences of this gene were found in the GenBank NCBI database (http://www.ncbi.nlm.nih.gov/BLAST), 7 of which were attributed to *Candidatus* Phytoplasma solani. Due to the small sample, *mapBN* primers were also rejected by us as candidates for use in the kit under development.

As a result, we chose the *SecY* gene, which encodes a protein involved in transmembrane transport, as a target to assess specificity of the of *Candidatus* Phytoplasma solani DNA detection. This gene is recommended by EPPO for the diagnosis of phytoplasma of wood blackening [24]. The *SecY* sequence analysis in the GenBank NCBI revealed 190 sequences, including 110 attributed to *Candidatus* Phytoplasma solani strains. The binding sites of the primers and a probe for qPCR we developed (*BN*) turned out to be strictly specific and allowed us to

detect all strains of *Candidatus* Phytoplasma solani, the DNA sequences of which were in the GenBank NCBI Nucleotide database on 01/16/2019 [27].

The results of DNA sequencing six grape samples, presumably infected by *Candidatus* Phytoplasma solani, carried out with primers to *SecY* gene to confirm the species affiliation of *Candidatus* Phytoplasma solani are presented in Figure 2.

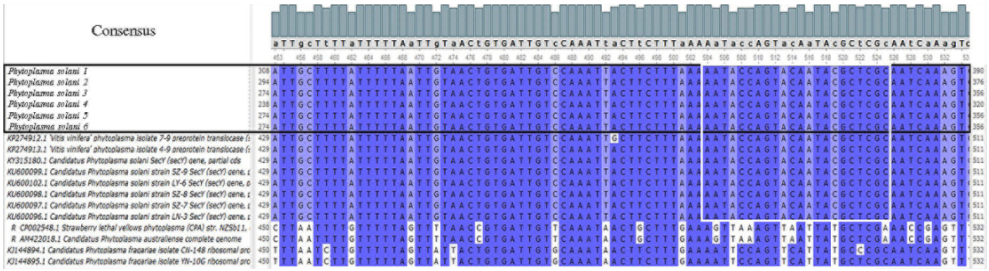

Fig. 2. Alignment of *Candidatus* Phytoplasma solani DNA sequences from the GenBank NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) with those from DNA sequencing of infected grape samples with primers to *SecY* gene. Alignments were generated in the Unipro UGENE program (Russia) version 1.31.1, the attachment zone for the forward primer *BN_F* is highlighted with a white frame. Phytoplasma solani 1-6 — samples collected in six different habitats. For a complete figure, see http://www.agrobiology.ru.

The analytical specificity of the reagent kit was assessed on 37 samples of closely related and accompanied organisms and six grape samples, presumably infected by *Candidatus* Phytoplasma solani according to visual signs. In determining the analytical specificity, positive results were obtained for all samples containing *Candidatus* Phytoplasma solani DNA, which was confirmed by DNA sequencing using *SolaSeq_F* and *So-laSeq_R* primers. We did not reveal false positive results with DNA of other phytoplasmas, including *Candidatus* phytoplasma convolvuli belonging, like *Candidatus* Phytoplasma solani, to the 16SrXII group, as well as with any associated organisms. The analytical specificity of the qPCR primers and probe to *SecY* gene for studied 37 samples was 100%.

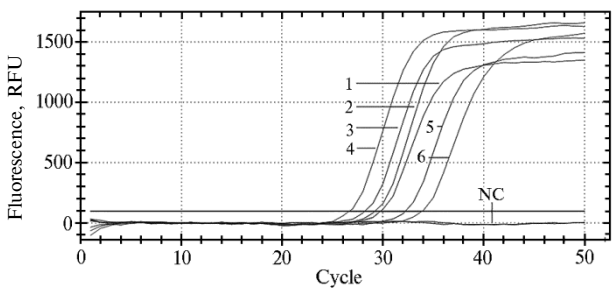

Fig. 3. Detecting *Candidatus* Phytoplasma solani DNA in six grape samples (1-6) **by real-time polymerase chain reaction (qPCR) with primers and probe to *SecY* gene** (NC — negative control; FAM detection channel, RFU — relative fluorescence units, a CFX-96 device, Bio-Rad, USA).

The analytical sensitivity of the reagent kit was evaluated by diluting plasmid DNA with the target insert of *Candidatus* Phytoplasma solani DNA. The initial plasmid concentration was 2×10^7 copies/μl. In PCR running, each reaction was duplicated. The calculated qPCR efficiency for a series of six 10-fold dilutions of the plasmid, from 10^7 to 10^2 copies per reaction, was E = 98.7%, with the slope of the kinetic curve A = –3.355, and the correlation coefficient $R^2 = 0.999$. Starting with the number of 50 copies of the plasmid per reaction, qPCR was performed in 24 replicates at dilutions of 50, 20, 15, 10, 5, and 1 plasmid copy per reaction. When diluting up to 10 copies per reaction, the result was unstable, and when diluting up to 1 copy of the plasmid per, a specific qPCR signal was not observed. The sensitivity of the reagent kit was established if at least 95% of the reactions gave a positive result. So the analytical sensitivity

of the reagent kit with *BN* primers was at least 15 copies per reaction

To test the developed kit for detecting *Candidatus* Phytoplasma solani DNA, we analyzed 194 grape samples from six territories (farms of the South-coastal agroclimatic region of the Crimean Peninsula). All tested samples yielded positive results for *Candidatus* Phytoplasma solani phytoplasm DNA (Fig. 3). DNA sequencing confirmed the obtained data.

The results for the developed system were confirmed with four devices for qPCR (Table 2). The qPCR efficacy was E = 97-100%, the slope of the kinetic curve was A = 3.31-3.39, the correlation coefficient R^2 = 1.000. Differences in threshold values averaged ±2 cycles, which is associated with a difference in the structure of optical modules, the heating and cooling rates of reactors, individual software for each device, and an algorithm for determining threshold cycle values.

To design primers and probe, we have chosen the *SecY* gene, encoding a translocase protein subunit which is characterized by high conservatism and specificity. This ensures differentiation of *Candidatus* Phytoplasma solani from closely related species. According to Davies et al. [9] and Lee et al. [10], this gene is the best marker to differentiate and classify strains of *Candidatus* Phytoplasma solani species. Target genes in existing and currently used diagnostic systems are the *16S rRNA* and *adk* gene regions [15]. The ribosomal RNA gene is inferior in specificity to both the gene encoding adenosine kinase (*adk*) and *SecY*. The analytical sensitivity of the kit we developed was at least 15 copies per reaction that is 10 times higher compared to the recommended diagnostic systems.

2. Reproducibility of qPCR results with primers and probe to *SecY* gene of *Candidatus* Phytoplasma solani on devices of different manufacturers

Samples, 10-fold dilutions	Threshold cycle, Ct			
	CFX96 (Bio-Rad, USA)	RG 6000 (QiaGen, USA)	DTprime 5 M1 (DNA Technology, Russia)	AHK 32 (IAI RAS, Russia)
10^{-1}	18.61	18.37	16.2	19.21
10^{-1}	18.73	18.26	16.2	19.27
10^{-2}	22.05	21.57	19.3	22.61
10^{-2}	21.94	21.72	19.3	22.75
10^{-3}	25.51	24.98	23.0	25.77
10^{-3}	25.45	24.92	22.8	26.05
10^{-4}	28.75	28.37	26.0	29.24
10^{-4}	28.66	28.18	26.1	29.10
10^{-5}	32.05	31.34	29.4	32.53
10^{-5}	32.46	31.46	29.1	32.57
Negative control	N/A	N/A	N/A	N/A
Negative control	N/A	N/A	N/A	N/A
Sloope, A	3.39	3.32	3.32	3.31
R^2	1.000	1.000	1.000	1.000
Effectiveness, E	97	100	100	100

N o t e. N/A means not available.

Successful testing of the proposed kit in qPCR with four devices of both Russian and foreign manufacturers allows us to recommend this system for diagnostic and screening in research laboratories.

Thus, a kit has been developed for qPCR detection of *SecY* gene fragment of *Candidatus* Phytoplasma solani, the causal agent of the black wood of grapevine. Analysis of field samples (leaves, roots and fragments of grapevine) which were presumably infected by *Candidatus* Phytoplasma solani with the proposed kit revealed DNA of this pathogen in all samples. qPCR data were confirmed by DNA sequencing. The developed qPCR system has a high sensitivity and specificity and is suitable for epiphytotic studies and evaluation of varietal resistance to this pathogen.

REFERENCES

1. Matyashova G.N., Zaets V.G. *Vestnik Rossiiskogo universiteta druzhby narodov. Seriya: Agronomiya i zhivotnovodstvo*, 2015, 4: 7-14 (in Russ.).
2. Risovannaya V.I., Gorislavets S.M., Volodin V.A. *Magarach. Vinogradarstvo i vinodelie*, 2013, 4: 6-8 (in Russ.).
3. Doi Y., Teranaka M., Yora K., Asuyama H. Mycoplasma- or PLT group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or Paulownia witches' broom. *Annals Phytopathological Society*, 1967, 33(4): 259-266 (doi: 10.3186/jjphytopath.33.259).
4. Duduk B., Bertaccini A. Phytoplasma classification: taxonomy based on 16S ribosomal gene, is it enough? *Phytopathogenic Mollicutes*, 2011, 1(1): 3-13 (doi: 10.5958/j.2249-4669.1.1.001).
5. Deng S., Hiruki C. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *Journal of Microbiological Methods*, 1991, 14(1): 53-61 (doi: 10.1016/0167-7012(91)90007-D).
6. Lee I.M., Davis R.E., Hiruki C. Genetic interrelatedness among clover proliferation mycoplasma-like organisms (MLOs) and other MLOs investigated by nucleic acid hybridization and restriction fragment length polymorphism analyses. *Applied and Environmental Microbiology*, 1991, 57(12): 3565-3569.
7. Duduk B., Perić P., Marčić D., Drobnjaković T., Picciau L., Alma A., Bertaccini A. Phytoplasmas in carrots: disease and potential vectors in Serbia. *Bulletin of Insectology*, 2008, 61(2): 327-331.
8. The IRPCM Phytoplasma/Spiroplasma Working Team — Phytoplasma taxonomy group. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *Internation Journal of Systematic and Evolutionary Microbiology*, 2004, 54(4): 1243-1255 (doi: 10.1099/ijs.0.02854-0).
9. Davies D.L., Lee I.-M. Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. *Phytopathology*, 1993, 83(7): 1008-1011 (doi: 10.1094/Phyto-83-1008).
10. Lee I.-M., Gundersen-Rindal D.E., Davis R.E., Bartoszyk I.M. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Internation Journal of Systematic and Evolutionary Microbiology*, 1998, 48(4): 1153-1169 (doi: 10.1099/00207713-48-4-1153).
11. Matyashova G.N., Zaets V.G. *Vestnik Rossiiskogo universiteta druzhby narodov. Seriya: Agronomiya i zhivotnovodstvo*, 2015, 4: 7-14 (in Russ.).
12. PM 7/133 (1) Generic detection of phytoplasmas. *EPPO Bulletin*, 2018, 48(3): 414-424 (doi: 10.1111/epp.12541).
13. Hren M., Boben L., Rotter A., Kralj P., Gruden K., Ravnikar M. Real-time PCR detection systems for Flavescence dorée and Bois noir phytoplasmas in grapevine: comparison with conventional PCR detection and application in diagnostics. *Plant Pathology*, 2007, 56(5): 785-796 (doi: 10.1111/j.1365-3059.2007.01688.x).
14. Li W., Hartung J.S., Levy L. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. *Journal of Microbiological Methods*, 2006, 66(1): 104-115 (doi: 10.1016/j.mimet.2005.10.018).
15. PM 7/079 (2) Grapevine flavescence dorée phytoplasma *EPPO Bulletin*, 2016, 46(1): 78-93 (doi: 10.1111/epp.12280).
16. Angelini E., Bianchi G.L., Filippin L., Morassutti C., Borgo M. A new TaqMan method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. *Journal of Microbiological Methods*, 2007, 68(3): 613-622 (doi: 10.1016/j.mimet.2006.11.015).
17. Berger J., Via J.D., Baric S. Development of a TaqMan allelic discrimination assay for the distinction of two major subtypes of the grapevine yellows phytoplasma Bois noir. *European Journal of Plant Pathology*, 2009, 124(3): 521-526 (doi: 10.1007/s10658-008-9424-9).
18. *Edinyi perechen' karantinnykh ob"ektov Evraziiskogo ekonomicheskogo soyuza*. S izmeneniyami i dopolneniyami ot 30 marta 2018 g. (izmeneniya ot 2 maya 2018 g. — Reshenie Soveta Evraziiskoi ekonomicheskoi komissii ot 30 marta 2018 g. N 25) [Single list of quarantine facilities of the Eurasian Economic Union. With amendments and additions of March 30, 2018 (changes of May 2, 2018 — Decision of the Council of the Eurasian Economic Commission of March 30, 2018 N 25)]. Available: https://vniikr.ru/edinyij-perechen-karantinnyix-obektov-evrazijskogo-ekonomicheskogo-soyuza. Accessed: 14.04.2019 (in Russ.).
19. Choueiri E., Jreijiri F., El Zammar S., Verdin E., Salar P., Danet J.L., Bové J., Garnier M. First report of grapevine "Bois Noir" disease and a new phytoplasma infecting solanaceous plants in Lebanon. *Plant Disease,* 2002, 86(6): 697-697 (doi: 10.1094/PDIS.2002.86.6.697A).
20. Franova J. Difficulties with conventional phytoplasma diagnostic using PCR/PFLP analysis. *Bulletin of Insectology*, 2011, 64(Suppl.): S287-S288.
21. Galetto L., Bosco D., Merzachi C. Universal and group-specific real-time PCR diagnosis of

flavescence dorée (16Sr-V), bois noir (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant hosts and insect vectors. *Annals of Applied Biology*, 2005, 147(2): 191-201 (doi: 10.1111/j.1744-7348.2005.00030.x).
22. Plavec J., Križanac I., Budinšćak Ž., Škorić D., Music M.S. A case study of FD and BN phytoplasma variability in Croatia: multigene sequence analysis approach. *European Journal of Plant Pathology*, 2015, 142(3): 591-601 (doi: 10.1007/s10658-015-0637-4).
23. Bogoutdinov D.Z., Kastal'eva T.B., Girsova N.V. *Tavricheskii vestnik agrarnoi nauki*, 2018, 2(14): 15-34 (doi: 10.25637/TVAN.2018.02.02) (in Russ.).
24. *European and Mediterranean Plant Protection Organization*. Available: https://www.eppo.int. Accessed: 11.02.2019 (in Russ.).
25. Doyle J.J., Doyle J.L. Isolation of plant DNA from fresh tissue. *Focus,* 1990, 12: 13-15.
26. Alekseev YA.I., Belov Yu.V., Varlamov D.A., Konovalov S.V., Kurochkin V.E., Marakushin N.F., Petrov A.I., Petryakov A.O., Rumyantsev D.A., Skoblilov E.Yu., Sokolov V.N., Fesenko V.A., Chernyshev A.V. *Nauchnoe priborostroenie*, 2006, 16(3): 132-136 (in Russ.).
27. *National Center for Biotechnology Information*. Available: http://www.ncbi.nlm.nih.gov. Accessed: 16.01.2019.
28. Alyapkina Yu.S., Moiseeva M.V., Ksenofontova O.V., Alekseev Ya.I. *Izvestiya TSKHA*, 2008, 3: 5-16 (doi: 10.26897/0021-342X-2018-3-5-16) (in Russ.).
29. Pelletier C., Salar P., Gillet J., Cloquemin G., Very P., Foissac X., Malembic-Maher S. Triplex real-time PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control. *Vitis*, 2009, 48(2): 87-95.
30. Quaglino F., Zhao Y., Casati P., Bulgari D., Bianco P.A., Wei W., Davis R.E. '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur- and bois noir- related diseases of plants. *International Journal of Systematic and Evolutionary Microbiology*, 2013, 63: 2879-2894 (doi: 10.1099/ijs.0.044750-0).
31. Yu X.M., Ai C.X., Wang J., Fu L., An M., Wang H.R., Sun Q.R. First report of a 16SrXII-A subgroup phytoplasma associated with *Artemisia scoparia* witches'-broom disease in China. *Plant Disease*, 2016, 100(7): 1494 (doi: 10.1094/PDIS-11-15-1251-PDN).
32. Girsova N.V., Bottner K.D., Mozhaeva K.A., Kastalyeava T.B., Owens R.A., Lee I.M. Molecular detection and identification of Group 16SrI and 16SrXII phytoplasmas associated with diseased potatoes in Russia. *Plant Disease*, 2008, 92(4): 654 (doi: 10.1094/PDIS-92-4-0654A).