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Original Article

Development of a multiplex real-time PCR method for the detection of *Pseudomonas savastanoi* pv. *glycinea* and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in soybean seeds

Desenvolvimento de método de PCR multiplex em tempo real para a detecção de *Pseudomonas savastanoi* pv. *glycinea* e *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, em sementes de soja

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Abstract

Multiplex real-time PCR with TaqMan® probes has been developed for the simultaneous detection of soybean pathogens *Pseudomonas savastanoi* pv. *glycinea* and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. The method specificity has been confirmed using 25 strains of target bacteria and 18 strains of other bacteria common to soybean seeds as endophytes. The multiplex real-time PCR developed has been shown to have high sensitivity – a positive result was achieved at 0.01 ng/µl of DNA for both target organisms, and at 100 CFU/ml of bacteria in soybean seed homogenate. The robustness of the multiplex real-time PCR developed has been verified by the detection of the pathogens in 25 commercial seed stocks, in comparison with previously published PCR protocols. In all tests, three seed stocks were positive and 22 were negative. The multiplex real-time PCR can be applied in diagnostic practice for the simultaneous detection of two important pathogens of leguminous plants.

Keywords: *Pseudomonas savastanoi*, *Curtobacterium flaccumfaciens*, PCR, soybean, bacterial diseases.

Resumo

A PCR Multiplex em tempo real com sondas TaqMan® foi desenvolvida para a detecção simultânea de patógenos da soja *Pseudomonas savastanoi* pv. *glycinea* e *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. A especificidade do método foi confirmada utilizando 25 cepas de bactérias alvo e 18 cepas de outras bactérias comuns a sementes de soja como endófitas. A PCR multiplex em tempo real desenvolvida demonstrou alta sensibilidade, uma vez que, um resultado positivo foi alcançado a 0,01 ng/µl de DNA para ambos os organismos – alvo e a 100 ufc/ml de bactérias em homogeneizado de sementes de soja. A robustez da PCR multiplex em tempo real desenvolvida foi verificada pela detecção dos patógenos em 25 estoques comerciais de sementes, em comparação com protocolos de PCR publicados anteriormente. Em todos os testes, 3 estoques de sementes foram positivos e 22 negativos. Dessa maneira, é possível afirmar que a PCR multiplex em tempo real pode ser aplicada em diagnóstico.

Palavras-chave: *Pseudomonas savastanoi, Curtobacterium flaccumfaciens*, PCR, soja, doenças bacterianas.

1. Introduction

Soybean (*Glycine max* (L.) Merr.) is a highly important staple for oilseed, and as a forage crop, throughout the World. The total harvest of soybean in Russia reached 6.3 million tons in 2022, which was 22.6% more than in 2021 (Russia, 2023). Soybean diseases significantly reduce the potential yield of the crop, and decrease the quality of the beans, oil and proteins. Globally, average financial losses due to soybean diseases amount to about 10% of the crop price (Bradley et al., 2021). The economic harm of bacterial diseases increases when seed infection above

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an epidemic threshold combines with weather conditions favourable for the disease's development (Koenning and Wrather, 2010; Wrather et al., 1995).

Bacterial blight of soybean can reduce yields by up to 40% (Jagtap et al., 2012; Tarakanov et al., 2022a). The causative agent of this disease is *Pseudomonas savastanoi* pv. *glycinea* (Coerper, 1919; Gardan et al., 1992) (syn. *Pseudomonas syringae* pv. *glycinea* (Coerper, 1919; Young et al., 1978)) (further - Psg) (Zhang et al., 2018). In seasons with wet, warm springs, bacterial blight occurs regularly in the North Caucasus and Far East regions of Russia, often with high severity. Under favourable weather conditions, the disease may cause great damage, especially in early-maturing cultivars (Tarakanov et al., 2022a). While Psg may infect all parts of the soy plant above the ground, only leaves and pods demonstrate typical symptoms of the disease: water-soaked or chlorotic spots further turning to necrotic lesions with a chlorotic halo (Ignjatov et al., 2007). The pathogen is preserved and spreads in infected seeds and plant debris, and significantly affects soybean quality and the soybean harvest (Shepherd and Block, 2017; Monteil et al., 2016).

In contrast, the reported yield loss of soy plants affected by bacterial tan spot will usually be less than 20% (Dunleavy, 1984). The pathogen is a Gram-positive bacterium *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff) (Hedges, 1922) that spreads in the vascular system of plants and causes leaf blight, wilting and the death of seedlings and adult plants of many legumes, including soybean plants (Osdaghi et al., 2020; Huang et al., 2009).

The bacterial pathogens' presence in seeds reduces their germination rate and energy, and can be the main reason for disease outbreak or the principal means for the pathogen's long-distance dissemination (Huang et al., 2007; Camara et al., 2009; Hsieh et al., 2006).

The control of soybean bacterial diseases mostly relies on prevention of the infection. Some positive results have also been achieved through the use of resistant cultivars (Monteil et al., 2016; Silva Júnior et al., 2012; Urrea and Harveson, 2014), chemical bactericides including copper compounds (Estefani et al., 2007), nanoparticles of chitosan with copper (Tarakanov et al., 2023), antagonistic bacteria (Völksch and May, 2001), Plant-Growth Promoting Rhizobacteria (PGPR) (Martins et al., 2013), bacteriophages (Tarakanov et al., 2022b, c) and essential oils and plant extracts (Rani et al., 2008; Tarakanov and Dzhalilov, 2022).

The use of pathogen-free seeds is, however, the most efficient way to reduce the risk of potential epidemics in field and harvest losses due to bacterial diseases (Shepherd and Block, 2017; Huang et al., 2009; Hsieh et al., 2006). Considering the zero-level tolerance (one infected seed per 5,000-9,000, depending on the seed's weight and method of detection) for seed infection by quarantine organisms, seed testing must be carried out using high-sensitivity methods, like PCR. Previously, PCR analysis with electrophoretic detection of the amplicons was developed for each pathogen separately: the method for Psg published by Bereswill et al. (1994), and the Cff assays reported by Tegli et al. (2002) and Guimaraés et al. (2001).

It is worth noting that the PCR protocols listed above were designed before the recent phylogenetic reclassification of phytopathogens, like *Pseudomonas syringae* (now *P. savastanoi*) (Gomila et al., 2017), and that classic PCR is less practical than PCR-RT for commercial application. Real-time PCR provides reliable data more quickly, has a high sensitivity for the detection of DNA, due to a combination of the amplification performed by the PCR step and the system of detection, and has the economic advantage of being able to laboratory test a large number of pathogens in one step (Zhang et al., 2021). The development of a multiplex real-time PCR for the detection of Psg and Cff in soybean seeds is the aim of the present work.

2. Materials and methods

2.1. Microorganisms and cultivation

Bacterial strains used in this work are listed in Table 1. They were obtained from the All-Russian Collection of Microorganisms (VKM; Pushchino, Russia), the French Collection of Phytopathogenic Bacteria (CFBP; Beaucouzé, France), the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany), the American Type Culture Collection (ATCC; Manassas, USA) and the National Collection of Plant Pathogenic Bacteria (NCPPB; London, United Kingdom). Some original strains had been isolated and identified in previous studies (Tarakanov et al., 2022b, d). Among 43 strains of different geographic origin, 25 represented target species (13 Psg strains and 12 Cff strains); 15 strains were members of related species and three strains belonged to other bacteria most common on soybean plants in Russia. Other controls included bacteria from 25 commercial seed stocks (three Psg- and Cff-positive and 22 – negative). All bacteria were preserved in 40% glycerol at -80 °C. All bacterial strains were cultured using King's B medium for 3 days at 28 °C.

2.2. Primers and probes construction

The primers and probes designed and applied in this work are presented in Table 2. Gene encoding 2-oxoglutarate-dependent ethylene/succinate-forming enzyme (*efe*) was chosen for the construction of the *Pseudomonas* pv. *glycinea* (Psg) primer set and the probe (Figure 1). The ethylene-producing ability of strains of *P. savastano*i pvs. *glycinea* and *phaseolicola* had previously been shown to be potentially related to their pathogenicity (Weingart and Volksch, 1997). This marker gene was used for the detection of ethylene-producing bacteria pathovars of *Pseudomonas* (Weingart and Volksch, 1997; Sato et al., 1997). A BLAST search using the database comprising all 847 NCBI GenBank genomic sequences of *Pseudomonas syringae* and *Pseudomonas savastanoi* and 59 sequences of their plasmids was conducted. The search indicated the presence of the *efe* gene in the draft genomic assemblies of 35 of the 43 strains of Psg, in three of the 45 strains of *P. savastanoi* pv. *phaseolicola* (Psp), in one of the eight strains of *P. syringae* pv. *aptata*, in one of the six strains of *P. syringae* pv. *papulans*, in 11 of the 13 strains of *P. syringae* pv. *pisi*, in both strains of *P. syringae* pv. *spinaceae* and in a genomic assembly labelled as *P. savastanoi* strain JD03 (NCBI Accession JANAKG010000000). According to the results of

Table 1. List of bacteria used in this study and PCR method specificity.

№	Target bacteria	Isolate	Average Ct ±SD for multiplex real-time PCR		Result of classic PCR analysis		Reference
			FAM (Psg-specific fluorophore)	R6G (Cff-specific fluorophore)	*cfl* (Bereswill et al., 1994)	*tlsp* (Tegli et al., 2002)	
			Target bacteria				
1.	*Pseudomonas savastanoi* pv. *glycinea*	2411^T	20.07 ±0.12	-	+	-	NCPPB
2.		G1	19.32 ±0.53	-	+	-	Tarakanov et al. (2022a)
3.		G2	19.07 ±0.02	-	+	-	
4.		G3	21.01 ±0.16	-	+	-	
5.		G4	21.87 ±0.59	-	+	-	
6.		G5	18.77 ±0.15	-	+	-	
7.		G6	19.65 ±0.19	-	+	-	
8.		G7	19.84 ±0.32	-	+	-	
9.		G8	19.93 ±0.26	-	+	-	
10.		G9	20.42 ±0.41	-	+	-	
11.		G10	18.50 ±0.09	-	+	-	
12.		G11	20.23 ±0.23	-	+	-	
13.		G17	19.98 ±0.07	-	+	-	
14.	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	3418	-	16.59 ±0.63	-	+	CFBP
15.		20129	-	15.28 ±0.06	-	+	DSM
16.		F-125-1	-	15.76 ±0.15	-	+	Tarakanov et al. (2022b)
17.		F-125-2	-	16.21 ±0.29	-	+	
18.		F-125-3	-	15.69 ±0.02	-	+	
19.		F-30-1	-	15.93 ±0.21	-	+	
20.		F-30-2	-	16.89 ±0.12	-	+	
21.		F-30-3	-	16.24 ±0.09	-	+	
22.		C101	-	15.26±0.32	-	+	Tokmakova et al. (2023) (in press)
23.		C122	-	14.08±0.45	-	+	
24.		C139	-	16.96±0.04	-	+	
25.		C142	-	17.91±0.6	-	+	
			Non-target bacteria				
26.	*Pseudomonas syringae* pv. *pisi*	2105	17.88 ±0.19	-	-*	-	CFBP
27.	*Pseudomonas fuscovaginae*	7231	-	-	-*	-	DSM
28.	*Pseudomonas fluorescence*	B-894	-	-	-*	-	VKM
29.	*Pseudomonas savastanoi* pv. *phaseolicola*	1429	-	-	+	-	CFBP
30.	*Pseudomonas savastanoi*	B-1546	-	-	-*	-	VKM
31.	*Pseudomonas syringae* pv. *aptata*	2-1-5	-	-	-	-	Ignatov et al. (2018)
32.	*C. flaccumfaciens* pv. *betae*	20141	-	-	-*	-	DSM
33.	*Curtobacterium flaccumfaciens* pv. *oorti*	1384	-	-	-*	-	CFBP
34.	*Curtobacterium herbarum*	14013	-	-	-*	-	DSM
35.	*Curtobacterium flaccumfaciens* pv. *poinsettiae*	2403	-	-	-*	-	CFBP
36.	*Curtobacterium citreum*	B-1207	-	-	-*	-	VKM
37.	*Curtobacterium albidum*	Ac-2186^T	-	-	-	-	VKM
38.	*Curtobacterium luteum*	Ac-2188^T	-	-	-	-	VKM
39.	*Curtobacterium pusillum*	19096	-	-	-		ATCC
40.	*Bacillus amyloliquefaciens*	MBI 600	-	-	-		Samaras et al. (2021)
41.	*Xanthomonas phaseoli* pv. *phaseoli*	2534	-	-	-*		CFBP
42.	*Clavibacter michiganensis* subsp. *nebraskensis*	2405	-	-	-		CFBP
43.	*Pectobacterium carotovorum* subsp. carotovorum	2046	-	-	-*		CFBP

Each test was repeated in triplicate. *Strains where PCR produces non-specific bands; ^{T} - The type strain of corresponding species; SD - standard deviation.

Table 2. Sequences of primers and probes used in the study.

Primer / probe	Nucleotide sequence (5'-3' direction)	Target region	Amplicon sequence for the type strain	Product size, bp	Source
Psg2F	CCT-CCA-GTC-GAG-GGA-GAG-AA	ethylene-forming enzyme *(efe)*	CCTCCAGTCGAGGGAGAGAAGCGTAAT CGTAACTGGTTGCCTGGT GAGAGCTCAGCAGGCATGTTTG AGCACGATGAACCTTGGACCTT CGTGACGCCCACCCCAGGCGTGTG GACAGTTTTCCCAGGTGATATCTT GCAGTTCATGACCGGCGGCCAGC TGCTTTCCACTCCGCACAAGGTTAAGCTCAATACCCGC GAACGTTTCGCCTGCGCTTATTTTCATGAGCCT AATTTTGAAGCATCCGCCTATCCGTTGTTCGA GCCCAGCGCCAATGAGCGTATTCATTATGGTGAGCACTTTAC CAACATGTTTATGCGTTGCTATCCAGATCGGATCACC	342	This study
Psg2R	GGT-GAT-CCG-ATC-TGG-ATA-GCA-A				
ProbePsg2	(FAM)AC-CCG-CGA-ACG-TTT-CGC-CTG-(RTQ1)				
Cff1F	GTT-ATG-AC(T/C)-GAA-CTT-CAC-TCC	trypsin-like serine protease (*tlsp*)	GTTATGACTGAACTTCACTCCTACAGTCTCGGCCCG TCCGAATTTCCGGCGGCTGACGGTGAGTCGAACC CGTTAGTGAAGGCTGACGAATCCGTCGATGC GGGATGGACACTAGCACCGACGGAACCAACGA CTCACAATAGTGAGACACGCCCCGTACGGA TGCAAACGACCGATAGCTCCATGTATTTCGGTCCT GCAGTTAGTCAGCAACGGACGCGCGCCG AGCGGTAGCAGCAACTATCGCCACGCCGCGA ACTATTCAATCAATTGCCGCCACCCGGTCAACTGAACACCGGGAACATCC	308	Tegli et al. (2002) (with modifications)
Cff1R	GGA-TGT-TCC-CGG-TGT-TCA-GT				
ProbeCff	(R6G)TC-AAT-TGC-CGC-CAC-CCG-GTC-(BHQ2)				

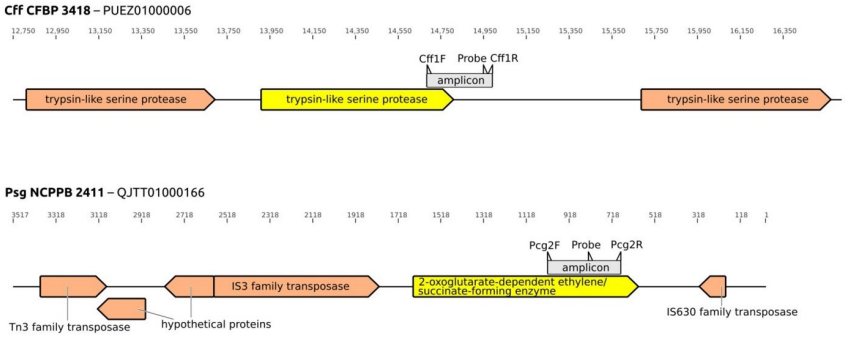

Figure 1. Genomic loci containing the target genes used for the construction of primer sets. Cff CFBP 3418 means the type strain of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*(T), Psg NCPPB 2411 means *Pseudomonas savastanoi* pv. *glycinea* NCPPB 2411(T). NCBI Accession Nos. are shown to the right of strain names. The ruler at the top of the sequences shows the number of the nucleotide base in the corresponding contig. Direction of translation is indicated by arrows.

average nucleotide identity (ANI) calculations, the latter can represent a strain of Psg (99.77% ANI compared with Psg type strain NCPPB 2411 = CFBP 2214 = ICMP 2189 = LMG 5066). All listed Psg strains are presented by draft assemblies, therefore the absence of *efe* genes can be explained by the incompleteness of genomes, or, possibly, by the presence of indigenous plasmids, as has been shown previously for other pathovars (Sato et al., 1997). In all strains, the target gene was found in one copy.

The PCR-diagnostics set of primers and a probe used for the *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff) detection were constructed based on the PCR assay developed by Tegli et al. (2002). The set employs the trypsin-like serine protease (*tlsp*) gene and the adjacent 3'-non-coding region located in pathogenicity islands found in Cff genome assemblies and plasmids, potentially related to Cff pathogenicity (Evseev et al., 2022; Figure 1). The 5'-end of the forward primer Cff1F was extended by three nucleotides, compared with Tegli et al. (2002), for equalizing the melting temperature of the Psg primers. The specificity of the Cff PCR set was checked, through the BLAST search, using all 277 NCBI GenBank genomic sequences of *Curtobacterium* spp. The search indicated the presence of the *tlsp* gene in 27 of the 39 Cff genomic sequences, most of which were draft assemblies, and in five plasmids. The search did not reveal the target region in the sequences other than Cff. The amplicon sequences were identical for most strains, except one insertion after position 119 in an amplicon sequence and a single polymorphism C/T in position 9, compared with the type strain. These differences were found for four novel Cff strains, Carlos1, Carlos2, Carlos4 and Carlos7. Analysis *in-silico* for the designed Cff-specific primers confirmed the absence of interaction with non-target microorganisms.

2.3. Optimization of duplex classical PCR using the modified Taguchi experiment design method

Duplex PCR was optimized using the modified Taguchi experiment design (Cobb and Clarkson, 1994). The Taguchi method is one of the best experimental methodologies for finding the main factors that affect traits, with the minimum number of experiments to be performed. This method helps to find the optimal parameters for the most important factors of PCR analysis in a factorial experiment within an orthogonal array (Ramakrishna et al., 2013). Four critical parameters of multiplex real-time PCR were chosen in nine combinations for the experiment: 1) $MgCl_2$, 2) dNTP, and primers concentration for 3) Psg and 4) Cff. Each factor had three levels (concentrations): $MgCl_2$ (1.25, 2.5 and 3.75 µM); dNTP (200, 300 and 400 µM); primers Psg2F+Psg2R and Cff1F+Cff1R (10.0, 20.0, and 30.0 pM per reaction for each pair) (Tables 3 and 4).

The time and temperature profiles of every stage are given in the next part of Materials and methods. All PCR reactions were performed using a commercial kit «Hot-start PCR Color» (Dialat Ltd, Moscow, Russia). PCR amplicons were separated by electrophoresis in 2% agarose gel stained with ethidium bromide, and documented with GelDocXR+ (Bio-RAD, Hercules, CA, USA). Images were assessed using the densitometric method with ImageJ (National Institutes of Health, Bethesda, MD, USA). The PCR mix with the best signal-to-noise ratio was used for further amplifications.

2.4. Optimization of annealing temperature

The range of annealing temperature from 58.0 to 63.0 °C was applied, for the purpose of finding the optimal temperature for PCR performance. All reactions were repeated five times. The temperature that provided the lowest average Ct was used for further experiments.

The resulting profile was the following: 95°C - 10 min (initial denaturation); 40 cycles of 95°C - 30 sec, 60°C – 2 min; 72°C – 30 sec; and final elongation at 72°C for 5 min. The fluorescence was measured after primer annealing for every cycle. Thresholds were chosen manually for each TaqMan® probe.

2.5. Specificity evaluation

Specificity of the multiplex real-time PCR analysis was tested on the 43 samples listed in Table 1. DNA for analysis was isolated from 72h-old bacterial cultures using the commercial kit «GS-Proba» (AgroDiagnostika, Moscow, Russia), according to the manufacturer's instructions. The concentration and purity of the DNA samples were evaluated with NanoDrop OneC (Thermo Scientific,

Table 3. Orthogonal scheme of classic PCR optimization experiment according to Taguchi model.

Reaction number	Tested factors				Factor codes			
	1. $MgCl_2$, μM	2. dNTP, μM	3. Primers Psg2F+Psg2R, pM	4. Primers Cff1F+Cff1R, pM	1	2	3	4
1	1.25	200	10.0	10.0	A	A	A	A
2	1.25	300	20.0	20.0	A	B	B	B
3	1.25	400	30.0	30.0	A	C	C	C
4	2.50	200	20.0	30.0	B	A	B	C
5	2.50	300	30.0	10.0	B	B	C	A
6	2.50	400	10.0	20.0	B	C	A	B
7	3.375	200	30.0	20.0	C	A	C	B
8	3.75	300	10.0	30.0	C	B	A	C
9	3.75	400	20.0	10.0	C	C	B	A

Table 4. PCR mix components for nine reaction variants according to Taguchi model.

Reaction number	μl per reaction (25 μl)							
	SmarTaq polymerase (C=1.25 U)	$MgCl_2$ (C=50 μM/μl)	Primers Psg2F+Psg2R (C=5 pM/μl)	Primers Cff1F+Cff1R (C=5 pM/μl)	dNTP, (C=100 μM/μl)	DNA (C=10 ng/μl) each	10x PCR buffer	Water for PCR
1	1	0.625	1+1	1+1	2	1+1	2.5	12.875
2	1	0.625	2+2	2+2	3	1+1	2.5	7.875
3	1	0.625	3+3	3+3	4	1+1	2.5	2.875
4	1	1.25	2+2	2+2	2	1+1	2.5	8.25
5	1	1.25	3+3	3+3	3	1+1	2.5	3.25
6	1	1.25	1+1	1+1	4	1+1	2.5	10.25
7	1	1.875	3+3	3+3	2	1+1	2.5	3.625
8	1	1.875	1+1	1+1	3	1+1	2.5	10.625
9	1	1.875	2+2	2+2	4	1+1	2.5	5.625

Waltham, MA, USA). All samples had the $A_{260/280}$ ratio about 1.8 and were adjusted to 10 ng/μl. Each specificity test was repeated three times.

All of the samples were verified by classic PCR analysis, using previously published protocols. The method developed by S. Bereswill was used for target gene *cfl* in Psg (Bereswill et al., 1994) and the method of S. Tegli *tlsp* gene was used for Cff (Tegli et al., 2002). The analysis was repeated three times and amplified DNA was analysed as described in sub-section 2.3 of Materials and methods. Genomic DNA of strains NCPPB 2411 (Psg) and CFBP 3418 (Cff) were used as positive controls, and sterile water as a negative control. The result of classic PCR was positive if the size of amplicons was ~650 bp for Psg and ~300 bp for Cff when measured in comparison with 100 bp Ladder (Dialat Ltd, Moscow, Russia).

2.6. Sensitivity evaluation

Real-time TaqMan® PCR analysis was performed with a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA), using time and temperature profiles as described in sub-section 2.4 of Materials and methods. PCR effectiveness was calculated using a standard curve method, with CFX Maestro™ Software (BioRad, Hercules, CA, USA).

2.6.1. Sensitivity of duplex PCR and multiplex real-time PCR

Purified genomic DNA of Psg (NCPPB 2411) and Cff (CFBP 3418) was adjusted using nuclease-free water to 100 ng/μl, and used for serial 10-fold dilutions. Each dilution was applied as a template for duplex PCR or in real-time PCR in three independent trials.

Concentrations of viable bacterial cells in the samples were estimated for Psg NCPPB 2411 and Cff CFBP 3418. Bacteria were collected using a glass spatula after 96 h growth on King's B agar (King et al., 1954) at 28 °C and suspended in aliquots of 10 μM PBS. Serial 10-fold dilutions were prepared in 10 μM PBS and 100 μl of each dilution was plated on King's B agar in Petri dishes. Bacterial suspension was spread using a sterile spatula and incubated at 28 °C for 96 h. Bacterial colonies were counted and used to calculate concentrations in each dilution. Each suspension

was used for qPCR analysis as described by Holeva et al. (2019). For this, 2 µl of bacterial suspension was added to 23 µl of the reaction mixture for PCR. The experiments were repeated in triplicate.

2.6.2. Sensitivity evaluation for multiplex real-time PCR in soybean seed extracts and seed samples

Pathogen-free soybean seeds collected in 2021, in the Ramon district of the Voronezh region, from the field of 2nd reproduction for cultivar Sultana (Societe Ragt 2n S.A.S., France) were used for extraction, according to the protocol of the European and Mediterranean Plant Protection Organization (EPPO) PM 7/102 (1), which was developed for Cff diagnostics in legume seeds (EPPO, 2011).

A seed sample (200 g) was placed into a plastic bag for homogenization with side filter BagFilter P (Interscience, Saint Nom la Brétèche, France); then, 300 ml of SPS-buffer was added and left overnight at +4.5 °C. After incubation, the sample was homogenized for 5 min using BagMixer 400 P (Interscience, Saint Nom la Brétèche, France). The filtrate was transferred into 50 ml Falcon tubes and centrifuged for 20 min at 10,000 g at +4 °C. The precipitate obtained was resuspended in 1.5 ml of SPS buffer. The samples (500 µl each) with different concentrations of the target bacteria were obtained by mixing 495 µl of seeds extract and 5 µl of bacterial suspension, to obtain final concentrations in the range 10^5 to 10^1 CFU/ml.

These samples were used for DNA extraction using a commercial kit with magnetic particles "Phytosorb" (Syntol LLC, Moscow, Russia), according to the manufacturer's instructions. To perform PCR, 2 µl of extracted DNA was added to 23 µl of the PCR reaction mixture. An extract from pathogen-free soybean seeds was applied as a negative control. Ct values of real-time PCR were used to plot the calibration curve for reaction efficiency. The experiment was repeated in duplicate.

A total of 25 commercial soybean seed samples produced in different geographic regions of Russia were analyzed using the methods described above. The results obtained for newly developed multiplex real-time PCR were verified using previously published methods for Psg (Bereswill et al., 1994) and Cff (Tegli et al., 2002).

Reference strains of Psg (NCPPB 2411) and Cff (CFBP 3418) were used as positive controls, and sterile SPS buffer was used as a negative control. The experiment was repeated three times.

3. Results

3.1. Standardization of duplex classic PCR using the modified Taguchi method

The optimal amplification conditions for each gene were calculated using the modified Taguchi method for the developed multiplex real-time PCR at the stage of classical PCR verification. The selection of optimal PCR parameters with an increased yield of amplification products for both genes, and minimal competition for dNTP, was the main purpose of this step.

Four critical factors were selected: concentrations of $MgCl_2$ and dNTP, and primers for Psg and Cff. The experimental design included nine reactions (see Materials and methods sub-section) and the reactions resulted in different intensities of amplification products (Figure 2). The presence of amplicons with the expected molecular weight (342 bp for the *efe* gene and 308 bp for the *tlsp* gene) was confirmed. No non-specific amplification products were observed.

The electrophoretic images obtained from densitometric analysis of agarose gels were analyzed, and the graphs (Figure 3) demonstrated a certain competition between the amplification of *efe* and *tlsp* genes in several variants of the reaction mixture. In addition, different variants showed distinct total yields of the amplification products.

The densitometry data analysis demonstrated that the highest yield of *tlsp* gene fragment amplification products was observed in variants 1, 6 and 8, while the largest number of *efe* gene amplicons was observed in variants 5, 4 and9.

Analysis of the amplification results showed variant 3 to be optimal, where the alignment of the number of amplification products (the ratio of the sum of signals to variance) was the greatest, amounting to 0.077, with an average output of 10,757-11,573 units (Table 4).

Thus, the optimal composition of the reaction mixture that was used in further experiments was as follows: 1.25 U SmarTaq polymerase, 1.25 µM $MgCl_2$, direct and reverse primers for the *efe* and *tlsp* genes at 30.0 pM for each pair, 400 µM each dNTP, 1+1 µl DNA of each species, 2.5 µl 10x buffers for PCR and water to a total volume of 25 µl.

3.2. Optimization of primers/probe annealing temperature

To switch to the real-time PCR mode, 0.5 µl of each probe (ProbePsg2 and ProbeCff) with a concentration of 20 pM/µl was added to the reaction mixture, and PCR was performed with annealing temperature ranging from 58 to 63 °C. Different fluorescence values were observed at every annealing temperature used (Figure 4). Thus, the maximum average fluorescence value (2144 RFU) was observed at 62.7 °C for the *tlsp* gene product, and 1058 RFU at 61.2 °C for the *efe* gene product. In the case of the Ct cycle values, the results were slightly different: the lowest average Ct cycle values were obtained at an annealing temperature of 60.0 °C for the products of both genes (19.05 for *efe* and 18.55 for *tlsp*). Thus, the annealing temperature of 60.0 °C was chosen for further experiments.

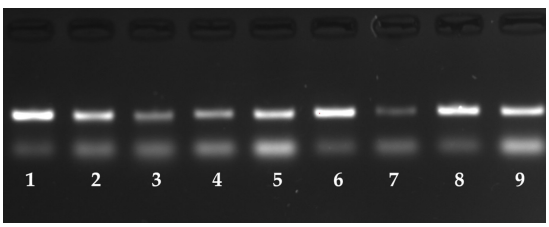

Figure 2. Classic PCR reaction for duplex analysis in nine reaction variants according to the Taguchi experiment model (See Table 3 for reaction variants description).

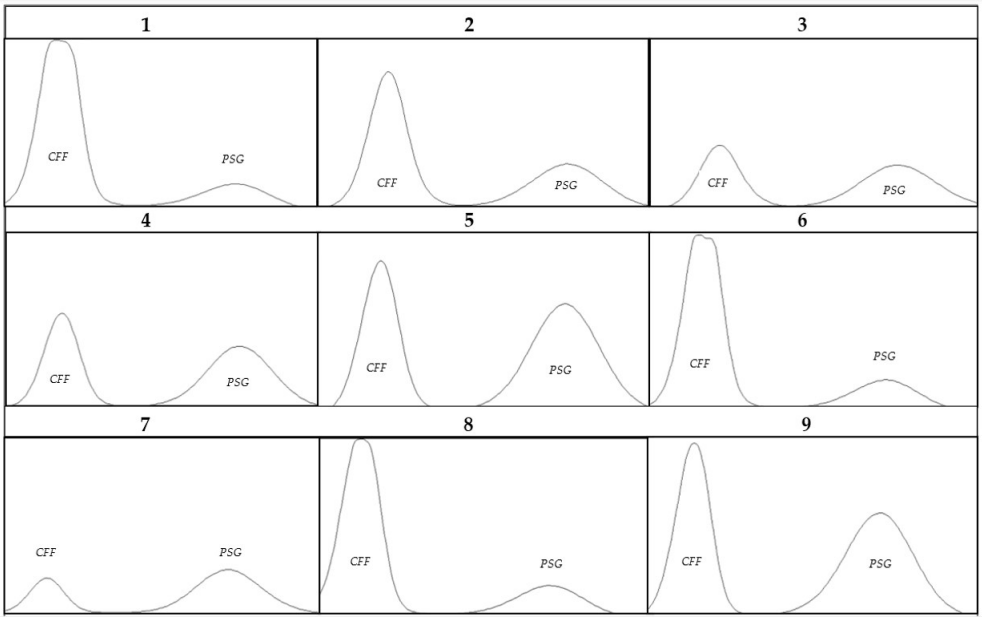

Figure 3. Duplex classic PCR amplicons quantification with program ImageJ for nine reaction variants, according to the Taguchi experiment model (See Table 3 for reaction variants description).

3.3. Specificity of multiplex real-time PCR

The multiplex real-time PCR protocol was tested using the bacterial isolates listed in Table 1. The specificity of the developed primers was tested on 25 previously identified target bacteria, 18 strains of related species (*P. syringae* pv. *pisi*, *P. syringae* pv. *aptata*, *P. savastanoi*, *P. savastanoi* pv. *phaseolicola*, *P. fuscovaginae*, *P. fluorescence*, *C. flaccumfaciens* pv. *betae*, *C. flaccumfaciens* pv. *oorti*, *C. flaccumfaciens* pv. *poinsettiae*, *C. herbarum*, *C. citreum*, *C. albidum*, *C. luteum*, *C. pusillum*, *Clavibacter michiganensis* subsp. *nebraskensis*), a non-target pathogen for soybeans (*Xanthomonas axonopodis* pv. *phaseoli*) and a mixture of 12 strains of non-target bacteria found as endophytes on plants (*Bacillus amyloliquefaciens*, *Pectobacterium carotovorum*, *Pantoea agglomerans* and *Agrobacterium radiobacter*).

A positive result of PCR amplification was achieved for all analyzed target strains – either Psg or Cff (Table 1). During the analysis, only the *P. syringae* pv. *pisi* strain showed false-positive amplification by the Psg-specific primer/probe (FAM channel). No strains with false-positive amplification were found for the Cff-specific PCR set (R6G fluorophore).

The same strains were used for classic PCR analysis using previously developed methods (Bereswill et al., 1994; Tegli et al., 2002), and some unexpected results were obtained.

While there were no discrepancies in the results of classic PCR with multiplex real-time PCR for Cff, non-specific amplification with DNA of *Pseudomonas syringae* pv. *pisi* and *Pseudomonas savastanoi* pv. *phaseolicola* strains was observed for classic PCR with *efe* gene primers (Table 5). The multiplex real-time PCR protocol produced no false amplification with DNA of the non-target bacteria. Meanwhile, when using the classic PCR protocol, nonspecific amplification was observed, resulting in products that differed from the target band (650 bp) (Table 5) in many non-target strains, which could cause problems for interpreting the results of PCR analysis. This also confirms the need to use real-time PCR to avoid possible false-positive results.

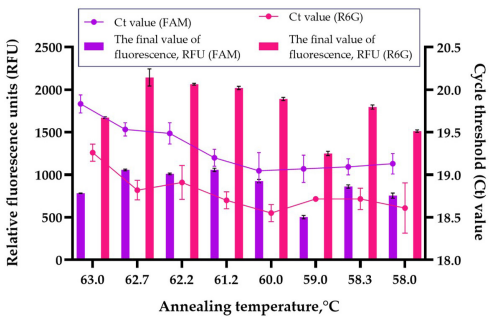

Figure 4. Primer annealing temperature effect on Ct values for FAM (Psg) and R6F (Cff). Average values with standard deviations for five independent experiments.

3.4. Protocol sensitivity

3.4.1. Simplex real-time PCR

The sensitivity of the simplex reaction was evaluated on serial dilutions of genomic DNA of strains Psg (NCPPB 2411) and Cff (CFBP 3418); the results are presented in Table 6 and Figure 5A. Psg detection analysis showed sequential amplification of the *efe* gene in the presence

Table 5. PCR amplicons analysis for nine reactions within Taguchi model-based experiment for classic duplex PCR method. Average values for three experiments are shown.

Reaction number	Average values of signal (n=3)		Modul of difference of signals	Ratio of Signals Sum to signal difference	Dispersion	Ratio of Signal Sum to Dispersion
	CFF	PSG				
1	31436.1	5634.2	25 760.6	1.4	331803972.8	0,000111928
2	22270.1	10537.8	11 298.4	2.9	63826412.9	0,000519752
3	10757.1	11573.9	763.5	29.3	291461.5	0,076800513
4	13223.6	14920.4	1 363.5	20.9	929574.3	0,030634814
5	22067.9	28590.5	6 162.6	8.2	18988622.2	0,00265168
6	27990.0	7082.2	20 796.4	1.7	216244627.4	0,000161673
7	4549.3	10567.5	6 018.2	2.5	18109443.9	0,000834749
8	25841.0	6818.7	19 021.1	1.7	180900818.3	0,000180532
9	23321.8	104285.9	3 959.0	12.8	7836856.3	0,006458295

Table 6. Sensitivity analysis in simplex and multiplex real-time PCR for serial dilutions of DNA of Psg and Cff. Average values of Ct for three experiments.

DNA amount per PCR reaction	simplex real-time PCR		multiplex real-time PCR	
	FAM (Psg NCPPB 2411)	R6G (Cff CFBP 3418)	FAM (Psg NCPPB 2411)	R6G (Cff CFBP 3418)
100 ng	17.41	15.74	18.39	15.86
10 ng	21.31	18.98	21.02	19.15
1 ng	24.86	22.49	24.31	22.72
0.1 ng	28.18	26.45	28.69	26.71
0.01 ng	31.3	29.72	31.2	28.79
Efficiency (%)	94.36	91.53	99.7	99.2
Slope	-3,465	-3,543	-3,329	-3,342
Y-intercept	24.612	22.676	34.71	32.672

of 0.01 ng of DNA per reaction or more. The detection sensitivity of Cff was approximately equal (but with lower Ct cycle values) to that of Psg (about 0.01 ng per reaction). The calculated reaction efficiency was 94.36% for Psg and 91.53% for Cff. Thus, the detection of Cff via the *tlsp* gene showed consistently lower Ct values than the analysis for Psg detection.

3.4.2. Multiplex real-time PCR

A multiplex real-time PCR sensitivity analysis showed that Psg and Cff DNA could be detected at a concentration of at least 0.01 ng per reaction. The reaction efficiency was calculated as 99.7% for Psg and 99.2% for Cff (Table 6, Figures 5B-5D). The use of the designed primers and probe also showed good results with dilutions of culture suspensions (from 10^6 to 10^1 CFU/ml) of Psg NCPPB 2411 and Cff CFBP 3418 strains. The results presented in Figures 6A, 6B indicate minor differences when using each species of bacteria separately as a matrix (simplex) or after mixing (multiplex).

For example, the average Ct values of the simplex real-time PCR at the concentration of 10^3 CFU/ml were 28.7 and 25.5 cycles for Psg and Cff, while for the multiplex real-time PCR the values averaged 28.9 and 27.4 cycles, respectively. Thus, simultaneous amplification of two target genes increased the value of the Ct cycle in multiplex real-time PCR, compared with simplex real-time PCR.

3.5. Detection of the pathogens in soybean seed extract

To simulate the analysis of soybean seeds for the presence of the target bacteria, serial dilutions of bacteria were added to the pathogen-free soybean seed extract homogenized in the buffer. Total DNA was isolated and used for PCR. The applied PCR protocol was able to detect the target bacteria with concentrations above 100 CFU/ml (Figure 6C).

The threshold values of the Ct cycle linearly correlated with the concentration of bacteria and enabled the plotting of a standard curve (Figure 6C). This calibration curve can be used for approximate quantitative assessment of the presence of bacteria in samples.

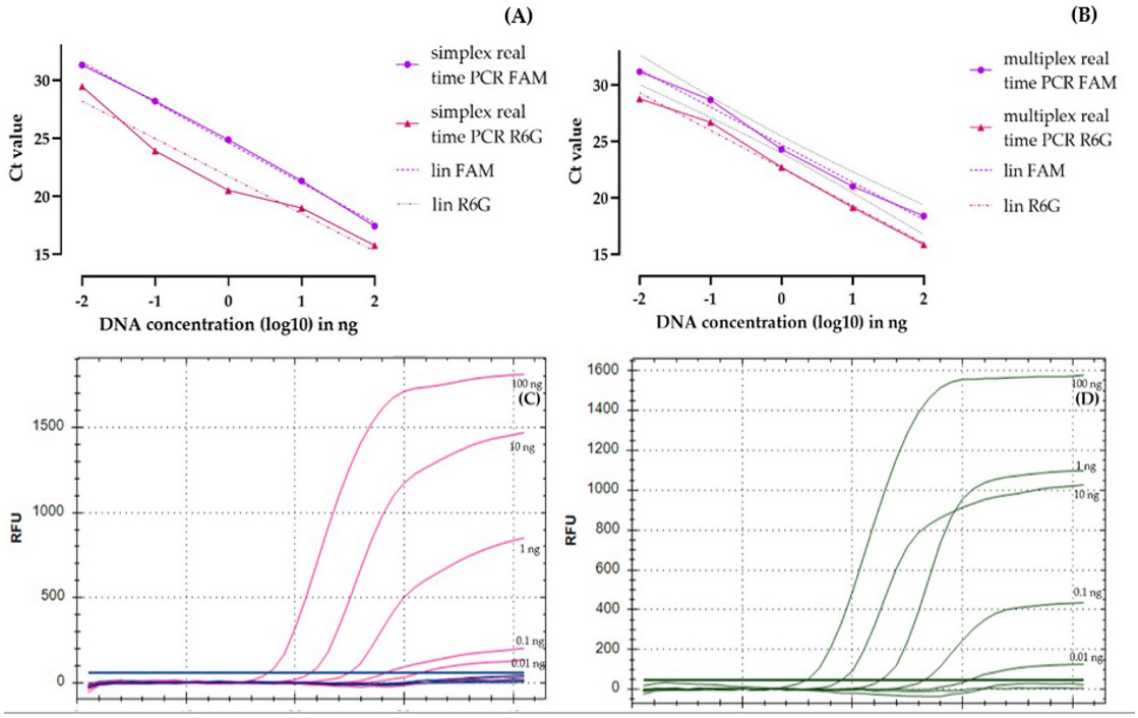

Figure 5. Sensitivity of DNA detection in simplex (A) and multiplex real-time PCR (B, C, D). Log10 of DNA concentrations (ng) are compared with the corresponding Ct values at (A) and (B). The average values were obtained in three experiments. Fluorescence curves for multiplex real-time PCR are shown for Psg (C, FAM) and Cff (D, R6G).

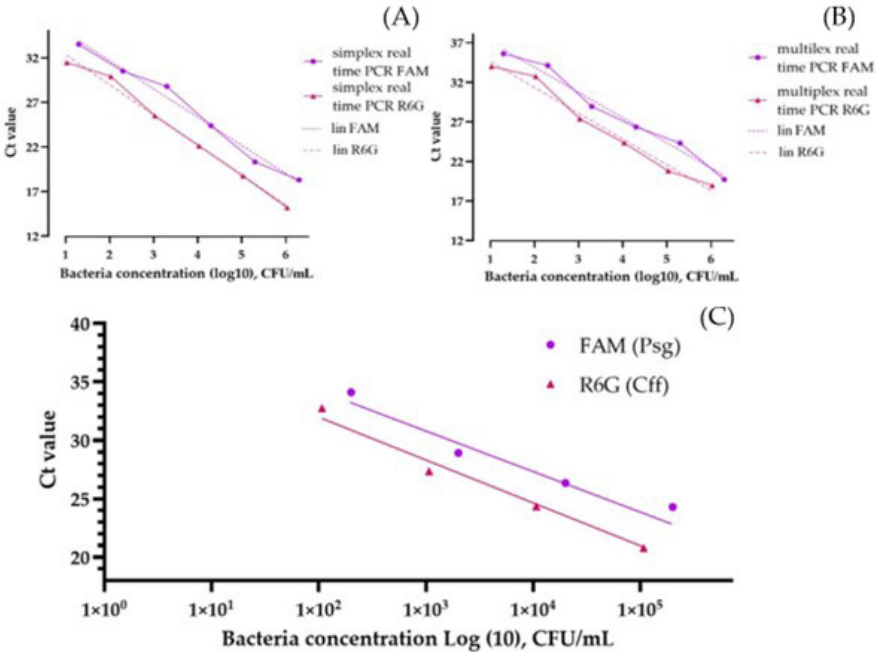

Figure 6. Sensitivity of Psg and Cff detection in simplex (A) and multiplex real-time PCR (B) reactions. Standard serial 10-fold dilutions of bacterial cells of Psg NCPPB 2411 (FAM) and Cff CFBP 3418 (R6G) were used for both (A) and (B). Simplex real-time PCR (A) employed each of the bacteria separately, multiplex real-time PCR (B) analysis included both bacteria in mix 1:1, and standard curve (C) for multiplex real-time PCR was obtained for soy seeds extract mixed with serial 10-fold dilutions of bacterial cells in 10 µM PBS. Average values for three experiments were calculated to get Log10 of bacterial concentrations (CFU/ml), shown on axis X, and the number of Ct (cycles), shown on axis Y.

3.6. Testing of the developed multiplex real-time PCR protocol using commercial soybean seeds

Using the multiplex real-time PCR protocol, 26 seed samples collected in 2022 from several regions of Russia were tested. Three samples demonstrated a positive signal in real-time PCR. A positive reaction to Psg was shown in a sample from the Amur region, and samples from the Tver and Voronezh regions showed a positive reaction to Cff. The same samples were verified using protocols of Bereswill et al. (1994) and Tegli et al. (2002) and the results were also positive. However, in the analysis of the protocol (Bereswill et al., 1994), in addition to the target product with a size of 650 bp, a non-specific amplification of non-target bands was observed.

4. Discussion

Currently, several assays to detect Psg and Cff are known. They include a microbiological method, analysis by ELISA and PCR (EPPO, 2011). For a microbiological assay, extracts of suspected plants are spread over selective nutrient media for isolation of a pure culture of the pathogen and diagnostics according to Koch's postulates. However, PCR is recommended as a secondary test, when the presence of bacteria has already been proved by another method and its confirmation is required (EPPO, 2011).

For detection of Psg using a direct plating method, KBC (modification of King's B medium) is prepared by the addition of boric acid, cephalexin and cycloheximide (Mohan, 1987). It can also be used for other pathogens of the species *Pseudomonas syringae* (*P. savastanoi*).

SSM and MSCFF are applied as selective media for Cff (Tegli et al., 1998). SSM contains rhamnose as a carbon source, and cycloheximide and polymyxin B as selective agents. MSCFF medium contains the selective agents chlorothalonil, thiophanate methyl, nalidixic acid, nitrofurantoin and oxacillin (Maringoni et al., 2006).

FAME analysis (fatty acid profiling) is not recommended for use in the diagnostics of Cff, since it requires isolation of a pure culture of pathogens, which is laborious and time-consuming. Also, the FAME profiles of pathogenic Cff and non-pathogenic *Curtobacterium* spp. are similar (Weller et al., 2000; Dickstein et al., 2001).

Serological methods are applied for the diagnostics of Psg (Suryadi and Machmud, 2006). A presented protocol based on the NCM-ELISA method enables the detection of the pathogen in a plant sample at concentrations above 10^4 CFU/ml. For the diagnostics of Cff, a number of serological assays have been described, for both infected plant material and pathogen pure culture (McDonald and Wong, 2000; Diatloff et al., 1993). All of these methods have, however, been shown to be unable to detect all known strains of the pathogen, and therefore they are used only sparingly (Guimaraés et al., 2001). Thus, systems based on serological methods have limited sensitivity and specificity, and cannot be used for commercial diagnostics of seeds.

Finally, there are several methods of analysis that use the classical PCR method. These include, in particular, separate systems developed by Bereswill et al. (1994) for Psg, and Tegli et al. (2002) and Guimaraés et al. (2001) for Cff.

Primers for the detection of Psg (Bereswill et al., 1994) were designed using a fragment of the *cfl* gene encoding an enzyme important for the biosynthesis of coronatine. These primers are able to react with all pathogens of the *Pseudomonas syringae* (*savastanoi*) species, and therefore cannot discriminate pseudomonads other than Psg in a phytopathological analysis of seed material.

P.M. Guimaraěs et al. described a classical PCR assay to detect Cff (Guimaraěs et al., 2001), but, according to S. Tegli, this system was not tested using infected plant material (Tegli et al., 2002). Primers for the diagnostics of Cff developed by S. Tegli (Tegli et al., 2002) are recommended for use in the EPPO protocol (EPPO, 2011). This assay amplifies the gene that is potentially important for pathogenicity. This system was chosen as a basis for improved primers for Cff, where two nucleotides were added into the reverse primer sequence to increase and balance the annealing temperature.

Since both bacteria are dangerous pathogens for soybean production, the use of real-time PCR methods results in a more accurate information about the health of the tested seeds. It is important from the economic point of view to use PCR for the simultaneous detection of several pathogens (at least two) using multiplex PCR (Elnifro et al., 2000).

At the moment, there are a number of multiplex diagnostic protocols for several important plant pathogens, usually found on the same crop. There exist PCR methods for detecting powdery and common scab of potatoes (pathogens *S. subterranea* and pathogenic *Streptomyces* sp.) (Qu et al., 2011), a complex of six important bacterial rice pathogens (Cui et al., 2016), soybean pathogens (*Colletotrichum truncatum, Corynespora cassiicola* and *Sclerotinia sclerotiorum*) (Ciampi-Guillardi et al., 2020) and some others. Thus, multiplex real-time PCRs have been developed and used to detect many important plant pathogens.

This is the first report on a multiplex real-time PCR with TaqMan® probes, which simultaneously detects Psg and Cff soybean pathogens. Both pathogens damage soybeans, and this includes simultaneous infection (Sammer and Reiher, 2012); they are transmitted by seeds and can be diagnosed within the framework of a single-step analysis, to reduce the cost of PCR diagnostics and increase lab productivity.

The developed assay demonstrates high specificity to all of the strains of target pathogens described earlier. In the case of Psg-specific primers, positive results were obtained with all 13 strains of the target pathogen and only one non-target strain - *Pseudomonas syringae* pv. *pisi*, which cannot infect soybean plants. The *tlsp* gene-specific primers reacted with all 12 strains of the target pathogen and did not interact with any non-target strain. The sensitivity of the system when using a commercial DNA extraction kit was 100 CFU/ml, which is suitable for in-line diagnostics in phytopathological laboratories.

Another important advantage of the system is its speed of obtaining results. For example, the analysis of 25 batches of soybean seeds using the commercial DNA isolation kit "Phytosorb" (Syntol, Moscow, Russia), the automatic DNA isolation station Auto-Pure 96 (Allsheng, Hangzhou, China) and the developed real-time PCR system

yielded results within 24 h. The suitability of the system for the diagnosis of soybean seed samples was shown for commercial samples of soybean seeds.

5. Conclusions

The multiplex real-time PCR protocol developed in this work was based on genomic analysis of data from the available genetic and genomic sequences of Psg and Cff. It was successfully tested and has demonstrated high sensitivity and specificity for use in practical seed health diagnostics, as an essential part of an integrated disease control system.

The method and data presented in this study can be used in studies of pathogen dissemination and in epidemiological studies to obtain new information about the pathogen's life cycle.

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