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Detection and identification of wheat and barley phytopathogens in the Russian Federation

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ABSTRACT

Grain export is an important branch of the food business in the Russian Federation. The countries of Europe, Asia, Africa, and South America are importers of Russian grain. Each importing country has its own requirements for the phytosanitary condition of imported products. One important requirement for importers is the absence of pathogens that can cause bacterial diseases of grain crops, such as *Pectobacterium rhapontici, Rathayibacter tritici, Pseudomonas fuscovaginae, Pseudomonas syringae* pvs.*, Acidovorax avenae* subsp. *avenae, Xanthomonas translucens* pvs., *Rathayibacter rathayi*, and *Pseudomonas cichorii*. Reliable information on the distribution of these bacterial strains in the Russian Federation is limited. Methods for the isolation and identification of these bacterial pathogens have not been developed to date, which increases the risk of the spread of phytopathogens that could cause significant economic harm to agriculture.

The purpose of this study was to isolate and identify the causative agents of bacterial diseases of wheat and barley. In order to do this, we collected samples of plant material of wheat and barley in the Rodionovo-Nesvetaysky, Myasnikovsky, Zernogradsky, Azovsky, and Martynovsky districts of the Rostov Oblast. Various bacterial strains were isolated from the obtained samples using the appropriate cultural media. The strains were tested by polymerase chain reaction (PCR) using primers designed for the 16S ribosomal RNA (PSF/PSR and 8UA/519B) and SyD1/SyD2 primers selected for the *Pseudomonas syringae* genome (GenBank CP047267.1) with subsequent sequencing according to the Sanger method. As a result, the following bacterial strains were isolated and identified from wheat and barley samples: *Curtobacterium* sp.*, Paenibacillus* sp.*, Enterobacteriaceae, Pseudomonas azotoformans, P. poae, P. azotoformans, P. hibiscicola, P. fluorescens, Stenotrophomonas* sp., *P. syringae* pv. *syringae, P. syringae* pv. *atrofaciens, Bacillus* sp.*, Erwinia* sp.*, Pantoea* sp., and *Pantoea agglomerans*.

INTRODUCTION

Since 2011, the Russian Federation (RF) is one of the three largest world exporters of cereal products, in particular wheat and barley [1]. In each of the countries purchasing Russian grain, there are requirements for the quality of imported grain crops. Particular attention is paid to the phytosanitary condition of the grain. Quarantine and plant protection organizations in different countries have Lists of pests recommended for regulation as quarantine pests (the List of quarantine pests). Section A1 of this List includes quarantine harmful organisms that are absent in this country, and section A2 includes quarantine harmful organisms present but not widespread in this country. Pests included in both section A1 and A2 of the List are controlled organisms in this country. In Europe, these tasks are carried out by the European and Mediterranean Plant Protection Organization (EPPO) [2]. Most countries importing Russian cereal products have a List of quarantine pests that includes a list of harmful microorganisms whose presence in grain crops is not allowed. It should be noted that, when specifying the taxonomic names of bacteria, synonyms can be used, which complicates the work of phytosanitary services. The following species are included in the List of quarantine pests of cereal diseases of genera *Triticum* L. (wheat) and *Hordeum* L. (barley) of the *Poaceae* family (cereals):

- *Pectobacterium rhapontici* (Millard 1924) Patel & Kulkarni 1951, the causative agent of pink grain of wheat and rye. Synonyms: *Erwinia rhapontici* (Millard 1924) Burkholder 1948 [3], *Erwinia carotovora* var. *rhapontici* (Millard) Dye [3]. This phytopathogen has not yet been described in the RF. Cereals are not the main host plants of this bacterium, but *P. rhapontici* is included on the Lists of quarantine pests of countries in East Africa and South Africa (the regulated product is wheat) [2];

- *Rathayibacter tritici* (Carlson & Vidaver) Zgurskaya, Evtushenko, Akimov & Kalakoutskii, causative agent of yellow slime disease of wheat. Synonyms: *Clavibacter tritici* (Carlson & Vidaver) Davis, Gillaspie, Vidaver & Harris, *Corynebacterium michiganense* pv. *tritici* (Hutchinson) Dye & Kemp, *Corynebacterium tritici* (Hutchinson) Burkholder, *Phytomonas tritici* (Hutchinson) Bergey, *Pseudomonas tritici* Hutchinson [2]. This bacterium infects wheat [4] and is included on the List of quarantine pests (section A1) in Brazil, Uzbekistan, Greece, Moldova, and the countries of the Eurasian Economic Union (EAEU), and is also on the List of quarantine pests in the US [2] as well as the People's Republic of Bangladesh [5];
- *Pseudomonas fuscovaginae* (ex Tanii *et al*. 1976) Miyajima *et al.* 1983 [3], the causative agent of the sheath brown rot of the leaf cover of cereal crops. It mainly affects wheat but is currently considered a plant pathogen for other cereals, including corn and sorghum [6]. The bacterium is on the List of quarantine pests (section A1) in Egypt [7], and belongs to the quarantine species in Nigeria. The microorganism is distributed in Asia (Japan, China, Malaysia, Iran, the Philippines, Nepal), Oceania (Australia), South America (Brazil), North America (Mexico), and Africa (Burundi and Madagascar) [8]. Information on its distribution in the RF is not available;
- *Pseudomonas syringae* pv. *atrofaciens* (McCulloch) Young, Dye & Wilkie [2], causative agent of basal glume rot of wheat. A synonym is *Pseudomonas atrofaciens* (McCulloch) Stevens. In addition to wheat, the phytopathogen affects rye, barley, and oats. Data on its distribution in the RF are not available. This causative agent belongs to the quarantine species in Mexico [2] and Egypt [7];
- *Pseudomonas syringae* pv. *coronafaciens* (Elliott) Young, Dye & Wilkie, causative agent of halo blight of rye. Bacteria can also affect oats. In the phytosanitary requirements of the countries importing cereals, the regulated product for this phytopathogen is wheat. This phytopathogen belongs to the quarantine species in Nigeria and Mexico and it is included in the List of quarantine pests, section A2 in East and South Africa. The bacterium is distributed in the RF [2];
- *Pseudomonas syringae* pv. *syringae* van Hall 1902 [2], causative agent of bacterial canker. It can affect plants of various species, including cereals. Exact data on its distribution in the RF are not available. It is included in the List of quarantine pests, section A1 in Brazil and in section A2 in Jordan as well as on the List of quarantine pests of Mexico [2];
- *Acidovorax avenae* subsp. *avenae* (Manns 1909) Willems *et al.* 1999 [9]; the causative agent of bacterial leaf streak. Synonyms: *Pseudomonas avenae* subsp. *avenae* Manns 1909, *Acidovorax avenae* (Manns) Willems, Goor, Thielemans, Gillis, Kersters & De Ley. The bacterium can cause diseases in many plants of economic importance, including rice, corn, oats, sugarcane, millet, and foxtail [10, 11]. It belongs to the quarantine species for wheat in Egypt [7] and Morocco and is in section A2 of the List of quarantine pests in East Africa and South Africa. It is believed that the bacterium is absent in the RF [2];
- *Xanthomonas translucens* pv. *cerealis* (Hagborg) Vauterin, Hoste, Kersters & Swings, causative agent of bacterial black chaff of rye. Synonyms: *Xanthomonas campestris* pv. *cerealis* (Hagborg) Dye, *Xanthomonas translucens* f. sp. *cerealis* Hagborg. It affects cereals such as wheat, rye, barley, and oats. There are no exact data on its distribution in the RF. It belongs to the quarantine species in Mexico [2];
- *Xanthomonas translucens* pv. *translucens* (Jones, Johnson & Reddy) Vauterin, Hoste, Kersters & Swings, causative agent of bacterial black chaff of barley. The name of this bacterium has many synonyms: *Pseudomonas translucens* (Jones, Johnson & Reddy) Stapp, *Xanthomonas campestris* pv. *hordei* (Hagborg) Dye, *Xanthomonas campestris* pv. *translucens* (Jones, Johnson & Reddy) Dye, *Xanthomonas translucens* (Jones, Johnson & Reddy) Vauterin, Hoste, Kersters & Swings, *Xanthomonas translucens* pv. *hordei* (Hagborg) Dye. The main host plant is barley. The pathogen can affect rye and wheat as well as herbs: timothy grass, bromus, and creeping wheatgrass. It is believed that the bacterium is distributed in the RF. It belongs to the quarantine species in Morocco and Nigeria, and is included in section A2 of the List of quarantine pests in Jordan and Turkey [2];
- *Xanthomonas translucens* pv. *graminis* (Egli, Goto & Schmidt) Vauterin, Hoste, Kersters & Swings, the causative agent of bacterial wilt of crops. Synonyms: *Xanthomonas campestris* pv. *graminis* (Egli, Goto & Schmidt) Dye, *Xanthomonas graminis* Egli, Goto & Schmid. It affects cereals, including wheat, rye, and barley. It can also infect wild species such as cocksfoot grasses. This phytopathogen is found in Europe (France, Germany, Switzerland, and Great Britain) [2]. Exact data on the distribution in the RF are not available. It belongs to the quarantine species in Egypt [7];
- *Xanthomonas translucens* pv. *undulosa* (Smith, Jones & Reddy) Vauterin, Hoste, Kersters & Swings [2], the causative agent of bacterial black chaff of wheat. Synonyms: *Phytomonas translucens* var. *undulosa* Stapp, *Xanthomonas campestris* pv. *undulosa* (Smith, Jones & Reddy) Dye, *Xanthomonas translucens* f. sp. *undulosa*. It is believed that this pathogen is distributed throughout the world and affects wheat and barley [12]. It is included in the List of quarantine pests in Nigeria;
- *Rathayibacter rathayi* (Smith) Zgurskaya, Evtushenko, Akimov & Kalakoutskii, gumming disease. Synonyms: *Corynebacterium michiganense* pv. *rathayi* (Smith) Dye & Kemp, *Corynebacterium rathayi* (Smith) Dowson, *Phytomonas rathayi* (Smith) Bergey *et al.* The bacterium infects rye, wheat, and the cocksfoot grasses. This species is widespread in many European countries (Austria, Cyprus, Denmark, Germany, Norway,

Romania, Sweden, the UK), but it is absent in the RF. It is included in section A1 of the List of quarantine pests in South Africa [2];

- *Pseudomonas cichorii* (Swingle) Stapp, the causative agent of bacterial blight of various crops. It infects mostly salad [13], but for those countries importing cereals, the regulated product is also wheat [7]. Other names: *Phytomonas cichorii*, *Pseudomonas endiviae*, *Pseudomonas papaveris.* The bacterium is widespread on all continents. It is included in the List of quarantine pests of Mexico, in the List of quarantine pests, section A1, in Egypt, and in section A2 in Jordan [2].

The causative agents of bacteriosis of cereal crops, except *Rathayibacter tritici*, are not considered as the quarantine species in the RF. Therefore, there were no attempts to identify them, and reliable data on their distribution in the RF are not available. In addition, there is no diagnostic techniques for a number of bacterial phytopathogens of cereals. Accordingly, the identification of pathogens of bacterioses (with the exception of *Rathayibacter tritici*) in batches of both exported and imported grain in the RF is not currently carried out. That leads to a high risk of the penetration of phytopathogens into the RF, which can cause significant economic harm to the agriculture.

Therefore, the development of diagnostic methods for the phytopathogens of cereals is an urgent task.

The purpose of this study was to isolate and identify the causative agents of bacterial diseases of wheat and barley in order to determine the phytosanitary quality of Russian grain products.

MATERIALS AND METHODS

Collection of samples

A total of 1,720 hectares (ha) of cultivated areas were examined, of which 1,536 ha were sown with winter wheat and 184 ha were sown with barley. During the survey, winter wheat and barley plants were in the heading phase. Plants with symptoms of spotting, the presence of strokes and stripes as well as signs of wilting and deformation of the leaves were examined first. The corresponding symptoms are common for diseases caused by various bacteria e.g. *Pseudomonas syringae* pvs. Attention was also paid to the presence of aphids, which is a potential carrier of bacteria *Xanthomonas translucens* pvs. [14]. Particular attention was paid to plants with rot and chlorotic-necrotic stripes on the leaves, since such symptoms can be caused by *X. translucens* pv. *translucens* [15]. Plants with mechanical damage have also been noted, since damage can facilitate the penetration of pathogenic bacteria, e.g. *X. translucens* pv. *graminis*, in protoxylem tissue, from where the pathogen subsequently migrates into vascular tissue [16]. One sample of the vegetative parts of cereal plants with the corresponding symptoms was taken from each plot with the addition of a sufficient amount of healthy tissue sample of the same plants. The collection and packaging of the samples was organized in a way that would prevent damage, contact with other samples, and contamination. The leaves and stems were placed between the layers of the filter paper in containers with air access. The samples were stored at a temperature of 2°C to 8°C prior to the study.

Preparation of analytical samples

Suspensions of the microbiota of all the samples (analytical samples) were obtained in the laboratory. Sampling was carried out from sites at the junction of symptomatic and healthy tissues. A laboratory specimen of 1.0 g to 2.5 g was taken from each sample and placed in a 100 ml container. The specimens were weighed on a laboratory electronic balance (AJH-4200CE, Vibra, Japan). Phosphate-buffered saline (PBS) [17] was added to the laboratory specimen (20:1, by weight), followed by shaking vigorously on a Unimax 2010 rotary shaker (Heidolph, Germany) at 200 rpm for 45-60 min. The extract was separated from impurities of plant tissues by gravity filtration using the fine filters "Blue Ribbon", pore size 3-5 μm. The resulting extracts were centrifuged for 10 min at 4°C (10,000 g, Allegra X-30R, Beckman Coulter, Denmark). Immediately after centrifugation, the supernatant was removed and the pellet was resuspended in 1 ml of PBS. The resulting suspension was used to isolate the bacteria.

Bacteria isolation

The cultures were isolated on CRL, CRL.2, and mCRL.2 media. The CRL medium contained the following components: peptone 12.0 g, glycerol 10.0 g, agar 18.0 g, $MgSO_4$ 0.7 g, K_2HPO_4 2.0 g, KH_2PO_4 2.0 g, glucose 2.5 g, yeast extract 2.0 g, meat peptone 2.0 g, sucrose 15.0 g, NaCl 2.0 g, $CaCO_3$ 20.0 g (PanReac AppliChem, Spain) per 1 liter of distilled water. The CRL.2 medium consisted of the same components, with the exception of $CaCO_3$. All of the ingredients were mixed, the pH was adjusted with a 20% hydrochloric acid solution to 7.0-7.2 using a pH meter (MP 220, Mettler Toledo, Switzerland) and sterilized at 121°C for 15 min (autoclave, MLS -3020U Sanyo, Japan). In order to obtain the mCRL.2 medium the following reagents were added to the medium CRL.2 (after sterilization and cooling to 50°C): an alcohol solution of cycloheximide (70%) to a final concentration of 200 mg per 1 liter of the medium and an aqueous solution of 2,3,5-triphenyltetrazolium chloride (TTX) to a final concentration of 50 mg per 1 liter of medium.

Analytical samples were used to prepare a series of tenfold dilutions in PBS. Dilutions of 10^{-3} and 10^{-4} in a volume of 100 μl were sown using a Drigalski spatula on Petri dishes with an mCRL.2 medium using the stretching method for 2 cups in two repeats (4 cups for each sample). After seeding, the Petri dishes were tightly wrapped with Parafilm and kept at 25°C in an incubator (MIR-254, Panasonic Healthcare Co., Ltd., Japan) for five days. After that, the colonies of various morphotypes were selected. Some colonies were reseeded into Petri dishes with the CRL and CRL.2 media using a bacteriological loop in order to obtain a pure bacterial culture. Petri dishes

wrapped with Parafilm were incubated at 25°C for 72 h. Separate colonies were then selected using a bacteriological loop and placed into microtubes with 200 µl PBS.

PCR and sequencing

In order to lyse the cells, the samples were incubated for 10 min at 96°C and cooled in the freezer for 5 min. The obtained preparations were used for PCR that was carried out in several steps. In the first step, the DNA of all the isolates was tested by classical PCR with PSF/PSR primers (PSF 5'-AGC CGT AGG GGA ACC TGC GG-'3, PSR 5'-TGA CTG CCA AGG CAT CCA CC-'3) specific for the genus *Pseudomonas*. *Pseudomonas syringae* pv *syringae* strains obtained from the Hungarian collection AOBC PPSCD were used as a positive control. The reaction mixture for one reaction contained 16.0 µl of deionized water, 5.0 µl of 5X MasDDTaqMIX-2025 master mix (ZAO Dialat, RF), and 1.0 µl of each primer at a concentration of 10 pmol. An aliquot of 2 µl of DNA was used for every reaction. The total volume of the reaction mixture was 25 µl. Amplification was carried out according to the following protocol: initial denaturation 95°C for 10 min, then 25 cycles: 95°C for 20 s, 64°C for 15 s, 72°C for 15 s, and then final elongation 72°C for 2 min.

Isolates that showed negative results in PCR with PSF/PSR primers were retested in PCR with universal 8UA/519B primers specific for the 16S-23S ribosomal RNA (rRNA) (8UA: 5'-AGA GTT TGA TCM TGG CTC AG-3 ', 519B: 5'-GTA TTA CCG CGG CKG CTG-3 '). The size of the amplification product was 500 base pairs (b.p.) Primers were obtained from LLC Evrogen (RF). Reaction mixture for one sample contained 14 µl of deionized water, 5 µl of a 5X MasDDTaqMIX-2025 master mix (ZAO Dialat, RF), and 2 µl of each primer at a concentration of 10 pmol. Then, 2 µl of the sample DNA template was added to the microtube. The total volume of the reaction mixture was 25 µl. Amplification was carried out according to the following protocol: initial denaturation 96°C for 10 min, then 35 cycles: 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, and then final elongation 72°C for 10 min.

The isolates that showed positive results in the PSF/PSR primer assay were further analyzed with SyD1/SyD2 primers (SyD1 5′-CAGCGGCGTTGCGTCCATTGC-3′, SyD2 5′-TGCCGCCGACGATGTAGACCAGC-3′) specific for *Pseudomonas syringae* pv. *syringae*. *Pseudomonas syringae* pv *syringae* strains were also used as a positive control. Reaction mixture for one reaction contained 17.4 µl of deionized water, 5 µl of a 5X MasDDTaqMIX-2025 master mix (ZAO Dialat, RF), and 0.3 µl of each primer at a concentration of 10 pmol. Then, 2 µl of the sample DNA was added to each tube. The total volume of the reaction mixture was 25 µl. Amplification was carried out according to the following protocol: initial denaturation 95°C for 10 min, then 25 cycles: 95°C for 20 s, 64°C for 15 s, 72°C for 45 s, and then final elongation 72°C for 7 min.

Bio-Rad T100 Thermal Cycler amplifier (Bio-Rad, USA) was used for all the PCR reactions. The identification of reaction products was accomplished on a 1.5% agarose gel using a Power Pac HV electrophoresis power supply and a Bio-Rad Imaging System (Bio-Rad, USA).

For each sample that was positive in one of the tests, Sanger sequencing was used for identification [18] according to the following protocol. Amplicons obtained by PCR were purified using the DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Then, in each sample containing purified amplicons, the DNA concentration was measured using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, USA). The DNA concentration of each sample was adjusted to the standard concentration, based on the length of the amplification product on an electropherogram (product length divided by 100). Water was used for dilution. Then, the amplification was carried out using the Big Dye Kit (Thermo Fisher Scientific, USA) that contains labeled dNTPs to produce a labeled chain. A reaction mixture for one reaction contained: 1 µl Big Dye 3.1, 1.5 µl Big Dye buffer, 2 µl primer (direct or reverse) at a concentration of 0.8 pmol, 4.5 µl water, and 1 µl DNA. Amplification was carried out according to the following protocol: 96°C for 1 min, then 25 cycles 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. After amplification, DNA was purified using the BigDye® XTerminator™ Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Then, 25 µl of purified DNA were added to the wells of an AB-3500 sequencer (Applied Biosystems, USA, Japan). The sequencing program was selected based on the length of the fragments. The results were processed using the BioEdit program. The obtained nucleotide sequences were compared with the bacterial sequences hosted in the GenBank using the BLAST service [19].

RESULTS

Identification of bacterial isolates

In this study, bacterial specimens were isolated from samples of wheat and barley. Samples of plant material were taken in May 2019 in the Rodionovo-Nesvetaysky, Myasnikovsky, Zernogradsky, Azovsky, and Martynovsky districts of the Rostov Oblast (Fig. 1). Plants with disease symptoms (spotting, streaks, and stripes) were selected (Fig. 2). Information about the examined crops varieties and the corresponding areas under crops is shown in Table 1. In total, 22 samples were selected. Colonies of various morphotypes were obtained by isolating bacterial cultures on the mCRL.2 medium (Fig. 3). For PCR tests and identification, 116 colonies were selected.

In order to select isolates belonging to the genus *Pseudomonas*, the DNA of all the isolates was tested by PCR with PSF/PSR primers specific for this genus [20]. As a result of the testing, amplification products with lengths from 420 to 750 b.p. were obtained for 43 DNA samples from 116 bacterial isolates (Fig. 4A). In 31 of the 43 cases, the length of the amplification products was 600 b.p., which corresponded to the positive control (Fig. 4B).

Fig. 1. Map of the Rostov Oblast. The areas of the conducted survey of grain crops are shown in blue.

Fig. 2. Symptoms of a bacterial disease in wheat plants.

Table 1. The studied cultivated areas sown with winter wheat and barley in different districts of the Rostov Oblast.

District	Cereal	Variety	Area (ha)
Rodionovo-Nesvetaysky	spring barley	Leon	18
	winter wheat	Kalym	47
		Alekseich	50
		Tarasovskaya 70	57
Myasnikovsky	winter wheat	Bezostaya 100	168
		Kalym	146
		Lydia	90
		Bagrat	88
	winter barley	Dostoinyi	15
		Espada	15
Zernogradsky	winter wheat	Tabor	100
		Tanais	81
		Tanya	252
		Tabor	114
		Bagrat	110
Azovsky	winter wheat	Sila	69
		Vassa	42
		Stan	46
		Yukka	42
		Grom	16
	winter barley	Timofey	136
Martynovsky	winter wheat	Bagrat	18

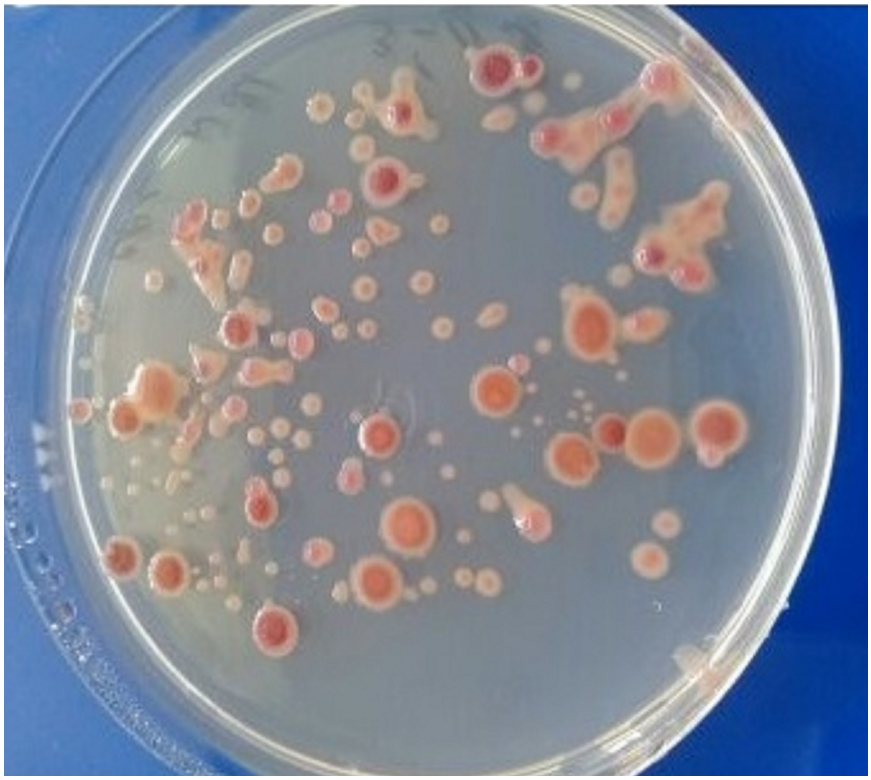

Fig. 3. Bacterial colonies of various morphotypes obtained on the mCRL.2 media. Colonies were obtained at 72 h post incubation of the microbiota suspension.

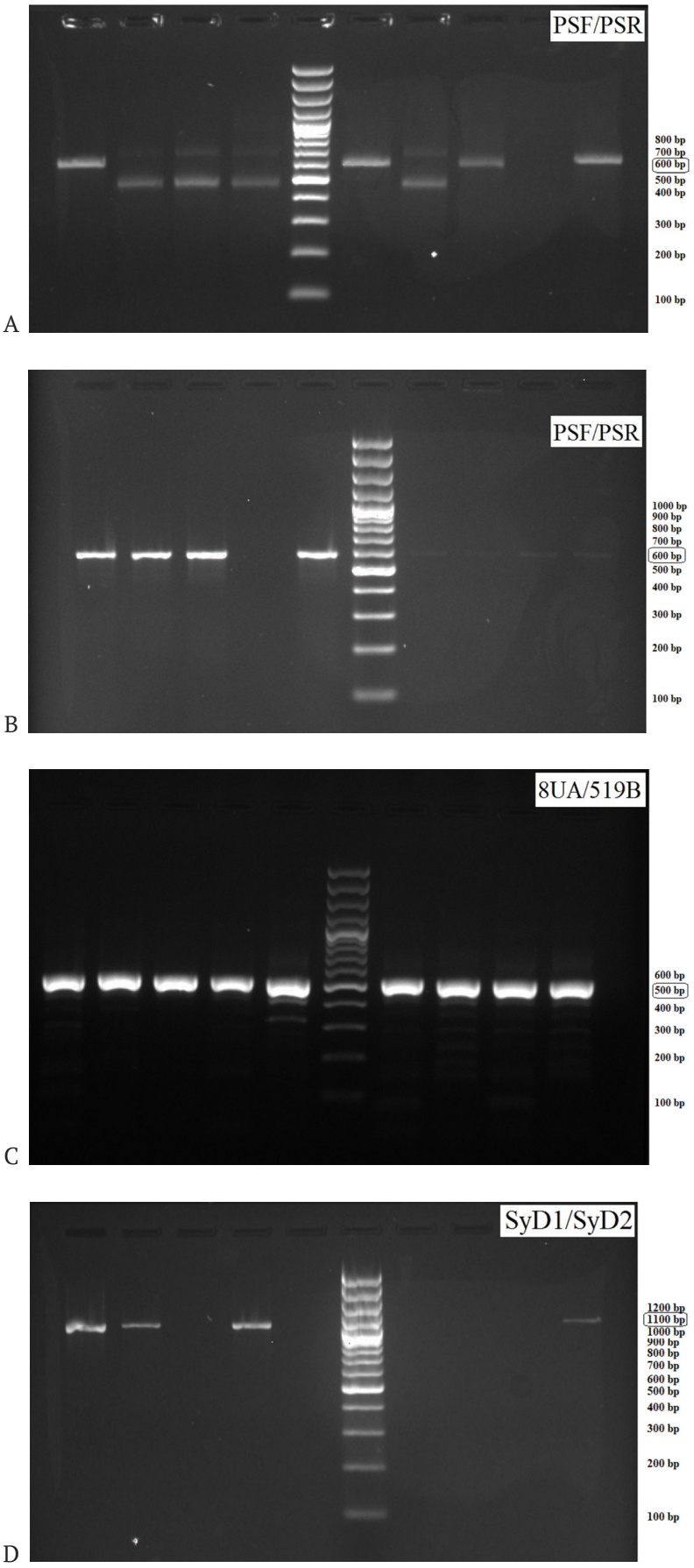

Fig. 4. PCR products obtained for the samples studied in the agarose gel. (A) PCR products obtained with PSF/PSR primers for the samples of bacterial isolates. (B) PCR products obtained with PSF/PSR primers corresponding to the genus *Pseudomonas* (600 b.p.). (C) PCR products obtained with 8UA/519B primers (500 b.p.). (D) PCR products obtained with SyD1/SyD2 primers. The positive control sample in Fig. 4 B, C is presented in the first well.

Specimens isolated from 73 bacterial samples that showed a negative result when tested with PSF/PSR primers were further analyzed in PCR with universal 8UA/519B primers specific for the 18S-23S rRNA [21]. The resulting amplification products had a length of 500 b.p. (Fig. 4C). Specimens isolated from 43 bacterial samples that showed a positive result when tested with PSF/PSR primers were further analyzed with SyD1/SyD2 primers specific for *Pseudomonas syringae* pv. *Syringae* [20]. As a result, 1,100 b.p. fragments corresponding to the positive control were obtained for 7 samples (Fig. 4D).

Therefore, we obtained 43 samples of amplified products with PSF/PSR primers, 73 with 8UA/519B primers, and 7 with SyD1/SyD2 primers for identification by sequencing. The results of the sequencing of these amplification products are shown in Table 2.

The following bacterial isolates were identified by sequencing the amplification products with PSF/PSR primers:

- *Pseudomonas fluorescens* (from the sample of Alekseich cultivar winter wheat, the Rodionovo-Nesvetaysky district as well as from the sample of Tabor cultivar winter wheat, the Zernogradsky district). The bacterial colonies on the mCRL.2 culture medium appeared as round circles with uneven edges, 0.02 mm in diameter, white;
- *P. poae* (from a sample of Alekseich cultivar winter wheat). The culture on the mCRL.2 medium appeared as round colonies of a regular shape of pink color, 0.05 mm in diameter;
- *Curtobacterium* sp. (from a sample of Kalym cultivar winter wheat, the Myasnikovsky district). The culture on the CRL.2 medium appeared as flat yellow-beige colonies of irregular shape, 1.0 mm in diameter. On the CRL medium, these colonies were bright orange;
- *P. azotoformans* (from a sample of Timothy cultivar winter barley of the Azovsky district). On the CRL medium, the bacterial colonies appeared as milky white, irregular in shape, convex, up to 5.0 mm in diameter;
- *P. hibiscicola* (from a sample of Yucca cultivar winter wheat, the Azovsky district). On the CRL medium, the culture colonies appeared as dull, creamy yellow, convex, round circles with smooth edges, from 1.0 to 5.0 mm in diameter.

The other amplification products obtained with PSF/PSR primers were identified as *Pseudomonas* sp. by comparison with sequences deposited in the GenBank.

The following bacterial isolates were identified by sequencing the amplification products with 8UA/519B primers:

- *Pantoea agglomerans* (from a sample of Leon cultivar spring barley, the Rodionovo-Nesvetaysky district). The colonies of this isolate on the mCRL.2 medium appeared as circles, 4 mm in diameter, peach in color with a bright crimson center. On the CRL.2 and CRL media, the culture appeared as yellow irregular spreading colonies;
- *Enterobacteriaceae* (from a sample of Leon cultivar spring barley). On the mCRL.2 medium, the culture appeared as round, slightly convex, raspberry-colored colonies, 3.0 mm in diameter.

In all the wheat and barley samples, the isolates that belong to the bacteria *Paenibacillus*, *Stenotrophomonas, Bacillus* sp., *Erwinia* sp., and *Pantoea* sp. were also identified.

Isolates of *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *atrofaciens* were identified in the samples of Leon cultivar spring barley, the Rodionovo-Nesvetaysky district and in Kalym cultivar winter wheat, the Myasnikovsky district by sequencing of the amplification products obtained with SyD1/SyD2 primers. On the CRL.2 and CRL media, cultures of these isolates appeared as white, matte, spreading colonies of irregular shape, characterized by rapid aggressive growth (Fig. 5).

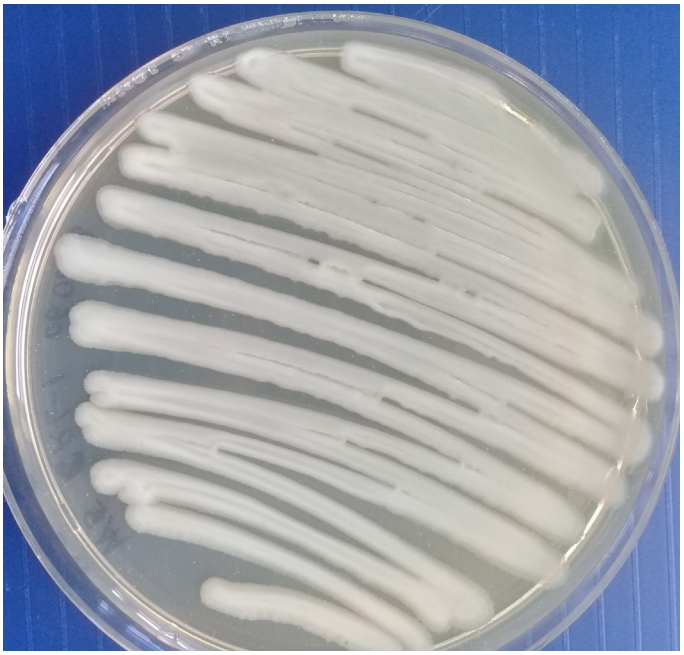

Fig. 5. Culture of *P. syringae* on the CRL.2 medium after incubation at 25°C for 48 h.

DISCUSSION

The isolation and identification of pathogens of bacterial diseases of wheat and barley was accomplished in this study. In the process of crops examining and collecting samples of winter wheat and barley in the Rodionovo-Nesvetaysky, Myasnikovsky, Zernogradsky, Azovsky, and Martynovsky districts of the Rostov Oblast, the presence of symptoms of bacterial diseases such as streaks, spotting, chlorotic and necrotic tissue sites were found. These observations serve as evidence of the presence of bacterial phytopathogens with high virulence on the grain crops in the Rostov Oblast.

Rapid bacterial growth was observed on all the experimental media. The use of the mCRL.2 medium containing cycloheximide prevented the overgrowth of Petri dishes with fungal colonies during inoculation. At the same time, cycloheximide did not exhibit bacteriostatic properties, which allowed for the isolation of a wide variety of different bacteria. Another component of the

Table 2. Bacteria isolated from the studied samples of winter wheat and barley identified by PCR with consecutive sequencing

Cereal	Variety	Bacteria identified by PCR with corresponding primers		
		PSF/PSR	8UA/519B	SyD1/SyD2
Spring barley	Leon	*Pseudomonas* sp.	*Pantoea agglomerans, Enterobacteriaceae, Paenibacillus, Stenotrophomonas, Bacillus* sp., *Erwinia* sp., *Pantoea* sp.	*P. syringae* pv. *syringae, P. syringae* pv. *atrofaciens*
Winter wheat	Kalym		*Paenibacillus, Stenotrophomonas, Bacillus* sp., *Erwinia* sp., *Pantoea* sp.	
	Alekseich	*P. fluorescens, P. poae, Pseudomonas* sp.		
	Tarasovskaya 70	*Pseudomonas* sp.		
Winter wheat	Bezostaya 100	*Pseudomonas* sp.	*Paenibacillus, Stenotrophomonas, Bacillus* sp., *Erwinia* sp., *Pantoea* sp.	
	Kalym	*P. curtobacterium* sp., *Pseudomonas* sp.		
	Lydia	*Pseudomonas* sp.		
	Bagrat			
Winter barley	Dostoinyi	*Pseudomonas* sp.		
	Espada			
Winter wheat	Tabor	*P. fluorescens, Pseudomonas* sp.	*Paenibacillus, Stenotrophomonas, Bacillus* sp., *Erwinia* sp., *Pantoea* sp.	
	Tanais	*Pseudomonas* sp.		
	Tanya			
	Tabor	*P. fluorescens, Pseudomonas* sp.		
	Bagrat	*Pseudomonas* sp.		
Winter wheat	Sila	*Pseudomonas* sp.	*Paenibacillus, Stenotrophomonas, Bacillus* sp., *Erwinia* sp., *Pantoea* sp.	
	Vassa			
	Stan			
	Yukka	*P. hibiscicola, Pseudomonas* sp.		
	Grom	*Pseudomonas* sp.		
Winter barley	Timofey	*P. azotoformans, Pseudomonas* sp.	*Paenibacillus, Stenotrophomonas, Bacillus* sp., *Erwinia* sp., *Pantoea* sp.	
Winter wheat	Bagrat	*Pseudomonas* sp.	*Paenibacillus, Stenotrophomonas, Bacillus* sp., *Erwinia* sp., *Pantoea* sp.	

medium – an aqueous solution of 2,3,5-triphenyltetrazolium chloride at a concentration of 50 mg/l – stained some bacterial colonies, which simplified the description of their morphology. The use of the CRL.2 and CRL media for the cultivation of pure cultures appeared to be promising when working with the pathogens of cereal crops.

During the PCR test with PSF/PSR primers, the reaction products of 752 b.p. length, described by the developers of these primers, were not obtained [17]. This PCR produces amplification products with a length ranging from 420 to 750 b.p. depending on the various types of bacteria of the genus *Pseudomonas* tested. The size of the amplification product for positive control of PCR, *P. syringae* pv. *syringae*, was 600 b.p. In this study, the following bacteria were identified: *Pseudomonas azotoformans, P. poae, P. hibiscicola*, and *P. fluorescens*. This test can be used as a method for determining the pseudomonas among selected colonies.

The use of 8UA/519B primers followed by sequencing allowed for the identification of bacteria of different genera, such as *Curtobacterium* sp., *Enterobacteriaceae, Pantoea agglomerans, Stenotrophomonas, Paenibacillus, Bacillus* sp., *Erwinia* sp., and *Pantoea* sp. It was impossible to identify species belonging to the genus *Pseudomonas* using these primers because the target genome site is identical in most pseudomonads.

According to the literature [17], the amplification of DNA of *P. syringae* pv. *atrofaciens* bacteria with

primers SyD1/SyD2 should lead to the formation of a reaction product with a length of 558 b.p. Despite this, we found that the size of the corresponding PCR product is 1,100 b.p., including the product for positive control, *P. syringae* pv. *Syringae*, which match the location of these primers in the target region of the *P. syringae* genome sequence (from 2966585 to 2967602 b.p., GenBank CP047267.1). The use of these primers followed by sequencing allowed for the identification of *P. syringae* pv. *atrofaciens* and *P. syringae* pv. *Syringae* bacteria that are on the Lists of quarantine pests of countries importing cereal crops.

The data shown in Table 2 show that each sample of winter wheat and barley contains bacteria of the *Pseudomonas, Paenibacillus, Stenotrophomonas, Bacillus, Erwinia*, and *Pantoea* genera in its microbiota. The bacteria *Pseudomonas, Erwinia,* and *Pantoea* potentially have the highest virulence among the found phytopathogens since exactly these bacterial genera contain the largest number of phytopathogenic species [22]. This may be due to the fact that the above-mentioned bacteria, being gram-negative, have secretion systems different from those of gram-positive bacteria [23]. The presence of the III, IV, and VI type secretion systems in gram-negative bacteria allow them to deliver the virulence factors through the cell membranes of the host plant [24]. Considering the presence of symptoms of bacterial diseases in winter wheat and barley crops in the course of the examination period, it can be assumed that isolated samples of bacteria, such as *P. syringae* pvs., are highly virulent for host plants [22].

P. syringae pv. *atrofaciens* and *P. syringae* pv. *syringae* were the only types of phytopathogenic bacteria identified in the studied samples that are included in the Lists of quarantine pests of cereal crops for importing countries. The identification methods used in this study may not be sufficient to identify some pseudomonads, such as *P. cichorii* and *P. fuscovaginae*, because when comparing the obtained nucleotide sequences with sequences deposited to the GenBank, a number of bacterial species of the genus *Pseudomonas* were not identified.

Nevertheless, the approach used in this study allows us to conclude that there were no bacteria, such as *Pectobacterium rhapontici, Rathayibacter tritici, R. rathayi, Acidovorax avenae* subsp. *Avenae*, and *Xanthomonas translucens* pvs. in the selected samples.

The data on the composition of the microbiota of wheat and barley obtained in this study can be used to determine the compliance of Russian grain products with the phytosanitary requirements of the countries importing cereal crops.

CONFLICT OF INTEREST

The author does not pursue commercial or financial interests.

CITATION

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