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Original Article

Genetic and phenotypical diversity of *Pseudomonas syringae* population in the Russian Federation

Diversidade genética e fenotípica da população de *Pseudomonas syringae* na Federação Russa

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Abstract

Proteobacteria comprising species of *Pseudomonas syringae* group cause diseases of many plants around the world. The phytopathogen has a complex taxonomic structure, which is constantly being revised due to the emergence of new molecular and biochemical diagnostic methods. Here for the first time, we describe the genetic and phenotypic diversity of 57 strains of *Pseudomonas syringae* isolated from affected soybeans, cereals, sunflowers, and other plants in the Russian Federation from 1950 to 2019. Genetic diversity was assessed by Multi Locus Sequence Analysis (MLSA) using fragments of the genes of *glyceraldehyde-3-phosphate dehydrogenase (gapdh),* the *DNA-directed RNA polymerase subunit D (rpoD), gyrase (topoisomerase) B subunit (gyrB)*, and *citrate synthase I (gltA).* The synthesis of syringomycin and coronatine by bacteria was assessed by the reaction of susceptible yeast culture, seedlings of barley, tomato, and sunflower, and by presence of toxin genes confirmed by PCR test. The pathogenicity of the strains was confirmed on seedlings of dicotyledonous and monocotyledonous plants of peas, soybean, sunflowers, barley and wheat, as the most affected crops. The sensitivity of bacteria to 10 antibiotics of the main mechanisms of activity and two bactericidal commercial products was tested by standard disc method. The obtained results showed a high genetic homogeneity of the Russian population of *P. syringae*, which infects various agricultural crops, and an increase in the proportion of antibiotic-resistant strains over the years.

Keywords: *Pseudomonas syringae*, MLSA, phytotoxins, antibiotics, resistance.

Resumo

Proteobactérias compreendendo espécies do grupo Pseudomonas syringae causam doenças de muitas plantas ao redor do mundo. O fitopatógeno possui uma estrutura taxonômica complexa, que está em constante revisão devido ao surgimento de novos métodos de diagnóstico molecular e bioquímico. Aqui, pela primeira vez, descrevemos a diversidade genética e fenotípica de 57 cepas de Pseudomonas syringae isoladas de soja, cereais, girassol e outras plantas afetadas na Federação Russa de 1950 a 2019. A diversidade genética foi avaliada por análise de sequência multilocus (MLSA) usando fragmentos dos genes da gliceraldeído-3-fosfato desidrogenase (gapdh), a subunidade D da RNA polimerase dirigida por DNA (rpoD), a subunidade B da girase (topoisomerase) (gyrB) e a citrato sintase I (gltA). A síntese de siringomicina e coronatina por bactérias foi avaliada pela reação de cultura de leveduras suscetíveis, plântulas de cevada, tomate e girassol, e pela presença de genes de toxina confirmados pelo teste de PCR. A patogenicidade das cepas foi confirmada em mudas de plantas dicotiledôneas e monocotiledôneas de ervilha, soja, girassol, cevada e trigo, como as culturas mais afetadas. A sensibilidade das bactérias a 10 antibióticos dos principais mecanismos de atividade e dois produtos bactericidas comerciais foi testada pelo método de disco padrão. Os resultados obtidos mostraram uma alta homogeneidade genética da população russa de P. syringae, que infecta várias culturas agrícolas, e um aumento na proporção de cepas resistentes a antibióticos ao longo dos anos.

Palavras-chave: *Pseudomonas syringae*, MLSA, fitotoxinas, antibióticos, resistência.

1. Introduction

Bacteria, yeast and fungi colonize the aboveground parts of plants, on the plant surface or within the plant itself. This habitat is known as the phyllosphere and its inhabitants are known as epiphytes. *Pseudomonas syringae*, a pathogenic bacterium is a leaf colonist that thrives on healthy plants by employing quorum sensing, virulence factors, and other traits. Group of γ-proteobacteria *Pseudomonas syringae* (*Psyr*) is one of the most important objects for studying the pathogenesis and resistance of plants to bacterial diseases. These bacteria infect a wide range of host plants, including

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hundreds of species of monocots, herbaceous dicots and woody dicots worldwide (Horst, 2013). On annual plants, reports of disease caused by the *Psyr* have increased within the last years. The main reasons for the increase of economic losses caused by *Psyr* in Russia are climate change, the use of susceptible plant varieties and hybrids, and the lack of effective bactericides for the disease control (Anderson et al., 2020; Jürisoo et al., 2021). *Psyr* strains are subdivided into 16 species, 4 genomospecies, and 63 pathological variants (pathovars), the names of which are historically associated with the host plant when the pathovar was first described, and into races, which are identified by the reaction of plants on a differentiating set of varieties and species of host plants (Shaydayuk and Gultyaeva, 2020). Despite this fact, several new disease reports contradict the concept of the pathovar, thereby raising the question of whether strains in the *Psyr* are mostly generalists rather than specialists as has been currently believed (Ichinose et al., 2020; Morohoshi et al., 2021).

Some *Psyr* strains are virulent against a large number of plant species, while others, on the contrary, are specialized (Nobori et al., 2018; Morohoshi et al., 2021). This bacterium is ubiquitous as an epiphyte on healthy plants, as a symbiont - in phytophagous insects, mites, and nematodes (Oukala et al., 2021), and found within all phases of the water cycle in nature, often in association with water plants and algae (Hieu and Thao, 2019). Most *Psyr* isolates show high phenotypic homogeneity and virulence in relation to different plants (Cheng et al., 2017). The classification of this species was first based on phenotypic traits, on the results of DNA-DNA hybridization, and recently - on the data of phylogenetic analysis of gene sequences (Multi Locus Sequence Analysis/Typing - MLSA/MLST), and comparison of complete genomes of bacteria (Horita et al., 2014; Rong and Huang, 2014; Jacques et al., 2015; Nowlan et al., 2020).

Some authors used MLST/MLSA to assess the genetic diversity of the *Psyr* population as a whole and for individual species/pathovars of this bacterium (Monteil et al., 2016).

Pseudomonas syringae was described by Van Hall at 1902 (Shabani et al., 2019) and several closely related species have since been described. Later, several other species closely related to *Psyr* were proposed and validated: *P. meliae*, *P. savastanoi*, *P. ficuserectae*, *P. avellanae*, *P. cannabina*, *P. tremae*, *P. congelans*, *P. asturiensis*, *P. cerasi*, and *P. caspiana*. The P. syringae species complex is usually considered to include all these taxonomically closely related species. These groups correspond to genome species previously identified using DNA-DNA hybridization (Gardan et al., 1999; Rabêlo et al., 2021), and are confirmed by genome-wide analysis of representatives of this species (Degrassi et al., 2019; Lalucat et al., 2020).

For *Psyr* the isolated strains are assigned to many pathovars, which, however, have no taxonomic significance (Berge et al., 2014). One of the newly isolated species, *Pseudomonas savastanoi* (*Ps*, genomic group 3), includes legume pathogens *Ps.* pv. *glycinea* (Psg) and *Ps.* pv. *phaseolicola*, reducing the yield of soybeans and beans by 40% under favorable for infection conditions (Marcelletti et al., 2011; Scortichini et al., 2013).

Various *Psyr* strains use an extensive arsenal of biochemical mechanisms of virulence towards certain host plants, including phytotoxins, ice condensation proteins (Bender et al., 1999), and effectors of third and fourth type secretion systems (T3SS, T4SS) which determine the specific nature of pathogen virulence (Abramovitch and Martin, 2004; Sultanov et al., 2016). *Psyr* patovars synthesize four main types of phytotoxins: coronatine, phaseolotoxin, syringomycin, and tabtoxin (Bender et al., 1999; Abramovitch and Martin, 2004; Panchal et al., 2016).

There are a few articles devoted to analysis of a limited number of *Pseudomonas* strains isolated from grain crops of the Russian Federation (Kahala et al., 2012; Yerlikaya et al., 2021; Zhang et al., 2022). According to the published data, the Russian population of *Psyr* was the variable in the presence/absence and activity of syringomycin. Syringomycin (lipodepsinonapeptide) causes the loss of electrolyte from the cytoplasm through pores in the membrane of the plant cell. Affected plant cells release of nutrients that occurs as a consequence of cellular lysis and benefit *Psyr* growth in plant tissues. The synthesis of syringomycin is under the control of several genes (*syrA-SyrE*), some of which (for example, *syrD*) were used for express diagnostics of a syringomycin-positive phenotype using PCR test. Coronatine, a potent bacterial phytotoxin, is a molecular mimic of the plant hormone jasmonoyl-L isoleucine. Coronatine activates jasmonic acid signaling, induces jasmonic-responsive genes, and antagonizes the action of the immune signal salicylic acid. Coronatine consists of two components, coronafacic acid and coronamic acid. The genes that encode for coronafacic acid and coronamic acid biosynthesis are not constitutively expressed in the bacterium. These genes are induced on the plant leaf surface, or *in vitro* when the bacterium is grown in inducing medium. *Psyr* activates coronatine genes at the pre-invasive phase of its life cycle to open stomates and infect plants at night time (in darkness) that favor bacteria movement into leaf tissues. This functional attribute of coronatine may provide epidemiological advantages for the bacteria on the leaf surface (Bender et al., 1999; Abramovitch and Martin, 2004; Marcelletti et al., 2011). Although toxins are potentially important for bacterial virulence, none of them are sufficient for the disease process (Panchal et al., 2016).

Pseudomonas bacteria are generally more resistant to biocides comparing to other plant pathogenic bacteria. A limited number of antimicrobials such as copper-based compounds (copper sulfate, copper hydroxide, cuprous oxide, copper oxychloride, copper ammonium carbonate, and copper octanoate), and a few antibiotics (streptomycin, kasugamycin) are used for bacterial diseases control. However, they have been used for many decades to curb the development of crop diseases caused by bacteria and oomycetes (Collmer et al., 2008; Matveeva et al., 2008). For the test, we selected 10 antibiotics of different mechanisms of activity and two commercial bactericides (antibiotic producents Streptomyces – Fitolavin-300, Fitoplasmin) approved for use as biocontrol agents in Russia

In this work, we for the first time studied the genetic structure of the population of 57 Russian *Psyr* strains by MLST using fragments of four genes: *glyceraldehyde-3-*

phosphate dehydrogenase (*gapdh*), the *DNA-directed RNA polymerase subunit D* (*rpoD*), *gyrase (topoisomerase) B subunit* (*gyrB*), and *citrate synthase I (gltA)*, and evaluated some physiological properties of the studied strains, including pathogenicity to the most common host plants, the activity of syringomycin, the phytotoxin synthesized by bacteria in the range of 20-28°C, and the sensitivity of bacteria to antibiotics of the main groups of the mechanism of action.

2. Material and Methods

2.1. Bacterial strains

A collection comprised of 57 strains isolated in 1950-2019 in various regions of Russia was provided by Russian State Agrarian University – Moscow Agricultural Academy named by K.A. Timiryazev, All-Russian Research Institute of Phytopathology, and RUDN University) (Table 1).

It covered in particular, the Southern region (Rostov, Voronezh regions, Republic of Crimea), the Central region (Moscow, Tula, Ryazan, Bryansk, Ivanovo regions); the North Caucasian region (North Ossetia-Alania, Krasnodar Territory, Stavropol Territory, Dagestan), the Central Black Earth Region (Tambov, Kursk, Belgorod, Lipetsk, Oryol Regions, Northwest Region (Leningrad, Novgorod Regions), the Volga Region, and the West Siberian region.

The MLST sequences of 12 type strains representatives of different species and genomic groups of *Psyr* were obtained from the Genbank [National Center for Biotechnology Information (NCBI, 2022)].

2.2. DNA isolation

The bacteria were cultivated on King's B agar medium (Lelliott et al., 1966; Schaad et al., 2001). Total DNA samples were isolated from 2-3 days-old cultures for using the method of sorption on magnetic particles (Miniprep kit, LLC "Sileks", Russia), according to the manufacturer's instructions.

2.3. Phenotypic analysis

The morphological, physiological, and biochemical characteristics (including LOPAT) of bacterial cultures were determined using the methods of phenotypic differentiation of the genus *Pseudomonas* described in the Manual for the identification of phytopathogenic bacteria (Green et al., 2010).

2.4. Assessment of bacterial response to antibiotics

Sensitivity of *Psyr* strains to antibiotics was checked by the method of antibiotic-containing discs. We used antibiotics representing different mechanisms of activity: 1) penicillin, 2) cephalosporin, 3) vancomycin, 4) nicomycin, 5) nystatin, 6) chloramphenicol, 7) polymyxin, 8) streptomycin, 9) erythromycin, 10) tetracycline (Research Center of Pharmtherapy, Moscow), and registered commercial bactericide for plant protection 11) Fitolavin-300, 12) Fitoplasmin (FarmBiomed Co., Moscow, Rus.). NBY agar medium supplemented after autoclaving with 50 ml of 10% glucose and 1 ml of 1 M magnesium sulfate (Green et al., 2010) was used for disk assay.). Bacteria were grown for 24 hours in a liquid LB medium, composition (g/l): yeast extract - 5, tryptone - 10, sodium chloride - 5. Petri plates were inoculated by spreading 100 µl of bacterial suspension (10^8 CFU/ml) with sterile glass spatula. The areas with no growth indicated a bactericidal effect. The result was recorded on the 5th day. The experiment was repeated 4 times. Analysis of obtained results was conducted by Ward cluster analysis using STATISTICA 6.0 (StatSoft, USA), and 5 groups of bacterial reaction were identified (Table 1) (Ignjatov et al., 2007).

2.5. Identification of syringomycin and coronatine activity on a model object and on plants

The synthesis of syringomycin on potato-dextrose agar (PDA) was assessed by inoculating the center of the plate with an aliquot of fresh bacteria culture, incubating the plates at 20 or 28°C for 48 hours and spraying with a spore suspension of the yeast species *Rhodotorula pilimanae* MUCL, followed by additional incubation at 20°C for 48 hours (Ward Junior, 1963). The syringomycin activity was assessed by the maximum radius of the zone of inhibition of yeast growth (Green et al., 2010). To assess the reaction of plants, sunflower and wheat seeds were soaked in a suspension of bacterial strains at a concentration of 10^8 CFU/ml obtained during 24 h of cultivation in Luria's liquid medium (Ward Junior, 1963). Counting was performed on days 3, 5, and 7 after incubation at room temperature.

For the coronatine production, bioassay on the potato slices were performed. Bacterial suspension was prepared in PDA and applied on potato slices. Visualization of coronatine production was confirmed by hypertrophy of the tissue slices caused by bacteria.

2.6. PCR amplification and sequencing of the syringomycin synthetase and coronafacate ligase genes

The PCR test for *syrD* gene was carried out in 25 µl of a reaction mixture containing 25 pmol of each primer syrD1 and syrD2 (Table 2), 0.5 U *Taq* DNA polymerase (Evrogen, Moscow). The mixture of nucleotides (dNTPs) was added in the amount of 0.2 mM.

The PCR test for *cfl* gene was carried out in 25 µl of a reaction mixture containing 25 pmol of each primer PsgFOR1 and PsgREV (Table 2), specific for fragment of *cfl*-gene (650 bp) (Bultreys and Gheysen, 1999). The program for amplification of the *syrD* and *cfl* gene fragments included 37 cycles according to the protocol: 1 cycle – pre-denaturation 93°C for 3 min, 2-37 cycles - 93°C for 1 min; annealing at 60°C - 1 min, synthesis - 72°C for 1 min in cycles 2-36, and 6 min - in the 37th cycle.

2.7. PCR amplification and sequencing for MLSA

PCR amplification and sequencing were performed using previously developed primers (Table 2) and optimized protocols.

The temperature-time curve of the reaction was used as follows: initial denaturation at 94°C - 2 min; then 30 cycles: 94°C - 30 s, 61°C - 30 s, 72°C - 1 min;

Table 1. Strains of *Pseudomonas syringae* studied in this work and type strains [8], the sequences of which were used as type ones to determine phylogroups and subgroups. 1 - South region, 2 - Central region, 3 - North Caucasian, 4 - Central Black Earth region, 5 - Northwest region, 6 - Volga region, 7 - West Siberian region.

No.	Strain	Crop, variety	Region	Year	syrD PCR	Syringomycin	*Cfl* PCR	Coronatine	Wheat / Sunflower	Antibiotic	MLST cluster
1.	37	Oleander	1	1954	-	0	-	0	R/S*	6	3
2.	38	Pea	1	1980	N/D	N/D	-	0	R/S	4	2b
3.	103	Pea	2	1952	+	8	-	0	R/S	3	2b
4.	P2001	Pea	2	1964	+	4	-	0	R/S	6	2b
5.	1249	Sunflower	2	2006	+	8	-	0	R/S	5	2b
6.	1398	Spring rape	2	2006	+	10	-	0	R/S	3	1
7.	1425	Tomato	2	2006	-	11	+	1	R/S	4	1
8.	1511	Sunflower	2	2007	+	11	-	0	R/S	5	3
9.	1513	Winter rape	2	2007	+	10	-	0	R/S	3	6
10.	1545	Cucumber	4	2007	-	0	-	0	S/S	4	2d
11.	1564	Sunflower	3	2007	+	10	-	0	R/S	6	6
12.	1570	Spring rape	2	2007	+	11	-	0	R/S	4	1
13.	1634	Radish	2	2008	+	5	-	0	R/S	4	1
14.	1649	Winter rape	2	2008	+	6	-	0	R/S	3	1
15.	1651	Turnip	2	2008	+	13	-	0	R/S	3	1
16.	1710	Spring rape	2	2008	+	5	-	0	R/S	6	2b
17.	1736	Sunflower	2	2008	+	5	-	0	R/S	6	1
18.	1746	Cucumber	6	2009	+	7	-	0	R/S	6	2b
19.	1785	Winter rape	2	2009	+	14	-	0	R/S	6	2b
20.	1840	Sunflower	6	2010	+	2	-	0	R/S	1	1
21.	1845	Sunflower	1	2010	+	5	-	0	R/S	1	2b
22.	1899	Winter rape	2	2010	+	4	-	0	R/S	5	3
23.	1910	Sunflower	6	2010	+	0	-	0	R/S	5	3
24.	1918	Cucumber	5	2010	-	0	-	0	R/S	4	2b
25.	1928	Winter rape	2	2010	+	11	-	0	R/S	6	1

syrD PCR, Syringomycin – PCR reaction with primers for *syrD* gene, yeast growth supression zone, mm. *Cfl* PCR, Coronatine - PCR reaction with primers for *cfl* gene, potato slice reaction, growth presence (1)/absence (0). Antibiotic - type of reaction. MLST cluster, see Figure 1. N/D: not determined; na: not available. *Wheat/Sunflower germination test, R - seed germination and plant development are not suppressed, S - toxic effect on the plant.

Table 1. Continued...

No.	Strain	Crop, variety	Region	Year	syrD PCR	Syringomycin	*Cfl* PCR	Coronatine	Wheat / Sunflower	Antibiotic	MLST cluster
26.	1986	Sunflower	1	2011	+/-	6	-	0	R/S	5	1
27.	2025	Tomato	3	2011	+	10	+	1	R/S	1	1
28.	2069	Spring rape	2	2011	+	4	-	0	R/S	6	1
29.	2070	Winter rape	2	2011	+	13	-	0	R/S	1	1
30.	2071	Sunflower	3	2011	+	14	-	0	R/S	6	1
31.	2072	Spring rape	2	2011	+	7	-	0	R/S	1	1
32.	2073	Sunflower	1	2011	-	0	-	0	R/S	1	3
33.	2074	Sunflower	1	2011	-	0	-	0	R/S	1	3
34.	2075	Wheat	4	2011	+	11	-	0	S/S	1	2b
35.	2076	Wheat	4	2011	+	12	-	0	S/S	1	2b
36.	2081	Barley	5	2011	+	11	-	0	S/S	4	2b
37.	2083	Oat	5	2011	-	0	-	0	S/S	2	2b
38.	2100	Barley	5	2012	+	12	-	0	S/S	6	2b
39.	2104	Buckwheat	2	2012	+	11	-	0	R/S	1	2d
40.	2105	Spring rape	2	2012	+	11	-	0	R/S	1	1
41.	2108	Sunflower	6	2012	-	0	-	0	R/S	5	2d
42.	2109	Sunflower	6	2012	+	13	-	0	R/S	2	2d
43.	22401	Soybean	3	2019	+/-	6	+	1	R/S	5	1
44.	22402	Soybean	3	2019	+	10	+	1	R/S	1	1
45.	22403	Soybean	3	2019	+	4	+	1	R/S	6	1
46.	22404	Soybean	3	2019	+	13	+	1	R/S	1	1
47.	22406	Soybean	3	2019	+/-	6	+	1	R/S	5	1
48.	22408	Soybean	3	2019	+	10	+	1	R/S	1	1
49.	22412	Soybean	3	2019	+	4	+	1	R/S	6	1
50.	22414	Soybean	3	2019	+	13	+	1	R/S	1	1

syrD PCR, Syringomycin – PCR reaction with primers for *syrD* gene, yeast growth supression zone, mm. *Cfl* PCR, Coronatine - PCR reaction with primers for *cfl* gene, potato slice reaction, growth presence (1)/absence (0). Antibiotic - type of reaction. MLST cluster, see Figure 1. N/D: not determined; na: not available. *Wheat/Sunflower germination test, R - seed germination and plant development are not suppressed, S - toxic effect on the plant.

Table 1. Continued...

No.	Strain	Crop, variety	Region	Year	syrD PCR	Syringomycin	*Cfl* PCR	Coronatine	Wheat / Sunflower	Antibiotic	MLST cluster
51.	22415	Soybean	3	2019	+/-	6	+	1	R/S	5	1
52.	22416	Soybean	3	2019	+	10	+	1	R/S	1	1
53.	22422	Soybean	3	2019	+	4	+	1	R/S	6	1
54.	22424	Soybean	3	2019	+	13	+	1	R/S	1	1
55.	22425	Soybean	3	2019	+/-	6	+	1	R/S	5	1
56.	22432	Soybean	3	2019	+	10	+	1	R/S	1	1
57.	22435	Soybean	3	2019	+	4	+	1	R/S	6	1
58.	DC3000	Tomato	na	na	na	na	na	na	na	na	1
59.	Cit7	N/D	na	na	na	na	na	na	na	na	2a
60.	H5E1	Pea	na	na	na	na	na	na	na	na	2b
61.	ST99	Grape	na	na	na	na	na	na	na	na	2b
62.	508	NP	na	na	na	na	na	na	na	na	2c
63.	B728A	Beans	na	na	na	na	na	na	na	na	2d
64.	1448A	Wild bean	na	na	na	na	na	na	na	na	3
65.	LMG10912	Rice	na	na	na	na	na	na	na	na	4
66.	LMG5067	Sunflower	na	na	na	na	na	na	na	na	6
67.	ES4326	Cabbage	na	na	na	na	na	na	na	na	5
68.	LMG2252	Primrose	na	na	na	na	na	na	na	na	7
69.	Pf-5	NP	na	na	na	na	na	na	na	na	Out

syrD PCR, Syringomycin – PCR reaction with primers for *syrD* gene, yeast growth supression zone, mm. *Cfl* PCR, Coronatine - PCR reaction with primers for *cfl* gene, potato slice reaction, growth presence (1)/absence (0). Antibiotic - type of reaction. MLST cluster, see Figure 1. N/D: not determined; na: not available. *Wheat/Sunflower germination test, R - seed germination and plant development are not suppressed, S - toxic effect on the plant.

Table 2. Primers used for multilocus genotyping of strains of the Russian population of *Pseudomonas syringae* according to Hwang et al. (2005).

Gene	Primer	Oligonucleotide sequence '5-3'	PCR fragment, bp	Annealing temperature, °C
syrD	*syrD1*	CAG CGG CGT TGC GTC CAT TGC	1040	60.0
	syrD2	TGC CGC CGA CGA TGT AGA CCA GC		
cfl	*PsgFOR1*	GGC GCT CCC TCG CAC TT	650	56.0
	PsgREV2	GGT ATT GGC GGG GGT GC		
gapA	*gapAF*	CCG GCS GAR CTG CCS TGG	633	57.5
	gapAR	GTG TGR TTG GCR TCG AAR ATC GA		
gltA	*gltAF*	GCC TCB TGC GAG TCG AAG ATC ACC	980	57.8
	gltAR	CGA AGA TCA CGG TGA ACA TGC TGG		
gyrB	*gyrBF*	TCB GCR GCV GAR GTS ATC ATG AC	780	55.7
	gyrBR	TTG TCY TTG GTC TGS GAG CTG AA		
rpoD	*rpoDF*	CAG GTG GAA GAC ATC ATC CGC ATG		56.4
	rpoDR	CCG ATG TTG CCT TCC TGG ATC AG	1098	

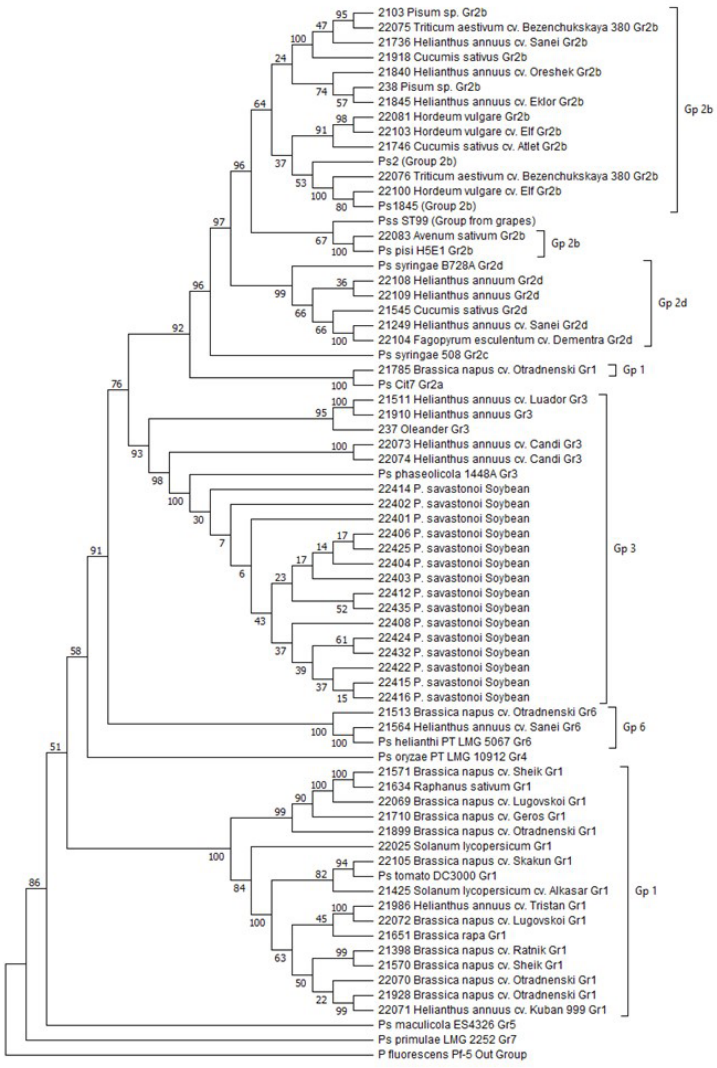

Figure 1. A phylogenetic tree based on the results of a comparative analysis of the combined nucleotide sequences of *gapdh*, *rpoD*, *gyrB*, and *gltA* (1760 base pairs in total) for 57 Russian strains of the *Psyr* species and 12 type strains for phylogroups 1-7 using the ME algorithm for bacteria of the *Pseudomonas syringae* species. The scale corresponds to 1 replacement per 100 bp (evolutionary distances). The numbers indicate the statistical significance of the branching order (%), determined using bootstrap analysis of 1000 alternative trees.

final elongation - 5 min at 72°C. PCR fragments were detected by electrophoresis in 1.5% agarose gel. Nucleotide sequences were determined on Genetic Analyzer 3130xl ABI (Applied Biosystems, USA) according to the manufacturer's instructions. The resulting sequences were deposited in the Genbank database (Table 3).

2.8. Analysis of nucleotide sequences

The primary comparative analysis of nucleotide sequences obtained in this work and presented in the Genbank database was carried out using the NCBI BLAST program (Bultreys and Gheysen, 1999). Sequence alignment was performed using CLUSTALW 1.75v (Altschul et al., 1990). Phylogenetic trees were constructed in the MEGA program (version 6.0) using the methods of nearest neighbors joining (NJ) and minimal evolution (ME) (Chenna et al., 2003; Tamura et al., 2013). The statistical significance of the branching order of the obtained trees was calculated using bootstrap analysis by constructing 1000 alternative replicas, or trees.

3. Result and Discussion

Total of 57 Russian strains and 12 strains typical for different *Psyr* genetic groups were analyzed (Table 1). Phylogenetic analysis was carried out according to the scheme proposed by Hwang et al. (2005) using sequences of fragments of four genes: *gapdh, rpoD, gyrB,* and *gltA*.

In 2014, an alternative scheme for MLST analysis was published (Sarkar and Guttman, 2004), describing the analysis of 763 strains representing a collection of over 7,000 *Psyr* isolates, characterized by key diagnostic features of the species. This work identified the presence of at least 13 phylogenetic groups within the *Pseudomonas* species, including *P. syringae, P. cichorii* and *P. viridiflava*. An alternative MLST scheme was used, based on fragments of four genes proposed by S. Morris [Plant Associated and Environmental Microbes Database (PAMDB, 2022)]: two were common with scheme of Hwang et al. (2005) (*rpoD* (RNA polymerase sigma factor gene - RNA polymerase sigma70 factor) and *gyrB* (subunit gene B gyrase - gyrase B) and two – different: *gapA* (glyceroaldehyde -3 gene - phosphate dehydrogenase - glyceraldehyde-3-phosphate dehydrogenase A) and *cts* (citrate synthase gene). Berge et al. (2014) also identified type strains for each phylogenetic group, the proximity to which means that the new *Psyr* isolates belong to one of 13 phylogroups. Many strains characterized were also previously used for MLST, which allows comparing the results of phylogenetic grouping by the Hwang method with the data of the latter method (Hwang et al., 2005; Berge et al., 2014).

We used the Plant Associated and Environmental Microbes Database (PAMDB, 2022) to select sequences of unique fragments of each of the *gapdh, rpoD, gyrB,* and *gltA* genes for 205 different strains of the species *Psyr* corresponding to the MLST scheme, for which a phylogenetic analysis was carried out. According to the phylogenetic tree built for 4 combined sequences of each strain, 7 phylogenetic groups (phylogroups) (1-7) out of 13 known from the results of Berge et al. (2014) were identified, and 4 subgroups for phylogroup 2 (2a-2d), corresponding subgroups of the same name in this work.

The analysis identified the reference strains for each group, the sequences of which were included in the phylogenetic analysis of the *Psyr* strains of the Russian population (Table 1).

Table 3. DNA sequence numbers of the studied *Pseudomonas syringae* strains deposited in the Genbank.

Gene	Sample No.
gapA	OP593331-OP593387
gltA	OP593445-OP593511
gyrB	OP585478-OP585534
rpoD	OP593388-OP593444

3.1. Phenotypic analysis of strains isolated in 1950-2019

The main biochemical properties of strains of phytopathogenic bacteria of the genus *Pseudomonas* were determined using the LOPAT system (Lamichhane et al., 2015). For further analysis, we used only strains that fully corresponded to biotype 1 (*P. syringae*) with the following indicators: levan (+), oxidase (-), potato maceration (pectolytic activity) (-), arginine (-), hypersensitivity reaction on tobacco plants and geranium (+), formation of sodium gluconate (+), reduction of nitrates (-), formation of acid from sucrose (+), oxidative metabolism (O).

The pathogenicity of bacteria was determined on seedlings of wheat (cv. "Moskovskaya 39") and sunflower (cv. "Gelios") (Table 1). Most of the strains did not possess specialization and actively suppressed the growth of the primary root and stem of plants, also causing necrotic damage to the stem and cotyledon leaves. 95% of the strains suppressed growth of dicotyledonous plants (sunflower), while 78% of the strains obtained from dicotyledonous plants did not inhibit the growth of the root and leaves of wheat seedlings.

3.2. Syringomycin & coronatine activity and PCR tests for syrD and cfl genes

The PCR test of the occurrence rate of syringomycin with syrD1/syrD2 primers showed that only 18.1% of the strains did not have the expected amplification product in 3 strains (2%), the resulting product differed in intensity from others, probably due to differences in the sequence of primer landing sites. More than 86% of phylogroup 2b strains, 84% of phylogroup 1 strains, 73% of phylogroup 3 strains, and 100% of phylogroup 6 strains were *syrD*-positive. Moreover, 71% of phylogroup 2d strains were *syrD*-negative.

We found a high rank correlation between the result of PCR test and the phenotypic manifestation of the effect of syringomycin (0 or >0) on the growth of the yeast *Rhodotorula pilimanae* (Spearman's R = 0.86, significant at 95% confidence level). According to the literature, the synthesis of syringomycin was observed in most strains of phylogroup 2.

The PCR test for *cfl* gene with PsgFOR1 and PsgREV primers showed that only 16 tested strains (28%) have the expected amplification product. All of positive bacteria were from tomato and soybean. We found a full agreement between the result of PCR test and the phenotypic effect of coronatine (0 or 1) on potato slices.

3.3. Antibiotic susceptibility of strains

Kanamycin, gentamicin, tetracycline, and streptomycin were most effective against the studied strains of *Psyr*. Analysis of the results of strains clustering in accordance with the response to antibiotics and bactericides in general did not reveal a correlation between the resistance of bacteria and the phylogenetic grouping of the studied strains. No significant correlation was found between the response of strains to individual substances. Six groups identified using the Ward method for the quantitative response of bacteria to antibiotics included 26, 20, 13, 28, 20, and 31 strains, respectively. Significantly higher resistance of the strains isolated after 2010 was noted to Fitolavin-300 (3.1 times), Fitoplasmin (2.4 times) and tetracycline (1.7 times), regardless of the phylogenetic group of the strain or the host plant.

This might be due to an increase in the volume of Fitolavin-300 and Fitoplasmin, which are an unpurified mixture of antibiotics synthesized by streptomycetes, for plant protection against bacterial diseases, and with the probable use of veterinary antibiotics (tetracycline) for treatment seeds and plants. Small differences were observed between the response of strains to a number of antibiotics and the affected crop - significant differences were found mainly between spring and winter plants, which probably indicates the importance of antibiotic resistance for survival in the rhizosphere of the host plant during the wintering period, when the phytopathogen faces strong competition from microorganisms - producers of antibiotics.

3.4. Phylogenetic relationships between strains based on MLSA results

Russian strains belonged to phylogroups 1, 2, 3, and 6. Phylogroup 2 included strains from subgroups 2a, 2b, and 2d. Analysis of previously published data and our own results (Table 1) shows that the distribution of phenotypic diagnostic characters for the selected phylogroups does not have absolute affinity to specific genotypes. The two main diagnostic features of phytopathogenic pseudomonads used in the isolation of these bacteria - the synthesis of the fluorescent pigment pyoverdin and hydrolysis of esculin, were found not to be characteristic of all strains of phylogroups 1 and 3, which include a large number of epidemic strains of *Psyr*. The genetic distance between groups of strains was greatest for groups 1 (pv. *lachrymans*, pv. *maculicola,* pv. *morsprunorum,* pv. *tomato*) and 6 (pv. *tagetis* and pv. *helianthi*). The minimum distance was within group 2 - between subgroup 2b (pv. *syringae*, pv. *aptata*, pv. *atrofaciens*) and 2d (pv *pisi,* pv. *solidagae*) (Table 4).

According to the published data, 35% of all studied strains, including all representatives of phylogroups 8, 11, 12, and 13 (not included in phylogroups 1-7 according to Hwang et al. (2005)) did not synthesize ice condensation protein, 27% of strains did not induce a reaction hypersensitivity to tobacco plants, 72% of the strains were not pathogenic or toxic to germinating pumpkin seeds (Sarkar and Guttman, 2004).

Table 4. The evolutionary distances between groups of 64 *Pseudomonas syringae* strains were determined by the Maximum Composite Likelihood model based on the comparison of 2009 nucleotides using the MEGA X program (Kumar et al., 2018).

Gene group	Gp_3	Gp_2b	Gp_2d	Gp_1
Gp_3				
Gp_2b	0.0649			
Gp_2d	0.0636	0.0275		
Gp_1	0.0894	0.0921	0.0902	
Gp_6	0.0753	0.0803	0.0764	0.0959

In general, the use of 11 phenotypic traits of bacteria (fluorescence, oxidase, esculin, levan, sucrose, maceration of potato slices, D (-) - tartrate, hypersensitive response on tobacco, ice condensation protein, pathogenicity on pumpkin seeds and syringomycin synthesis) made it possible to correctly determine (with 95% confidence) the strains belonged to phylogroups 1, 2, 7, 8, 9, 10, 11, and 13. Phylogroups 3, 4, 5, and 12 included strains variable for these traits.

Phylogroups 8 (*P. viridiflava*) and 11 (*P. cichorii*) belonged to other canonical species of the genus Pseudomonas, identified by biochemical characteristics, and phylogroups 9, 11, 12, and 13 did not have generally accepted microbiological species names.

Psyr phylogroup 1 includes mainly phytopathogenic strains, although some of them were isolated from the environment (water, soil). The strains of this group have genes for the degradation of aromatic substances, which apparently gives them the ability to infect plants that form a large number of secondary metabolites (stone nuts, kiwi) (Matveeva et al., 2008). In Russia, this phylogroup is represented by strains isolated from cabbage and nightshade crops, rarely from sunflower (Table 1).

Psyr phylogroup 2 includes the largest number of strains isolated from a wide variety of habitats grouped into three genetic subgroups (clades) 2a, 2b, and 2c described earlier (Lamichhane et al., 2015). Subgroup 2b includes typical strains of such common phytopathogens as *pv. syringae, P. s. pv. aptata, P. s. pv. atrofaciens*, while subgroup 2c includes non-pathogenic *P. syringae* strains, with some similarity to *P. viridiflava.*

In general, the strains of phylogroup 2 were distinguished by the highest frequency of hypersensitive response on tobacco plants, damage to wheat and sunflower seedlings, and the synthesis of syringotoxin. In Russia, this phylogroup represents more than 56% of all studied strains isolated from legumes, sunflower, cereals, cucumber and grapes (Table 1).

Psyr phylogroup 3 includes pathovars of a newly defined species *P. savastanoi.* This group includes many

pathogens of woody or leguminous plants, which, like strains of phylogroup 1, are capable of destroying aromatic substances that play an important role in the formation of plant immunity to phytopathogens (Bartoli et al., 2015). In Russia, this phylogroup is represented by strains isolated from sunflower, grain crops, and grapes (Table 1).

Psyr phylogroup 4 includes pathovars, which mainly affect monocotyledonous plants, including economically important cereals. Phylogroup 5 includes pathovars with very different phenotypes, which infect a variety of plants, such as hemp, cauliflower, cabbage, and coriander. Strains of this group were not found in the studied collection. Phylogroup 6 includes pathovars *P. s. pv. tagetis* and *P. s. pv. helianthi*, infecting Compositae crops and papaya. In the Russian population, this phylogroup is represented by strains isolated from sunflower. Phylogroup 7 includes two pathovars *P. s. pv. ribicola, P. s. pv. primulae*, and most of the previously studied strains of *P. viridiflava*. The main diagnostic feature of this phylogroup is the ability of bacteria to synthesize syringomycin, cause damage to pumpkin seedlings and maceration of potatoes (soft rot), which indicates the presence of a set of pectolytic exoenzymes in them. Strains of this group are not represented among the studied Russian collection.

4. Conclusion

Plant pathogenic bacteria belonging to *Pseudomonas syringae* group of species cause disease in agricultural and wild plants of numerous genera and families around the World. The bacteria have a complex taxonomic structure, which is constantly being revised due to the emergence of new molecular and biochemical diagnostic methods. To the date, genetic diversity of the *Psyr* population in the Russian Federation remains unexplored. Here we report the genetic and phenotypic diversity of 57 strains of *Pseudomonas syringae* isolated from affected legumes, cereals, sunflowers, and other plants in the Russian Federation from 1950 to 2019. Multi Locus Sequence Analysis using fragments of *gapdh, rpoD, gyrB,* and *gltA* showed a high genetic homogeneity of the Russian population of *Pseudomonas syringae*, from various agricultural crops, and an increase in the proportion of antibiotic-resistant strains over the years.

Although the taxonomy of *Psyr* group has been extensively analyzed, significant uncertainties remain regarding the species composition of this taxon. Strains were originally identified phenotypically as members of the *Psyr* complex by LOPAT test (fluorescent pseudomonads, positive for levan sucrase activity, negative for oxidase activity, unable to rot potato, able to produce arginine dihydrolase and able to cause a hypersensitive response on tobacco) (Bartoli et al., 2015). Such classification led to increased taxonomic confusion, as more *Psyr* strains have been isolated from different environments besides diseased plants (Morris et al., 2010). MLST data are very useful in delineation of phylogenomic species that merits the species status. The concept of *Psyr* species complex is useful for many practical issues (disease control, plant breeding for resistance, etc.), although a proper naming of bacterial species is essential in order to establish a truly systematic taxonomy.

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