Antibacterial activity of rhizosphere pseudomonads against economically significant phytopathogens

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Abstract. The work determined the antimicrobial activity of two pseudomonad species (*Pseudomonas protegens, P. aeruginosa*) isolated from the rhizosphere of wild plants in the Orenburg region. It was found that the studied strains suppress the growth of the most significant phytopathogens (*Agrobacterium tumefaciens, Curtobacterium flaccumfaciens, Pseudomonas syringae, Xanthomonas campestris*) to varying degrees. It was found that the *P. protegens* 1 strain inhibits the growth of all the studied bacteria, indicating a large set of antagonism factors. It was found that *P. aeruginosa* strains differ in the severity of antagonism to phytopathogens. The study revealed new potential candidates for creating agents to combat bacterial pathogens in plants.

1 Introduction

According to the Food and Agriculture Organization of the United Nations, plant pests and diseases pose a serious threat to food security, food trade, and livelihoods worldwide. Up to 40 percent of agricultural crop yields are lost annually due to plant diseases and pests (https://www.fao.org/plant-production-protection).

Various means are used to combat plant pathogens. Antibiotics have been widely used since the 1950s; they are usually produced as powders with an active substance content of 17–20%, which are dissolved or suspended in water at a concentration of 50–300 ppm [1]. There are 11 antibiotics in common use, belonging to 8 classes: aminoglycosides (streptomycin, gentamicin and kasugamycin), tetracyclines (tetracycline, oxytetracycline), quinolones (oxolinic acid), heptacenes (aureofungin), penicillins (amoxicillin), cephalosporins (cefadroxil), nucleoside antibiotics (ningnanmycin) and validamycins (validamycin). They are most often used to treat crops (in descending order) of rice, tomatoes, citrus fruits, capsicum, potatoes, cabbage, eggplant, pumpkin, onions and corn [2]. The choice of antibiotic depends on the pathogen and is regulated by national legislation; the main limiting factor in the use of antibiotics is the development of antibiotic resistance in pathogens and the adverse effects on human health.

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Another group of biological agents for the prevention and treatment of bacterial plant diseases include bacterial viruses - bacteriophages. Phages are "precision weapons", being specific to their bacterial hosts and not suppressing normal and beneficial rhizosphere microflora and soil microbiota. Lysis of target bacterial cells reduces the population of pathogenic bacteria, and coevolution with their bacterial targets allows overcoming bacterial resistance. The widespread use of bacteriophages is hampered by sensitivity to environmental conditions (high and low temperatures, ultraviolet radiation, drying). When phages are used together with herbicides, insecticides, fumigants, grain and seed dressings, the latter can inactivate phages. The problems of dosage, frequency and time of phage application, their delivery to the necessary plant organs remain unresolved [3]. These circumstances limit the widespread use of bacteriophages, although there are highly effective agents based on bacterial viruses both in monoform and in a mixture in the form of lytic cocktails.

Perhaps the most promising direction for combating plant infections is the use of rhizosphere plant growth-stimulating bacteria. A special place in this group of microorganisms is occupied by representatives of the genus *Pseudomonas*, differing from others by its presence in the rhizosphere of various plants and a large number of species. By releasing phosphates, synthesizing phytohormones and fixing atmospheric nitrogen, pseudomonads have a positive effect on the growth and development of plants. Thus, bacteria of the species *P. putida* and *P. fluorescens* reduce the destructive effect of stress caused by water deficiency and increase the length of roots and the number of leaves of black henbane (*Hyoscyamus niger*); increase the iron content in rice grain; increase the length of plants and improve the quality of pepper and spinach; increase plant biomass, stem length, number of leaves and the curcumin content in turmeric (*Curcuma longa*). *P. putida* improves the growth and yield of fresh and dried tomatoes under stress from seawater irrigation. *P. nitroreducens* show significant growth improvement (especially in terms of biomass) in *Arabidopsis thaliana* and *Lactuca sativa*. *Pseudomonas* sp. improve growth, nodule formation, chlorophyll and leghemoglobin content in chickpea (*Cicer arietinum*) [4].

Pseudomonas ensure plant development, resistance to stress and protection from external, including biological, factors by regulating the secretion of various phytohormones (auxins, gibberellins, indole-3-acetic acid), flavonoids, enzymes (1-aminocyclopropane-1-carboxylate deaminase, phenyl-alanine ammonia lyase) [5], however, numerous specific metabolites that determine the strength and spectrum of antibacterial and antifungal activity play a direct role in protection against microbial pathogens.

The aim of this work was to study the antimicrobial activity of Russian rhizosphere strains of pseudomonads against economically significant bacterial pathogens of plant diseases.

2 Materials and methods

2.1 Microorganisms and Nutrient Media

The work used pseudomonad cultures isolated from the rhizosphere of wild plants by the staff of the Laboratory of Microbial Ecology and Dysbiosis of the Institute for Cellular and Intracellular Symbiosis of the Ural Branch of the Russian Academy of Sciences - *Pseudomonas protegens* 1, *Pseudomonas aeruginosa* (35 and 42), as well as the museum strain *Staphylococcus aureus* ATCC 6538. The phytopathogen cultures were obtained from the collection of the Laboratory of Molecular Genetic and Microbiological Methods of the Federal Research Center of the Kazan Scientific Center of the Russian Academy of

Sciences (Kazan): *Agrobacterium tumefaciens* MGMM176, *Xanthomonas campestris* MGMM179, *Curtobacterium flaccumfaciens* MGMM181, *Pseudomonas syringae* MGMM182.

For growing microorganisms, nutrient media from HiMedia Laboratories Pvt. Ltd. (India) were used – Schaedler Agar (M291) and Schaedler Broth (M292). For the preparation of soft agar, Schaedler Broth was used with the addition of a hardener (agar-agar) at a concentration of 6 g/l.

2.2 Determination of antimicrobial activity

The plate method (delayed antagonism principle) was used to determine antimicrobial activity. The biomass of the overnight culture of the studied pseudomonads was applied to the surface of dried 1.5% Schaedler agar with a bacteriological loop, forming a spot (patch) with a diameter of about 5 mm. The dishes were incubated in a thermostat at 30 °C for 48 hours. The grown cultures were killed with chloroform: a filter paper disk was placed in the lid of the Petri dish, moistened with 2-3 ml of chloroform and kept for 45 minutes. After evaporation of the chloroform, the dishes were turned over and a mixture was prepared for pouring the second layer. For this purpose, 5 ml of soft (0.6%) melted and cooled agar was mixed with 0.1 ml of broth overnight culture of the phytopathogen, shaken on a vortex and applied to the surface of the agar, distributing it over the entire surface of the first layer so that the soft agar completely covered the pseudomonad culture. After the agar had solidified, the dishes were turned over and incubated in a thermostat at 30 °C for 24 hours.

The results of the experiments were assessed by examining the Petri dishes in transmitted light. The appearance of a growth inhibition zone (GIZ) of the phytopathogen above and around the pseudomonad patch indicates the presence of antimicrobial activity in the producer culture. For comparative characteristics of the strains, the coefficient of antimicrobial activity (CAA) was determined as the ratio of the diameter of the growth inhibition zone of the indicator culture to the diameter of the producer colony, expressed in arbitrary units.

3 Results and Discussion

Analysis of the obtained results showed that all the studied pseudomonad strains had antagonistic activity (Figure 1). Zones of growth inhibition with a diameter of 14 to 37 mm were observed above the patches and around the producer colonies, usually with smooth edges and complete enlightenment; the exceptions were the zones of growth inhibition of *A. tumefaciens* MGMM176 and *P. syringae* MGMM182 cultures around the colonies of *P. protegens* 1.

Comparison of the coefficients of antimicrobial activity of pseudomonads revealed that the culture of *P. protegens* 1 has a more pronounced activity; the maximum level of AAA was noted in relation to *Staphylococcus aureus*. Strains of *Pseudomonas aeruginosa* did not suppress the growth of *P. syringae* MGMM182 and differed in the severity of antagonism: a higher AAA was noted for the strain *P. aeruginosa* 42 in relation to the indicator cultures of *X. campestris* MGMM179 and C. *flaccumfaciens* MGMM181.

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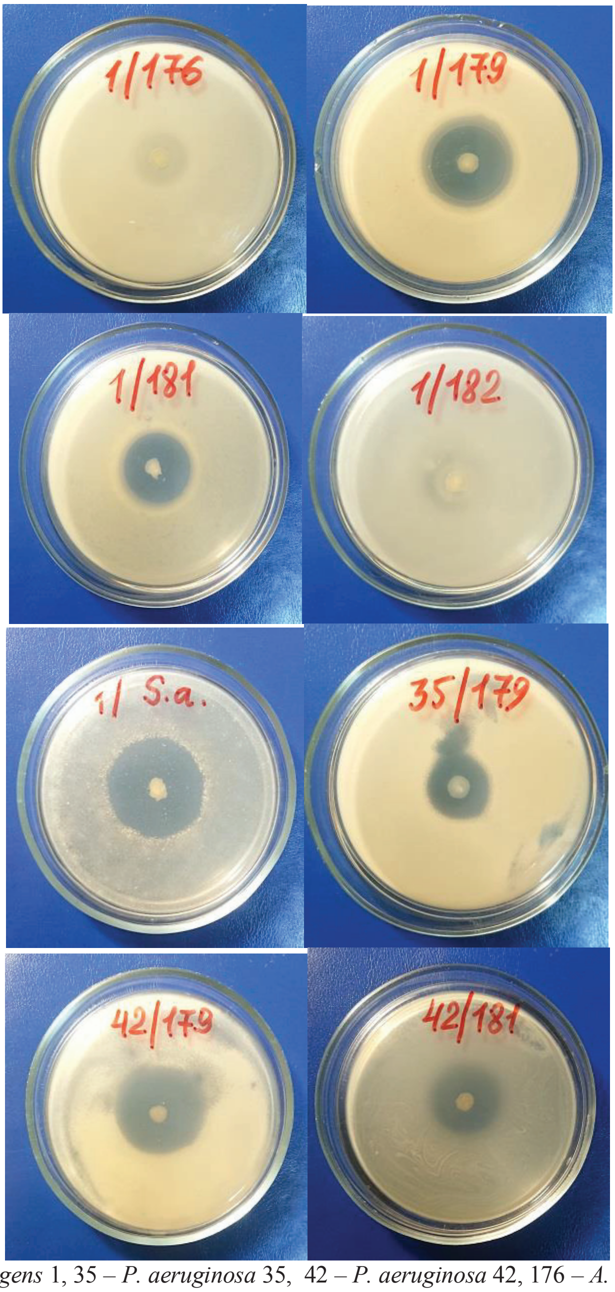

1 – *P. protegens* 1, 35 – *P. aeruginosa* 35, 42 – *P. aeruginosa* 42, 176 – *A. tumefaciens* MGMM176, 179 – *X. campestris* MGMM179, 181 – *C. flaccumfaciens* MGMM181, 182 – *P. syringae* MGMM182, S.a. – *S. aureus* ATCC 6538.

Fig. 1. Antimicrobial activity of pseudomonads. Digital designation on the Petri dish: in the numerator – the producer number, in the denominator – the indicator culture number.

Table 1. Antimicrobial activity coefficient of pseudomonads, a.u.

Indicator culture	Pseudomonas producer strain		
	***P. protegens* 1**	***P. aeruginosa* 35**	***P. aeruginosa* 42**
A. tumefaciens MGMM176	3.14	did not determine	did not determine
X. campestris MGMM179	4.0	2.86	4.57
C. flaccumfaciens MGMM181	3.71	2.0	3.43
P. syringae MGMM182	3.43	0	0
S. aureus ATCC 6538	5.29	did not determine	did not determine

The choice of indicator test cultures for assessing the antimicrobial activity of pseudomonads in this study was not random: a survey of the international expert community conducted by the journal Molecular Plant Pathology to nominate bacterial pathogens that could be placed in the "Top 10" based on scientific/economic importance showed that the list includes, in ranking order: (1) *Pseudomonas syringae* pathovars; (2) *Ralstonia solanacearum*; (3) *Agrobacterium tumefaciens*; (4) *Xanthomonas oryzae* pv. *oryzae*; (5) *Xanthomonas campestris* pathovars; (6) *Xanthomonas axonopodis* pathovars; (7) *Erwinia amylovora*; (8) *Xylella fastidiosa*; (9) *Dickeya* dadantii and *D. solani*; (10) *Pectobacterium carotovorum* (and *Pectobacterium atrosepticum*) [6]. This list does not mention the causal agent of bean leaf spot *C. flaccumfaciens* pv. *flaccumfaciens*, but it has been included in the European and Mediterranean Plant Protection Organization (EPPO) List A2 of quarantine objects (https://www.eppo.int).

The choice of *Pseudomonas aeruginosa* as a putative producer of antimicrobial substances in this study was determined by the fact that, existing as part of multispecies consortia, it has a large set of antagonism factors. In addition to contact-dependent mechanisms mediated by secretion systems of types V and VI, contact-independent antimicrobial factors are known. The latter include bacteriocins (pyocins of types S, L and M; tailocins of types R and F), signal molecules of the quorum sensing system, serine protease LasA (elastase A), hydrocyanic acid (HCN), rhamnolipids, phenazines (pyocyanin, etc.), siderophores (pyoverdin, pyochelin). This allows representatives of the *P. aeruginosa* species to influence the viability of a wide range of microorganisms, including phytopathogens. For example, bacteria (*Burkholderia glumae, Ralstonia solanacearum, Pectobacterium carotovorum, Xanthomonas oryzae pv. oryzae, Xanthomonas euvesicatoria pv. perforans, Clavibacter michiganensis subsp. michiganensis, Acidovorax avenae subsp. avenae, Pseudomonas asplenii*) and chromists (*Phytophthora colocasiae, Phytophthora infestans, Phytophthora capsici*) are sensitive to *Pseudomonas aeruginosa. Pseudomonas aeruginosa* has an antifungal effect against both ascomycetes (*Fusarium verticillioides, Fusarium graminearum, Fusarium tricinctum, Fusarium oxysporum, Alternaria alternata, Botrytis cinerea, Neocosmospora solani, Cladosporium cladosporioides, Pestalotiopsis neglecta, Pyricularia oryzae, Sclerotinia sclerotiorum, Ustilaginoidea virens, Sarocladium oryzae*) and basidiomycetes (*Rhizoctonia solani, Ceratobasidium cereale*).

Another species, *Pseudomonas protegens*, was described in 2011; initially, fluorescent pseudomonads resistant to black root rot (causative agent - *Thielaviopsis basicola*, Ascomycota) isolated from the surface of tobacco roots in Switzerland were classified as this species [7]. Later, antifungal activity was also detected against representatives of other ascomycetes. Thus, volatile organic compounds of the endophyte *P. protegens* QNF1 inhibit the growth of *Monilinia fructicola* mycelium by 95.35% compared to the control, reducing the development of brown rot on post-harvest peach fruits by 98.76% [8]. The strain *P. protegens* B21-059 is capable of inhibiting the growth of *Sclerotinia sclerotiorum* (Ascomycota), the causal agent of white rot of lettuce, *in vitro* [9]. The Chilean strains *P. protegens* Ca10A and ChB7 provide control of the chicory root rot pathogen *Boeremia exigua* var. *exigua* (Ascomycota); the importance of these crops is determined by the fact that there is currently no chemical or varietal control for this disease [10]. The culture suspension and cell-free supernatant of the *P. protegens* strain ML15 significantly suppress fungal growth (53.0 ± 0.63%) and mitigate disease development (52.8 ± 1.5%) in cherry tomatoes four days after inoculation with *Botrytis cinerea*, the causal agent of tomato gray mold [11].

The antibacterial activity of *P. protegens* has been studied much less. For example, it is known that cell-free fractions of the *P. protegens* PBL3 strain exhibit inhibitory activity (*in vitro* and *in planta*) against *Burkholderia glumae*, the causative agent of bacterial rot of rice panicles [12]. The *P. protegens* CLP-6 strain exhibits pronounced antagonistic activity against another representative of the same family (*Burkholderiaceae*), *Ralstonia solanacearum*, and has been proposed for use as a safe and additive fumigant to combat bacterial wilt of tobacco and other crops of the nightshade family [13]. The crude extract of rhizosphere *P. protegens* Pf-5 exhibits pronounced activity against gram-negative rods of the *Erwiniaceae* family - *Pantoea ananatis* DZ-12, the causative agent of brown rot of corn leaves [14]. The results of the present study allowed us to expand the list of sensitive bacterial targets.

The spectrum of antimicrobial activity of *P. protegens* is determined by the diversity of secondary metabolites: in addition to the initially known compounds (2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, phenazine(s), hydrogen cyanide) [7], new substances were also discovered that determine the antimicrobial effect. A new cyclic lipopeptide, orfamide H, from the culture fluid of the type strain of *P. protegens* CHA0 inhibits the formation of appressoria of the fungus *Magnaporthe oryzae*, the causative agent of rice blast [15]. After the addition of N-acetyl-D-glucosamine to the cultivation medium, the strain *P. protegens* Cab57 begins to synthesize protegenins A-D, unique bacterial polyines that have a conjugated C≡C bond starting from the terminal alkyne; protegenins have activity against the oomycete *Pythium ultimum* OPU774 [16]. As a result of a spontaneous reaction between the biosynthetic intermediates of two well-studied metabolites of *P. protegens*, pyochelin and pyrrolnitrin, pyonitrins are formed - molecules with chimeric structures in which 10 of the 20 carbon atoms are quaternary, and 7 of them are adjacent. Despite weak antifungal activity *in vitro*, pyonitrins exhibit significant activity against the yeast *Candida albicans* under *in vivo* conditions [17].

4 Conclusion

The obtained results indicate that new rhizosphere strains of pseudomonads suppress the growth of phytopathogens belonging to four different families (*Rhizobiaceae, Lysobacteraceae, Microbacteriaceae and Pseudomonadaceae*) *in vitro* and are of interest from both scientific and practical points of view. Studying the conditions of formation of antimicrobial metabolites and evaluating the effectiveness of these bacteria in field conditions will allow selecting the best candidates for the creation of biopreparations.

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