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PSEUDOMONADS ASSOCIATED WITH SOIL LUMBRICIDES AS PROMISING AGENTS IN ROOT ROT BIOCONTROL FOR SPRING GRAIN CROPS

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Abstract

Currently, crop yields can be increased by high farming standards which include environmentally friendly use of chemical fertilizers and pesticides, as well as their replacement by bioformulations having similar activity. That is why both search for new promising species, strains and isolates of bacterial antagonists for their potential use as biocontrol agents, and study of antifungal activity mechanisms, particularly the relationship between the activity in model tests and in agrocenoses, are relevant. The aim of this study was to estimate bacterial isolates from redworm coprolites as potential bioactive agents to control phytopathogenic fungi causing root rot of crops. The experiments were conducted in 2013-2015. In the preliminary laboratory screening for fungistatic and growth-promoting activity we selected two strains, *Pseudomonas* sp. GS4 and *Pseudomonas* sp. PhS1, and assessed their ability to decrease the growth rate of fungal colonies in Petri dish test on nutrient agar medium and to reduce seed infestation of soft wheat (*Triticum aestivum* L., Irgin cultivar) in sterile paper roll test. Seeds soaked in distilled water served as control. As a standard, we used seed treatment with a chemical fungicide Dividend® Star («Syngenta AG», Switzerland) (30 g/l difenoconazole, 6.3 g/l cyproconazole) at recommended rates. In field tests, we recorded root rots in soft wheat Irgin cultivar plants and in barley (*Hordeum vulgare* L.) Acha cultivar plants during tillering and beginning of blooming. The laboratory tests showed a statistically significant ($p < 0.05$) 1.5-2.5-fold decrease in the growth rate of phytopathogenic fungi *Fusarium oxysporum*, *Bipolaris sorokiniana* and *Alternaria* spp. as compared to control. In all experiments with bacterization, there was a 53-76 % decrease ($p < 0.05$) in total seed infestation by pathogens as compared to non-bacterized plants. The effect of the bacteria in planta was assessed in small model systems. The obtained data show a statistically significant ($p < 0.05$) reduction in the root rot disease incidence in bacterization with *Pseudomonas* sp. GS4 (by 33-37 %) and *Pseudomonas* sp. PhS1 (by 57-60 %). Root rot disease severity decreases 2.1-2.4-fold and 3.3-3.5-fold, respectively. In 2015, we revealed a tendency towards a 19-70 % increase in the total number of rhizosphere microorganisms at the beginning of plant blooming depending on the crop and type of bacterization. The number of phosphate-mobilizing bacteria in the rhizosphere under bacterization was, on average, 5.5-7.2-fold higher in wheat and 2.1-3.2-fold higher in barley than that without bacterization. Our results of root rot field study in the 2013-2015 showed the efficacy of both monocultures and complex bacterization which provided a decrease in wheat and barley root rot disease severity by 6.5-57.6 % and 18.6-50.0 %, respectively, depending on the bacterial culture and the weather conditions. The maximum biological efficacy of the isolates is noted at the beginning of blooming.

Keywords: biocontrol, rhizobacteria, *Pseudomonas*, *Eisenia fetida,* antifungal activity, phytopathogen, *Bipolaris*, *Alternaria*

Antifungal activity is a relatively common bacterial feature, which gives an environmental advantage in the environments that can support the growth of

mixed bacterial and fungal flora. There are a number of mechanisms, by which one organism suppresses the growth of its competitor: competition for the limited nutrient supply, production of siderophores [1-3], antibiotics, enzymes, and sundry compounds [4, 5]. Bacterial activity in vitro usually has a positive correlation with their ability to inhibit phytopathogen growth as well as with their stimulating activity in vivo [1, 6, 7]. However, the fungistatic and plant-growth stimulating effects of bacteria, albeit shown in the laboratory, are not always confirmed by in vivo experiments [8-10].

Despite the fact that bioformulations based on antagonistic bacteria are widely used in agriculture and have positive effects on plant growth and development, early studies noted the instability of results [11, 12]. A more detailed study showed [13-15] that the effectiveness of bioformulations depends on various factors: microorganism strain culturing parameters, preparative form, storage methods and time, soil properties, agro-climatic conditions, the host plant, the strain's capability of a strong symbiosis with this or that cultivated crop, as well as the microbial community status at the sowing of seeds treated with the bioformulation.

Several researchers have successfully protected plants by combining different bacterial strains [16, 17]. It was shown that the most effective way to control disease progression is to use a combination of strains and bi-component biofungicides [18, 19]. Pairing microbial strains sometimes allows for a reduction in the bioformulation concentration while improving its quality [7, 14]. In most such papers, Pseudomonas bacteria were one of the bioformulation components. This is due to their high antifungal activity and multifaceted effects on plant growth and development [1, 4, 11]. The second component is any bacterium widespread in the plant rhizosphere and capable of stimulating the growth and development of plants and of the primary component.

Active use of bioformulations in state-of-the-art agricultural technology requires searching for efficient strains that could be used for biological control of phytopathogens under various agrocenotic conditions. An in-depth study of the connection between the manifestations of bacterial bio-activity in the lab and in the field will contribute to the development of such bioformulations.

This paper is the first to find that *Pseudomonas* strains isolated from coprolites help reduce the infestation of soft wheat with infectious seed pathogens in a laboratory setting, which correlates with their ability to suppress the development of fungi (root rot pathogens) in an agrocenosis.

The purpose hereof is to evaluate the feasibility of using bacteria isolated from earthworm coprolites as the basis for bioformulations intended to control fungal phytopathogens (grain-crop root rot pathogens).

Techniques. Laboratory and field tests were carried out in 2013-2015. Bacterial cultures of *Pseudomonas* sp. GS4 and *Pseudomonas* sp. PhS1 were isolated from coprolites of earthworms (*Eisenia fetida*) after one-month culturing on a 1:2 peat and dung substrate, inoculated directly onto a growth medium based on fish hydrolyzate (FH broth) (State Research Center for Applied Microbiology and Biotechnology, Russia). Strains were selected after preliminary screening for fungistatic and growth-stimulating activity in laboratory tests [9].

Liquid enrichment cultures were produced by growth in flasks with FH broth (250 flasks, each containing 100 ml of the medium) using an ES-20/60 shaker (Biosan, Latvia) at 180 rpm and 28 ± 0.5 °C until the number of bacteria reached 1×10^9-9×10^9 cells/ml. FH broth contained pancreatic hydrolyzate of fish meal (8 g/l), enzymatic peptone (8 g/l), and sodium chloride (4 g/l). The bacterial population was controlled by counting in a Goryaev chamber (×400 magnification).

The test objects used to assess the antagonistic activity of bacteria were phytopathogenic fungi *Fusarium oxysporum*, *Bipolaris sorokiniana*, and *Alternaria* spp. (provided by the Department of Biological Plant Protection, Novosibirsk State Agrarian University). Those were cultured in 2% potato-glucose agar (0.23 l potato extract, 20.0 g glucose, and 0.77 l tap water). To determine the antifungal activity, bacterial suspensions were streaked on solid agar media edge-to-edge in Petri dishes, one suspension per sample. The dishes were incubated for two days. A cylindrical agar block, 2 mm in diameter, containing 6-day fungus mycelium, was placed in the center of the unoccupied medium surface. The dishes were then placed in a thermostat at 24±1.0 °C, and every 24 hours the radius of the fungal colony growth towards the streak was measured (the control sample was a fungal colony not exposed to bacteria). The fungistatic effect was evaluated by reduction of fungal colony growth on a dense growth medium in the bacteria-streaked samples as compared to the controls [20].

In laboratory experiments, a soft wheat (*Triticum aestivum* L.) cultivar Irgin seeds were soaked for 20 min in the bacterial suspension of test-strain monocultures (1×10^7-5×10^7 cells/ml). In case of co-inoculation, the amount of each strain-specific suspension was halved to 5×10^6-25×10^6 cells/ml. The controls were seeds soaked in tap water; for reference, the seeds were treated with Dividend® Star chemical fungicide (Syngenta AG, Switzerland) in the recommended dosage. This chemical is allowed for use in the Russian Federation and contains the following active ingredients: difenoconazole, 30 g/l; cyproconazole, 6.3 g/l. The fungistatic effect of the bacteria was evaluated by the reduction of seed infestation with the seed infection pathogens as detected by phytopathological assay using rolls of sterile filter paper [10, 20]. The effects of bacteria in *in planta* experiments were studied in small model ecosystems, each consisting of three parts: substrate (sterile coarse river sand), host plant (wheat), and bacteria. Bacteria were added to the system in a dose of 1×10^6 cells/seed. Control and experimental pots were placed in a GC-300TLH climatic chamber (Jeio Tech, Korea) for 10 days at 10 klx lighting, 16-hours light period, and 22 to 24 °C. After that, the signs of root rot in seedlings were scored [20].

Field trials (Siberian Agriculture and Peat Research Institute, Luchanovo, Tomsk Region) were carried using Irgin wheat cultivar and barley (*Hordeum vulgare* L.) Acha cultivar. The soil was gray podzolic mid-loamy, pH 5.0, humus content 4.87%, 24.9 mEq of absorbed bases per 100 g of absolutely dry matter (adm), $N\text{-}NH_4$ = 2.66, $N\text{-}NO_3$ = 8.48, P_2O_5 = 236.5, and K_2O = 99.2 mg/kg adm. The experiments were arranged according to Dospekhov's methodology [23], with 3-fold repetition and systematic placement of 40 m^2 plots (32 m^2 evaluated area per plot). The dose of nitrogen fertilizer (urea) was 45 kg/ha by nitrogen (N_{45}). Before sowing, seeds were bacterized with enrichment cultures (working titer of 1×10^7-5×10^7 cells/ml) at a rate of 100 ml/10 kg of grains. The seeding rate was 6.5 million seeds/ha for wheat, 5.5 million seeds/ha for barley.

To assess the fungistatic activity of the tested bacteria at tillering phase and at the flowering onset, at least 100 plants were randomly collected for each repetition/sample. The numbers of healthy, affected, or dead plants, and the damage score were used to calculate the disease prevalence and progression indices [10, 22].

The amount of microorganisms in the rhizosphere was determined by plating soil suspension serial dilutions on elective growth media. Total bacterial count was determined on FH agar, the phosphate-mobilizing bacteria were counted on Muromtsev agar (0.2 g/l K_2SO_4, 0.2 g/l $MgSO_4\cdot7H_2O$, 10 g/l glucose, 1.0 g/l asparagine, 3.3 g/l $CaCl_2$, 3.8 g/l Na_3PO_4, and 15 g/l agar-agar), and micromycetes were counted on Czapek medium (1.0 g/l K_2HPO_4, 2.0 g/l

$NaNO_3$, 0.5 g/l KCl, 0.1 g/l $FeSO_4 \cdot 7H_2O$, 20 g/l glucose, 0.5 g/l $MgSO_4 \cdot 7H_2O$, and 15 g/l agar-agar, HCl to adjust to pH 5.5).

The obtained data were processed with STATISTICA 6.0 software (StatSoft, Inc., United States). The growth rate of the fungal colony was calculated by linear regression method. The results of the phytopathological tests, as well as the prevalence of diseases during field vegetation are shown given Fisher's test for probabilities below 25% and above 75%. Statistical significance was evaluated by comparing sampling fractions given Student's *t*-test at 95% significance level for probabilities ranging from 25% to 75%; for any other probability, Fisher's test was applied. Root rot indices and microbial counts are presented as arithmetic means (M) with the confidence interval ($\pm\sigma$) taking into account Student's *t*-test for a 95% significance level, and compared by Student's *t*-test, with a significant threshold of $p < 0.05$.

Results. Laboratory experiments are an integral part of developing bioformulations to protect plants; such experiments help launch the production of new formulations faster. Promising microbial introducents and the introducent-based bioformulations should be further tested over several growing seasons that featured different weather.

Using standard streaking, we examined the effect of the bacterial isolates from coprolites on growth and development of some phytopathogenic fungi. Bacterial cultures isolated from earthworm coprolites are classified as *Pseudomonas* [23] by their morphological, physiological, and biochemical properties. The bacteria are short, single, motile, asporogenous, gram-negative bacilli that grew well on organic media. They liquefied gelatin, hydrolyzed starch, possessed catalase activity, reduced nitrates to nitrites, and produced acid on glucose- and sucrose-containing media. Besides, they are aerobic, and one of the selected strains, *Pseudomonas* sp. PhS1, can produce yellow water-soluble pigment and mobilize sparingly soluble phosphates.

It was found out that in the presence of bacteria, the growth rate of all the fungi under analysis is statistically significantly ($p < 0.05$) lower than that of the controls (a 1.5- to 2.5-fold reduction, see Table 1). Since the reduction in the growth rate of fungal colonies did not require direct contact of the fungal hyphae and bacterial steaks while the micromycetes stopped growing without reaching it, it is safe to say that the antifungal effect was associated with the soluble and media-diffusible bacterial metabolites.

1. Effects of *Pseudomonas* strains isolated from earthworms coprolites on the radial growth rate (mm/h) **of pure phytopathogenic fungi cultures** ($M\pm\sigma$, lab test)

Strain	*Fusarium oxysporum*	*Bipolaris sorokiniana*	*Alternaria* spp.
Control (water)	0.24±0.014	0.22±0.021	0.30±0.019
Pseudomonas sp. GS4	0.15±0.012*	0.09±0.006*	0.15±0.046*
Pseudomonas sp. PhS1	0.11±0.010*	0.07±0.005*	0.11±0.011*

* Difference from the controls statistically significant at $p < 0.05$.

According to literature data, the inhibition of fungal growth by the *Pseudomonas* bacteria is first of all related to various bacterial antibiotics, including phenazine-1-carbolic acid, derivatives of phloroglucinol, pyrrolnitrin, and other compounds [1, 5, 13]. Aside from antibiotics, siderophores (iron-transporting yellow-green pigments) produced by such bacteria are also important for inhibition. These bind ferric ions and form stable complexes with such iron, which deprives fungi of a necessary nutrient, inhibiting their development [4, 5, 15]. On MPA, *Pseudomonas* sp. PhS1 produced yellow medium-diffusing pigment. Apparently, in this case siderophores play an important role in inhibiting growth of the phytopathogenic fungi. *Pseudomonas* sp. GS4 had no pigmentation; apparently, their antifungal activity is related to the production of other antibiotics.

2. Reduction in seed colonization due to bacterization and chemical treatment as a percent of the untreated seed colonization ($M \pm \sigma$, soft wheat *Triticum aestivum* L., Irgin cultivar, lab test)

Variables	Total infection prevalence	Helminthosporium disease	Alternaria spot	Bacteriosis
Pseudomonas sp. GS4	54.0±2.0*	35.5±3.4	61.3±22.4	64.5±21.0
Pseudomonas sp. PhS1	76.2±2.1*	100.0±1.0*	29.0±7.8	100.0±1.0*
Pseudomonas sp. (GS4 + PhS1)	64.5±1.9*	100.0±1.0*	58.0±23.0	35.5±9.0
Dividend® Star, SC	74.1±1.7*	69.7±7.2*	25.4±7.9	100.0±1.0*

* Difference from the controls statistically significant at $p < 0.05$.

A statistically significant ($p < 0.05$) reduction in the overall seed infections was observed in all the bacterized samples, see Table 2. Treatment of wheat seed with a *Pseudomonas* sp. PhS1 monoculture was the most effective, resulted in the greatest reduction in infection and was comparable to the effect of a chemical protectant.

Vegetation modeling experiments *in planta* also demonstrated high biological activity of the analyzed bacteria. The prevalence of root rot was lower ($p < 0.05$) in samples bacterized with *Pseudomonas* sp. GS4 (33% to 37%) as well as with *Pseudomonas* sp. PhS1 (57% to 60%); the root rot progression index was 2.1 to 2.4 or 3.3 to 3.5 times lower than in the controls, respectively.

The weather conditions of the growing season 2013 were quite favorable for the growth and development of the crops used in the field tests. At the beginning and end of the growing season, temperatures were low while precipitation was frequent and intense; mid-July and early August were hot while precipitation was scarce. The early 2014 season was rather unfavorable, with low temperatures in May and early June accompanied by intense and frequent precipitation. The rest of the season, like 2013 season, was quite favorable for plant growth and development. In 2015, the sum of effective temperatures was accumulated from April to September; precipitation was above average in May, July, August, and September, while June was dry.

Microbiological analysis of the wheat and barley rhizosphere identified an upward trend in the microbial counts in the test samples. A marked reduction in the total number of lower fungi combined with a sharp increase in *Trichoderma* counts indicated a decrease in abundance of potentially hazardous microorganisms in the microbial rhizosphere community of the bacterized crops, while the proportion of biocontrol agents grew. The abundance of phosphate-mobilizing bacteria testified to highly competitive and successful colonization of the applied *Pseudomonas* sp. PhS1 culture in the plant roots. Thus, the counts of this microbial group in the rhizosphere of the test plants were 5.5 to 7.2 times higher for wheat and 2.1 to 3.2 times higher for barley as compared to the controls.

3. Microbial counts (million CFU/g of soil adm) **in the rhizosphere of soft wheat (*Triticum aestivum* L.) Irgin plants and barley (*Hordeum vulgare* L.) Acha plants after seed inoculation with bacteria isolated from earthworm coprolites** ($M \pm \sigma$, Luchanovo, Tomsk Province, 2015)

Variables	Total counts	Phosphate-mobilizing bacteria	Lower fungi	
			total number	*Trichoderma*
Wheat				
Control	102.0±26.5	2.1±0.2	0.71±0.01	0.01±0.002
Pseudomonas sp. PhS1	121.0±38.4	15.1±1.6*	0.24±0.03*	0.01±0.005
Pseudomonas sp. (PhS1 + GS4)	174.0±20.7*	11.6±2.9*	0.75±0.02	0.05±0.008*
Barley				
Control	60.0±2.1	5.0±0.2	1.42±0.27	0
Pseudomonas sp. PhS1	59.0±1.9	10.7±0.9*	0.58±0.01*	0.07±0.007*
Pseudomonas sp. (PhS1 + GS4)	94.0±1.5*	16.0±1.5*	0.67±0.07*	0.05±0.002*

* Difference from the controls statistically significant at $p < 0.05$.

Literature review [12] as well as the results of phytosanitary survey carried out annually by the Rosselhoztsentr, Tomsk Province, indicate that Helminthosporium disease and Fusarium root rot caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker and *Fusarium oxysporum* Schleht remain the most harmful crop diseases in the region [22, 24, 25]. The disease damages durum wheat, barley, soft spring wheat, and winter rye the most. Diseases reduce the wheat yield by 19% to 20%, the barley yield by 25% to 30%, or even more [22, 24, 25].

In 2013, there was a slight difference in the effectiveness of different bacterization options applicably to wheat and barley, see Table 4. Thus, wheat yielded the best results when treated with a mixture of bacterial cultures, with a statistically significant ($p < 0.05$) 34% reduction in root rot at the phase of earing and early flowering. Barley displayed the greatest reduction in root rot affection when bacterized with *Pseudomonas* sp. GS4 monoculture: 32% to 41%, and 39% to 44% during tillering and flowering, respectively.

4. Key indicators of root rot progression in wheat (*Triticum aestivum* L.) Irgin cultivar and barley (*Hordeum vulgare* L.) Acha cultivar after seed inoculation with bacteria isolated from earthworm coprolites ($M \pm \sigma$, Luchanovo, Tomsk Region)

Crop	Variables	Tillering		Flowering onset	
		incidence, %	severity, %	incidence, %	severity, %
		2013			
Wheat	Control	49.2±3.9	17.0±2.3	51.7±3.6	19.9±3.8
	Pseudomonas sp. GS4	56.4±2.7	16.4±1.0	44.4±2.6*	18.6±3.5
	Pseudomonas sp. (PhS1 + GS4)	56.7±5.1	18.0±1.2	36.7±5.7*	13.2±2.6*
Barley	Контроль	82.7±2.4	39.5±2.6	44.4±2.9	14.7±2.6
	Pseudomonas sp. GS4	53.3±7.6*	23.2±2.8*	24.4±8.1*	8.3±2.5*
	Pseudomonas sp. (PhS1 + GS4)	70.7±3.7*	27.3±3.6*	41.0±7.8	13.3±2.5
		2014			
Wheat	Control	66.3±14.2	26.7±4.8	60.6±8.9	20.4±3.7
	Pseudomonas sp. PhS1	74.4±6.4	27.3±3.6	52.2±12.6	17.2±8.5
	Pseudomonas sp. (PhS1 + GS4)	72.5±12.3	25.9±3.3	54.4±10.2	20.8±7.4
Barley	Control	84.0±6.7	41.3±4.0	86.7±12.9	43.6±10.6
	Pseudomonas sp. PhS1	84.0±6.7	37.5±7.6	73.3±10.1	35.5±7.6
	Pseudomonas sp. (PhS1 + GS4)	80.8±5.1	36.9±4.1	71.1±12.6	30.5±10.7
		2015			
Wheat	Control	55.6±17.8	31.2±3.8	61.1±17.5	21.7±6.6
	Pseudomonas sp. PhS1	55.6±17.8	27.5±4.0	50.0±17.9	15.8±4.2
	Pseudomonas sp. (PhS1 + GS4)	50.0±17.9	26.7±7.6	26.6±15.8*	9.2±3.7*
Barley	Control	55.6±17.8	21.7±5.5	66.7±16.9	28.3±3.7
	Pseudomonas sp. PhS1	57.8±17.7	22.5±6.1	46.7±17.8	14.2±5.0*
	Pseudomonas sp. (PhS1 + GS4)	51.7±17.9	22.5±7.8	57.8±17.7	20.8±3.8*

* Difference from the controls statistically significant at $p < 0.05$.

The root rot progression indicators were much higher in the growing season of 2014 as compared to 2013, see Table 4. Greater prevalence and progression of root rot was associated with the unfavorable climate conditions, as cold weather and frequent precipitation do not benefit crops in any way. Furthermore, efficient root colonization by inoculated bacteria requires a minimum temperature of 12 °C, while the optimum temperature range is 14 to 16 °C [13]. Lack of heat in May, with a monthly average temperature of 8.1 °C (2.5 °C below normal), which apparently prevented bacteria from successful colonization of the plant rhizosphere while inhibiting their ability to compete with the natural inhabitants and to fully manifest their defensive and stimulating properties. As a result, the 2014 growing-season field experiment did not reveal any statistically significant inoculation-attributable suppression of root rot in crops. Over the entire season, there was only a slight downward trend in the infestation of wheat and barley, with no clear correlation with the bacterization type or inoculation method. The favorable weather conditions of 2015 did have a positive effect on the phytosanitary situation: the prevalence of root rot was lower in both the controls and in the experimental samples as compared to the 2014 and 2014

values. A significant ($p < 0.05$) reduction in the root rot progression index was noted in wheat and barley at the flowering onset (55% to 59% and 25% to 28%, respectively) in the samples bacterized with a mixture of cultures, whereas in barley, significant reduction was also observed in the *Pseudomonas* sp. PhS1 monoculture-treated samples (by 48% to 51%).

Experimental root rot data prove the efficiency of complex bacterization and the feasibility of developing microbial bioformulations based on the complementary associations of microbial cultures that combine various functions, from providing plants with readily available nutrients to stimulating the growth and protection against phytopathogens [18, 19]. Similar data were obtained in the field experiments in 2011 [26]. Bacterial isolates helped reduce the prevalence of root rot in soft wheat and barley crops by 18% to 63%. Bacterization with *Pseudomonas* sp. GS4 was comparable to Dividend Star in terms of reducing the progression of root rot in wheat (at tillering and earing phase) as well as in barley (tillering phase). Bacterization with *Pseudomonas* sp. GS4 increased the wheat and barley yield by 19.6%, and the grain protein and gluten content by 0.9% to 1.3 % as compared to the Dividend Star-treated samples.

Therefore, *Pseudomonas* strains isolated from earthworm coprolites reduce the overall infestation of wheat seeds with seed infections in lab tests. The lowest infestation level is observed in samples treated with *Pseudomonas* sp. PhS1 (only a quarter of the control indicators). The laboratory-identified antifungal activity of bacteria correlates with their ability to suppress the development of root rot pathogen fungi in an agrocenosis. Over the three growing seasons, inoculating wheat and barley seeds with experimental strains helped reduce the root rot progression index; however, the observed fluctuations in the efficiency (5% to 58% depending on the bacterial culture and on the weather) necessitate further research to improve the stability of bacterial preparations and optimize their application.

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