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BACTERIAL STRAINS ANTAGONISTIC TO *Pyrenophora tritici-repentis* in vitro DEMONSTRATE DIFFERENT EFFICACY ON WHEAT SEEDLING IN GREEN HOUSE

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Abstract

Yellow leaf spot is a wide spread diseases of soft and hard wheat. Numerous publications and the authors' own research show that its epiphytotics occur in different countries (Australia, Canada, USA, India, England, Belgium, Romania, Czech Republic, Kazakhstan) with crop losses reaching 65 %. In Russia the disease is most common in the North Caucasus. Development and application of new high-performance environmentally friendly biological products is regarded as one of the most effective biologized approach to wheat protection against the disease. In searching potential bacterial agents for use as protective means, their in vitro antagonistic activity should be accompanied by the ability to provide effective protection for seeds and seedlings. Herein we studied the repression of yellow spot development during early stages of plant vegetation in a greenhouse as influenced by the bacterial strains which were in vitro antagonistic to *P. tritici-repentis* (Died.) Drechsler. Winter wheat cultivar Bat'ko susceptible to the pathogen was used as test plant. Six isolates of the *Bacillus* family (*Bacillus* sp. BZR 18, *B. subtilis* BZR 336 s, *B. subtilis* BZR 336 g, *B. subtilis* BZR 436, *B. subtilis* BZR 517, B. *licheniformis* BZR 59), as well as *Ochrobactrum* sp. BZR 417 from the collection of All-Russian Research Institute of Biological Plant Protection were used as candidate bio agents. For comparison, liquid Fitosporin-M was used as a biological preparation (LLC Scientific Innovation Enterprise «BashInkom», Russia) and Prozaro emulsion concentrate was used as a chemical fungicide («Bayer CropScience», Germany). Liquid cultures of antagonistic bacterial strains were applied in three modes, namely before inoculation (prophylactic treatment), at early signs of the disease (on the day 3 after inoculation) and by their combination. All treatments were performed with inlaying and without inlaying grain with liquid bacterial culture. All studied bacterial strains except *Ochrobactrum* sp. BZR 417 showed considerable biological efficacy of leaf spot inhibition. The *Bacillus* sp. BZR 18 and *B. subtilis* BZR 517 were the most inhibiting strains which repressed leaf spot development at 68.5 to 83.0 % and 55.6 to 64.0 % rate, respectively, in all variants except treatment at early signs of the infection without inlaying grains when the efficiency was 26.8 and 35.9 %, respectively. *B. licheniformis* BZR 59 provided for 52.6 to 68.9 % leaf spot inhibition. Liquid culture of *B. subtilis* BZR 336 g strain ensured efficiency from 51.5 to 58.3 % in all variants except a preventive treatment without inlaying grain when the efficiency was 40.9 %. *B. subtilis* BZR 336 s, if used at early signs of infection with inlaying grain and in combination of prophylactic treatment with application at early signs of infection and inlaying grain, caused 60.2 and 60.3 % leaf spot repression; in other cases a 14.7 to 44.3 % repression was observed. BZR 436 *B. subtilis* effectiveness was from 28.4 % under preventive treatment with preliminary grain inlaying to 73.4 % under preventive treatment together with application at early signs of infection and inlaying grain. The efficiency of *Ochrobactrum* sp. BZR 417 strain does not exceed 45.4 % in all variants. Depending on antifungal activity of the bacterial agent, a combination of grain pre-treatment, prophylactic treatment and treatment at early signs of infection proved to be the most effective.

Keywords: *Pyrenophora tritici-repentis*, antagonistic bacteria, biological effect, winter wheat, tan spot disease, seedlings.

Yellow spot of wheat leaves is economically important in many regions where this crop is cultivated, such as Australia, Canada, USA, India, England, Belgium, Romania, Czech Republic, Kazakhstan, etc. [1-8]. In Russia, this dis-

ease is most common in the North Caucasus. In the Krasnodar Territory, the pathogen was first found in 1985 [9] and has been noted every year since then [10-12]. In case of epiphytotic development, crop losses may achieve 40-65 % [13, 14]. The causal agent of this disease is homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechsler, the imperfect stage of *Drechslera tritici-repentis* (Died) Shoem. The pathogen has a wide range of gramineous host plants, including both cultivated and various wild-growing forms.

At present, pesticides are the most popular means of plant protection (including against yellow spot of wheat leaves). However, as toxic agents, they have a negative influence on agrobiocenoses, which gives rise to serious concern. The existing limitations on the use of pesticides have encouraged the search for alternative means of plant protection with the emphasis on biological preparations based on living microorganism cultures. The range of the biological preparations known for their activity against the phytopathogens of the genus *Pyrenophora* (*Drechslera*) is quite narrow. According to the List of Pesticides and Agrochemicals Permitted for Use in the Russian Federation [15], Planriz, Alirin B, Baktofit and Gamair are recommended against barley net blotch (helminthosporiosis), however none of them is referred to preparations against yellow spot. At the same time, it is reported that some biocontrolling microorganisms exhibit antifungal activity in respect to both the causal agents of this disease and other representatives of the genus *Pyrenophora* (*Drechslera*).

Thus, T. Taechowisan et al. [16] report about antifungal action of the 3-methylcarbazoles produced by endophytic strain *Streptomyces* sp. LJK109 with regard to a number of phytopathogens, including *Drechslera* sp. Several strains of *Trichoderma* spp. were tested against *P. tritici-repentis* (*D. tritici-repentis*) by Argentinean researchers in field conditions. The use of bioagents for presowing treatment of seeds and vegetation treatment led to reduction in spread of yellow spot disease by 16-56 %, depending on the conditions of the year, methods of application and variety of the wheat [17]. Earlier, studying the microflora of wheat leaves, the same authors extracted 13 isolates of fungi, two isolates of yeasts and one strain of *Bacillus* (Bw/97), which, in laboratory tests, turned out to be active against the pathogens causing leaf diseases, in particular, *Alternaria triticimaculans*, *Bipolaris sorokiniana*, *Drechslera tritici-repentis* and *Septoria tritici*. The largest inhibiting effect in these studies was observed for *Aspergillus niger*, Bw/97 and *Nigrospora sphaerica* [18]. Substantial antifungal activity of strains of *Pseudomonas fluorescens* bacteria against fungi of the genus *Pyrenophora* was noted with regard to the barley. It must be stressed that the efficiency of bioagent application in greenhouse experiments depended on treatment time, in fact, protective action was more pronounced in case of use before pathogen inoculation [19].

Endophytic micromycete *Chaetomium globosum* also exhibits biological activity against the yellow spot pathogen. It has been established that culture filtrate on its basis promoted the production of extracellular protein and, therefore, retardation of yellow spot development on wheat leaves [20, 21].

In general, however, it can be concluded that information about biocontrol of the diseases caused by pathogens of the genus *Pyrenophora* (*Drechslera*) is quite fragmentary and scarce, and the biological preparations recommended for use are, as a rule, not referred to target ones. For example, one of such products is Planriz, which was developed based on *Pseudomonas fluorescens* AP-33 (Institute of Genetics and Cytology of the National Academy of Sciences of Belarus, Minsk). At present, it is produced by some Russian companies (PA Sibbiopharm Ltd, Berdsk; Biotekhagro LLC, Timashevsk) and widely used on various crops in different regions of the country, including for control of leaf spot diseases of grain

crops. In the studies of V.P. Borovaya [22], biological efficiency of Planriz against barley net blotch was 82 %. The same author reports about high efficiency of Psevdobakterin-2 based on *Pseudomonas aureofaciens* (84 %) and Baktofit based on *Bacillus subtilis* (70 %) against the mentioned pathogen. Cedomon (Bio-Agri, Sweden) based on *Pseudomonas chlororaphis* (strain MA 342) can be distinguished among foreign preparations effective against the causal agents of the genus *Pyrenophora* (*Drechslera*). Its antifungal action is mainly related to production of phenazine antibiotics. The preparation is intended for control of barley and oat seed infections and used for presowing treatment. Manufacturer Cedomon states that such treatment on the barley is more efficient than the application of chemical treaters, such as Fungazil A, Panoctine Plus 400, Cevex 300, Robust [23-25].

Along with antagonistic activity in vitro, an important property of potential protective bioagents is their capability to provide efficient protection of seeds and seedlings.

In this connection, in a greenhouse trial, we have studied the influence of the bacterial stains exhibiting antagonistic properties with regard to *Pyrenophora tritici-repentis* in vitro on yellow spot development in case of wheat plants in the seedling phase.

Technique. The objects of study were represented by seven promising bacterial strains from microorganism collections of the All-Russian Research Institute of Biological Plant Protection, which exhibited antagonistic properties with regard to the pathogens causing fusarium disease, *Fusarium graminiarum* Schwabe and *F. culmorum* (Sm.) Sacc., and yellow spot of wheat leaves (*Pyrenophora tritici-repentis*) in vitro [26-28]. Six of the studied strains belonged to the genus *Bacillus* (*Bacillus* sp. BZR 18, *B. subtilis* BZR 336 s, *B. subtilis* BZR 336 g, *B. subtilis* BZR 436, *B. subtilis* BZR 517, *B. licheniformis* BZR 59), and one was from the genus *Ochrobactrum* (*Ochrobactrum* sp. BZR 417).

The plants of winter soft wheat variety Batko susceptible to the pathogen were hydroponically grown in greenhouse conditions (under daylight, at 20-24 °C) using Knop's solution to the 2-leaf stage and were infected by virulent isolates of *P. tritici-repentis* from the collection of the All-Russian Research Institute of Biological Plant Protection using water spore suspension with the density of 5×10^3 conidia/ml.

Treatment with liquid culture based on antagonistic strains was carried out in three ways: 1 — before inoculation (preventive treatment), 2 — upon appearance of the first signs of disease (on the 3rd day after inoculation), 3 — preventive treatment and application of the preparation upon appearance of the first signs of disease development. These treatments were carried out both with and without inlaying of grains with the liquid culture based on bacterial strains. The liquid culture based on the promising strains was applied at the rate of 1-3 l/ha. Chemical and biological standards were fungicide Prozaro (emulsion concentrate, 0.6 l/ha) (Bayer CropScience, Germany) and Phytosporin-M (liquid, 1 l/ha) (NVP Bashinkom LLC, Russia), respectively. A total of 50 plants were accounted for in each trial case. Disease development on the plants infected and treated with liquid bacterial culture was compared with the control (without treatment). The number of spots was counted; a reaction type was assessed according to the 5-point scale of Rees et al. (1987), and disease development percentage (2) was evaluated for one plant on the 7^{th} day after inoculation. Biological efficiency was calculated by Ebbot's formula [29].

Statistical processing was carried out using the standard Microsoft Excel software.

Results. The reduction of point-based reaction type evaluation allows us to assess the degree of inhibition of plant tissue colonization by the fungal

pathogen; decrease in the number of spots characterizes the bacterial strain ability to inhibit *P. tritici-repentis* infection on plants, and the variation of disease development percentage reflects the capability of limiting infection contamination and pathogen development in plants.

In case of infection, the average number of spots per plant in a control (without treatment) was 15.6 pcs with the reaction type under disease development at 4.6 points and the disease development of 26.4 % (Table 1).

1. The characteristic of protective action of the strains exhibiting antagonism in vitro with regard to *Pyrenophora tritici-repentis* on wheat plants of sensible variety Batko in the seedling phase in greenhouse conditions ($X \pm s$)

Antagonistic strain, preparation	Treatment					
	preventive		upon the first signs of disease		preventive + upon the first signs of disease	
	1	2	1	2	1	2
Number of spots per plant, pcs						
Bacillus sp. BZR 18	6.1±1.2	7.8±1.2	5.7±0.9	12.5±1.4	6.2±1.1	7.3±0.9
Bacillus subtilis:						
BZR 436	10.9±1.8	8.8±1.2	9.6±1.2	9.1±1.3	6.8±1.8	10.4±1.5
BZR 517	4.5±1.1	7.5±1.7	10.2±1.1	12.4±1.7	8.8±1.9	9.7±1.2
BZR 336 s	10.1±1.0	10.8±1.5	8.0±1.3	12.3±2.1	8.5±1.3	10.1±1.1
BZR 336 g	9.0±1.1	9.5±1.7	7.2±1.5	8.4±1.8	13.3±1.4	11.8±1.3
B. licheniformis BZR 59	9.4±1.2	10.3±1.1	7.0±1.3	6.7±1.1	8.2±1.5	7.9±0.9
Ochrobactrum sp. BZR 417	10.5±1.9	10.5±1.1	11.9±1.8	12.3±1.7	10.1±1.1	10.3±1.1
Phytosporin-M, liquid	7.7±0.9	10.5±1.2	9.7±1.4	8.3±1.3	7.9±1.1	11.3±1.4
Prozaro, emulsion concentrate	–	4.3±1.0	–	6.9±1.1	–	4.3±1.1
Control (without treatment)	15.6±1.8	15.6±1.8	15.6±1.8	15.6±1.8	15.6±1.8	15.6±1.8
Type of reaction to infection contamination, points						
BZR 18 *Bacillus* sp.	3.2±0.4	3.2±0.5	2.8±0.5	4.0±0.4	2.7±0.5	3.2±0.5
Bacillus subtilis:						
BZR 436	4.0±0.5	3.8±0.5	3.5±0.4	3.8±0.6	3.1±0.5	3.8±0.5
BZR 517	3.4±0.6	3.6±0.8	3.6±0.8	4.1±0.6	3.2±0.5	3.6±0.5
BZR 336 s	4.3±0.5	4.5±0.6	3.9±0.5	4.4±0.6	3.8±0.6	4.3±0.7
BZR 336 g	4.1±0.9	4.1±0.5	3.6±0.7	3.9±0.8	4.1±0.8	4.0±0.8
B. licheniformis BZR 59	4.1±1.0	3.6±0.4	3.6±0.5	4.0±0.8	3.4±0.7	3.6±0.6
Ochrobactrum sp. BZR 417	4.1±0.9	4.2±0.7	3.8±0.6	4.3±0.5	3.6±0.6	4.1±0.6
Phytosporin-M, liquid	3.9±0.5	4.0±0.7	3.6±0.5	3.6±0.6	3.5±0.5	3.6±0.5
Prozaro, emulsion concentrate	–	2.4±0.5	–	3.3±0.6	–	2.3±0.5
Control (without treatment)	4.6±0.5	4.6±0.5	4.6±0.5	4.6±0.5	4.6±0.5	4.6±0.5
Disease development on one plant,%						
BZR 18 *Bacillus* sp.	4.4±2.5	8.2±4.5	4.6±3.1	19.3±4.8	4.6±3.5	8.3±4.3
Bacillus subtilis:						
BZR 436	18.9±4.7	11.5±4.5	10.1±4.1	13.3±4.2	7.0±4.0	13.8±4.5
BZR 517	9.5±3.6	9.5±4.0	11.7±3.9	16.9±4.5	9.4±4.8	11.4±4.0
BZR 336 s	14.7±4.1	22.0±6.1	10.5±4.0	22.5±5.2	10.5±4.5	22.5±5.8
BZR 336 g	11.8±3.9	15.6±5.5	12.8±3.5	11.0±4.7	12.0±4.6	12.5±3.8
B. licheniformis BZR 59	11.4±4.0	12.5±5.1	8.9±5.0	8.2±3.8	8.5±4.8	8.5±4.0
Ochrobactrum sp. BZR 417	15.0±4.1	15.5±3.9	14.4±4.5	18.5±4.0	14.8±5.2	15.0±4.6
Phytosporin-M, liquid	14.0±4.3	15.8±5.0	15.3±5.1	16.2±4.5	14.7±3.5	16.0±4.2
Prozaro, emulsion concentrate	–	4.5±2.8	–	7.7±3.0	–	5.1±3.1
Control (without treatment)	26.4±5.6	26.4±5.6	26.4±5.6	26.4±5.6	26.4±4.6	26.4±4.6

Note: 1 — with additional seed inlaying, 2 — without inlaying. Dashes mean that the variant was not used. In control no preparations were used.

The analysis of biological efficiency of bacterial preparations has revealed differences depending on the properties of the strain used and modes of application. The highest inhibition of spot formation and development was noted for *Bacillus* sp. BZR 18 (68.5-83.0 %) and *B. subtilis* BZR 517 (55.6-64.0 %) in all cases, but treatment upon appearance of the first signs without preliminary inlaying of grains (in this case, the indicator was equal to 26.8 and 35.9 %, respectively), as well as for *B. licheniformis* BZR 59 (52.6-68.9%) in all variants (Table 2).

2. The biological efficiency (relative to the control,%) **of the strains exhibiting antagonism in vitro with regard to *Pyrenophora tritici-repentis* on wheat plants of sensible variety Batko in the seedling phase in greenhouse conditions**

Antagonistic strain, preparation	Treatment					
	preventive		upon the first signs of disease		preventive + upon the first signs of disease	
	1	2	1	2	1	2
By number of spots on leaves						
Bacillus sp. BZR 18	60.8	50.0	63.4	19.8	60.2	53.2
Bacillus subtilis:						
BZR 436	36.0	43.6	38.5	41.6	56.4	33.3
BZR 517	71.1	51.9	34.6	20.5	43.5	37.8
BZR 336 s	35.8	30.7	48.7	21.1	45.5	35.8
BZR 336 g	42.3	39.1	53.8	46.1	46.7	43.5
B. licheniformis BZR 59	39.7	33.9	55.1	57.0	47.4	49.3
Ochrobactrum sp. BZR 417	32.7	32.7	23.7	21.1	35.8	33.9
Phytosporin-M, liquid	50.6	32.7	37.8	46.8	49.3	27.6
Prozaro, emulsion concentrate	–	72.4	–	55.7	–	72.4
By type of reaction to infection contamination						
BZR 18 *Bacillus* sp.	30.4	30.4	39.1	13.0	41.3	30.4
Bacillus subtilis:						
BZR 436	13.0	17.3	23.9	17.3	32.6	17.3
BZR 517	26.0	21.7	21.7	10.8	30.4	21.7
BZR 336 s	6.5	2.1	15.2	4.3	17.3	6.5
BZR 336 g	10.8	10.8	21.7	15.2	10.8	13.0
B. licheniformis BZR 59	10.8	21.7	21.7	13.0	26.0	21.7
Ochrobactrum sp. BZR 417	10.8	8.6	17.3	6.5	21.7	10.8
Phytosporin-M, liquid	15.2	13.0	21.7	21.7	23.9	21.7
Prozaro, emulsion concentrate	–	47.8	–	28.2	–	50.0
By disease development degree						
BZR 18 *Bacillus* sp.	83.0	68.9	82.5	26.8	83.0	68.5
Bacillus subtilis:						
BZR 436	28.4	56.4	61.7	49.6	73.4	47.7
BZR 517	64.0	64.0	55.6	35.9	64.3	56.8
BZR 336 s	44.3	16.6	60.3	14.7	60.2	14.7
BZR 336 g	55.3	40.9	51.5	58.3	54.5	52.6
B. licheniformis BZR 59	56.8	52.6	66.2	68.9	67.8	67.8
Ochrobactrum sp. BZR 417	43.1	41.2	45.4	29.9	43.9	43.1
Phytosporin-M, liquid	46.9	40.1	42.0	38.6	44.3	39.4
Prozaro, emulsion concentrate	–	82.9	–	70.8	–	80.6

Note: 1 — with additional seed inlaying, 2 — without inlaying. Dashes mean that the variant was not used. In control no preparations were used.

Treatment with the liquid culture based on strain *B. subtilis* BZR 336 g provided efficiency within 51.5-58.3 % in all cases, except for preventive treatment without preliminary treatment of grains (40.9 %); for *B. subtilis* BZR 336 s, this indicator was almost equal and quite high (60.2 and 60.3 % in treatments upon appearance of the first signs with preliminary inlaying of grains and in case of preventive application in combination with treatments upon appearance of the first signs and with preliminary inlaying, respectively), whereas, in the other cases, it was significantly lower and strongly varied (from 14.7 to 44.3 %). The efficiency of strain *B. subtilis* BZR 436 ranged from 28.4 % (preventive treatment with preliminary grain inlaying) to 73.4 % (preventive treatment in combination with application upon appearance of the first signs and preliminary inlaying). The efficiency of strain *Ochrobactrum* sp. BZR 417 did not exceed 45.4 % in all cases.

The chemical standard has shown maximum protective effect against the yellow leaf spot pathogen with regard to the number of spots (55.7-72.4 %), type of disease manifestation (28.2-50.0 %) and the disease development level (to 78.0-82.9 %). The biological efficiency of Phytosporin-M (liquid) was less than that of the chemical standard, but matched the efficiency of other strains, particularly from 27.6 to 50.6 % with regard to decrease in the number of spots,

from 13.0 to 21.7 % with regard to the type of reaction to disease development, and from 38.6 to 46.9 % with regard to the disease development.

It has been noted that basic mechanisms for biocontrol of phytopathogens by rhizobacteria, including the studied new agents, comprise the following: competition for ecological niches and nutrient sources; enzyme activity leading to lysis of phytopathogen cells; production of substances of antibiotic nature, and induction of resistance to phytopathogens [30, 31].

The previously obtained data allow us to assume that the substantial protective effect in all cases of winter wheat plant treatment with test samples of biological preparations upon appearance of the first signs of yellow leaf spot is associated with the synthesis of mycolitic enzymes of chitinase, lipase and protease groups, as well as with the production of antibiotic substances [26, 27].

It is important to note that high protective action indicators under preventive treatment with test samples of developed biological preparations may be presumably associated with a capability of biological agents to cause Induced Systematic Resistance (ISR) of plants [32].

Thus, all the studied bacterial strains, except for *Ochrobactrum* sp. BZR 417, on average, exhibited biological efficiency above 50 % with regard to a capability of restraining the development of yellow spot of wheat leaves in various variants of treatments in the seedling phase. The best results were observed for the combination of preliminary seed inlaying with subsequent preventive treatment and application of preparation upon appearance of the first signs of disease (depending on the antifungal activity of the bacterial agent).

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