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## Bacillus megaterium 501<sup>rif</sup> AS ANTIDOT OF HERBICIDE PROMETRYN IN CROPS OF OATS AND CORN

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## Abstract

The application of the herbicide prometryn (4,6-bis-(isopropylamino)-2-methylthio-1,3,5triazine) for weed control, makes many agrotechnological and ecological problems due to relatively higher persistence in the environment. It is well known that many microorganisms are capable of decomposing the herbicide. There has been some attempt to use microorganisms for bioremediation of soils. Bacillus megaterium is of particular interest because it produces many physiologically active substances that increase the efficiency of photosynthesis, stimulates growth, and accelerates the formation of plants reproductive organs, as well as decomposes some pesticides. In this article, we present new data on the effect of *B. megaterium*  $501^{rif}$  inoculation upon plant resistance to the herbicide prometryn. There was shown that the *B. megaterium*  $501^{\text{rif}}$  brings down the phytotoxicity of the herbicide and decomposes it in the rhizospheres of oats and corn. The purposes of the work were to study the survival rate of *B. megaterium* 501<sup>rif</sup> in the rhizosphere of oats and maize and to estimate its effect on plant resistance to prometryn, as well as ability to effectively decomposition of this herbicide in the soil. B. megaterium 501<sup>rif</sup> was cultured on a rotary shaker for 48 hours at 30 °C, 140 rpm. The bacterial titer was  $5 \times 10^8$  CFU/ml and included at least 90 % of the alive cells. Seeds of oats (Avena sativa L.) cultivar Pobeda and maize (Zeal mays L.) cultivar Ross 199 MV were inoculated with a 2-days liquid culture of *B. megaterium* 501<sup>rif</sup> and were sown in vegetative pots. The soil was soddy-podzolic, medium loam, with an organic matter content of 2.3 %, pH 5.8. An aqueous suspension of wetting powder of prometryn (Panama Agrochemical Inc., Panama) was applied in the quantity of 0.12, 0.22, 0.67, and 1.23 mg/kg in the experiment with oats and 3.4, 6.8 and 20.4 mg/kg in the experiment with corn. In last case there was a variant with prometryn (6.5 mg/kg) but without plants. Plants were grown in the light chamber Phytos-4 (PHYTOS, Russia) at a temperature of 22-25 °C. The dry weight of plants, the quantity of prometryn in the soil, and the number of bacteria in the rhizosphere were determined 30 days after the sprouts appeared. The field experiment was conducted in the experimental field of the All Russia Institute for Agricultural Microbiology (Pushkin, Leningrad region). The soil was soddypodzolic medium loam, with an organic carbon content of 2.3 %, pH 5.6. The herbicide prometryn was applied to the soil at a dose of 500 mg/m<sup>2</sup>, which approximately corresponded to 1,5 mg/kg. The seeds of corn were not inoculated in the control. The dry weight of plants and the quantity of herbicide in the soil were determined 30 days after the sprouts appeared. B. megaterium 501rif took root well in the rhizospheres of oats and corn. The number of bacteria were 300 to 500 thousand CFU/g soil, and from 58 to 80 % of them were physiologically active cells. The weight of oats increased by 11 %, corn — by 20 %, when seeds were inoculated with *B. megaterium*  $501^{\text{rif}}$  culture. The resistance of plants to herbicide significantly increased and quantity of prometryn decreased 2-3-fold in the soil under oats, and 20-fold under corn. In the field experiment the weight of the corn plant was 11.6 % higher, while the herbicide quantity in the soil was 3 times lower than in the control. We suppose that the higher resistance of plants inoculated with bacteria to the herbicide is due to a positive effect of their metabolites, in particular poly-beta-hidroxibutyrate, produced by bacteria as well as active participation of bacteria in the degradation of the herbicide. Thus, B. megaterium 501<sup>rif</sup> like an antidote takes off the phytotoxic effect of the herbicide on plants and increases their productivity. Corn, when inoculated with bacteria, decomposes prometryn effectively and can be used for bioremediation.

Keywords: *Bacillus megaterium*, protector, antidote, prometryn, herbicide degradation, oats, corn, soil bioremediation

The herbicide prometryn (4,6-bis-(isopropylamino)-2-methylthio-1,3,5-triazine) is widely and effectively used to control annual dicotyledonous and cereal weeds in corn, cotton, soybeans, potatoes, as well as vegetables and green crops [1, 2]. Its persistence  $(T_{50})$  in soil, depending on the dose, soil and climatic conditions, and agrotechnical methods, ranges from several weeks to 18 months [3, 4]. The long-term persistence of the herbicide in the soil causes agrotechnological and ecological problems. From an agronomic point of view, they are primarily associated with the alternation of crops in the crop rotation. There is a significant risk of reduced yield and even death of herbicide-sensitive plants sown after the prometryn-treated precursor. From an ecotoxicological point of view, it should be noted that, despite the relatively low mobility, prometryn and its transformation products are washed out of the soil into the surrounding water bodies, having a negative effect on aquatic vegetation and undermining the food base of aquatic organisms [5, 6].

Microorganisms and physicochemical properties of the soil are the main factors providing degradation of herbicides [6, 7]. Bacteria utilizing prometryn as a source of carbon [8], nitrogen [9, 10], and sulfur [11] were isolated from the soil. According to Kruglov et al. [12], an accelerated decomposition of the herbicide occurred in the soil inoculated with *Bacillus megaterium* 501<sup>rif</sup>. Some *B. megaterium* strains produce poly-beta-hydroxybutyric acid. It has been found that the treatment of crops with a preparation containing this substance increases plant resistance to stress, including some pesticides [13]. The *B. megaterium*-based preparations have been created that are used in crop production to increase crop yields [14, 15]. However, there are practically no works in which the influence of these microorganisms on plant resistance to herbicides widely and universally used in modern agricultural technologies has been studied.

This paper provides new data on the effects of *B. megaterium*  $501^{rif}$  inoculation on plants with regard to their resistance to the herbicide prometryn. It is shown for the first time that *B. megaterium*  $501^{rif}$  culture reduces phytotoxicity of the herbicide and degrades it in the rhizosphere of oats and corn

The work aimed to study the survival rate of *B. megaterium*  $501^{rif}$  in the rhizosphere of oats and maize plants, and to assess its effect on plant resistance to prometryn and the efficiency of decomposition of this herbicide in soil.

*Materials and methods.* Pot tests were carried out at the experimental base of the All-Russian Research Institute of Agricultural Microbiology (St. Petersburg–Pushkin).

Mutant rifampicin resistant strain *B. megaterium* 501<sup>rif</sup> was obtained by gradient selection from the original strain previously isolated by us from ordinary chernozem (Kokchetav region, Kazakhstan). The original strain is deposited in the Collection of Non-Pathogenic Beneficial Microorganisms for Agricultural Purposes (All-Russian Research Institute of Agricultural Microbiology) [16].

*B. megaterium* 501<sup>rif</sup> was cultured for 48 h on a rotary shaker (140 rpm, 30 °C). The nutrient medium was as follows (g/l): K<sub>2</sub>HPO<sub>4</sub> – 1.6; KH<sub>2</sub>PO<sub>4</sub> – 0.4; NH<sub>4</sub>NO<sub>3</sub> – 0.5; MgSO<sub>4</sub> – 0.2; CaCO<sub>3</sub> – 0.05; FeSO<sub>4</sub> – 0.025; yeast extract – 0.2; sucrose – 10.0; pH 6.8. The bacterial titer was  $5 \times 10^8$  CFU/ml (at least 90% of vegetative cells).

Formation of poly- $\beta$ -hydroxybutyrate intracellular granules in *B. megaterium* 501<sup>rif</sup> was investigated by phase contrast microscopy (Axio Lab. A1, Carl Zeiss, Germany).

Seeds of oat (*Avena sativa* L.) variety Pobeda and corn (*Zeal mays* L.) variety Ross 199 MB were inoculated with a 2-day liquid culture of *B. mega-terium*  $501^{rif}$  and then planted in 2.0 kg pots. The soil is soddy-podzolic medium loamy, with a 2.3% organic matter content, pH<sub>sal</sub>. 5.8. Prometryn (an

aqueous suspension of a wettable powder, Panama Agrochemical Inc., Panama) was added at 0.0 (no herbicide), 0.12; 0.22; 0.67 and 1.23 mg/kg for oats and 0.0 (without herbicide), 3.4; 6.8 and 20.4 mg/kg for corn. In the latter case, an additional option introduced was bare fallow soil containing prometryn (6.8 mg/kg). The soil was thoroughly mixed and packaged. Similar variants without inoculation served as control.

The plants were grown in a Phitos-4 light chamber (Phitos, Russia) at 22-25 °C and 50-60% soil moisture content of total moisture capacity. The experiment was repeated 3 times.

The dry biomass of plants, the prometryn content in the soil, and the *B.* megaterium  $501^{rif}$  abundance in the rhizosphere of oats and maize were assessed 30 days after the emergence of seedlings. The bacteria were counted by serial dilution method [17] with a mineral salt agar medium of the above composition with the addition of 2.5% agar (Difco, USA) and 0.02 g/l rifampicin. For differentiated determination of bacterial spores, the soil suspension was pasteurized for 10 min at 80 °C prior to plating on the nutrient medium. The herbicide was extracted from the soil with acetone, followed by quantitative determination on a Tsvet-106 gas chromatograph (NPO Khimavtomatika, Russia) with a thermoionic detector [18]. The amount of prometryn extracted from soil was 65-70% of the calculated amount.

In a field trial (experimental field, the All-Russian Research Institute of Agricultural Microbiology, Pushkin, Leningrad region, 2010; 1 m<sup>2</sup> plots in three replicates), the soil was soddy-podzolic medium loamy, with a 2.3% organic carbon content, pH 5.6. Prometryn (500 mg/m<sup>2</sup>, which, according to the analysis, corresponded to 1.5 mg/kg) was incorporated into the topsoil at a 0-10 cm depth. Prior to sowing, seeds of maize variety Ross 199 MB were inoculated with liquid culture of *B. megaterium* 501<sup>rif</sup> (5×10<sup>8</sup> CFU/ml) (no inoculation in control). The aboveground part weight of plants, the herbicide content in the soil, and the bacterial titer were determined 30 days after the emergence of seedlings.

The data were statistically processed using Microsoft Excel software. The mean values of the indicators (M) and standard deviations ( $\pm$ SD) were calculated. The reliability of the results was assessed at the P<sub>0.95</sub> confidence level.

*Results.* In the pot tests, 0.12 mg/kg prometrine slightly stimulated oat plant growth. The phytomass increased by 11% (P<sub>0.05</sub>) compared to that in plants not treated with the herbicide (Fig. 1). Doses of prometryn above 0.22 mg/kg inhibited plant growth, and 1.23 mg/kg prometryn caused plant death in 2 weeks.

Oat seed inoculation with *B. megaterium* 501<sup>rif</sup> culture decreased the phytotoxic effect of prometryn and a significantly (P<sub>0.95</sub>) increased the aboveground phytomass as compared to the control (without inoculation), regardless of the herbicide content in the soil. *B. megaterium* 501<sup>rif</sup> colonized the oat rhizosphere well, and physiologically active vegetative cells comprised from 58 to 80% of bacteria. A month after the emergence of seedlings, the prometryn content in the soil decreased, and, moreover, in the variants with inoculated seeds it was 1.5-3.0 times less than in the control (without inoculation) (Table). Thence, *B. megaterium* 501<sup>rif</sup> had a protective effect on oat plants, increasing their resistance to the herbicide, and also reducing the herbicide content in the soil, which, in turn, reduced its phytotoxic effect.

Prometryn had a similar effect on corn, but its resistance was an order of magnitude higher than that of oats (see Fig. 1). The herbicide manifested its inhibitory effect at a concentration of 20.4 mg/kg, while lower doses stimulated plant growth. Corn seed inoculation with bacteria completely eliminated the phytotoxic effect of the herbicide. In addition, the phytomass increased 1.5-3.0 times ( $P_{0.95}$ ) as compared to non-inoculated corn plants.

*B. megaterium*  $501^{rif}$  colonized the corn rhizosphere well, up to 335 thousand CFU g/soil (see Table). Thirty days after germination, the content of the herbicide in the soil under corn plants decreased 20 times, and with bacterial inoculation 60 times compared to bare fallow soil. Therefore, both plants and *B. megaterium*  $501^{rif}$  bacteria from the rhizosphere, were involved in the herbicide degradation.



Fig. 1. Dry weight of oat (*Avena sativa* L.) variety Pobeda (A) and corn (*Zeal mays* L.) variety Ross 199 Mv (B) plants as influenced by herbicide prometryn upon seed inoculation with *Bacillus megaterium* 501<sup>rif</sup> culture (30 days after the emergence of seedlings): a - control (without inoculation), b - inoculation. Test in pots (vertical bars mean root-mean-square deviations).

The soil concentration of prometryn and abundance of bacterial inoculant *Bacillus* megaterium 501<sup>rif</sup> in oats (*Avena sativa* L.) variety Pobeda and corn (*Zeal mays* L.) variety Ross 199 MB (30 days after the emergence of seedlings) (pot tests,  $M\pm$ SD)

Soil	Prometryn mg/kg			B. megaterium 501 <sup>rif</sup> ,	
	initial	day 30		CFU n 10 <sup>3</sup> /g	
		without	inoculation with	total number	spores, % of the
		inoculation	B. megaterium 501 <sup>rif</sup>		total number
Oats	$0.22 \pm 0.03$	< 0.02	< 0.02	$500 \pm 70$	20.0
Oats	$0.67 \pm 0.04$	$0.59 \pm 0.02$	$0.18 \pm 0.03$	$550\pm65$	27.0
Oats	$1.23 \pm 0.03$	$0.93 \pm 0.15$	$0.58 \pm 0.07$	350±33	42.0
Corn	$6.80 \pm 0.05$	$0.22 \pm 0.02$	$0.07 \pm 0.02$	$335 \pm 40$	12.0
Soil without corn	$6.80 {\pm} 0.05$	$4.50 \pm 0.07$			

Given the high efficiency of herbicide degradation under the cover of corn plants, the effect of bacteria on the accumulation of green phytomass and the prometryn degradation in soil was also assessed in plot tests. Thirty days after the emergence of seedlings, the plant weight in the control was  $257\pm15$  g/m<sup>2</sup>, while under inoculation with *B. megaterium* 501<sup>rif</sup> it was  $287\pm20$  g/m<sup>2</sup>, or 12% more than in the control. There was practically no difference between the variants of the experiment. The soil concentration of the herbicide upon inoculation decreased almost 3 times compared to the control,  $0.15\pm0.03$  vs.  $0.45\pm0.10$  mg/kg, which is consistent with the pot test data.

Considering the reasons for the positive effect of *B. megaterium*  $501^{\text{rif}}$ , one should pay attention to the fact that in plants inoculated with bacteria, the architecture of the root system changes significantly due to the more intensive development of lateral roots [19], the generative organ formation is accelerated [20], and the total concentration of photosynthetic pigments in leaves rises [21].

It was found that these microorganisms produce auxins and B vitamins [22], as well as poly-beta-hydroxybutyric acid [23-26] which had a positive effect on root formation and photosynthesis in plants. The content of poly-beta-hydroxymatic acid in *B. megaterium* cells ranged from 10 to 80% of the mass of bacteria, depending on the strain and cultivation conditions [23-25]. The strain *B. megaterium* 501<sup>rif</sup>, obtained by us, during its growth in a liquid medium also intensively synthesized and accumulated poly-beta-hydroxybutyric acid granules

in vegetative cells (Fig. 2).



Fig. 2. Bacillus megaterium 501<sup>rif</sup> cells filled with polybeta-hydroxybutyric acid beads (Axio Lab. A1 microscope, Carl Zeiss, Germany, phase contrast).

The trial of Albit biological (Scientific and Production Company Albit LLC, Russia), the main active ingredient of which is poly-betahydroxybutyric acid, has shown its high efficiency in a number of agricultural crops as compared to vatious chemical herbicides [13, 27]. Zlotnikov et al. [13, 27] consider Albit as a universal anti-stress preparation with antidote properties towards pesticides used in agriculture. According to the authors, the mechanism of protective action is primarily

associated with the positive effect of poly-beta-hydroxybutyrate on photosynthesis. Based on this, it can be assumed that the protective effect of *B. megaterium*  $501^{rif}$  we observed is associated, with a high degree of probability, with the positive effect of bacterial products, primarily poly-beta-hydroxybutyric acid.

The results for corn are of particular interest. It is known that corn absorbs and degrades triazine herbicides. In this case, the enzymes involved in the hydroxylation play a significant role [28]. It was found that during the transformation of prometryn by plants and microorganisms, significant amounts of metabolic products are formed among which sulfoxide, sulfone, hydroxypropazine occupy the main place, and, with the subsequent transformation of hydroxypropazine, the production of ameline, amelide and cyanuric acid is possible. The products of hydrolysis and N-dealkylation of sim-triazines form conjugates with glutathione and sugars [6, 28].

Until now, the main attention of researchers has been focused on the study of the herbicidal effect of prometryn and the products of its transformation [29]. There is very little evidence of their beneficial effect on plants. Thus, Lebedev reported [30, 31] that small doses of prometryn increased the net productivity of photosynthesis and the absorption of nitrogen and phosphorus by Scots pine and Siberian cedar seedlings. Nadar et al. [32] observed a stimulating effect of simtriazine herbicides, including methylthio derivates (prometryn and ametryne, on the growth of callus and protein synthesis in sorghum, which allowed the authors to concluded about the hormonal kinin-like action of low concentrations of simtriazines on plants. Triazine herbicides affect the ionic balance of plants and, thence, the synthesis of DNA, proteins, and enzymes [33]; therefore, it is possible that the high efficiency of corn plant inoculation with *B. megaterium* 501<sup>rif</sup> upon prometryn application is due to the synergistic effect of the bacterial excretions, the herbicide and products of its transformation.

Thus, inoculation of oat and maize seeds with *Bacillus megaterium* 501<sup>rif</sup> has a positive effect on plant growth. Moreover, the efficiency of inoculation is higher upon prometryn application than in the control. Also, the plant resistance to prometryn increases and its decomposition in the rhizosphere accelerates. Consequently, the *B. megaterium* 501<sup>rif</sup> and its metabolites, in particular poly-beta-hydroxybutyric acid, serve as protectors, or antidotes, removing the phytotoxic effect of prometryn. The pot tests and plot trials showed that the corn plants inoculated with *B. megaterium* 501<sup>rif</sup> possesses properties of a biomeliorant, effectively removing herbicide prometryn from soil. This opens up prospects for the use of corn inoculated with *B. megaterium* in bioremediation of the soils

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