# SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA [AGRICULTURAL BIOLOGY], 2014, № 5, pp. 107-112 ISSN 313-4836 (Online)

### Soil microbiology

UDC 631.147:579.64

doi: 10.15389/agrobiology.2014.5.107rus doi: 10.15389/agrobiology.2014.5.107eng

# Bacillus megaterium 501<sup>rif</sup> INTRODUCED INTO THE SOIL: FACTORS AFFECTING THE RATE OF SURVIVAL, SPORULATION AND DECOMPOSITION OF THE HERBICIDE PROMETRYN

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#### Abstract

Introduction of microorganisms into the soil is a fundamental problem in application of microbial preparations in agriculture, crop production and ecology. Its effectiveness depends on many factors (i.e. the type and strain of microorganism, the physical and chemical properties of the soil, plant cover, climate and so on), which are not well understood, leading to a high variability of the results of application of microbial preparations. In this article, there is reported the first investigation of survival and sporulation dynamics of Bacillus megaterium 501rif introduced into sodpodzolic soil, depending on the temperature of the soil, organic matter content and herbicide prometryn, the 2-methylthio-4,6-bis (iso-propylamino)-sim-triazine, application. In laboratory experiments it was found that the optimal conditions for the *B. megaterium* 501<sup>rif</sup> introduced into the soil are achieved at a temperature of 20 °C. After the third day after inoculation the titer of bacteria in the soil increased almost 10 times and remained at that level until the end of the experiment (32 days). B. megaterium 501rif throughout the experiment was mainly in the form of a physiologically active cells and fits well into the soil microbiom. Under these conditions an additional incorporation into the soil of the organic matter, such as cereal straw and corn flour, leads to 10-100-fold increase of the amount of B. megaterium 501<sup>rif</sup>. At 37 °C the total number of B. megaterium 501<sup>rif</sup> reduced several times, and stabilized at this level until the end of the experiment. In this case, 100 % of *B. megaterium* 501<sup>rif</sup> revert into spores and are physiologically inactive. The temperature below the minimum for physiological reproduction of B. megaterium (4  $^{\circ}$ C) inhibits spore formation and leads to a rapid loss of physiologically active bacterial cells introduced into the soil. The total number of bacteria decreases 1000 times within a month. These data lead to the conclusion that the «low-temperature shock» reduces the competitiveness of B. megaterium to the indigenous microflora and its resistance to bactericidal metabolic products of soil biome. It is shown that at the optimum temperature (20 °C) of soil the B. megaterium inoculation and the use of additional organic matter such as straw accelerate the decomposition of herbicide prometryn, can potentially be used in a biotechnology of remediation of the soil, contaminated by herbicide.

Keywords: Bacillus megaterium, sporulation, introduction, soil, temperature, organic matter, prometryn herbicide.

Bacterial preparations are used in agriculture to stimulate plant growth and development, to protect plant against phytopathogens and pests, and as well for cleaning the soil from xenobiotics (1-4). Aerobic sporulating bacteria of the genus *Bacillus*, i.e. *B. thuringiensis* (5, 6), *B. subtilis* (7), *B. megaterium* (8-10) are widely applied as they are well manufacturable, long stored and suitable for use in the field. However, the efficiency of these preparations varies in a wide range due to poor knowledge of the behavior of the bacilli introduced into the soil.

Spore forming bacteria are common inhabitants in different soils. Their number depends on the soil and climatic conditions and vegetation, ranging from  $10^3$  to  $10^6$  CFU/g (11). Some researchers (11, 12) consider them to be free living soil microorganisms, while the others (13), on the contrary, designate these bacteria as a common inhabitant of the rhizosphere, at least in some plants, and *B. subtilis* and *B. megaterim* are referred to as the facultative endo-

phytes (14, 15). According to A.I. Melent'ev (16), a survival rate of spore forming bacteria in the rhizosphere of cereals depended on the number, species and strain of introduced microorganisms. Their number on the roots of plants varied from  $10^4$  to  $10^7$  CFU per 1 g of the roots, being lower by the end of vegetation compared to initial period. Most bacterial cells were transformed to spores which were not physiologically active and thus unable to affect plant growth and development.

As to spore forming aerobic bacteria inoculated into the soil, it is little known about their survival. Poor knowledge results in lack of the approaches to optimization of the inoculation procedure to make the conditions favorable for the bacteria and allow effective application of their preparations for plant growth stimulation and soil bioremediation in environmentally friendly agrotechnologies.

We studied the survival of *Bacillus megaterium* introduced into the soil and herbicide Promentryn degradation as influenced by different conditions.

*Technique*. Rifampicine resistant *B. megaterium* 501<sup>rif</sup> mutant obtained by gradient selection of resistant forms (17) of parental *B. megaterium* 501 strain isolated from medium loamy ordinary chernozem (Kokchetav region, Kazakhstan). *B. megaterium* 501 is deposited in Collection of Agricultural Microorganisms (VKSHM, All Russian Research Institute of Agricultural Microbiology). *B. megaterium* 501<sup>rif</sup> was cultivated in Erlenmeyer flasks on a rotary shaker at 140 rpm (UMVT-12-250, Ellion, Russia) using nutrient medium that consists of (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, Ca-CO<sub>3</sub> – 0.025, FeSO<sub>4</sub> – 0.025, yeast extract – 0,2, sucrose – 10, pH 6.8-7.0. Sod-podzolic sandy loam soil (pH 6.7) was filled into plastic containers and inoculated 2-day liquid culture of *B. megaterium* 501rif, containing not less than 90 % of the living cells. Soil moisture was maintained between 50-60% of the total capacity.

Effect of temperature was studied at 4, 20  $\mu$  37 °C. To estimate the influence of organic substances, the chopped oat straw (0.2 mm) and corn flour were added in an amount of 20 g per 1 kg of soil. To examine the effect and degradation rate of Prometryne, the 2-methylthio-4,6-bis(isopropylamino)sym-triazine, 50 % water emulsion of powder herbicide (Panama Agrochemicals Inc., Panama Republica) was added to the soil at 4 and 20 mg/kg.

Total number of *B. megaterium* 501<sup>rif</sup> (CFU/g of soil) was evaluated by serial dilution of bacterial suspension (19) spread onto nutrient media consisting of (g/l):  $NH_4NO_3 - 0.5$ ,  $K_2HPO_4 - 1.6$ ,  $KH_2PO_4 - 0.4$ ,  $MgSO_4 - 0.2$ ,  $CaCO_3 - 0.025$ ,  $FeSO_4 - 0.025$ , sucrose - 10, yeast extract - 0,2, agar - 20, rifampicine - 0,02. The spores number was assessed in suspensions after their pasteurization at 80 °C for 10 min.

Prometryn was extracted from soil using acetone method and analyzed by gas-liquid chromatography (GLC) (20).

Statistical analysis was performed at  $P_{0.05}$  level (21).

**Results.** In nonsterile soil at low temperature (Fig. 1) a sharp decrease of *B. megaterium* 501rif number was observed after inoculation. The most of bacteria were in the form of vegetative cells. At 4 °C, being lower than growth minimum for *B. megaterium* 501<sup>rif</sup>, the sporulation delayed and therefore most of vegetative cells died. After 1 month the total number of *B. megaterium* 501<sup>rif</sup> was 1000 times less than at the beginning of the experiment. Activation of *Fusarium* and *Penicillium* growth was detected. Therefore, the law temperature shock reduces protective functions and competitiveness of *B. megaterium*.

Relatively high survival of *B. megaterium*  $501^{rif}$  was found at 37 °C. After 32 days the total number of inoculated bacteria in soil decreased 3 times compared to initial level, and the sporulation frequency reached 100 %

after 12 days.





Most favorable conditions for *B. megaterium*  $501^{rif}$  in nonsterile soil were formed at 20 °C. Already 3 days after inoculation the bacteria increased in number almost 10 times and the level remained high until the end of the experiment after 32 days. Sporulation was observed on the day 12, but the proportion of the spores was less than 30 %.

Therefore, in the microbiome of sod-podzolic soil the *B. mega*-

terium 501rif remained active for all the time of observation.

When chopped straw added, the number of inoculated *B. megaterium*  $501^{rif}$  increased 10 times after 12 days incubation (Fig. 2). High titers were detected during a month, with the number of vegetative cells more than  $10^5$  CFU/g. The same was also found when corn flour added. However, in this case there was intensive sporulation, and after a month the number of spore reached 100 %.



Fig. 2. Dynamics of survival and spore formation in *Bacillus* megaterium 501<sup>rif</sup> after inoculation as related to extra organic matter: a, b, c - control, oat straw and corn flour, respectively; grey and white color means spore number and cell number, respectively.

A positive effect of straw and corn flour on *B. megaterium*  $501^{\text{rif}}$  is obviously due to more nutrients in soil promoting more vital activity in the microorganism. Also it could result from positive effect of these substrates on formation of microbe association favorable for *B. megaterium*.

For several decades the interrelation between microorganisms and xenobiotics is being under con-

sideration. Herbicides are such xenobiotics widely used in agriculture for weed control (18). These chemicals impact on soil microbiomes, and soil fertility in general, also detoxication and degradation of pesticides in nature are considered an urgent problem because many of them are dangerous for the environment, people and animals.

We studied the interrelation between *B. megaterium*  $501^{rif}$  inoculated into the soil and Prometryn, the herbicide widely used for weed control on potato, soybean, carrot and other crops. It was shown that Prometryn applied at the doses dozens times more than those used during commercial crop cultivation did not affect *B. megaterium*  $501^{rif}$ , and its number even increased significantly in the presence of oat straw (Table).

Dynamics of survival and spore formation (CFU,  $10^3$ /g) of *Bacillus megaterium* 501<sup>rif</sup> after inoculation into soil as influenced by herbicide Prometryn and oat straw ( $X \pm x$ )

Days	Control	Prometryn		
		4 mg/kg of soil	20 mg/kg of soil	20  mg/kg of soil + straw (2%)
0	<u>80±1.2</u>	<u>80±1.2</u>	<u>80±1.2</u>	<u>80±1.2</u>
	13±0.5	13±0.5	$13 \pm 0.5$	13±0.5
3	$200 \pm 3.4$	<u>135±24.2</u>	<u>90±6.0</u>	$1600 \pm 100.8$
	150±12.6	65±0.9	40±6.2	$500 \pm 8.0$
12	<u>135±5.3</u>	<u>114±3.5</u>	$100 \pm 10.0$	<u>1200±150.0</u>
	115±1.5	$112 \pm 4.0$	54±2.7	$1000 \pm 21.0$
32	$110\pm10.7$	$140 \pm 1.5$	<u>118±9.4</u>	<u>1200±63.6</u>
	$90 \pm 2.7$	$140 \pm 10.3$	115±1.5	1100±49.5
$\overline{C}$ o m m e n t s. Above line: the total number of bacteria, under line: number of spores.				



Fig. 3. Dynamics of Prometrine degradation in soil under *Bacillus megaterium* 501<sup>rif</sup> inoculation: 1 - control, 2 - in-oculation, 3 - inoculation in the presence of oat straw (2 %).

In the soil inoculated with *B. megaterium*  $501^{rif}$  the rate of Prometryn degradation increased significantly (Fig. 3). Most active degradation was observed when we added the oat straw. A month after inoculation the concentration of herbicide was 10 times lower if compared to control, thus correlating with an increase of the total *B. megaterium*  $501^{rif}$ number.

These results lead to the conclusion that applying organic matter, particularly the plant residues enriched with cellulose, and the inoculation with microorganisms capable to degrade the herbicide, are essential and probably the main conditions for improving bioremediation under soil pollution with Prometryn. So, it is shown that vegetative cells of

*Bacillus megaterium* 501<sup>rif</sup> inoculated into soil remain physiologically active for rather long time. When organic matter, such as cereal straw or corn flour, is added, it results in a rapid bacterial growth. Their number increases hundreds times, being kept stable. Active sporulation starts 12 days after the inoculation. The result of introduction and physiological state of the bacteria depend directly on soil temperature. The most favorable temperature for *B. megaterium* survival, provided optimal moisture, varies from 20 to 37 °C. Organic matter enriched with cellulose, such as the chopped straw, crop residues, etc., also contribute to the survival of bacteria. The isolated strain *B. megaterium* 501<sup>rif</sup> significantly accelerates the Prometryn degradation and can be useful in biotechnological remediation of soils polluted with this herbicide.

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