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MICROBIAL PREPARATIONS ON THE BASIS OF ENDOPHYTIC AND RHIZOBACTERIA TO INCREASE THE PRODUCTIVITY IN VEGETABLE CROPS AND SPRING BARLEY (*Hordeum vulgare* L.), AND THE MINERAL FERTILIZER USE EFFICIENCY

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Abstract

We use an approach involving a combination of endophytic and rhizobacteria in the development of microbial preparations for crop production, which allows to create self sufficient plant-microbe systems, enhancing agricultural production and reducing the environmental burden. The article presents data on the properties of promising strains of endophytic and rhizobacteria and their efficiency in pot and field experiments. Rhizobacteria were isolated from the roots of tomato plants (*Solanum lycopersicum* L.) cultivar Bella, endophytic bacteria from internal tissues of hogweed (*Heracleum sphondylium* L.) stem. Identification of the studied bacterial strains was determined by nucleotide sequences of the 16S rRNA gene, and on cultural and biochemical properties. Middle grade lettuce (*Lactuca sativa* L.) variety Eralash and early maturing variety Duro of radish (*Raphanus sativus* L.) were used in pot experiments. It was shown that the *Bacillus subtilis* strain TR6 was active against all tested phytopathogenic fungi. Zones of inhibition varied from 17.4 to 48.2 mm. Almost all of the studied strains were capable of producing auxins, except strain TR7. Highest auxin production was observed in strains TR1 and TR9 — 12.3 and 20.1 rg/ml of medium respectively. Three of the five studied strains (*B. subtilis* HC8, *B. subtilis* B2G and *Azotobacter chroococcum* AZ7) significantly (by 15.2-34.2 %) increased the biomass of the lettuce. The strains of *B. subtilis* Ch-13 and *B. subtilis* TR6 increased the yield of lettuce but insignificantly. Among the studied strains, *B. subtilis* HC8 should be noted, which significantly (by 15.2-42.1 %) increased harvest of radishes and lettuce in pot experiments. Application of microbial preparations based on endophytic and rhizobacteria can be effective promising element of modern technologies of barley (*Hordeum vulgare* L.) cultivation, significantly reducing production costs and improving the efficiency of application of mineral fertilizers for its cultivation. Thus, the grain yield of barley variety Danuta (2-year field experiments) due to application of microbial preparations on the basis of endophytic and rhizobacteria has been increased by 23.8-43.9 % compared to the control and by 11-29 % compared to mineral fertilizers. Due to inoculation of barley seeds with promising strains of the endophytic and of rhizobacteria the efficiency of nitrogen, phosphorus and potassium use from mineral fertilizers increased by 11.7-22.1 %, 5.1-10.3 %, and 10.2-19.4 %, respectively.

Keywords: endophytic and rhizobacteria, microbial preparations, bacilli, fungicidal activity, plant growth promotion, grain yield, mineral fertilizers use efficiency

Fertilizers and plant protection products are widely involved in modern high-efficiency agriculture. Thus, extensive use of mineral fertilizers, especially nitrogen chemicals, made it possible to raise major crop yields in developed countries more than 5 times over the last 50 years. But production and use of mineral nitrogen fertilizers are the most energy-intensive and consume from 30 to 50 % of all energy spent in the agricultural production. Consequences of mineral nitrogen application are negative for human health and soil microorganism biodiversity, deteriorating soil fertility, increasing greenhouse gas emissions, and in Europe alone, it requires annual compensation costs of 70 to 320 billion EUR [1]. Biological nitrogen is an alternative cheap and safe source of nitrogen for agricultural production. This is especially relevant for Russia, since application of mineral fertilizers has decreased 3-5 times over the last 20 years. At the same time, the situation is exacerbated by expensiveness of mineral fertilizers leading to the usage the reserves of nitrogen, phosphorus and potassium in the soil without replenishment, which results in the loss of soil fertility. With regard to the structure of cultivated areas in the Russian Federation and long-term testing microbial preparations efficacy, highly efficient biologicals are required to provide treatment of at least 20 million hectares [2-5].

The researchers' attention is drawn to plant associations with beneficial microorganisms regarding not only the study of the fundamentals of their interaction, but also the possible practical use of such interactions in environmentally oriented adaptive crop production. Most studies focus on rhizosphere microorganisms [6-8]. But there are microorganisms, the endophytic bacteria, that exist within the plant, including the aerial parts and seeds. Bacteria may be called endophytic when they are capable of colonizing internal plant tissues without adverse effects on the plant development and without causing diseases [9, 10)]. Each of the 300,000 plant species existing on the Earth is the host for one or more endophytic bacteria species [11]. Currently, just few plant species have been studied sufficiently for the presence of endophytic bacteria. There are great promises for finding, isolating and studying endophytic bacteria species that affect plant development positively, to create more efficient biologicals for adaptive crop production [12]. In a plant, bacterial endophytes colonize the same ecological niches as phytopathogenic microorganisms, so they are also considered as promising biocontrol agents against plant pathogens [8, 12].

We used an approach involving a combination of endophytic bacteria and rhizobacteria in the development of biologicals for self-sufficient plant-microbe systems, enhancing agricultural production and reducing the environmental load.

The purpose of this research was to study commercially valuable properties of endophytic and rhizobacteria in the roots and internal tissues of cultivated and wild plants, to develop the samples of strain-based microbial preparations and compare the effects of perspective and existing biologicals in barley and vegetable crops in pot and field experiments to identify effective options.

Technique. Methods of general and technical microbiology were used as described [13, 14].

Rhizobacteria were isolated from the roots of tomato plants (*Solanum lycopersicum* L.) cultivar Bella. Roots were rinsed in tap water, placed in flasks with 300 ml sterile water and kept on a shaker (200 rpm) for 2 hours. An aliquot 1 ml was taken from each flask to prepare serial dilutions (10^{-1} to 10^{-5}), 100 μl of each dilution were plated onto 20-fold diluted tryptone soy agar (TSA, Sigma, USA) (0.5× TSA). Dishes were cultured for 3 days at 28 °C. To isolate endophytic bacteria from the internal tissues of hogweed (*Heracleum sphondylium* L.) stem, surface sterilization method was used. Cleaned stem sample was washed in 70 % ethanol for 10 minutes, in 10 % sodium hypochlorite for 5 min, and 5 times in sterile tap water. After sterilization, the sample was smashed aseptically using sterile pestle and mortar. To confirm successful sterilization, swabs from the surface of tissue were performed, and the samples obtained were plated on potato agar medium (PDA, Sigma, USA). A series of dilutions were prepared of hogweed sap and plated on potato medium in Petri dishes (3 replicates).

Protease activity of endophytic and rhizobacteria strains was studied in the dishes with 0.5× TSA medium enriched with 5 % milk. Lipase activity was estimated on plates with 0.5× TSA agar supplemented with 2 % Tween 80, glucanase activity was tested on 0.5× TSA with lichenan, for cellulase activity estimation the 0.5× TSA with Na-carboxymethylcellulose was used, and chitinase activity was assessed on plates with chitin [15[. Auxin production was assayed colorimetrically [16]. Test strains were added to liquid King medium (Sigma, USA) containing tryptophan (250 μg/ml) and incubated at 28° C on a shaker (150 rpm) for 4 days. The resulting cultures were centrifuged at 13,000 rpm for 10 min, 1 ml of supernatant was added to a tube with 2 ml Salkowski reagent and 25 μl of orthophosphoric acid. The mixture was left at room temperature for 30 minutes, and the intensity of pink color was measured using a spectrophotometer Specord S100 (Analytik Jena AG, Germany) at $\lambda = 530$ nm. The level of indole-3-acetic acid (IAA) was estimated by a calibration curve.

Identification of endophytic and rhizobacteria strains was performed by 16S rRNA gene nucleotide sequences [17]. Universal eubacterial primers FD1 and RD1 were used for PCR [18]. Amplified DNA fragments were isolated from agarose gel [19], and their nucleotide sequences were determined using an automated CEQ2000 XL DNA sequencer (Beckman Coulter, USA) according to manufacturer's instructions. Nucleotide sequences obtained were compared with GeneBank database sequences using BLAST software [20].

Total endophytic and rhizobacteria strain titers were measured by serial dilution method on agar medium with TSA (Sigma, USA). To monitor commercially valuable properties in promising endophytic and rhizobacteria strains, growth promoting biological tests were used according to the description [18] in maize (*Zea mays* L.) and wheat (*Triticum aestivum*) seedlings, along with the pathogenic fungi *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL), *F. solani* and *Phytium ultimum* LBOP17 growth suppression tests (estimation of growth inhibition areas by well method in agar).

Efficiency of microbial preparations in pot and field experiments was studied by the common method [19].

Middle grade lettuce (*Lactuca sativa* L.) variety Eralash and early maturing radish (*Raphanus sativus* L.) variety Duro were used in pot experiments. When preparing substrate, sod-podzolic sandy loam soil from the experimental field of All-Russian Research Institute for Agricultural Microbiology (ARRIAM) was mixed with peat soil Terravita (MNPP Fart CJSC, Russia) (20 % + 80 %) and supplemented with NPK solution (NPK 16:16:16, Akron JSC, Russia) at the rate of 100 % NPK. The 3 liter pots with substrate (5 kg per pot) were shed with tap water once (700 ml). Radish seeds were germinated at 28 °C for 48 hours. Immediately prior to planting, lettuce seedlings were soaked in a rhizo- and endophytic bacteria suspension (10^7 CFU/ml) for 15 minutes. Immediately prior to planting, lettuce seeds (no germination) were soaked for 15 minutes in bacterial suspension (10^7 CFU/ml) of the following strains: *Bacillus subtilis* Ch-13 (standard, Extrasol-producing agent), *B. subtilis* B2G (standard, Z24-producing agent), *B. subtilis* TR6, *B. subtilis* HC8, *Azotobacter chroococcum* Az7. Control seeds were soaked in sterile water. Seeds were planted in the prepared substrate at 1.5 cm depth (2 pcs. per well). Well location was of the envelope pattern (5 wells per pot). After planting, a single watering was performed (500 ml). After lettuce and radish germination, three plants per pot were left. During vegetation, 100 ml watering was performed every 3 days. Lettuce crop yield (aerial parts of plants) was recorded in 44 days, radish (tubers) crop yield was harvested after 35 days of vegetation (collected samples were weighed using electronic scale at the accuracy of 0.1 g).

Field experiment (Ryazan Province, 2013-2014) in barley (*Hordeum vulgare* L.) variety Danuta, with winter wheat variety Mironovskaya 808 as the predecessor, was performed according to the common method [19] in 4 replicates in the total area of 460.8 m^2 (one plot area of 9.6 m^2). The plot soil was gray forest heavy loam with a high level of nutritional elements. Soil analysis was performed according to GOST 26483-85, GOST 26213-19, GOST25336, and GOST 26207-9 (state standards). Agrotechnics conformed that generally accepted for the crop in the southern part of the Non-Chernozem zone of the Russian Federation. Mineral fertilizers were scattered superficially in spring before sowing (active material, kg/ha: diammophoska — 23, ammonium nitrate — 37). Microbial preparations were used as recommended by the developer. Experiment pattern: variant 1 — no fertilizers (control); variant 2 — $N_{60}P_{60}K_{60}$ (background); variant 3 — Extrasol (*Bacillus subtilis* Ch-13, standard; developed by All-Russian Research Institute of Agricultural Meteorology; soaking seeds in 10 % preparation solution); variant 4 — Extrasol + $N_{60}P_{60}K_{60}$; variant 5 — *B. subtilis* TR6; variant 6 — *B. subtilis* TR6 + $N_{60}P_{60}K_{60}$; variant 7 — *B. subtilis* HC8; variant 8 — *B. subtilis* HC8 + $N_{60}P_{60}K_{60}$. Soil samples were taken from each plot (5 random samples which constituted an average sample). The effectiveness of fertilizer application was determined by the difference method [5].

The statistical significance of differences was assessed according to B.A. Dospekhov [21].

Results. A total of seven strains of rhizobacteria from tomato roots and an endophytic bacteria strain HC8 were isolated. Determination of gene 16S rRNA nucleotide sequences in isolates made it possible to identify strains TR6 and HC8 as *B. subtilis* [23].

Fungicidal activity test showed that strain TR6 only was active against all the used phytopathogenic fungi *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl), *F. solani*, *Pythium ultimum*. The diameter of the inhibition zone was 17.4 to 48.2 mm.

The study of rhizobacteria enzymatic activity demonstrated protease activity in five of the seven strains studied (except TR9 and TR1), lipase activity in two ones (TR5 and TR6), and glucanase activity in three ones (TR10, TR7 and TR6). Two strains only (TR7 и TR6) had cellulase activity and none exhibited chitinase activity. The study of IAA production in the presence of tryptophan as IAA precursor confirmed that almost all the strains tested (except TR7) were capable of producing auxins. The highest auxin production was observed in strains TR1 and TR9 (12.3 and 20.1 μg/ml of medium, respectively). *B. subtilis* was selected for further studies for commercially valuable properties (biocontrol, growth promotion, enzymatic activity).

Endophytic bacteria *B. subtilis* strain HC8 isolated from hogweed internal tissues has been shown to have complex commercially valuable properties and be of interest for the use as a microbial preparation which increases crop productivity [23-25].

In this regard, we studied the effects of the previously selected five promising endophytic and rhizosphere bacteria *Bacillus* and *Azotobacter* strains on the productivity of radish and lettuce. *B. subtilis* Ch-13 (standard, Extrasol-producing agent) and *B. subtilis* HC8 (hogweed endophyte) strains provided the greatest significant increase in radish crop yield of 27.7 and 42.1 %, respectively (Table 1). *B. subtilis* strain TR6 selected previously as a producer of antifungal and growth promoting substances decreased radish tuber yield significantly (by 23,9 %). Other strains, *A. chroococcum* AZ7 and *B. subtilis* B2G (standard, Z24-producing agent) decreased radish tuber crop yields insignificantly (by 15.3 % and 11.9 %, respectively).

1. Productivity of radish (*Raphanus sativus* L.) and lettuce (*Lactuca sativa* L.) inoculated with promising endophytic and rhizosphere bacteria strains (pot experiment)

Variant	Redish (variety Duro)			Lettuce (variety Eralash)		
	tuber weight, g	increase in crop yield		plant biomass yield, g	increase in crop yield	
		g	%		g	%
Control	9.91			65.0		
Bacillus subtilis Ch-13 (Extrasol producing agent)	12.66*	2.75	27.7	71.5	6.5	10.0
Bacillus subtilis B2G (Z24 producing agent)	8.73	–1.18	–11.9	75.9*	10.9	16.8
Bacillus subtilis TR6 (rhizobacteria)	7.54	–2.37	–23.9	70.6	5.6	8.6
Bacillus subtilis HC8 (endophyte)	14.08*	4.17	42.1	74.9*	9.9*	15.2
Azotobacter chroococcum AZ7 (rhizosphere bacteria)	8.39	–1.52	–15.3	87.2*	22.2	34.2
LSD_{05}	1.90			9.3		

* Differences with control are significant at $P < 0.05$.

The selected lettuce variety was more responsive to inoculation with promising endophytic, rhizosphere and rhizobacteria strains studied. Three of the five strains studied (HC8, B2G, and AZ7) increased lettuce biomass significantly (by 15.2-34.2 %). Strains *B. subtilis* Ch-13 and *B. subtilis* TR6 increased lettuce green biomass yield insignificantly. Among the studied strains, *B. subtilis* HC8 should be noted, which significantly (by 15.2-42.1 %) increased the crop yields of radishes and lettuce in pot experiments.

Thus, pot experiments with vegetables (lettuce and radish) demonstrated that the effects of endophytic, rhizosphere and rhizobacteria strains previously selected for complex commercially valuable properties on crop productivity are not always significant.

Continued studies of their efficacy in small plot field experiments with barley variety Danuta (2-year experiments, Ryazan Province) showed that promising endophytic and rhizobacteria *B. subtilis* HC8 and *B. subtilis* TR6 strains, like the standard *B. subtilis* strain Ch-13, increased barley crop yields significantly (by 13.1-17.6 %) (Table 2).

2. Barley (*Hordeum vulgare* L.) variety Danuta crop yields in a small plot field experiment under inoculation with promising endophytic and rhizosphere bacteria strains (Ryazan region, average over the years of 2013-2014)

Variant	Grain crop yield, t/ha	Gain, t/ha	
		to control	to background
No fertilizers (control)	2.44		
$N_{60}P_{60}K_{60}$ (background)	2.72	0.28	
Bacillus subtilis Ch-13, microbial preparation Extrasol (standard)	2.76	0.32	0.04
B. subtilis Ch-13 + $N_{60}P_{60}K_{60}$	3.51	1.07	0.79
B. subtilis TR6 (rhizobacteria)	2.87	0.43	0.15
B. subtilis TR6 + $N_{60}P_{60}K_{60}$	3.28	0.84	0.56
B. subtilis HC8 (endophyte)	2.80	0.36	0.08
B. subtilis HC8 + $N_{60}P_{60}K_{60}$	3.02	0.58	0.30
LSD_{05}	0.26		

Interestingly, the effects of all strains tested were greater than that of mineral fertilizers ($N_{60}P_{60}K_{60}$) at the recommended dose. Thus, the use of promising endophytic and rhizobacteria *B. subtilis* strains resulted in a significant increase in barley grain yield by 23.8-43.9 % versus control and by 11-29 % compared to mineral fertilizers. Thus, application of microbial preparations to treat barley seeds is a promising element of modern cultivation technologies, considering the cost of mineral fertilizers, especially.

A natural question arises about the mechanisms of promising endophytic and rhizobacteria strains effects on barley plants. Previously, we have demonstrated that the investigated strains of these bacteria are capable of producing some phytohormones [22, 23] that may promote root growth, root hairs especially. Development of the root system and root hairs of inoculated plants contributes to a significant improvement of absorption capacity and, consequently, results in improved use of fertilizers applied. This has been confirmed in our small plot field experiments in the calculation of fertilizer utilization performed by the difference method (Table 3). Due to inoculation of barley seeds with promising strains of endophytic and rhizobacteria, the efficacy of mineral fertilizer nitrogen, phosphorus and potassium use increased by 11.7-22.1 %, 5.1-10.3 %, and 10.2-19.4 %, respectively. This explains a significant increase in barley crop yield in the field experiment.

3. Efficacy of fertilizer use by barley (*Hordeum vulgare* L.) variety Danuta in a small plot field experiment under inoculation with promising endophytic and rhizosphere bacteria strains (Ryazan Province, average over the years of 2013-2014)

Variant	Utilization rate, %		
	N	P_2O_5	K_2O
$N_{60}P_{60}K_{60}$ (background)	12.9	5.7	11.4
Bacillus subtilis Ch-13 (microbial preparation Extrasol) (standard) + $N_{60}P_{60}K_{60}$	35.0	15.4	30.8
B. subtilis TR6 + $N_{60}P_{60}K_{60}$	27.9	12.3	24.6
B. subtilis HC8 + $N_{60}P_{60}K_{60}$	24.6	10.8	21.6

So, efficacy of previously developed and proposed biologicals based on endophytic bacteria and rhizobacteria isolated from the roots of cultivated and wild plants compared in pot and field experiments indicates the prospects of combined application of these two groups of bacteria in modern biotechnologies for cultivation of barley and vegetable crops (lettuce and radishes). Combining endophytic and rhizobacteria in the development of microbial preparations for plant makes it possible to arrange self-sufficient plant-microbial systems, reducing the cost of production and providing more effective utilization of mineral fertilizers used in crop cultivation.

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