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Herbicide-Resistant Strain *Pseudomonas plecoglossicida* CH5%2 to stimulate wheat growth under herbicide stress

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In this study, the bacterial strain CH5%2 was isolated from soil contaminated with petrochemical waste and identified as *Pseudomonas plecoglossicida.* The bacterium was viable in the presence of herbicides: Octapon (commercial name) based on 2, 4-dichlorophenoxyacetic acid (2,4-D) and Nanomet (commercial name) based on metsulfuron-methyl. The strain synthesized indolyl-3-acetic acid, solubilized phosphates from insoluble phosphorus compounds, fixed atmospheric nitrogen, and showed antagonism against microscopic fungi of *Fusarium*, *Alternaria*, *Bipolaris* and *Rhizoctonia* genuses. In laboratory conditions, bacterial strain *P. plecoglossicida* CH5%2 decreased stress effects in wheat plants sprayed with herbicide. It maintained roots and shoots growth no lowers than that of control plants, and normalized chlorophyll and proline content in the leaves. A field experiment was conducted on experimental plots in the arid conditions of the trans-Ural steppe to test the effect of these bacteria on the wheat yield under herbicide treatment and without it. The results obtained in the field experiment confirmed laboratory data on the ability of *P. plecoglossicida* CH5%2 to mitigate herbicidal stress. Against the background of herbicides Octapon and Nanomet the use of bacterial culture increased yield of up to 25%. This study showed that the bacteria *P. plecoglossicida* CH5%2 significantly mitigate herbicidal stress in wheat and are able to stimulate its growth and increase productivity under stress.

Keywords: *Pseudomonas plecoglossicida*; herbicide resistance; herbicidal stress; anti-stress activity; wheat; plant growth stimulation.

INTRODUCTION

The productivity of agricultural plants is directly dependent on the use of measures for their integrated protection from stressful effects: diseases, pests and drought. The high demand for increased agricultural productivity is also accompanied by the widespread use of plant protection chemicals and the discovery of new pesticides (Sands et al. 2009). Pesticides are an important element in intensive plant cultivation technologies, at least 35% of which are herbicides (Kiely et al. 2004; Aktar et al. 2009). Traditionally, much attention is paid to the control of crop contamination in the cultivation of agricultural land. Chemical herbicides are the main method of controlling unwanted vegetation along with pre-sowing soil treatment and crop rotation. The use of pesticides, and in particular herbicides, not only

leads to environmental pollution and affects the dynamics of biogeochemical cycles, soil fertility, damages the soil microbiota (Cycon et al. 2013), but also has another significant stress effect on the cultivated plants (Kutuzova et al. 2006, Kumar, Singh, 2010, Su et al. 2018). Thus, the use of pesticides raises two problems: reducing the period of persistence of pesticides in the soil, speeding up the process of their natural destruction and reducing the negative impact on cultivated plants. Microbiological transformation and detoxification of pesticides is presented in the literature quite fully (Silva et al. 2007, Martins et al. 2011, Kanissery, Sims, 2011), but the potential of bacteria in mitigating pesticide stress in plants (PSMB-plant stress mitigating bacteria) has not been studied and not shown clearly enough. The ability of the bacterial component to effectively combine with chemical herbicides, resistance to them and maintain a complex effect on the plant in their presence comes to the fore (Chetverikov, 2019). It is known that Plant Growth Promoting Bacteria (PGPB) have a beneficial effect on the growth and development of crops and prevent the spread of infections. The action of such bacteria is associated with their useful properties, such as the ability to nitrogen fixation, dissolution of phosphates, and the synthesis of phytohormones and antimicrobial compounds (Spaepen et al. 2009; Bakaeva et al. 2017; Arkhipova et al. 2019), competition with pathogens for nutrients and space of colonization (Kloepper et al. 2004). There are very few other reports on the alleviating of plant pesticide stress by bacteria (Ahemad, Khan, 2010; Nahi et al. 2016; Bourahla, 2018; Chennappa, 2018). Therefore, the search for bacteria for mitigating pesticide stress (PSMB) in agricultural crops is an urgent task. Based on the results of screening, a bacterial strain CH5%2 selected as the object of research. It was isolated from the soil from the territory of petrochemical plant (Republic of Bashkortostan, Russia). The strain CH5%2 is resistant to herbicides based on synthetic auxins and sulfonylureas and able to grow in their presence.

The purpose of this work is to study the properties of this strain and determine its potential as PSMB in cereals.

MATERIALS AND METHODS

As mentioned above, the object of research is the bacterial strain CH5%2 isolated from the soil from the territory of petrochemical plant (Republic of Bashkortostan, Russia), resistant to herbicides based on synthetic auxins and sulfonylureas and able to grow in their presence (Chetverikov, 2019).

Pure culture of the strain was described by its cultural, morphological, physiological and biochemical characteristics using well-known methods (Gerhardt et al. 1981; Garrity et al, 2005).

Cell morphology was studied using a scanning probe microscope "Solver Pro-M" ("NT-MTD", Russian Federation).

Total DNA was isolated using the method described in the paper (Wilson et al. 1995). Amplification of the 16s rRNA gene fragment was performed using bacterial primers 27F 27F (5` AGAGTTTGATC(A/C)TGGCTCAG 3`) and 1492R (5` ACGG(C/T)TACCTTGTTACGACTT 3`) on the "My Cycler" amplifier ("Bio-Rad Laboratories", USA). Isolation and purification of PCR products were obtained from low melting point agarose using a set of reagents "Wizard PCR Preps" ("Promega", USA) according to the manufacturer's recommendations. Sequencing of the obtained PCR fragments of the 16S rRNA gene was performed using a set of reagents “Big Dye Terminator V. 3.1” (“Applied Biosystems Inc”, USA) on an automatic sequencer “ABI PRIZM 3730” (“Applied Biosystems Inc”, USA) according to the attached manufacturer's instructions.

Search of homologous sequences was carried out with the use of EzBioCloud databases (http://www.ezbiocloud.net/eztaxon). The dendrogram of phylogenetic similarity was built in the program MEGA version 7 (http://www.megasoftware.net) by the neighbor-Joining method (Saitou, Nei, 1987) using the Kimura model (Kimura, 1980).

To determine the nitrogenase activity, an acetylene method was used, in which ethylene is formed when the enzyme nitrogenase effects on acetylene and then chromatographically determined (Hardy et al. 1973; Korshunova et al., 2013). The ability of the strain to synthesize IAA was determined using enzyme immunoassay as described (Bakaeva et al. 2020).

Solubilization of phosphates was assessed by the formation of transparent zones around bacterial colonies in the Pikovskaya medium (Pikovskaya, 1948).

Antagonism to phytopathogens was determined by the method of joint cultivation of bacteria and fungi in Petri dishes (Chetverikov, Loginov, 2009). The diameters of the fungal growth inhibition zones were measured (in mm). The test objects were following mycelial fungi: *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Helminthosporium sativum* Pam., King et Bakke),

Fusarium culmorum (W.G. Smith) Sacc. VKM F-844, *F. gibbosum* Appel et Wollenw VKM F-848, *F. graminearum* Schwabe VKM F-1668, *F. solani* (Mart) Sacc. VKM F-142, *F. oxysporum* Schltdl VKM F-137, *F. nivale* (Fr.) Ces. Ex Sacc. VKM F-3106, *F. semitectum* BKM F – 1938, *F. avenaceum* VKM F – 132, *Alternaria alternate* (Fr.) Keissl. VKM F-3047*, Rhizoctonia solani* J.G. Kuehn VKM F-895.

Herbicides from the following groups were used to treat plants and create herbicide stress: 1) on the base of the synthetic auxin 2,4-D (commercial name: Octapon), 2) on the base of sulfonylureas - metsulfuron-methyl (commercial name: Nanomet). The concentration of herbicides in the medium corresponded to their maximum permissible content in the working solutions were used for spraying.

Plants of soft spring wheat (*Triticum aestivum L*.) of the Kinelskaya Yubileynaya variety were grown in a phytotron in two-liter plastic containers filled with a mixture of sand and soil (humus - 8.50 %, nitrogen (total) - 0.62 %, P_2O_5 (total) - 0.19 %, absorbed calcium and magnesium 28.2 and 8.1 mg•eq/100 g of soil, respectively, salt pH 5.99, water pH 6.58) in the ratio of 1:9, at the photon flux density FAR 190 μmol•m^{-2}•s^{-1}, 14-hour photoperiod and a temperature of 26°C. Soil moisture was maintained at the level of 60-80% of the total moisture capacity. On the seventh day after germination, plants were sprayed with herbicide, bacterial suspension of the studied strain or a mixture of herbicide and bacterial suspension in the amount of 1.8 μl Octapon , 26 μg Nanomet, 1•10^8 CFU strain CH5%2 per container (concentrations of herbicides is designed taking into account the field application standards). Containers with control plants were not sprayed.

The proline content was determined using a ninhydrin reagent as described earlier (Bates et al. 1973). The calibration curve was plotted according to a standard, using chemically pure L-proline (“Sigma”, USA) as the standard. The content of proline in the material was expressed in micrograms per gram of dry weight. 30 plants were grown for each variant of the experiment.

The chlorophyll was extracted of 96% ethanol and quantified by spectrophotometric method (Vernon, 1960). The chlorophyll amount was expressed in milligrams per gram of dry weight.

Growth and weight characteristics of shoots and roots were determined after 14 days after spraying.

The field experiment was conducted in 2019 on wheat plants of the same variety as in the laboratory experiment in the arid climate trans-Urals steppe zone (52°41'15.2" North and 58°11'25.8" East; Fig. 1). Vegetating plants were treated once in the third leaf phase with herbicides Nanomet at a dose of 10 g/ha, Octapon at a dose of 0.7 l/ha, a suspension of bacteria of the investigated strain CH5%2 (2 l/ha with a titer of 10^9 CFU/ml) or a tank mixture of bacteria and herbicides using a knapsack sprayer. The consumption of the working solution was 200 l/ha. The area of each plot is 60 m^2, each variant of the experiment was performed three time. Wheat productivity was evaluated using the method described by Dospehov (Dospehov, 1979).

The data was expressed as average values calculated using MS Excel software. The reliability of differences between the average indicators was analyzed using the t-test.

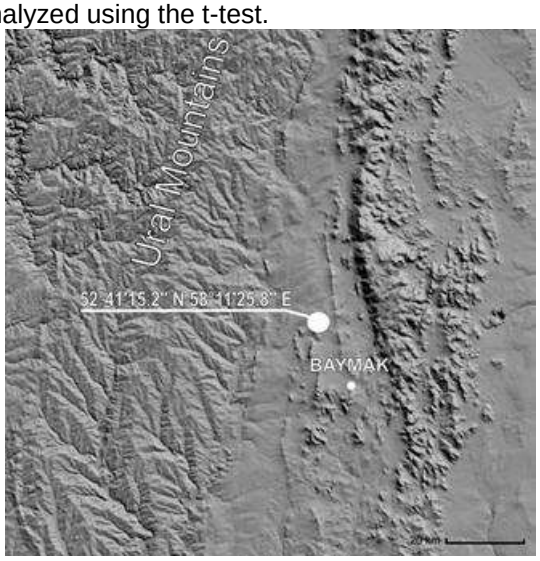

Figure 1: Geographical location of experimental fields in the trans-Ural steppe, Republic of Bashkortostan, Russia.

RESULTS

The cells of the investigated strain CH5%2 are gram-negative, mobile, and rod-shaped with a diameter of 1 μm and a length of 1.7-2.0 μm (Fig. 2). When grown on agarosed meat peptone broth, it forms white-cream, round, convex colonies with a diameter of 4-5 mm. Bacteria has respiratory metabolism, catalase-positive, synthesizes oxidase, does not have denitrifying activity, does not hydrolyze casein, gelatin, lecithin and starch. It does not synthesize lipolytic enzymes: is not able to grow on a medium with twin-80.

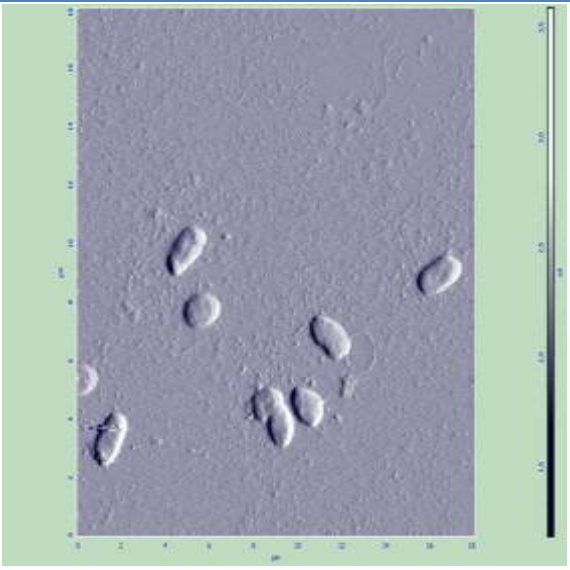

Figure 2: Cell image of strain CH5%2, performed using scanning probe microscopy.

Test for arginine dihydrolase is positive. The optimal temperature for growth is 26-30°C, the optimal pH value is 6.8-7.2. There is an intensive growth at a concentration of 0-5% NaCl, there is a weak growth with a higher concentration of up to 10% NaCl. Strain CH5%2 does not use as a sole source of carbon: glucose, sucrose, mannitol, fructose, sorbitol, Inositol, maltose, arabinose, xylose, mannose, galactose, lactose, rhamnose, meso-Inositol, starch, levan, potassium tartaric acid. The consumed carbon sources are: succinate, malate, citrate, 2-ketogluconate, ethanol, n-butanol, propylene glycol, L-leucine, L-lysine, L-valine, L-alanine, L-arginine, L-aspartate, L-histidine. The cells of the strain synthesize a fluorescent pigment. It is not pathogenic.

For the isolated strain, the sequence (1408 BP) of the 16s rRNA encoding gene corresponded to positions 28 to 1435 in the *E. coli* nomenclature was determined and subsequently deposited in GenBank under the number MT703879. The closest to the studied sample are the 16s rRNA gene sequences of bacteria *Pseudomonas plecoglossicida*, *P. juntendi*, *P. monteilii*, *P. asiatica* and *P. taiwanensis*. The level of similarity of sequences of strains CH5%2 and *P. plecoglossicida* NBRC 103162 was 99.86%, with P*. juntendi* BML3 99.79%, and with *P. monteilii* NBRC 103158, *P. asiatica* RYU5 and *P. taiwanensis* BCRC 17751 - 99.72%. To clarify the phylogenetic position of the strain, a comparative analysis of the nucleotide sequences of the 16S rRNA gene of close-lying pseudomonad species was performed with the construction of a dendrogram (Fig. 2). The data obtained in this way allowed us to identify the studied strain as *Pseudomonas plecoglossicida* CH5%2. Bacteria of this species are not particularly represented as PGPB (Plant Growing Promoting Bacteria). In this paper (Rameshkumar et al. 2012) *P. plecoglossicida* bacteria were described, which contributed to the growth of sugar cane plants due to the production of IAA, solubilization of phosphates, the ability to denitrify and the production of antifungal metabolites. Also known strain *P. plecoglossicida* PSB-5 that solubilizes phosphates, produces IAA and siderophores. As a result of a field study to test the effectiveness of its use to enhance the growth and yield of corn and wheat grown on an organic farm, a significant increase in grain yield was found, phosphorus uptake during inoculation with this strain against the background of phosphorous fertilizer from the natural mineral phosphorite compared to controls where only this fertilizer was used (Kaur, Reddy, 2014). The strain *P. plecoglossicida* 2,4-D capable of PFOS (perfluorooctanesulfonate) transformation (Chetverikov et al. 2017), isolated as a destructor of 2,4-D-based herbicides, was also able to produce IAA (Bakaeva et al. 2020).

The study of the fungicidal properties of the *P. plecoglossicida* strain CH5%2 showed its antagonism to phytopathogens (Fig. 3). Currently, there are many reports of *Pseudomonas* bacteria that have antagonistic properties against a wide range of phytopathogenic fungi (Sun et al. 2017; Patkowska, 2018). In terms of antagonistic activity, the studied strain is no higher than the *Pseudomonas* strains we studied earlier (Asabina et al. 2009). But in contrast, the CH5%2 strain is stable and able to grow in the presence of herbicides based on volatile 2,4-D esters (Octapon, 10 ml/l) and metsulfuron-methyl sulfonylureas (Nanomet, 1 g/l).

In addition, the studied bacteria showed other which are characteristic of PGP-bacteria: nitrogenase complex, solubilization of phosphates, synthesis of phytohormones, also in the presence of herbicides. The studied strain *P. plecoglossicida* CH5%2 synthesized IAA at the level of 323±16 ng/ml, while the nitrogenase activity was 19.8 nmol $C_2H_4 \bullet h^{-1} \bullet ml^{-1}$, which correlates well with the values of other known nitrogen fixators (Bakaeva M. et al. 2020). In the regulated working concentrations of the studied herbicides, the level of nitrogenase activity does not decrease, while the production of phytohormone decreased by no more than 10%. A similar decrease in IAA secretion was observed for the closely related glyphosate-resistant *Burkholderia cepacia* PSBB1 (Shahid, Khan,

2018). *P. plecoglossicida* CH5%2 is also capable of mobilizing phosphates. After five days growing on the Pikovskaya medium, transparent zones with a radius of 18 mm were formed around colonies of the strain. In the presence of herbicides, the ability of the strain to solubilize phosphates did not change. Also, it was previously noted for strain *Burkholderia sp*. L2 (Tripti et al. 2015) and *Pseudomonas sp.* PS6 (Shahid, Khan, 2017), in which this activity decreased with a multiple increase in the concentration of herbicides.

Thus, according to the set of characteristics presented above, strain CH5%2 can be recommended for creating a biological product with the properties of biofungicide and biofertilizer and for use in classic tank mixtures with herbicides.

Modern technologies for cultivating agricultural crops provide required methods to protect them from diseases, such as seed treatment and spraying crops with pesticides. However, these substances have an extremely negative impact on the environment and have a negative effect on plants, including protected crops. They can cause oxidative stress in them, slowing down growth processes, which negatively affects productivity (Light et al, 2005). Herbicide treatment is an important method of controlling weeds on an industrial scale and the problem of reducing the toxic stress from their use on cultivated plants remains open. It is believed that herbicides based on 2,4-D are not able to affect monocotyledonous plants, but if they are used before the onset of the tillering stage, wheat plants showed a slowdown in growth processes (Kumar, Singh, 2010). Also, in some cases, they do not cope with the toxic effects of herbicides based on sulfonylureas (Barrett, 1989).

In the laboratory experiment, treatment of plants with the herbicide Nanomet suppressed their growth. There was a decrease in the mass of shoots by 24.5% with a slight decrease in their length. The reduction in root weight reached up to 8% relative to untreated plants (Fig. 4 I, Fig. 4 II). Studied bacteria reliably alleviated the impact of Nanomet: there was a significant increase in the mass of the shoot (by 16%) and the root (by 14%), the length of the shoot (up to 10%) compared to the control variant. A different response was in plants to treatment by Octapon. This herbicide, with an active substance from the class of synthetic auxins, worked in our case as a natural auxin. It stimulated plants to increase root mass (by 31 % relative to untreated plants), at the same time without significantly affecting the shoots growth. Similar cases are mentioned in the review (Pazmiño et al. 2012). When combined with Octapon, the bacteria somewhat leveled such a one-sided increase in root growth. It should be noted that only bacterial treatment did not have any stimulating or negative effect on the growth characteristics of plants.

When the plant is exposed to different stresses, they form and accumulate some low-molecular compounds, one of which is the amino acid proline. According to sources, an increase in the concentration of proline in wheat leaves occurs in response to various abiotic stresses (Mwadzingeni et. al., 2016, Kolupaev et. al. 2016). In our work, we clearly observed raising in the proline concentration in wheat leaves on the third day after treatment by herbicides Octapon and Nanomet to 73% and 155%, respectively (Fig. 4 III), which indicates that plants are really under stress. However, additional treatment with *P. plecoglossicida* CH5%2 mitigated this stress effect. In the “Octapon+bacteria” amount of proline in wheat leaves was similar to control variant (Fig. 4 III). Similar effects of bacterial exposure were also observed when stress was induced by herbicides paraquat (Agafonova et al. 2016) and glyphosate (Shahid, Khan, 2018). Bacterial treatment was not perceived by plants as stress.

In addition to proline the content of chlorophyll amount in leaves is an important indicator of the plants condition under stress (Ashraf, Harris, 2013). It can be stated unequivocally: bacterial treatment with the CH5%2 strain was not stressful for the tested plants (Fig. 4 IV). Spraying with *P. plecoglossicida* CH5%2 promoted to the reduction of negative effect of herbicides on the photosynthetic apparatus. This was expressed in the quantity of pigments in wheat leaves. In all variants inoculation with bacteria led to an increase in the total amount of chlorophyll.

In a laboratory experiment the strain CH5%2 did not show a stimulating effect on plants during individual treatment, but it has shown itself as an anti-stress agent in relation to herbicide stress during joint treatment, reducing the toxic effect on plants. Field tests in the arid climate of the trans-Urals clearly confirmed the results obtained in the laboratory.

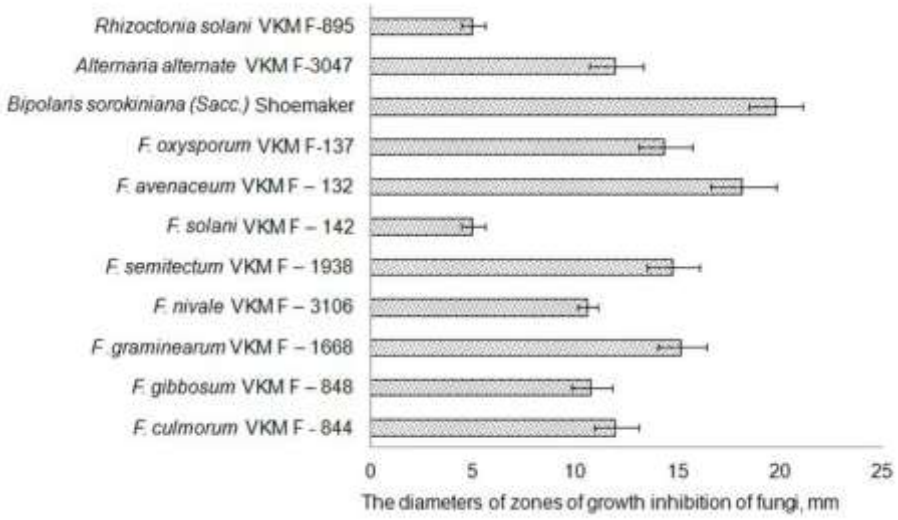

Figure 3: Spectrum of antagonistic activity of the bacterial strain *P. plecoglossicida* CH5%2; average values with a trust intervals are shown.

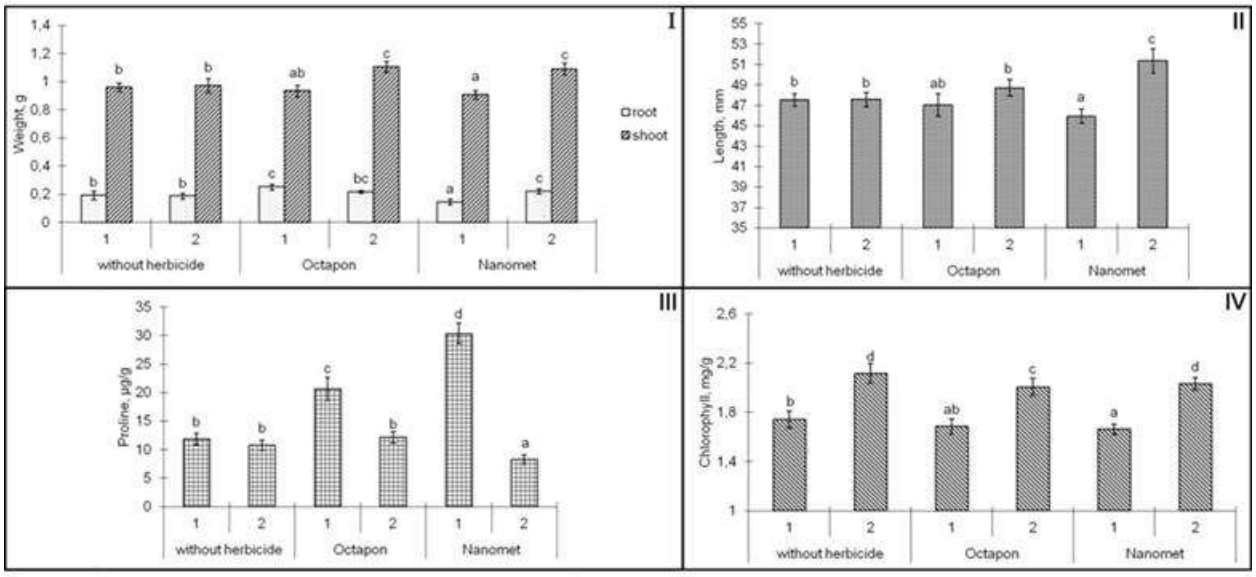

Figure 4: Effect of treatment on plants, I – root and shoot dry weight; II – shoot length; III – proline amount, IV – chlorophyll amount in leaves. 1 – without bacteria, 2 – with bacteria *P. plecoglossicida* CH5%2; average values with a trust intervals are shown; significantly different means of each parameter are marked with different letters (p≤0.05, t-test).

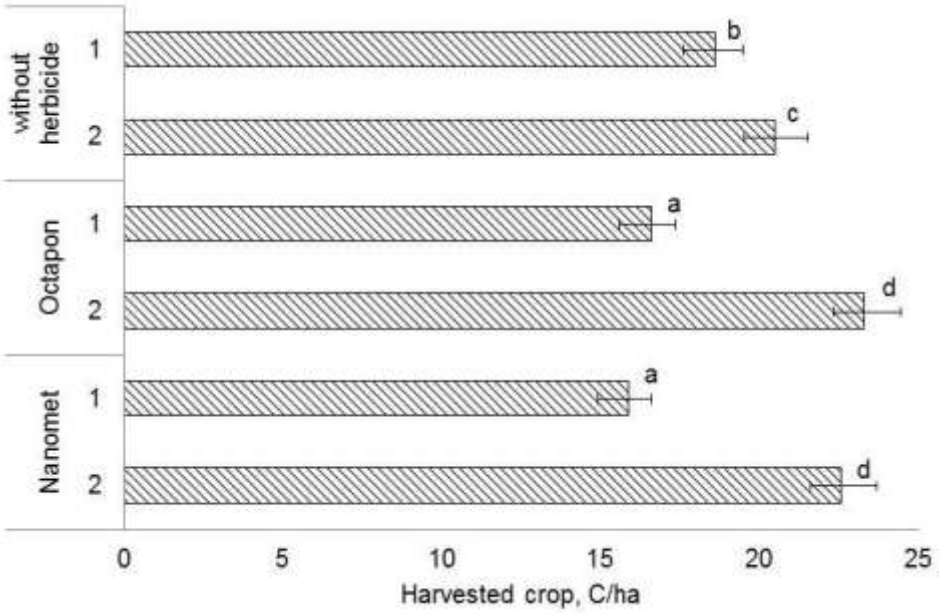

Figure 5: The effect of treatment on harvested crop of wheat. 1 – without bacteria, 2 – with bacteria *P. plecoglossicida* CH5%2; average values with a trust intervals are shown; significantly different means of each parameter are marked with different letters (p≤0.05, t-test).

The effect of strain CH5%2 on wheat yield was studied when treated with herbicides Octapon, Nanomet and a single treatment with a suspension of bacteria (Fig. 5). As expected, only herbicide treatment negatively affected the quantity of harvested grain, the decrease in yield relative to the control variant reached up to 15%. Summarizing the results of long-term tests of the herbicide 2,4-D showed a considerable number of cases of negative effects of this herbicide on wheat growth (Kumar, Singh, 2010). It is also shown that the use of herbicides can be more effective when combined with anti-stress growth promoting regulators (Naumov et al. 2019). Under field conditions, bacterial treatment showed a 10% stimulating effect. Treatment with a tank mixture had a synergistic effect on yield, increased it by 22 and 25% in the variants with Nanomet and Octapon, respectively. Given that the average wheat yield in the trans-Urals climate rarely exceeds 18 C/ha, the use of bacteria *P. plecoglossicida* CH5%2 in tank mixtures not only leads to an improvement in the physiological parameters of plants, but also a significant increase in yield.

CONCLUSION

As a result of our research, it was shown that the bacterial strain identified as *P. plecoglossicida* CH5%2 was resistant to the herbicide Octapon based on 2,4-D and Nanomet based on metsulfurone-methyl. It is capable, in the presence of herbicide, to suppress phytopathogenic micromycetes, synthesis of IAA, fix nitrogen and solubilize phosphates. The combined treatment of wheat plants with herbicide and CH5%2 strain had a positive effect, which was expressed in maintaining the growth of roots and shoots, at the level not lower than that of control plants, and normalization of the chlorophyll and proline quantity in leaves (laboratory experiment) as well as increasing producrivity (field experiment). Thus, the bacteria *P. plecoglossicida* CH5%2 played an important role in mitigating the herbicide stress in wheat, was able to stimulate its growth and increase crop capacity under stress.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

SC, GH, MB designed study, performed the statistical analysis, wrote and editing the manuscript. DS, AK, DC, MT, SS carried out the experiments and analyzed samples; ZS, TR, AF conducted field experience. All authors read and approved the final version.

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