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## ФИЗИОЛОГИЯ И БИОХИМИЯ РАСТЕНИЙ

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# Effect of bacterization with *Aeromonas media* GS4 and *Pseudomonas extremorientalis* PhS1 on wheat seedlings under different abiotic conditions*

*We studied the effect of soft wheat seed treatment (Triticum aestivum L.) with two bacterial strains (Aeromonas media GS4 and Pseudomonas extremorientalis PhS1) isolated from earthworm coprolites on the growth and development of wheat seedlings in a 12-day laboratory experiment, as well as on root rot disease and the activity of guaiacol-dependant peroxidase under optimal conditions and abiotic stress (elevated and low temperatures and moisture content). We established that growing nonbacterized wheat plants under stress abiotic conditions reduced the height of plants compared to growing under optimal abiotic conditions, and seed bacterization with P. extremorientalis PhS1 strain increased wheat plant height (by 9-15%) under stress abiotic conditions compared to the nonbacterized plants. Bacterization with both strains decreased infestation of wheat seedlings (2.5-4 times) by root rots under unfavorable abiotic conditions compared to nonbacterized plants. In addition, under optimal and arid conditions, bacterization with P. extremorientalis PhS1 strain was the most effective, and under humid conditions it was bacterization with A. media GS4 strain. We showed that the activity of guaiacol-dependant peroxidase correlates with the development of plant resistance to abiotic stress. In our experiments, plant bacterization resulted in a 2-fold increase in peroxidase activity both in leaves and roots of wheat plants compared to the nonbacterized plants. As the result, the ability of bacteria to activate peroxidase can serve as an information indicator of strengthening protective mechanisms of plants during bacterization.*

*The paper contains 4 Figures and 34 References.*

**Key words:** *Triticum aestivum*; abiotic stressors; temperature; moisture content; peroxidase activity; root rot disease.

## Introduction

Plants undergo various stresses throughout their life, for example, drought and flooding, salinity, heat and cold, heavy metals, etc. [1-2]. Human needs for

* The paper was translated by the authors.

high-quality products are growing and it is becoming hard to find suitable lands for agriculture and to obtain good low-cost crops [3]. Despite the vast territory of Russia, significant agricultural lands are in the zone of risky farming. In the West Siberia, abiotic factors that are the most stress-inducing for plants include low temperatures and high soil moisture at the beginning of crop vegetation and, on the contrary, extremely high temperatures and drought during budding and flowering. Abiotic stresses cause a number of morphological, physiological and molecular changes; disturb energy metabolism and regulation systems, which influence the growth and productivity of plants, as well as can lead to their death [4-6]. Under the influence of stress factors, both non-specific plant responses and production of specific compounds were observed [7-10]. Thus, one of the main plant responses to stress is accumulation of reactive oxygen species (ROS), particularly, hydrogen peroxide ($H_2O_2$) [7, 9-10]. Stress activates enzymatic and non-enzymatic systems of protection against highly active ROS in plants. When plants are subjected to stresses for a long time, protective systems may fail to cope with their functions, and the balance between ROS formation and quenching is disturbed, which results in oxidative damage. The main enzymes protecting from the damaging effect of hydrogen peroxide in the plant organism are catalase and peroxidase mobilizing it to $H_2O$ [4, 6]. In a living cell, peroxidases play a key role in keeping molecules in a reduced state, which is one of the main conditions for normal existence of living organisms [8, 11]. Thus, the main function of peroxidases is to protect a plant organism from a negative impact of ROS forming during photosynthesis and respiration [10-11]. The compensatory effect of peroxidases at the level of ROS during its increasing under the influence of various stress factors should be highlighted [4-5, 10-12], and the activity dynamics of these enzymes correlates with the development of plant resistance to abiotic stresses [4, 6, 8, 11].

Application of some microorganisms improving nitrogen and phosphorous nutrition of plants, having a stimulating effect and reducing infestation by phytopathogens, is one of the effective methods to enhance resistance of agricultural plants to stress abiotic factors [1, 2, 5, 13-16]. Therefore, a positive effect of plant treatment with different bacterial strains under abiotic stress is demonstrated in several studies. They show an increase in plant growth and biomass, induction of systemic resistance, and an increase in the activity of antioxidant enzymes in wheat, *Arabidopsis*, tomato and other plants when treated with *Enterobacter ludwigii* and *Flavobacterium* sp. [1], *Azotobacter chrocoocum* and *Pseudomonas putida* [6], *Bacillus licheniformis* [17], *Bacillus amyloliquefaciens* [3; 18], etc.

We have isolated several bacterial strains from earthworm coprolites. It was established that three isolates had antifungal activity *in vitro* and stimulated plant growth of some crops in laboratory tests, two of them being effective in field experiments. The aim of this research was to assess the effect of bacterization of wheat seeds on the activity of soluble guaiacol-dependant peroxidase under abiotic stress (elevated and low temperatures, substrate overmoisture and lack of moisture) in model experiments.

## Materials and methods

**Objects.** The objects of the research were two bacterial strains, *Aeromonas media* GS4 and *Pseudomonas extremorientalis* PhS1, and wheat plants (*Triticum aestivum* L., "Iren'" cultivar, widely grown in West Siberia). These bacteria were isolated from earthworm coprolites and demonstrated a significant antifungal and growth-promoting activity in screening [19-20] and were described as *Pseudomonas* spp. (isolates GS4 and PhS1) according to their morpho-physiological characteristics. Types of bacteria were identified by 16S rRNA Gene Sequence Analysis (Russian National Collection of Industrial Microorganisms, Moscow, Russia). The bacterial culture is maintained in the collection of the BIOCEN-TR (Siberian Research Institute of Agriculture and Peat, Tomsk, Russia). In the experiments, we used a 24-hour liquid bacterial culture grown in 250-ml flasks with 100 ml of GRM-broth on a shaker (180 rpm, "Biosan ES-20/60", Latvia) at 28…29°C. Culture density was determined using the colony-forming unit (CFU) method.

**Experiment conditions.** We studied the effect of bacterization on peroxidase activity in leaves and roots of wheat plants under different abiotic conditions using a simplest terrestrial ecosystem model consisting of three links: sand - host plant - bacterial strain [21]. Seeds pretreated with 70% ethanol for 3 min were washed with sterile water and grown at 18…20°C. Once the embryo (1 mm) had appeared, the seeds were bacterized with suspensions of experimental strains at a rate of $10^4$ cells/seed for 20 min. In control, seeds were soaked in distilled water. After treatment, the seeds were placed in plastic pots (1200 ml) (45 seeds each), filled with coarse sterile river sand (800 ml), evenly moistened with sterile Knop's solution for hydroponic and sand cultures, g/l: $Ca(NO_3)_2$ - 1.0, $KH_2PO_4$ - 0.25, $MgSO_4 \times 7H_2O$ - 0.125, $KNO_3$ - 0.25, and $FeCl_3$ - 0.012 [22].

For the first 6 days, we grew plants in a growth chamber (GC-300TLH, Jeio Tech, Korea) with 18…20/14…16°C (day/night), 16/8-h photoperiods at 200 μmol quanta × $m^{-2}$ $s^{-1}$ PAR and 60% moisture of total water capacity. Starting from the 7th day, in each of the three experiments, we placed plants under conditions corresponding to the required climatic parameters: in the experiment with optimal conditions, temperatures were 18…20/14…16°C (day/night), 60% substrate moisture; in the experiment with arid conditions, temperatures were 30…31/23…24°C, 40% substrate moisture; in the experiment with humid conditions, temperatures were 16…18/8…10°C, 80% substrate moisture. Under all conditions, the photoperiod was 16/8; the illumination intensity was 200 μmol quanta × m-2 s-1 PAR.

**Experiment assessment.** The total duration of each series of experiments was 12 days. Then, we recorded germination and measured the height of each plant and peroxidase activity in the leaves and roots. We registered the intensity of plant infestation by root and near-root rots by assessing the degree of browning of the stem base and the root system on the five-point scale, where 0 was the absence of

infestation signs and 4 was the death of plants from the disease [21, 23]. Based on the obtained data, we calculated root rot incidence and severity in the experiments [23]. Previously conducted experiments had repeatedly established successful plant colonization by microbial strains [20].

**Enzyme activity detection.** The total activity of guaiacol-dependant peroxidase was detected in the experiments according to Chupakhina [24] with some modification (sample preparation was carried out at +4°C). A sample (200 mg) of fresh plant tissue was homogenized in 1 ml of cold 0.15 M phosphate buffer (pH 5.4) at 4 °C. Then, the obtained volume was buffered to 25 mL in a volumetric flask and incubated at 4 °C for 10 min. The homogenate was centrifuged at 5000 rpm (OPn-8, Russia) for 10 min. The supernatant was used to detect the enzyme activity spectrophotometrically (SF-102, Russia): 1.5 mL 0.15 M K,Na-phosphate buffer (pH 5.4), 0.5 mL 0.15% hydrogen peroxide (Reakhim, Russia), 0.5 mL 0.05% guaiacol (Sigma, United States) (molar extinction coefficient for tetraguaiacol E470 = 26.6 $mM^{-1}$ $cm^{-1}$) and 0.5 mL of plant extract (concentration in the reaction mixture of peroxide and guaiacol 7.35 and 0.672 mM, respectively) at 25 °C immediately after obtaining the extract. Changes in the adsorption of the reaction solution at 470 nm were recorded in the linear portion of the reaction for 1 min. The enzyme activity was calculated according to Chance and Maehly [25] considering the molar extinction coefficient of tetraguaiacol and was expressed in μmol of guaiacol oxidized within one minute with one gram of fresh weight tissue (μmol of guaiacol/(min×g fr wt).

**Statistical analysis.** The experiments were performed in three independent biological replications. Each replication had 35 to 45 plants per treatment. The height and infestation were estimated for every plant (total plant number was 100-110/sample) and expressed as the arithmetic mean with confidence interval using the Student's t-test for 95% significance level. Statistical significance was estimated by the Student's t-test for 95% significance level. One sample for enzyme activity included leaves and roots of 5-7 plants. Measurements were performed three-four times in the average sample of each treatment of each replication. Thus, one treatment had 9-12 measurements of the enzyme activity. The data are expressed as the arithmetic mean with standard error. Statistical significance was estimated by the Mann-Whitney U test ($p < 0.05$).

## Results and discussion

Plant growth is possible in a relatively wide range of temperatures and soil moisture, and is determined both by geographical origin of the species, varietal characteristics and the combined influence of numerous environmental factors. Temperature demands of plants change with age and are not the same for different organs (leaves, roots, etc.). Temperature affects biochemical processes of respiration, photosynthesis and other metabolic systems of plants; and the diagrams of dependence of plant growth and enzyme activity from temperature have a similar

shape (bell-shaped curve). Thus, there exist zones of optimum where the factor activity provides the best plant productivity and zones of maximum and minimum where plant development is inhibited [26]. The optimum temperature for wheat growth and development is 12-25°C [27]: higher or lower temperatures have a negative impact on wheat growth, development and productivity. The amount of available moisture in the substrate shows the same pattern. Also, a combination of several negative abiotic factors, as usual, intensifies a negative effect on plant growth and productivity.

It is illustrated by the data on wheat height, which we obtained when growing plants under optimal (18…20/14…16°C (day/night), 60% substrate moisture), arid (30…31/23…24°C, 40% substrate moisture) and humid (16…18/8…10°C, 80% substrate moisture) conditions (Fig. 1).

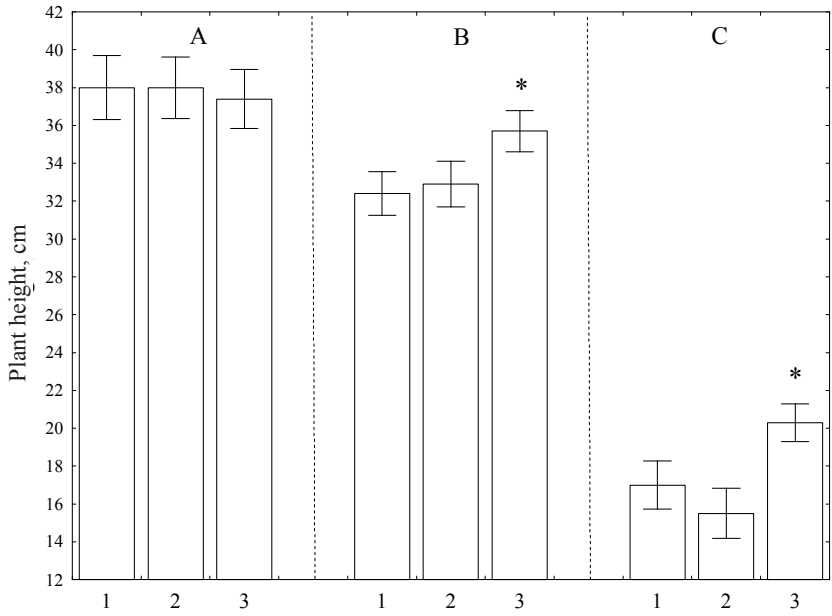


**Fig. 1.** The height of 12-day wheat plants in the experiments:
A - Optimal conditions, B - Arid conditions, C - Humid conditions;
1 - Without bacterial treatment (control), 2 - Inoculation with *Aeromonas media* GS4 strain, 3 - Inoculation with *Pseudomonas extremorientalis* PhS1 strain.
The data are expressed as the mean with confidence interval using Student's t-test ($p < 0.05$).
*Statistically significant difference from the control ($p < 0.05$)

Growing wheat plants under stress abiotic conditions decreased plant height compared to growing under optimal abiotic conditions: plant height decreased from 14 to 200%. Bacterization with both microbial strains under optimal conditions was not statistically significant for plant height, but there was an increase in the height of wheat plants (9-15%) during seed bacterization with *P. extremorien-*

*talis* PhS1 compared to nonbacterized ones. Previously conducted studies, including in the field, had demonstrated a stable effect of bacterization. The results of field experiments for different years showed that inoculation usually stimulates the growth of wheat and barley plants, which is expressed in the accelerated development at the main plant development stages, as well as in a statistically significant increase in the basic morphometric parameters of plants (height, number of productive stems, number of leaves, dry green mass) compared to non-inoculated plants [19].

Reducing the stressful impact of unfavorable temperatures and the lack of excess of soil moisture under bacterization corresponds, in general, to already known data [1-3, 5-6, 13-15] and is attributed to the release of growth-promoting substances by bacteria (hormones, vitamins, etc.) [3, 13, 17], improvement of mineral nutrition [13], or activation of antioxidant enzyme systems [13, 15].

In some studies, bacteria *A. media* and *P. extremorientalis* were also applied to promote the growth of wheat, tomato and fodder galega. In Arraktham et al. report [28], *A. media* ATCC 33907T strain, isolated from the intestines of the endogenic earthworm *Metaphire posthuma* collected from agricultural lands, produced a high amount of indole-3-acetic acid (IAA). *A. media* isolate P29 produced IAA and siderophores and solubilized inorganic phosphates [29]. The greenhouse experiment of Egamberdieva et al. [30] showed that co-inoculation with *P. extremorientalis* strain TSAU20 and *Rhizobium galegae* HAMBI 540, isolated from the rhizosphere of wheat growing in salinized soil, improved plant growth and nitrogen content, and was able to produce IAA. *P. extremorientalis* strain TSAU20 increased plant height and fruit yield of tomato under stress factors (saline soil condition). The bacterial inoculant also stimulated antioxidant enzymes' (superoxide dismutase, ascorbate peroxidase, catalase, glutathione reductase) activities, thereby, preventing ROS-induced oxidative damage in plants, and proline concentrations in plant tissues [31].

Despite the fact that we did not introduce phytopathogens in the given model system, we observed infestation of wheat seedlings by root rot pathogens. It is known that a plant seed is surrounded by the zone with an increased microbial activity called the spermosphere [32]. Microbial representatives of this area include not only beneficial microorganisms but also phytopathogens causing root rots and leaf infections. Fig. 2 shows data on root rot disease severity in different tests under optimal and stress-inducing abiotic conditions.

The given data demonstrate a statistically significant decrease ($p < 0.05$) in root rot disease severity (2.5-4 times) during bacterization under different abiotic conditions compared to nonbacterized plants. At the same time, bacterization with *P. extremorientalis* PhS1 is the most effective under optimal and arid conditions, whereas under humid conditions it is that with *A. media* GS4 strain. Root rot disease severity is a complex parameter including both the number of plants with root rot signs (root rot disease incidence), and the intensity of seedling infestation by pathogens. In our experiments, we detected a 2 times or more decrease in

disease incidence during bacterization compared to appropriate nonbacterized wheat plants.

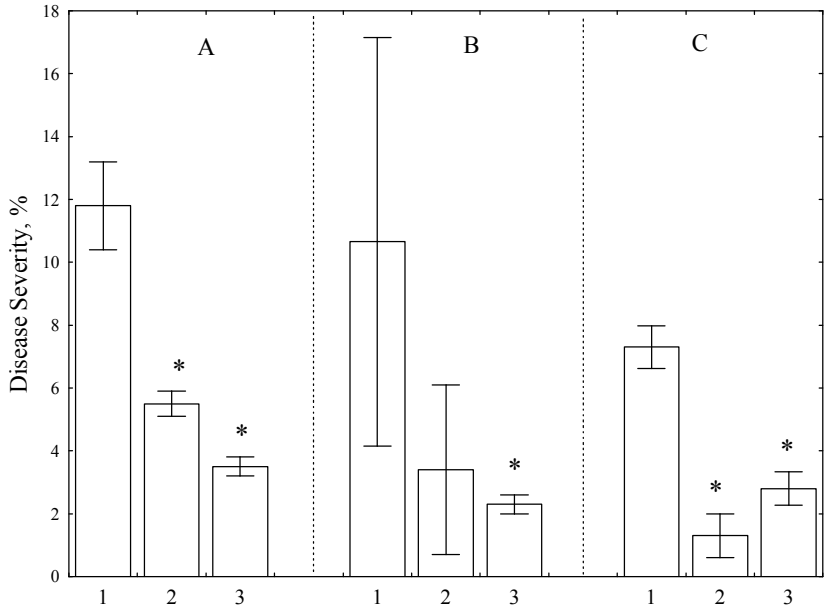


**Fig. 2.** Root rot disease severity of 12-day wheat plants in the experiments:
A - Optimal conditions, B - Arid conditions, C - Humid conditions;
1 - Without bacterial treatment (control), 2 - Inoculation with *Aeromonas media* GS4 strain, 3 - Inoculation with *Pseudomonas extremorientalis* PhS1 strain.
The data are expressed as the mean with confidence interval using Student's t-test ($p < 0.05$).
*Statistically significant difference from the control ($p < 0.05$)

Literature has amply described reduction in the number of plants with signs of damage caused by root rot pathogens during bacterization due to the release of antibiotics, siderophores, biosurfactants, cyanides and other biologically active substances by bacteria, and also due to induction of systemic resistance to phytopathogens in the plant [1-3, 13-15]. We have also repeatedly demonstrated antagonistic activity for the indicated strains in our previous studies [16, 20]. We noted a decrease in root rot disease severity in the laboratory experiments with wheat plants 2-4 times when treated with *P. extremorientalis* PhS1 and 2.1-2.4 times when treated with *A. media* GS4 [16, 20], as well as by 34% when treated with a mixture of strains in the field experiment compared to nonbacterized plants [20]. Besides, in *in vitro* experiments, strains manifested a stable inhibitory effect on such phytopatogens as *Fusarium oxysporum, Bipolaris sorokiniana* and *Alternaria* sp. [20].

Many authors have shown that application of microorganisms under stress conditions promotes plant growth and improves yield. For example, exposure to

*Bacillus licheniformis* CH102, functioning as a fungal antagonist and a promoter of plant growth and abiotic stress tolerance, increased the tolerance towards high temperatures and water deficits in *Arabidopsis* seedlings [17]. In their research, Gontia-Mishra et al. [1] demonstrate that PGPR strains (*Enterobacter ludwigii* IG 10 and *Flavobacterium* sp. IG 15) can proficiently alleviate drought stress in wheat through various mechanisms: improving plant growth (shoot length, root length and number, shoot and root fresh and dry weight of wheat seedlings), water status, membrane integrity and accumulation of compatible solutes; altering expression of stress-responsive genes and PGPR inoculation also had positive effects on recovery of drought-stressed wheat plants.

In our experiments, the given data on plant height and seedling infestation by root rot pathogens prove the effectiveness of bacterization to induce resistance of wheat plants to stress abiotic conditions in the laboratory experiments. A positive effect was noted both due to stimulating the development of plants, and due to decreasing their infestation by phytopathogenic micromycetes, which, generally, may positively contribute to the productivity of wheat plants under natural conditions when the rhizosphere is successfully colonized by the applied bacterial strains.

To evaluate physiological adaptation of plants to unfavorable environmental conditions, we analyzed the activity of free and weekly bound guaiacol-dependant peroxidase in wheat leaves and roots.

It is known that stress activates ROS in plant cells, among which hydrogen peroxide is immensely important [1, 4, 7-10]. In the studies of Javadiana et al. [4] and Kasim et al. [5], this fact is demonstrated for wheat plants under low temperature and under drought stress. In a plant cell, $H_2O_2$ activates (or represses) genes of many protective enzymatic proteins, signal proteins, as well as proteins regulating cell oxidative status. Besides, $H_2O_2$ activates calcium channels, causes stomatal closure, and participates in lignification and induction of apoptosis, lipid peroxidation, etc. [7-8, 10, 15]. Pathological consequences for plants arise from excessive accumulation of ROS, peroxides and their derivatives under stress. ROS concentration under stress can rise 3-10 times [33]. In detoxification reaction of hydrogen peroxide, iron-containing enzymes - catalase and different peroxidases - play an important role [10].

Peroxidase was detected in different cell compartments in the form of a soluble fraction and in the form bound both with the cell wall and other membranes. A distinctive feature of all peroxidases is their multifunctional role in a multitude of biochemical reactions: in reactions of oxydase, peroxidase and oxygenase oxidation of substrates, in the process of growth, in the mechanisms of forming plant responses to environmental factors, etc. [10, 33-34]. In plant cells, there is a special type of organelles containing peroxidase and performing a protective antimicrobial function. In addition, peroxidase is involved in lignin synthesis and destruction [15, 31]. The presence of the enzyme in chloroplasts indicates its involvement in redox reactions during photosynthesis, and peroxidase detection in mitochondria shows its participation in the energy metabolism of the cell [10,

11]. Antioxidant protection associated with hydrogen peroxide reduction is carried out, mainly, by glutathione and ascorbate peroxidases. In plant vacuoles, peroxide detoxification is effectively provided by guaiacol peroxidase.

Guaiacol peroxidases, or "classical peroxidases", catalyze oxidation of a great number of aromatic compounds using hydrogen peroxide as an electron acceptor [33]. Besides participation of guaiacol peroxidases in lignification, auxin breakdown and other processes, they also play an essential part in cell protection from oxidative stress.

Peroxidase activity in leaves and roots of 12-day wheat seedlings demonstrated that the obtained experimental data illustrate an increase in the activity of these enzymes in plant tissues under abiotic stress (Fig. 3 and 4).

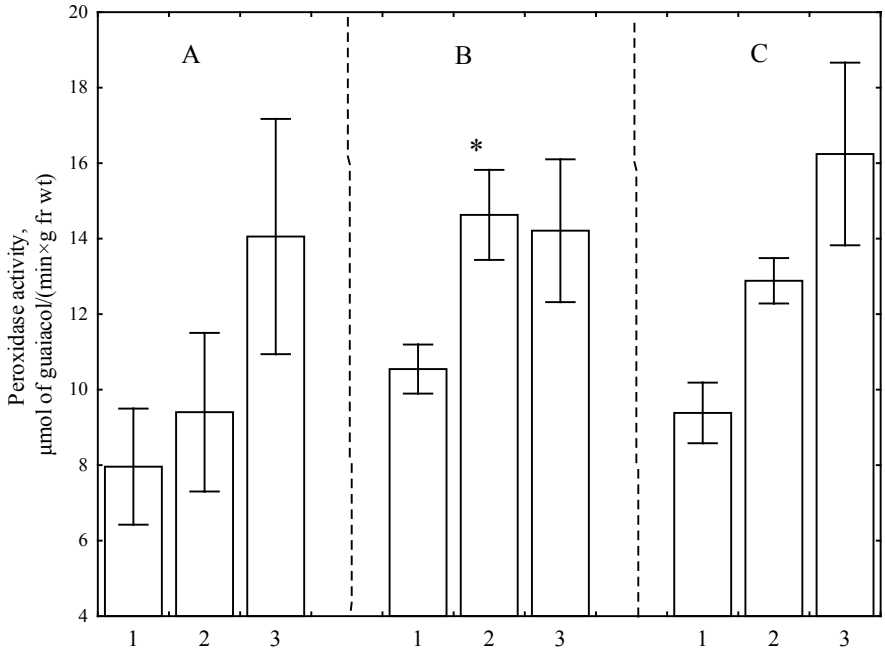


**Fig. 3.** Peroxidase activity in 12-day leaf tissues of wheat plants in the experiments:
A - Optimal conditions, B - Arid conditions, C - Humid conditions;
1 - Without bacterial treatment (control), 2 - Inoculation with *Aeromonas media* GS4 strain, 3 - Inoculation with *Pseudomonas extremorientalis* PhS1 strain.
The data are expressed as the mean with standard error.
*Statistically significant difference from the control ($p < 0.05$)

According to literature sources, root cells are more exposed to temperatures than aboveground vegetation organs [33]. We only noted this fact for humid conditions where there was an increase in peroxidase activity in the tissues of nonbacterized wheat plants by 19% and 3.5 times for leaves and roots, respectively, compared to nonbacterized seedlings grown under optimal conditions. Under arid conditions,

there was an increase in peroxidase activity in leaf tissues (31%), and the activity remained almost unchanged in root tissues.

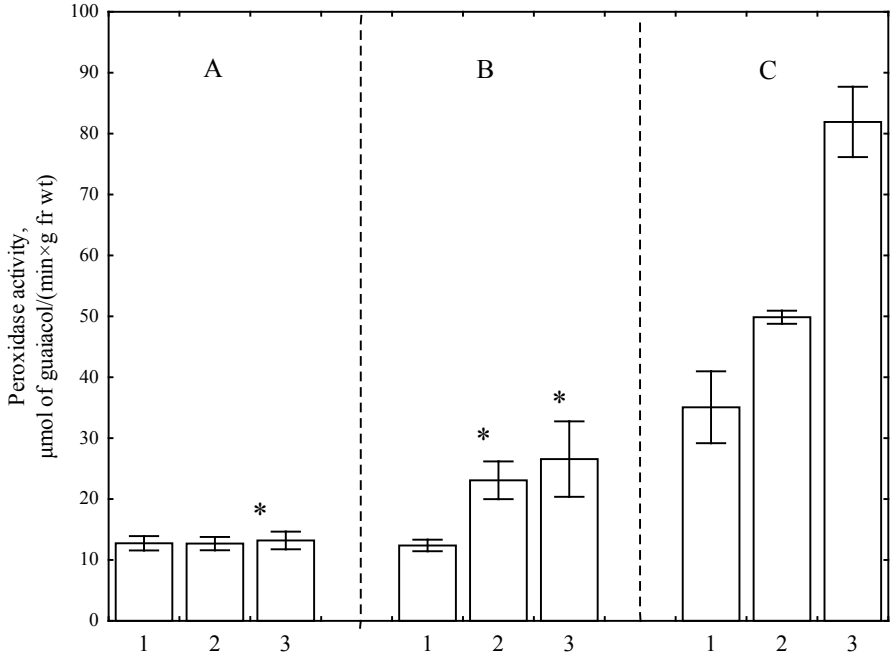


**Fig. 4.** Peroxidase activity in 12-day wheat roots in the experiments:
A - Optimal conditions, B - Arid conditions, C - Humid conditions; 1 - Without bacterial treatment (control), 2 - Inoculation with *Aeromonas media* GS4 strain, 3 - Inoculation with *Pseudomonas extremorientalis* PhS1 strain.
The data are expressed as the mean with standard error.
*Statistically significant difference from the control ($p < 0.05$)

Plant bacterization resulted, on average, in a 2-fold increase in peroxidase activity both in leaves and roots of plants compared to nonbacterized plants grown under appropriate conditions (Fig. 3 and 4). The largest increase in the enzyme activity in plant leaves and roots was due to wheat bacterization with bacteria *P. extremorientalis* PhS1. In this test, we almost always observed the greatest plant height and the least seedling infestation by root rot pathogens, which indicates the presence of a link between the given parameters and peroxidase activity in plant tissues. We noted an increase in the activity of this enzyme (from 4 to 76%) during bacterization with the given bacterial strains when growing bacterized and control plants with and without phytopathogen [16].

It is known that plants have evolved very efficient antioxidant systems for stress protection [4, 6, 7-9, 33-34]. In Shalygo et al. experiments [7], overwatering of barley seedlings changed the activity of all antioxidant enzymes (ascorbate

peroxidase, glutathione reductase, catalase) and activated synthesis of stress proteins (heat shock proteins Hsp70 and dehydrins), which improved their resistance to cold stress. Javadian et al. [4] showed that there is a correlation between peroxidase activity and low temperature tolerance of wheat plants which suggests the functioning of this enzyme as an internal protective mechanism preventing oxidative damage of wheat plants.

## Conclusion

Bacterization of soft wheat plants with microbial cultures *Aeromonas media* GS4 and *Pseudomonas extremorientalis* PhS1 showed a statistically significant decrease in wheat seedling infestation by root rot pathogens and, simultaneously, an increase in the activity of free guaiacol dependant peroxidases in wheat leaves and roots under different abiotic factors. Under optimal and arid conditions, the greatest resistance to root rot pathogens was due to wheat bacterization with *P. extremorientalis* PhS1, maximally intensifying the activity of free peroxidases in plant roots and leaves. Under humid climatic conditions, the lowest incidence was observed during bacterization with *A. media* GS4. Based on the obtained data, we can conclude that it is possible to improve wheat productivity under stress conditions by seed bacterization with *P. extremorientalis* PhS1 due to their growth-promoting and antifungal activity. The data on the activity of guaiacol-dependant peroxidase in the tissues of roots and leaves of bacterized wheat plants show an increase in the activity of this enzyme correlating with a decrease in seedling infestation by root rot. The ability of bacteria to stimulate the activity of this enzyme can serve as an information indicator of strengthening plant protective mechanisms under bacterization.

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