The phytostimulating activity of metal-resistant *Bacillus* strains isolated from Spolic Technosol of Lake Atamanskoe

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Abstract. This study was devoted to the isolation and identification of metal-resistant strains of microorganisms from soils contaminated with heavy metals, as well as their assessment as potential remediators of contaminated agricultural land. The soils with the highest concentration of zinc were selected as the source of the strains. In the course of the research, 10 strains with the highest zinc resistance were selected. For all strains, the phytostimulating and phytotoxic properties were assessed with the help of morphobiometric indicators. In the course of the study, three strains were selected and the phytostimulating potential was assessed in a model experiment. In the variant with the application of the selected strains, all the assessed characteristics significantly increased, in comparison to the plants grown in the contaminated soil.

1. Introduction

Special attention is currently being paid to the problem of soil pollution with heavy metals. These pollutants are characterized by the high level of stability in the environment. As a result, they cannot be removed from the soil during natural biological and chemical transformations. Heavy metals and metalloids, termed as potentially toxic elements (PTE), are necessary for stimulating the synthesis of proteins, fats and carbohydrates, because they are incorporated in the active cites of many enzymes. At the same time, the same elements in high concentrations have a detrimental effect on the physiological processes of plants. Growth inhibition is the most common manifestation of the PTE toxicity for plants, which is primarily associated with their direct effect on cell division and elongation [1]. Therefore, the measurement of morphobiometric indicators is a relevant and simple way to assess the degree of soil toxicity for plants.

An important indicator for assessing the toxicity of PTE is not only their content in the substrate, but also the form in which they are present, which affects the degree of bioavailability of the pollutant. Remediation methods based on the direct extraction of pollutants from the soil have many limitations; therefore, it is expedient to convert the pollutants into the forms inaccessible to plants. Some strains of microorganisms are capable of converting pollutants into insoluble compounds, which makes them valuable for soil remediation.
Studies of the resistance of microorganisms to contamination with heavy metals and the interaction of resistant strains with the root system of a plant have shown that microorganisms are able to convert heavy metals into compounds inaccessible to plants and, thus, reduce the load [2]. This fact allows the use of strains of microorganisms for the remediation of contaminated soil. The growth and development of plants depends on the activity of bacteria. Therefore, it is advisable to compare the plants grown on contaminated soil, where only native species of bacteria are present, and plants grown on soil with the introduced strains of metal-resistant bacteria.

The aim of this study was to assess the phytostimulating activity of metal-resistant bacteria, isolated from highly polluted soils.

2. Materials and methods of research
The objects of the study were the strains of metal-resistant bacteria, seedlings and barley plants under the conditions of a model experiment. Bacterial strains were isolated from the most contaminated soils chosen on the basis of previous monitoring observation [3]. After that, their selection, identification and assessment of the effect on the morphobiometric parameters of seedlings and plants of spring barley at the stage of stemming were carried out.

2.1. Microbiological soil analysis

2.1.1. Isolation of resistant strains from the most contaminated soils. To determine the resistance of microorganisms to heavy metals, soil samples were taken from the chemozems of the dry lake Atamanskoe with the highest pollution level. Since zinc was the dominant pollutant in these soils, the primary isolation of metal-resistant bacterial strains from the studied soils was carried out on nutrient agar with the addition of ZnSO₄. The soil was mixed with sterile water at a ratio of 1:10 and crushed with a rubber pestle to separate the bacterial cells from the soil particles. The resulting soil suspension was shaken in conical flasks on a rotary shaker for 30 min. To isolate spore-forming bacteria, the soil suspension was heated to 80 °C for 20 minutes. After cooling to room temperature, dilutions were prepared and plated on the nutrient agar. Zinc was added to the culture medium in the form of ZnSO₄ ∙ 7 H₂O. Sterile Zn salt was added to the culture medium after autoclaving. Colonies of metal-resistant bacterial strains were counted after 5 days of incubation at 30 °C. The pure cultures of bacteria were isolated and transferred to the same medium. The strains were then tested for metal resistance. The following series of zinc concentrations in the nutrient medium was used: 0, 1, 5, 10, 15, 20, 25 MPC (for mobile forms), corresponding to 0, 23, 115, 230, 345, 460, 575 mg/kg Zn in the form of soluble salt. The dilute suspension of the tested strains was streaked on the plates with the increasing Zn concentration, and after the incubation for 48 h, the plates were checked for visual growth of the bacterial culture.

2.1.2. Selection of the most metal-resistant bacterial strains. Further selection of the most resistant strains was performed by the method of gradient agar plates, as described in [4] with modification. Subsequently, the isolated metal-resistant strains were plated on Petri dishes containing certain concentrations of lead (in the form of Pb(NO₃)₂), copper in the form of CuSO₄, and cadmium in the form of CdCl₂. The presence or absence of growth was noted to determine the minimum inhibitory concentration.

2.1.3. Identification of bacterial strains. Identification of bacterial strains was carried out using a polyphasic approach [5], which included both genotypic and the phenotypic characterization of the strains. Genotypic characterization of the strains was based on sequencing of the 16S rRNA gene, and phenotypic characterization was performed using MALDI-TOF mass spectrometry on a Bruker Daltonics Biotyper instrument.
2.1.4. Seed treatment with bacterial suspensions. Bacterial cultures were grown on plates with nutrient agar, then the biomass was washed off with 10 ml of sterile water and the suspension was vortexed. Then the initial suspension was diluted with water 1:10 and the optical density of the resulting suspension was measured using a densitometer. After recalculation, the initial suspension was diluted with sterile water to obtain a cell concentration corresponding to $10^8$ CFU/ml. The barley seeds were soaked in this suspension for 30 minutes, then they were laid out on filter paper, and 3 ml of this suspension was added to each Petri dish or container. The same suspension was used for soaking seeds when determining the germination energy and germination ability. In the control variant, sterile water was used instead of bacterial suspension.

2.2. Model experiment
The scheme of the model experiment included the control - meadow-chernozem soil from the edge of an agricultural field, the highly polluted chernozem soil from the bottom of Atamanskoe lake, and the variant with the introduction of bacterial strains into the polluted soil. The soil was placed in pots, layer by layer soaked with a suspension of microorganism strains to a final dose of $10^{10}$ CFU/kg soil. The amount of water was calculated to adjust the soil moisture up to 60% of the maximum moisture capacity. Spring barley (*Hordeum vulgare* L.) was grown in pots for 51 days, after which the plants were harvested and the main morphobiometric parameters were analyzed.

2.3. Morphobiometric analysis of plants
The seed vigor and germination speed were determined on the 3rd and 5th day, respectively, in accordance with GOST 10968-88 [6]. The germination ability was determined on 7th day according to GOST 12038-84, 2011 [7]. The morphobiometric parameters of seedlings were measured on the 7th day. Root and shoot length were measured for each plant. The method of measuring morphobiometric indicators was carried out according to the method described by Zerling [8].

2.4. Mathematical data analysis
The determination of all the studied parameters was carried out in triplicate. Data are presented as mean values with a standard error. The parameters were compared with one-way ANOVA, and Tukey test as a post-hoc analysis. All calculations were performed using the STATISTICA 10.0 program.

3. Results and discussion

3.1. Isolation of resistant strains from the most contaminated soils, selection of isolated strains in laboratory conditions
To isolate metal-resistant bacterial strains, soil samples with maximum contamination levels were selected from the sampling sites 11, 12, and 13. In all these samples, the total zinc content exceeded 60,000 mg/kg, and the content of forms extractable with the acetate-ammonium buffer (pH 4.5) was more than 10,000 mg/kg of soil. Nevertheless, when inoculating on nutrient agar with the addition of zinc sulfate, the growth of bacteria was observed only up to a concentration corresponding to 15 MPC (table 1). These results suggest that in a real soil environment, the concentration of free zinc in the pore space is significantly lower than that obtained during the preparation of extracts.

The growth of aerobic spore-forming bacteria was observed at concentrations of 0, 1, 3, and 5 MPC. Despite the presence of bacterial strains with greater resistance, it was decided to choose aerobic spore-forming bacteria (order Bacillales) for further isolation and selection, in view of the fact that representatives of spore-forming bacteria are most convenient for use as remediation agents and for the development of bacterial biological preparations and the creation of biotechnological enterprise [9, 10].

The selection of colonies of aerobic spore-forming bacteria was carried out after inoculation of pasteurized soil suspensions on Petri dishes with zinc concentrations of 3 and 5 MPCs based on colony morphology with control by light microscopy.
Table 1. The number of ammonifying bacteria capable of growing on a medium with different zinc concentrations, 10^6 CFU/g abs. dry soil.

| Zn, mg/kg | MPC | Sampling site | № 11 | № 12 | № 13 |
|-----------|-----|---------------|-------|-------|-------|
| 0         | 0   |               | 4.31 ± 0.26 | 11.72 ± 2.32 | 7.87 ± 0.12 |
| 23        | 1   |               | 4.02 ± 0.18 | 10.06 ± 1.14 | 7.00 ± 0.30 |
| 115       | 5   |               | 2.16 ± 0.43 | 5.14 ± 0.72  | 3.89 ± 0.51 |
| 230       | 10  |               | 0.87 ± 0.08 | 2.35 ± 0.62  | 1.79 ± 0.03 |
| 345       | 15  |               | 0.09 ± 0.02 | 0.20 ± 0.03  | 0.17 ± 0.06 |
| 460       | 20  |               | 0.00 ± 0.00 | 0.00 ± 0.00  | 0.01 ± 0.01 |
| 575       | 25  |               | 0.00 ± 0.00 | 0.00 ± 0.00  | 0.00 ± 0.00 |

As a result, 50 strains of spore-forming bacteria were isolated, which were then subjected to selection on gradient agar plates. As a result, 10 strains that exhibit the highest resistance to zinc were selected (table 2). These strains were identified by 16S RNA gene sequencing and MALDI-TOF mass spectrometry.

Table 2. The results of identification of metal-resistant bacteria.

| Strain | Minimal inhibitory concentrations of Zn, mg/kg | Species according to 16S rRNA gene analysis | Species according to MALDI-TOF results |
|--------|-----------------------------------------------|---------------------------------------------|---------------------------------------|
| TR 1.5 | 136.5                                        | *Bacillus subtilis*                         | *Bacillus subtilis*                   |
| TR 2   | 91                                           | *Bacillus megaterium*                       | *Bacillus megaterium*                 |
|        |                                               | *Bacillus aryabhattai*                      |                                       |
| TR 3.1 | 182                                          | *Bacillus pumilus*                         | *Bacillus pumilus*                    |
| TR 3.2 | 91                                           | *Bacillus pumilus*                         | *Bacillus pumilus*                    |
| 20.1   | 182                                          | *Bacillus proteolyticus*                    | *Bacillus cereus*                     |
|        |                                               | *Bacillus cereus*                          |                                       |
|        |                                               | *Bacillus wiedmannii*                      |                                       |
| TR 3.3 | 182                                          | *Bacillus atrophaeus*                      | *Bacillus atrophaeus*                 |
| 20.2   | 91                                           | *Bacillus thuringiensis*                   | *Bacillus thuringiensis*              |
|        |                                               | *Bacillus toyonensis*                      |                                       |
| TR 4.1 | 136.5                                        | *Bacillus thuringiensis*                   | *Bacillus mycoides*                   |
|        |                                               | *Bacillus toyonensis*                      |                                       |
| TR 4.2 | 91                                           | *Bacillus stratosphericus*                 | *Bacillus pumilus*                    |
| TR 1.1 | 91                                           | *Bacillus thuringiensis*                   | *Bacillus cereus*                     |
|        |                                               | *Bacillus toyonensis*                      |                                       |

It was found that among the identified bacteria strains 20.1 (*B. cereus*), TR 3.1 (*B. pumilus*) and TR 3.3 (*B. atrophaeus*) demonstrate the highest level of Zn tolerance, which reached 182 mg/kg\(^{-1}\) as a result of the selection.

Subsequently, the resistance of the isolated strains to other PTE was assessed. The strains that showed the highest resistance to zinc were also characterized by higher resistance to other heavy metals. This is due to the presence of nonspecific resistance in bacteria, which makes it possible to use the same defense mechanism against the toxic effects of different PTE. The mechanisms of such resistance include active transport of ions from the cell using molecular pumps with low specificity or metal-binding proteins [11, 12].
3.2. Evaluation of phytotoxic or phytostimulating properties of the selected strains

To assess the phytostimulating properties of the isolated strains of bacilli, experiments were carried out to determine the seed vigor, the germination speed and germination ability of spring barley seeds, and the effect of seed treatment with the studied bacterial strains on morphobiometric parameters was determined. The data are presented in table 3.

Table 3. Indicators of germination energy, germination ability and germination of barley seeds when treated with a suspension of *Bacillus* strains.

| Variant                       | Seed vigor (3rd day), % | Germination speed (5th day), % | Germination ability (7th day), % |
|-------------------------------|-------------------------|-------------------------------|-------------------------------|
| The control                   | 27±3                    | 60±4                          | 88±2                          |
| *Bacillus pumilus* TR 3.1     | 25±2                    | 68±3                          | 95±2                          |
| *Bacillus cereus* TR 1.1      | 10±2                    | 42±4                          | 92±3                          |
| *Bacillus subtilis* TR 1.5    | 35±4                    | 75±4                          | 95±2                          |
| *Bacillus megaterium* TR 2    | 18±2                    | 58±2                          | 97±3                          |
| *Bacillus pumilus* TR 3.2     | 17±2                    | 77±4                          | 97±2                          |
| *Bacillus atrophaeus* TR 3.3  | 27±3                    | 82±3                          | 100±1                         |
| *Bacillus mycoides* TR 4.1    | 22±4                    | 52±3                          | 97±2                          |
| *Bacillus pumilus* TR 4.2     | 8±2                     | 50±4                          | 97±3                          |
| *Bacillus cereus* 20.1        | 27±2                    | 75±2                          | 98±2                          |
| *Bacillus thuringiensis* 20.2 | 10±1                    | 45±2                          | 95±2                          |

As can be seen from the data in the table, the strains *Bacillus pumilus* TR 3.1, *Bacillus atrophaeus* TR 3.3, and *Bacillus mycoides* TR 4.1 did not affect the germination energy of barley, *Bacillus subtilis* TR 1.5 strain had a weak stimulating effect, and all other bacillus strains reduced the seed vigor, some strains – very significantly (up to 3 times lower than the control). However, by the 5th day, the number of depressing strains decreased to three, while 5 strains had a stimulating effect. By the 7th day, almost all the studied *Bacillus* strains demonstrated a higher seed germination rate as compared to the control.

The observed picture can be explained by the peculiarities of the process of seed colonization by the bacteria. Many bacteria start producing secondary metabolites only after the formation of a stable biofilm, which may partially explain the “delayed action” of the studied bacterial strains.

Nevertheless, in our opinion, it is impossible to speak about a pronounced phytotoxic or phytostimulating effect of the studied strains based only on the rate of seed vigor, therefore, the analysis of the morphobiometric parameters of plants on the 7th day of their development was also carried out.

The study of the effect of seed treatment with *Bacillus* strains on the length of barley roots showed that the strains differ significantly in this effect. Figure 1 shows data based on the results of a one-way analysis of variance. It can be noted that strains *Bacillus cereus* TR 1.1 and *Bacillus thuringiensis* 20.2 significantly reduced the root length. The same strains significantly decreased the seed vigor and the germination ability in barley. Thus, these strains showed a moderate phytotoxic effect on barley plants.

As for the effect on shoots, there is a tendency (p=0.07) to the phytostimulating effect of the *Bacillus cereus* strain 20.1 on barley. At the same time, the *Bacillus cereus* TR 1.1 strain significantly reduced the shoot length in barley. These results clearly demonstrate intraspecific differences in the phytostimulatory activity of bacilli strains belonging to the same species, but isolated from different soils. The effect of bacteria on the length of shoots in the absence of differences in the length of the roots can be associated with the synthesis of gibberellins or substances with a similar mode of action.

Based on the data of the bacterial strains action upon barley seedlings and their overall PTE resistance, three strains were selected for the pot experiment, namely *Bacillus pumilus* TR 3.1, *Bacillus atrophaeus* TR 3.3 and *Bacillus cereus* 20.1.
Figure 1. The effect of seed treatment with the studied bacterial strains on the length of the roots (left) and the length of the shoots (right).

The toxic effects of PTE and the results of the bacterial strains application can be clearly seen in figure 2.

Figure 2. Visual symptoms of PTE toxicity in the leaves of *H. vulgare* L. (A – control; B – contaminated soil; C – contaminated soil + bacteria).

Normal growth is observed in uncontaminated soil (figure 2 A) and weak growth in contaminated soil (figure 2 B). Similar visual symptoms are common in plants grown on soils contaminated with heavy metals [13]. These visual symptoms can indicate the violation of the internal structure of the leaf and other plant tissues.

Analysis of plant growth parameters (figure 3) showed that there was a pronounced negative impact of contaminated soil on *H. vulgare* L., and the growth and development of plants were severely impaired. However, the addition of metal-resistant bacteria had a significant impact on plant growth.

The high efficiency of metal-resistant bacteria in reducing the stress effects of the PTE on *H. vulgare* L. cannot be fully explained by passive adsorption of HMs on bacterial cells. The mass of inoculum is approximately equal to 10 mg/kg of soil, assuming an average bacterial mass of $10^{-12}$ g. It is likely that the presence of an active PTE immobilization mechanism underlies the observed positive effects.

4. Conclusion
The selected strains of spore-forming bacteria showed a high level of resistance to zinc contamination, and their resistance to other PTE was also higher in comparison to other bacterial strains.
Figure 3. Influence of soil contamination on the length of shoots (A), roots (B), leaves (C) and dry weight (D).

Regarding the effect on plants, among the isolated strains, the most promising for remediation are the following: *Bacillus cereus* 20.1, *Bacillus pumilus* TR 3.1, and *Bacillus atrophaeus* TR 3.3. Some other bacterial strains can also be considered for their phytostimulating effects. For instance, *Bacillus subtilis* TR 1.5 is the only strain that significantly increases the seed vigor for barley even though it is less metal-resistant than the first three strains.

The present study gives an example of an effective system for isolation and testing bacterial strains with valuable properties. The selection on gradient plates allows to increase the resistance almost two-fold. The preliminary testing on barley seedlings can help to sort out the strains possessing undesirable phytotoxic properties and reduce the number of strains for pot experiments. Finally, the experiment with growing barley in naturally contaminated soil has shown the effectiveness of the selected strains in conditions similar to real soil remediation. The use of an aqueous suspension of metal-resistant microorganisms significantly reduced the negative effect of pollutants contained in the soil. Thus, the use of microorganisms strains for remediation of soils contaminated with PTE is a promising approach.

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