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EXPERIMENTAL ARTICLES

Interactions between Plants and Associated Bacteria in Soils Contaminated with Heavy Metals

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Abstract—Interactions were studied between oat (*Avena sativa*) and two bacterial species, *Bacillus subtilis* and *Pantoea agglomerans*, in soils contaminated with heavy metals (HM), cadmium (50 mg/kg), and lead (200 mg/kg). Exposure to HM resulted in decreased (by 30–50%) length, mass, and ratio of shoot to root dimensions. Inoculation with bacteria lead to restoration and further enhancement of plant productivity, raising it above the level achieved via inoculation of oat in uncontaminated soils. It also reduced HM accumulation by plants. Pure cultures of *P. agglomerans* accumulate HM more intensively than those of *B. subtilis* (adsorbing activity was studied for both cells and extracellular metabolites). After the introduction of bacteria, lead, and cadmium content in soil decreased four- to fivefold and two- to threefold, respectively. Protection from HM is attributable to reorganizations in the populations of root-associated bacteria: cell number increases in the rhizoplane while decreasing in the rhizosphere.

Key words: plant–microbial communities, plant growth promoting rhizobacteria (PGPR), heavy metals (HM), microbiological protection from stress, rhizosphere, rhizoplane.

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One of the most effective approaches to diminishing the effect of heavy metals (HM) on plants is their inoculation with plant growth promoting rhizobacteria (PGPR) [1–3]. Effective mechanisms of plant protection against HM are implemented as part of a large network of symbiotic interactions in the root zone: the bacteria improve plant nutrition (N_2 fixation, mobilization of insoluble phosphorus compounds), stimulate production of stress-specific proteins, and promote development of organic–mineral complexes in soil [2, 4–8]. The application of biopreparations under conditions of HM-intoxication may result in an increased productivity of cereal, vegetable, feeding, and industrial crops [9–11]. However, the mechanisms of formation of rhizobacterial communities under conditions of HM-intoxication remain poorly investigated.

In earlier works [12, 13], the results of mathematical simulation suggested that improved HM resistance of plants inoculated with PGPR might be due to significant changes in the spatial structure of bacterial populations in the root zone. The goal of the present work was a test of the above hypothesis, aimed at investigation of the populational and physiological mechanisms underlying the adaptation of an associative system to stress. For this purpose we studied the dynamics of two PGPR species (*Pantoea agglomerans* and *Bacillus subtilis*) in oat rhizosphere and rhizoplane under HM contamination and determined the effect of inoculation on plant productivity and the ability of bacteria to adsorb HM in soil and in pure culture.

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MATERIALS AND METHODS

The studied bacteria, earlier identified by numerical taxonomy as *Bacillus subtilis* (strain Ch13) and *Klebsiella mobilis* (strain 880), were obtained from the collection of the Research Institute for Agricultural Microbiology [14]. Strain Ch13 is used in production of the preparation extrasol, which exhibits growth-stimulating and phytoprotector activity [15]. Strain 880 was used in creating the biopreparation mobilin, which showed high effectiveness in trials conducted by the Geographical Net of the Research Institute for Agricultural Microbiology [11, 16, 17].

The bacteria were cultured in DAS medium (Dobereiner medium) containing the following (g/l of distilled water): K_2HPO_4, 0.1; KH_2PO_4, 0.4; $MgSO_4$, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; $FeCl_3$, 0.01; Na_2MoO_4, 0.002; malic acid, 2.5; sucrose, 1; glucose, 1; and yeast extract, 1; pH was 6.6.

Concentrations of cadmium and lead free ions in the samples were measured using ion-selective electrodes (IS-Cd, IS-Pb). The total HM content in plants and HM immobilization by pure bacterial cultures were determined using a Perkin-Elmer (HGA74) atomic-adsorption spectrophotometer. To study HM adsorption by pure bacterial cultures, they were grown in agarized TSA medium [18] for 48 h at 25°C, suspended in distilled water (10^6 cells/ml), and 0.1 ml of the suspension was added to the vials containing 20 ml of DAS medium with $Pb(NO_3)_2$ (0–5 mM Pb) or $CdCl_2$ (0–2 mM Cd). The vials were incubated at 258C for 48 h in five replications. Bacterial cells were centrifuged, washed twice with 0.8% NaCl, and resuspended in 5 ml of 0.8% NaCl [19]. Metal content in the biomass and the supernatant was then determined.

The number of bacteria colonizing the rhizosphere was measured per unit of filter surface [20], and the cell number in the rhizoplane, per unit of root mass. In both cases plating was carried out on selective agarized DAS medium with nalidixic acid (50 mg/l), because spontaneous mutants of the studied strains, resistant to the specified antibiotic, were used as control.

Suspensions of bacterial vegetative cells (10^6 CFU/ml) were applied to membrane filters. Seeds of oat (*Avena sativa*, Borus cultivar) were placed on the filters, wrapped in synthetic tissue (which protected the seeds and filter from mechanical damage, but did not interfere with migration of bacteria and diffusion of soil solutions) and put into the soil. At the starting moment and subsequently at specified intervals the filters were removed and cell numbers of the introduced bacteria were determined. The cells were washed from the filter surface (rhizosphere) or roots (rhizoplane) and counted by the MPN method in a selective medium (5 replications).

Vegetation experiments were conducted in plastic film greenhouses under natural illumination. The experiments were carried out in the vessels containing 0.5 kg of sod-podzolic soil (total nitrogen, 0.1%; K_2O, 2.8 mg/100 g; carbon, by Turin, 0.79%; humus, 1.36%; hydrolytic acidity, 12.05 mg-eq/100 g, pH_{KCl} 5.4). Fertilizers were added prior to the set up of the experiment (mg/kg soil): NH_4NO_3, 25; K_2HPO_4, 80; $MgSO_4$, 15; $ZnSO_4$, 1; $CuSO_4$, 1; H_3BO_3, 1; Na_2MoO_4, 0.5. Lead in the form of $Pb(NO_3)_2$ was added at 200 mg/kg soil, cadmium in the form of $CdCl_2$, at 50 mg/kg soil. These concentrations correspond to 10 MPC (maximum permissible concentration) [21].

For mathematical treatment of the data, dispersion analysis and Student's *t*-test were used.

For identification of bacterial strains based on analysis of 16S rRNA gene sequences, genomic DNA was isolated according to the standard procedure of SDS lysis with proteinase K [22]. Genome fragments of the studied strains containing 16S rRNA gene sequences were amplified using universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1: (5'-AAGGAGGTGATCCAGCC-3') [23]. PCR fragments of about 1500 bp were isolated from agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, United States) kits, ligated into a T-vector pTZ57R/T (MBI Fermentas, Lithuania) and cloned. The nucleotide sequences were identified with an ABI Prism 377XL automated sequencer using ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit and the standard primers M13for(–20) and M13rev(–26) as per the manufacturer's instructions (Applied Biosystems, United States). Sequence alignments were performed using BLAST (http://www.ncbi.nlm.nih.gov) [24], Bio Informatic Bacteria Identification (Bibi) Data Bases (http://umr5558-mg1.univ-lyon1.fr/qm?page=BibiDataBases), and Ribosomal Database Project II (http://rdp.cme.msu.edu).

RESULTS

Bacterial species identification. Comparative sequence analysis of 16S RNA gene fragments (200–500 bp) isolated from strain 880 confirmed that it belongs to the family *Enterobacteriaceae*. Although the strain was initially identified as *Klebsiella mobilis* [14], it exhibited the highest similarity (100%) to the *Pantoea agglomerans* B1 strain (GenBank accession number DQ133596).

Strain Ch13 was shown to belong to the family *Bacillaceae* with the maximum similarity (100%) to a range of *Bacillus subtilis* strains, e.g., ZJ06 (GenBank accession number EU26607), which compares favorably to the results of its numerical identification.

Effect of bacterial inoculation and HM exposure on plant development. The results of vegetation experiments showed that addition of HM to the soil inhibited development of oat, which is manifested in decreased length and mass of the plants (Table 1; Figs. 1, 2a); notably, cadmium exhibited greater toxicity than lead. Development of the shoot was more sensitive to inhibition by HM than root development: in the presence of cadmium, the ratio of root to shoot (R : S) by mass was 2.33, while in the control it is was 1.46 (Fig. 1). After inoculation with bacteria, the R : S ratio not only normalized, but changes in favor of the shoot (1.30–1.39). A similar tendency was observed for the length of plants (Table 1): the R : S ratio was 1.46 in the control, 1.77 for exposure to cadmium, and 1.20–1.21 for cadmium-treated plants inoculated with bacteria. By contrast, in rice, impaired root development is the primary manifestation of lead inhibition [25].

It is worth mentioning that inoculation of plants with bacteria under HM contamination resulted in a more active plant development as compared to the control (without HM and bacteria) (Table 1). At early stages (5 days), inoculation in the presence of HM increased the length of germ plants more effectively (by 80–178%) than it did in the absence of stress (by 31–

Table 1. Effect of inoculation with *B. subtilis* and *P. agglomerans* on length of oat (mm) as dependent on addition of heavy metals

Variants	3-day germ plants	Shoots of 20-day plants	Roots of 20-day plants
Control (without HM and bacteria)	8.0	160	171
Control + *P. agglomerans*	11.0 (+)	215 (+)	230 (+)
Control + *B. subtilis*	10.5 (+)	205 (+)	218 (+)
Cd	3.2 (–)	135 (–)	157 (–)
Cd + *P. agglomerans*	9.4 (+)	174 (+)	193 (+)
Cd + *B. subtilis*	8.9 (+)	168 (+)	190 (+)
Pb	5.6 (–)	154 (–)	167
Pb + *P. agglomerans*	10.8 (+)	210 (+)	235 (+)
Pb + *B. subtilis*	10.1 (+)	198 (+)	217 (+)
$HCP_{0.05}$	0.8	6	10

Note: (+) and (–) indicate reliable deviations from the blank control.

38%). After 20 days, inoculation produced a uniform effect in HM-treated and control plants (an increase in length by 25–35%). In the presence of lead, inoculation restored plant development to the level of inoculation without HM; in the presence of cadmium, full restoration did not occur. By day 27, the effectiveness of associative interaction increased to an even higher degree (Fig. 2a): inoculation resulted in reliably greater plant lengths in the case of lead intoxication, than it did without lead; addition of cadmium restored plant growth to the level of inoculation without HM.

The improvement of plant development correlated with a four- to fivefold decrease in HM accumulation in plants (Fig. 2b).

HM adsorption by bacteria in soil and in pure culture. Introduction of PGRP to HM-contaminated soil (without plants) resulted in a significant decrease in

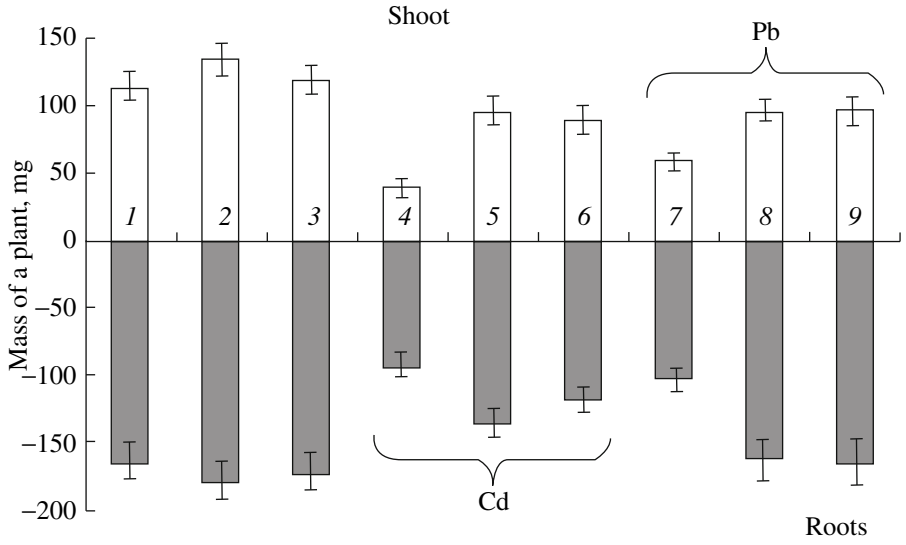

Fig. 1. Effect of inoculation with bacteria on shoot and root mass (mg) of 15-day plants of oat, grown in HM-contaminated soil. Control (HM- and bacteria-free) (*1*); Control + *P. agglomerans* (*2*); Control + *B. subtilis* (*3*); Cd (*4*); Cd + *P. agglomerans* (*5*); Cd + *B. subtilis* (*6*); Pb (*7*); Pb + *P. agglomerans* (*8*); Pb + *B. subtilis* (*9*).

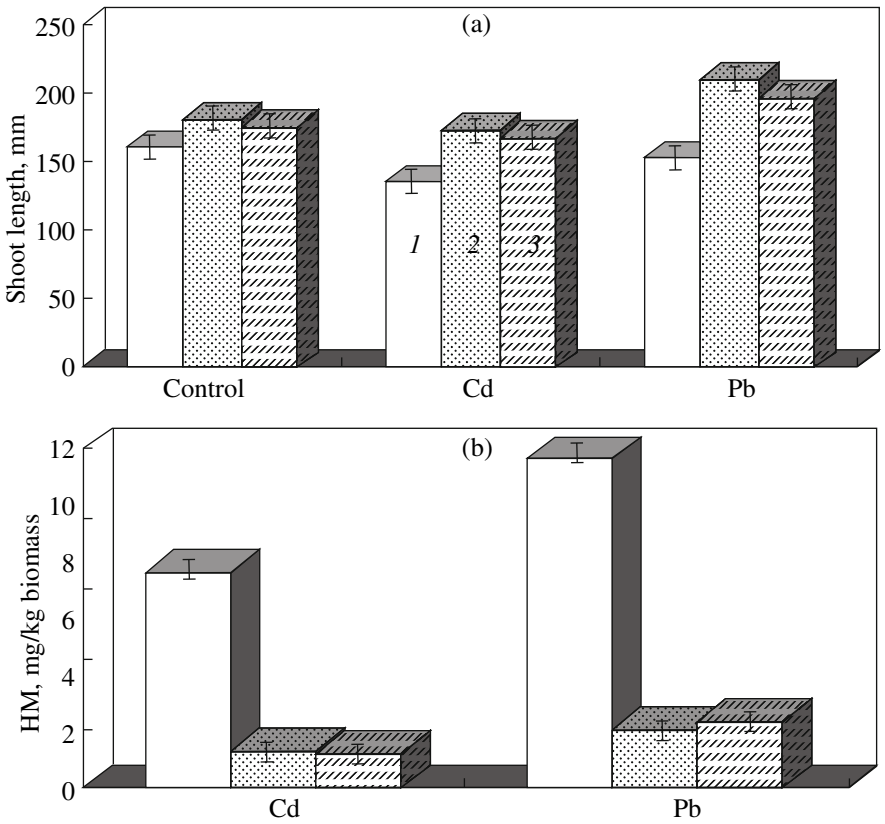

Fig. 2. Effect of inoculation with bacteria on shoot length (a) and HM accumulation in plants (b): control (*1*); *P. agglomerans* (*2*); *B. subtilis* (*3*).

HM mobility (Fig. 3): as the result of sorption processes, the content of mobile HM forms decreased by an average of twofold by day 5. By day 27, lead content in soil decreased by an average of fivefold, the cadmium content, by an average of twofold.

When grown in pure culture, both bacterial species adsorbed lead in greater quantities than cadmium, which is revealed in examination of adsorption activity of the cells (Fig. 4a) and of the extracellular metabolites in the culture liquid (Fig. 4b). For *P. agglomerans*, accumulation of both HM proved to be higher than for *B. subtilis*, which may be related to a greater HM resistance of the former [14].

Bacterial dynamics in soil and in the root zone. Under the action of HM, *B. subtilis* populations in soil declined more sharply (especially at the early stages of incubation) than those of *P. agglomerans* (Table 2). These results confirm the literature data concerning high sensitivity of the members of the genus *Bacillus* to HM contamination [5, 26]. Gram-negative bacteria, such as flavobacteria and enterobacteria, are more resistant to HM than gram-positive bacteria [8, 27].

To reveal the populational mechanisms of plant protection from HM toxicity, the populations of both bacterial species in the rhizosphere and rhizoplane of oat was studied. It was found (Tables 3, 4) that bacterial population exposed to HM increased in the rhizoplane and declined in the rhizosphere. The parameters of *Bacillus* population dynamics in the rhizosphere and soil were similar (a peak was observed on day 5), but differed significantly from the rhizoplane parameters (a peak on day 13). The genus *Pantoea* exhibited another tendency: populational dynamics were similar in the rhizosphere and rhizoplane (a peak on day 13), but differed from that in soil (a peak on day 5). For *Bacillus*, the population densities in soil and in the rhizosphere were of the same order of magnitude (both in the presence and in the absence of HM), while for *Pantoea,* the density in the rhizosphere was much higher than in soil. It appears evident that *Pantoea s*pecies can use the association with plants for adaptation to stress more effectively than *Bacillus*.

DISCUSSION

Our results suggest that protection of plants against HM by inoculation with bacteria is based on two mechanisms: adsorption of mobile HM forms from soil and formation of numerous bacterial populations on the root surface. The functioning of the first mechanism involves HM adsorption by both bacterial cells and their extracellular metabolites. The functioning of the second mechanism is defined by rearrangement of bac-

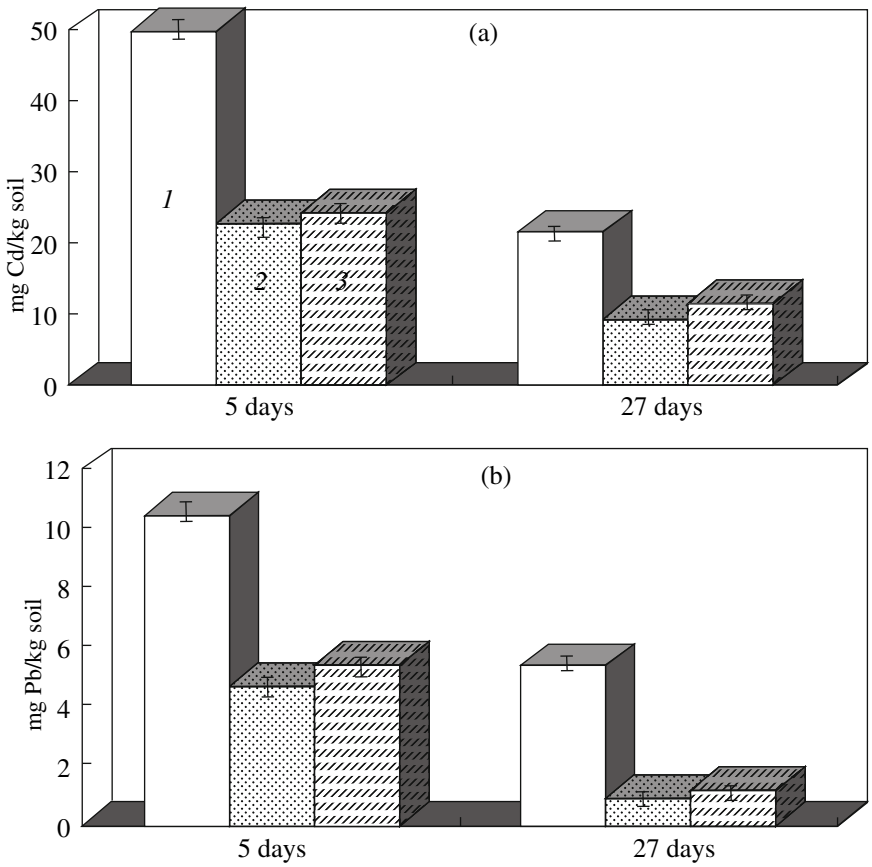

Fig. 3. Effect of inoculation with bacteria on cadmium (a) and lead (b) content in soil. Variants are denoted as in Fig. 2.

teria in the ecological niches of the root zone, i.e., their populations increase in the rhizoplane and decrease in the rhizosphere. Even in spite of this rearrangement, bacterial density in the rhizosphere in the presence of HM may be significantly higher than in soil without plants; this is an indication of an important role of interaction for the adaptation of both the plant and microbial component of the associated system to stress.

The populational redistributions induced by HM with different mechanisms of action (cadmium, lead) and by inoculation of plants with unrelated bacterial species (*Pantoea agglomerans*, *Bacillus subtilis*) were

Table 2. Dynamics of bacterial populations in soil ($\times 10^4$ CFU/g) contaminated by HM

Bacteria	HM	day 5	day 13	day 20	day 27
B. subtilis	No HM	80 ± 4.5	20 ± 1.4	0.65 ± 0.08	0.048 ± 0.003
	Lead	15 ± 1.2	10 ± 0.8	0.30 ± 0.02	0.030 ± 0.001
	Cadmium	10 ± 0.8	5 ± 0.3	0.15 ± 0.02	0.012 ± 0.001
P. agglomerans	No HM	420 ± 15	360 ± 12	50 ± 2.7	17 ± 1.2
	Lead	350 ± 8	320 ± 7.3	30 ± 2	14 ± 1.4
	Cadmium	120 ± 5	130 ± 4.6	25 ± 1.8	12 ± 0.9

Note: At the starting moment (0 days) the titer was 10^6 cells per gram of soil for both bacterial species.

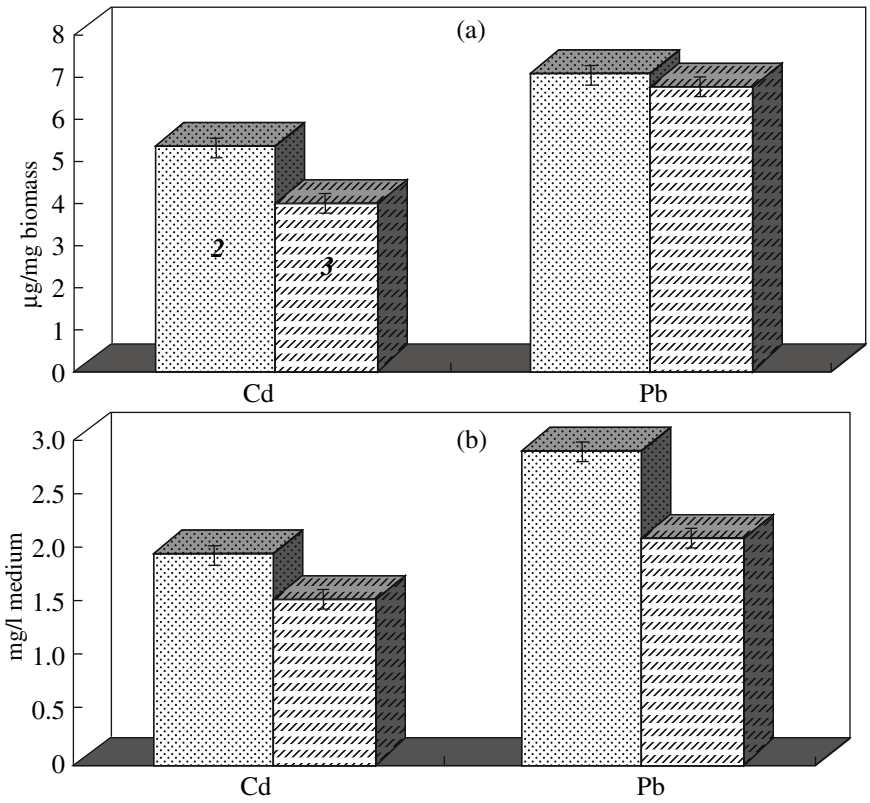

Fig. 4. Immobilization of heavy metals by bacteria in pure culture: by cells, µg per mg of biomass (a); by extracellular metabolites of the culture medium, mg per l of the medium (b). Bacteria are denoted as in Fig. 2.

similar. Variations in the root-zone bacterial dynamics are therefore caused primarily by plant-related factors activated in stress conditions. The data obtained are confirmed by analysis of the mathematical simulation, according to which the action of HM on a microbial–plant association alters dynamics of reproduction, dying, and migration of bacterial cells in the ecological niches of the root zone [12, 13].

Our investigations provided an important observation that exposure to HM coupled with inoculation allows to reach and, sometimes, to exceed the level of plant productivity characteristic of inoculation in the absence of HM; this is supported by the data obtained earlier [28]. Among the mechanisms of such increase is a decrease of HM concentrations to the levels (about 3 MPC) promoting plant development [29].

Another mechanism may be stress-induced activation of the physiological processes that provide high effectiveness of associative symbiosis. It is known that the studied strain 880 *P. agglomerans* is an indoleacetic acid (IAA) producer [14], and a *B. subtilis* strain Ch13 produces, apart from that, indole-3-lactic acid, indole-3-carboxylic acid, and indole aldehyde [15]. As was shown earlier [12], cadmium stress causes rhizobacteria to intensify IAA synthesis, which is among the mechanisms of adaptation of the microbial–plant system to stress. IAA synthesized by the bacteria may promote excretion of carbon compounds from roots and lectin production. Carbon compounds are nutrient substrates for rhizosphere bacteria, and lectins are essential for effective bacterial colonization of roots [30]. In summary, HM action may expand the carrying capacity of the root zone econiches, and thus promote their colonization by bacteria, which in turn increases stress sustainability in both components of the microbial–plant associated system, as is consistent with the view on symbiosis as a mechanism of adaptation of organisms to stress [29, 31].

When the stress factor is no longer present (decrease of HM concentrations to nontoxic levels), these mechanisms may provide higher plant productivity than in the variants with no stress. Such enhancement may also be related to the fact that the strains of rhizobacteria used to decrease the negative effects of HM, were selected beforehand for resistance to HM [9, 31]. Although native PGRP strains may exhibit significant growth-promoting activity, they are usually sensitive to increased HM content; consequently, their competitiveness during rhizosphere and rhizoplane colonization is low [29].

Our results enable us to recommend microbial preparations incorporating rhizobacteria *P. agglomerans* 880 and *B. subtilis* Ch13 for use in ecologically clean production on HM-contaminated soils together with

Table 3. Population dynamics of *B. subtilis* in the rhizosphere ($\times 10^4$ CFU per g of soil) and rhizoplane ($\times 10^4$ CFU per mm^2 of root surface) of oat in HM-contaminated soil

Econiche	HM	day 5	day 13	day 20	day 27
			Lead		
Rhizosphere	–	230 ± 13	41 ± 3	2.0 ± 0.1	0.27 ± 0.02
	+	18 ± 2	13 ± 1	0.85 ± 0.03	0.14 ± 0.01
Rhizoplane	–	18 ± 1.8	230 ± 14	7.5 ± 0.5	0.29 ± 0.01
	+	38 ± 3	600 ± 35	20 ± 2.1	0.71 ± 0.02
			Cadmium		
Rhizosphere	–	120 ± 6	20 ± 2	0.24 ± 0.08	0.12 ± 0.03
	+	20 ± 1.3	6 ± 1.4	0.08 ± 0.002	0.02 ± 0.001
Rhizoplane	–	18 ± 3	130 ± 10	2.3 ± 0.2	0.14 ± 0.05
	+	38 ± 6	400 ± 27	8.1 ± 0.6	0.48 ± 0.12

Table 4. Population dynamics of *P. agglomerans* in the rhizosphere ($\times 10^5$ CFU per g of soil) and rhizoplane ($\times 10^5$ CFU per mm^2 of root surface) in the context of HM-intoxication

Econiche	HM	day 5	day 13	day 20	day 27
			Lead		
Rhizosphere	–	360 ± 23	790 ± 37	86 ± 4	3.4 ± 0.05
	+	59 ± 5.1	120 ± 9	43 ± 2	1.4 ± 0.07
Rhizoplane	–	5.4 ± 0.14	37 ± 2.4	1.8 ± 0.02	0.23 ± 0.004
	+	76 ± 3	91 ± 6.3	23 ± 2	0.81 ± 0.006
			Cadmium		
Rhizosphere	–	60 ± 4	290 ± 14	5.4 ± 0.07	0.17 ± 0.002
	+	5.9 ± 0.12	14 ± 2.6	2.3 ± 0.02	0.05 ± 0.001
Rhizoplane	–	1.7 ± 0.02	12.5 ± 1.2	0.14 ± 0.002	0.16 ± 0.004
	+	25.3 ± 1.2	36.1 ± 2.6	2.1 ± 0.03	0.40 ± 0.006

other strains selected earlier for resistance to HM, that provide a significant decrease of HM content in plants [1, 29, 32]. This approach surpasses the currently recommended agrotechnical techniques [32, 33] in agronomic and economic effectiveness. Since phytoprotection effects revealed in our studies become apparent at early stages of plant development, the employed method of membrane filters can be modified for creation of a test-system allowing express estimation and selection of PGRP strains capable of effective plant protection from HM toxicity. Associated systems obtained on the basis of such strains can be used for creation of highly effective, economic, and environmentally safe technologies for remediation of polluted soils.

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