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EXPERIMENTAL ARTICLES

Experimental and Mathematical Simulation of Population Dynamics of Rhizospheric Bacteria under Conditions of Cadmium Stress

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Abstract—The method of membrane filters was used to study the population dynamics of bacteria belonging to the genera *Arthrobacter, Flavobacterium*, and *Klebsiella* in barley (*Hordeum vulgare*) rhizosphere under conditions of cadmium stress (5–15 mg Cd/g soil). Mathematical simulation allowed us to demonstrate that the phytoprotective effect is implemented via the following succession of events: the bacteria synthesize phytohormones (IAA and ethylene) ⟶ root excretory activity increases ⟶ the number of the bacteria in the rhizoplane grows ⟶ the flux of bacteria migrating from the rhizoplane to the rhizosphere increases ⟶ the number of bacteria binding cadmium ions in the rhizosphere grows ⟶ the amount of free ions entering the plant decreases. Among the bacteria studied, *K. mobilis* 880 displayed the highest migration and immobilization activity and the best survival rate under conditions of cadmium stress. Consequently, *K. mobilis* 880 is recommended for use in biopreparations for stimulating plant growth under conditions of heavy metal pollution.

Key words: rhizosphere, rhizoplane, rhizospheric bacteria, associative symbiosis, barley, heavy metals, mathematical model of bacterial population dynamics, immobilization of cadmium.

Over 60 million hectares of land in Russia are now polluted by discharges from production plants [1], and the polluted areas are constantly expanding. Cultivated plants accumulate heavy metals from soils, which renders the plants toxic to humans and animals [2]. Biological preparations involving rhizospheric bacteria are among the most efficient tools for decreasing the content of heavy metals in plants [3, 4]. These bacteria are capable of reducing the level of metals in plants by binding the metals into insoluble complexes inaccessible to plants [5–7]. The mechanism underlying protection of plants from heavy metals is implemented within a network of interactions between bacteria and plants in the rhizosphere [8, 9]. It is known that bacteria are able to decrease the content of ethylene in plants [10], thereby considerably elevating plant productivity [10]. Bacteria may protect plants by inducing stress-specific proteins, increasing tolerance to pathogens, or ameliorating mineral nutrition [4, 11].

To clarify specific features of microbial–plant interactions under cadmium stress, we used the method of membrane filters [12]. The bacterial dynamics was assessed according to cell counts on membrane filters placed in the plant rhizosphere. Concurrently, the dynamics of bacterial population on the root surface (in the rhizoplane) was monitored. A customized mathematical model allowed us to resolve the dynamics of the bacterial population on the filter surface into three components. In our work, particular attention was paid to the component related to the migration of bacteria from the rhizoplane, the intensity of which depends on cadmium concentration in soil. This approach allowed us to reveal the sequence of events that lead to joint adaptation of plants and bacteria to cadmium stress.

MATERIALS AND METHODS

In the experiments we used the strains *Flavobacterium* sp. L-30, *Arthrobacter mysorens* 7, and *Klebsiella mobilis* 880, selected from the collection at the All-Russia Research Institute for Agricultural Microbiology due to their increased ability to attenuate lead and cadmium accumulation in plants [3, 13].

IAA production was determined by photoelectrocalorimetry using the Salkovski reagent [14]; ethylene production, by gas chromatography (a Tsvet 100 chromatograph). Bacteria were cultivated in DAS medium, containing 0.4 g/l KH_2PO_4, 0.1 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.02 g/l NaCl, 0.02 g/l $CaCl_2$, 0.01 g/l $FeCl_3$, 0.002 g/l Na_2MoO_4, 0.013 g/l NH_4Nl, and 2.5 g/l malic acid. The concentration of free cadmium ions in soil was measured by an ion-selective electrode (IS-Cd, Russia) [15].

The vegetative experiments were performed in the summer of 2000 in film greenhouses with natural illumination using the barley (*Hordeum vulgare*) cultivar

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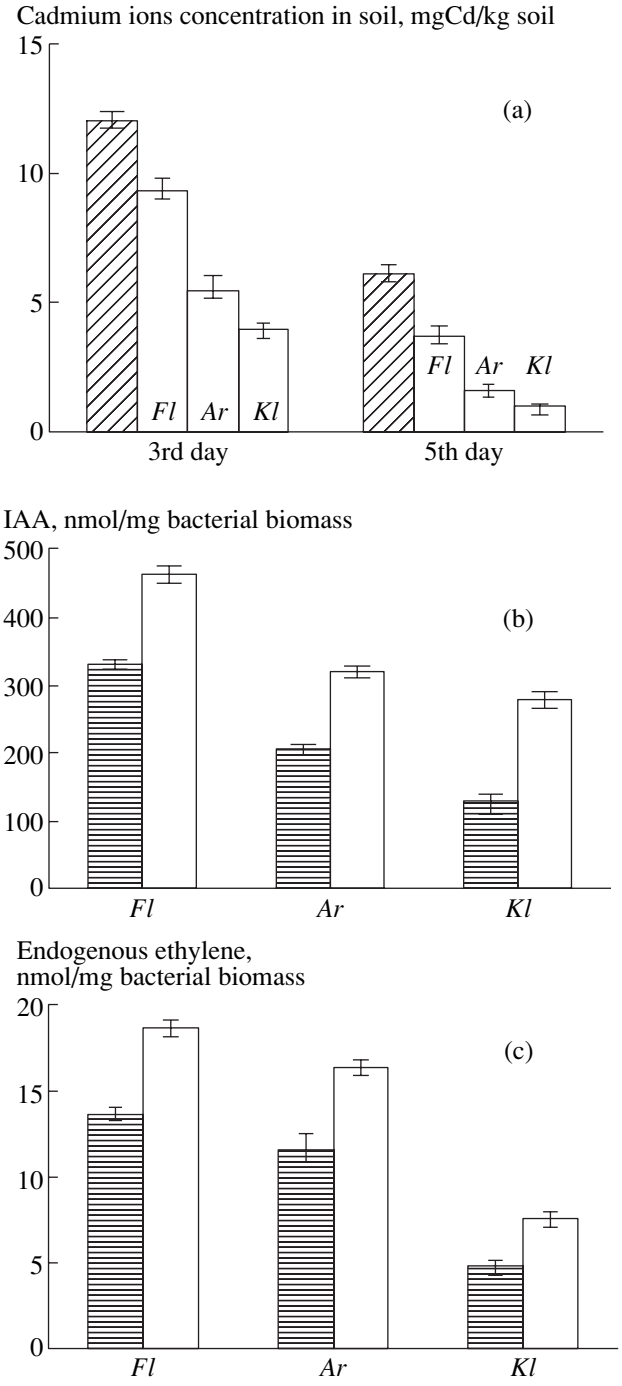

Fig. 1. Biological properties of bacteria under conditions of cadmium stress, including (a) immobilization of cadmium ions by bacteria, (b) IAA secretion, and (c) ethylene secretion: *Fl, Flavobacterium* sp. L-30; *Ar, A. mysorens* 7; *Kl, K. mobilis* 880; ▨, the absence of bacteria; ▤, and the absence of cadmium. $CdCl_2$ was added to the cultivation medium at a concentration of 20 μM/l.

Belogorsky, which exhibits high tolerance to cadmium [16]. In these experiments, 5-kg containers with soddy–podzolic soil (1.1% humus, 0.07% total nitrogen, 15 mg K_2O/100 g soil of exchangeable potassium, and 75 mg P_2O_5/100 g soil of exchangeable phosphorus; hydrolytic acidity of 12.2 mg equiv/100 g soil; and pH 6.4). The fertilizers were added to the soil before the beginning of the experiment in the following amounts: 25 mg/kg NH_4NO_3, 80 mg/kg K_2HPO_4, 15 mg/kg $MgSO_4$, 1 mg/kg $ZnSO_4$, 1 mg/kg $CuSO_4$, 1 mg/kg H_3BO_3, and 0.5 Na_2MoO_4. Cadmium in the form of the salts $CdCl_2$ and $Cd(NO_3)_2$ was added at concentrations of 5 and 15 mg/1 kg soil.

To clarify specific features of microbial–plant interactions during cadmium stress, the method of membrane filters [12] was used. Before being placed into the soil, the surface of a sterile nitrocellulose membrane filter (Vladipor of MFA-MA type, Tasma, Russia, with a size of 50 × 50 mm) was covered with a 3-day-old bacterial suspension (density, 10^8 cells/ml). Barley seeds were also placed on the filter after 3-min sterilization in a mixture of ethanol and hydrogen peroxide (1 : 1). At the beginning of the experiment, the membrane filters with the bacteria and barley seeds were placed vertically into the soil-filled containers. Membrane filters with plant roots were removed on days 3, 6, 14, and 20 to perform microbiological assays.

First, the roots were removed from the filter surface for further examination, and disks (d = 4 mm) were cut from the filter to place them into flasks containing 50 ml of sterile water (to desorb bacteria). Bacterial counts in suspension were determined by plating on agar medium, followed by dividing the number of bacterial cells by the area of the cut disks (number of cells/mm^2 of the membrane filter, i.e., cells/mm^2). The bacterial number on the filter was considered a function of the size of the rhizospheric subpopulation.

The plant roots withdrawn from the membrane filters were weighed, homogenized, and placed into flasks containing 50 ml of sterile water. Bacterial counts in root homogenate were determined by plating on agar medium. The counts obtained were divided by the root weight (measurement unit, number of cells/1 g of fresh root, i.e., cells/g roots). These counts were considered as a function of the size of the rhizoplane subpopulation.

The colonies of bacteria studied were distinguishable by their characteristic coloration on DAS agar medium. The colonies of *K. mobilis* CIAM 880 were white and mucous; the colonies of *Flavobacterium* sp. L-30, yellow–orange; and the colonies of *A. mysorens* 7, green and opalescent. The colonies were randomly tested by immunodiffusion [17], to exclude the accompanying resident microflora and confirm their attribution to the species in question. The protocol for obtaining the sera is detailed in [18].

Mathematical simulation was used to resolve the bacterial population dynamics measured on the surface of the membrane filter into components. The components of the population dynamics were described using standard mathematical formulas: the exponential decreasing function characterizing the dying-off of bacteria and the hyperbolic tangent function describing

Table 1. Values of the coefficients of the mathematical model

Coefficient	Cadmium concentration, mg/kg soil				
	0	$Cd(NO_3)_2$		$CdCl_2$	
		5	15	5	15
Klebsiella mobilis 880, N_0 = 1367 cells/mm^2					
μ_C, days^{-1}	0.390	0.290	0.310	0.320	0.450
μ_N, days^{-1}	1.880	1.700	1.600	1.700	1.400
μ_R, days^{-1}	0.295	0.349	0.397	0.342	0.390
r	6585	22317	8049	20488	3146
Flavobacterium sp. L-30, N_0 = 174 cells/mm^2					
μ_C, days^{-1}	1.80	1.90	1.92	2.00	2.10
μ_N, days^{-1}	4.90	4.20	4.10	5.00	4.80
μ_R, days^{-1}	1.00	0.75	1.20	1.20	2.50
r	103	586	394	575	402
Arthrobacter mysorens 7, N_0 = 8200 cells/mm^2					
μ_C, days^{-1}	0.580	0.610	0.620	0.560	0.600
μ_N, days^{-1}	1.580	1.480	1.420	1.600	1.500
μ_R, days^{-1}	0.700	0.900	1.200	0.900	1.200
r	1.098	4.512	1.463	3.902	1.341

Note: For all strains, $a = 0.75$, $b = 10^6$, and $c = 10^4$.

colonization by bacteria of a niche with a limited nutrient resource (solution of the logistic model [20]). The parameters of the mathematical model were calculated by the least squares method (computational medium, MathCad 2000), which allows finding the values of the parameters at which the root mean square deviations of the calculated values from the corresponding experimental data are minimal.

RESULTS AND DISCUSSION

The experiments demonstrated that the bacteria studied immobilized 24–68% of cadmium ions from the soil suspension, while *K. mobilis* 880 displayed the highest immobilization activity (Fig. 1a). All the bacteria increased their production of IAA and ethylene 1.5–2.0-fold when influenced by cadmium stress (Figs. 1b and 1c). This suggests that under conditions of cadmium stress, plants respond to an increase in bacterial IAA and ethylene production by secreting higher amounts of root exudates into the rhizosphere. The experiment confirmed that the bacterial population in the rhizoplane increased by one to two orders of magnitude (during cadmium stress as compared with the control). All bacterial populations in the barley rhizoplane reached their maximal size by day 20 of plant growth.

In constructing the mathematical model, we proceeded from the assumption that the population in the root area comprises subpopulations of the rhizosphere and rhizoplane, where the cells reproduce, die off, and migrate. In the rhizoplane, bacteria are near the source of exudates and therefore propagate the most intensively. Excessive bacteria migrate into the rhizosphere and bind cadmium. Three dynamic processes differing in their character were taken into account when analyzing the bacterial population dynamics on the surface of membrane filter. The population dynamics in question, $N(t)$, was represented as a sum of three items:

$$N(t) = N_C(t) + N_N(t) + N_M(t), \quad (1)$$

where $N_C(t) = N_0(1 - a)\exp\{-\mu_C t\}$ (cells/mm^2)

is a monotonically decreasing function reflecting the population dynamics of the bacteria that failed to adapt to the environmental conditions;

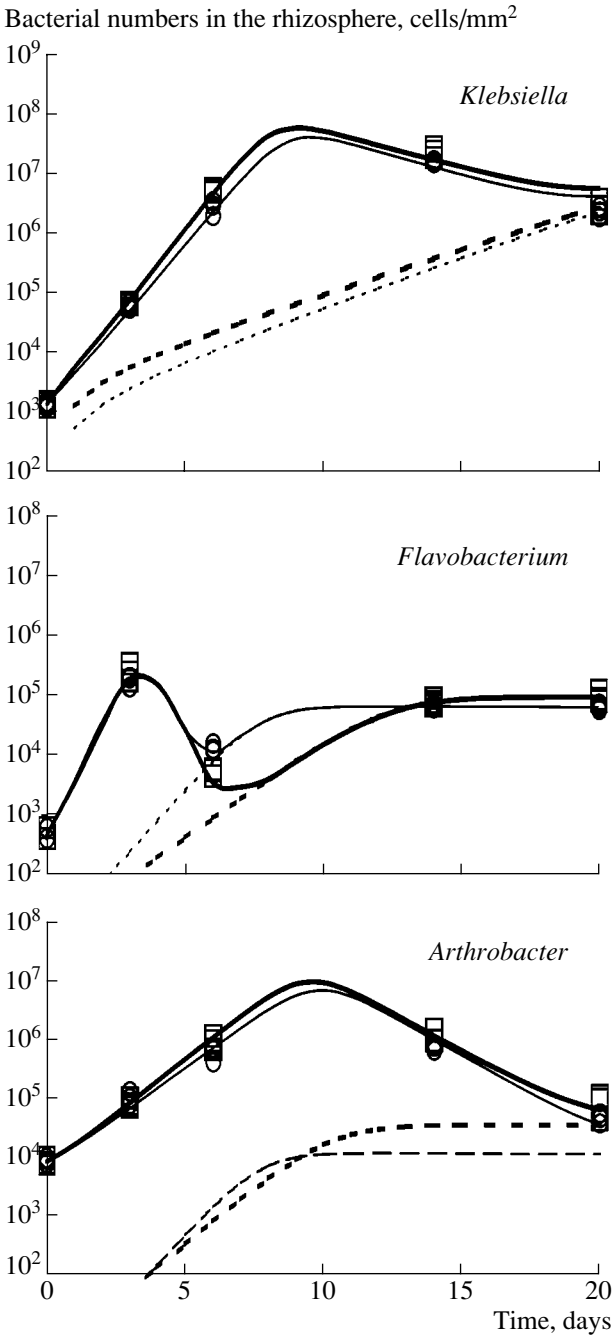

Fig. 2. Results of mathematical simulation of the bacterial population dynamics in the rhizosphere. Notations:

Cadmium doses	Experimental data	$N(t)$	$N_M(t)$
5 mg Cd/kg soil	○	———	– — –
15 mg Cd/kg soil	□	———	- - - -

$$N_N(t) = aN_0\frac{(b+1)\exp\{-\mu_C t\}}{b\exp\{-\mu_N t\}+1},\ (\text{cells/mm}^2)$$

is a nonmonotonic function reflecting the population dynamics of the bacteria that adapted to the environmental conditions; it has a lag phase, a saturation phase, and a phase of population decrease (we called this type of dynamics the population wave in the rhizosphere);

$$N_M(t) = \frac{rN_0}{c}\left(\frac{c+1}{c\exp\{-\mu_R t\}+1}-1\right)\ (\text{cells/mm}^2) \quad (2)$$

is a monotonically growing function that reflects the population dynamics of the bacteria with a lag phase and a saturation phase (but lacking the phase of population decrease), which corresponds to conditions of bacterial population development in the rhizoplane;

t is the observation time starting from the introduction of the membrane filter into the rhizosphere (a variable; days);

a is the coefficient of adaptation stability (a dimensionless constant);

N_0 is the initial cell count on the filter surface (a constant; cells/mm^2);

μ_C is the dying-off rate of bacteria on the membrane filter (a constant; days^{-1});

b is the coefficient characterizing the maximal increase in the bacterial population during the population wave (a dimensionless constant);

μ_N is the specific propagation rate of bacteria on the membrane filter (a constant; days^{-1});

r is the level of bacterial migration from the rhizoplane onto the membrane filter (a dimensionless constant);

μ_R is the specific propagation rate of bacteria in the rhizoplane (a constant; days^{-1}); and

c is a coefficient determining the maximal number of bacteria in the rhizoplane (a dimensionless constant).

The values of coefficients of the mathematical model (a, N_0, μ_C, b, μ_N, r, μ_R, and c) which provide the best fit between the model and the experiment (Table 1, Fig. 2) were found by comparing the experimental data and the calculations made using Eq. (1).

The coefficients μ_C and μ_N characterize the dynamic properties of bacteria. Among the bacteria studied, *Flavobacterium* sp. L30 (μ_C = 2.0 – 2.4 days^{-1} and μ_N = 3.8 – 4.5 days^{-1}) displays the highest specific rates of propagation and dying-off compared with *K. mobilis* 880 and *A. mysorens* 7 (μ_C = 0.38 – 0.6 days^{-1}, μ_N = 1.35 – 1.6 days^{-1}). Consequently, the population wave $N_N(t)$ formed by *Flavobacterium* sp. L-30 attenuates earlier (on days 7–8) than the population waves formed by *K. mobilis* 880 and *A. mysorens* 7, which attenuate on days 17–21. By the moment of attenuation of the *Flavobacterium* sp. L-30 population wave, the migration of bacteria from the rhizoplane to the filter is insignificant. Therefore, the population curve of *Flavobacterium* sp. L-30 at that time has a minimum. *K. mobilis* 880 and *A. mysorens* 7 form population waves extended in time whose tails are disguised by bacterial migration from the rhizoplane to the filter. Hence, the minimums

of the *K. mobilis* 880 and *A. mysorens* 7 population curves were not found.

According to Eq. (2), the product rN_0 gives the maximal number of bacteria that migrated to the filter from the rhizoplane and retained the ability to reproduce because they were not involved in cadmium immobilization (F-bacteria). The calculations performed (Table 1) demonstrate that the number of F-bacteria at a cadmium content of 5 mg is higher than in variants where cadmium was either absent or present in amount of 15 mg. This nonmonotonic dependence is accounted for by the fact that, on increasing cadmium concentration from 0 to 5 mg per 1 g soil, the growth in the number of bacteria migrating towards the filter exceeds that of bacteria immobilizing cadmium and failing to reach the filter. A further decrease in cadmium concentration from 5 to 15 mg per 1 g soil considerably activates the binding of this metal by bacteria. Therefore, the growth in the number of migrating bacteria is initially compensated for by the increase in the number of bacteria that immobilize cadmium in the rhizosphere and, at higher cadmium concentrations, the migration to the filter may be blocked completely. This means that such a concentration of cadmium may be reached at which all the bacteria that migrated from the rhizoplane will be involved in binding this metal and will not reach the filter ($r = 0$).

Evidently, the more that the bacteria bind cadmium, the less cadmium enters the plants and the less the number of bacteria found on the filter; that is, the decrease in the migration level, r, reflects the intensity of immobilization processes in the rhizosphere. To compare the bacteria with reference to this characteristic, we introduced the coefficient of migration and immobilization activity (Y_{Cd}). This coefficient shows the number of cells by which the population of F-bacteria reduces on increasing the concentration of cadmium by 1 mg/g soil (for a concentration range of 5–15 mg Cd/g soil). The coefficient Y_{Cd} is calculated from the following equation:

$$Y_{Cd} = N_0 \frac{r_5 - r_{15}}{Cd_{15} - Cd_5} \left[\frac{\text{cells kg soil}}{\text{mm}^2 \text{ mg Cd}} \right], \qquad (3)$$

where Cd_5 = 5 mg Cd/kg soil and Cd_{15} = 15 mg Cd/kg soil are variants of cadmium concentration in soil; r_5 and r_{15} are levels of migration at the corresponding cadmium concentrations. The calculation results obtained using Eq. (2) and the data listed in Table 1 are shown in Table 2.

According to Table 2, the migration and immobilization activity is three orders of magnitude higher in the population of *K. mobilis* 880 than in populations of *Flavobacterium* sp. L-30 and *A. mysorens* 7. In addition, *K. mobilis* 880 has a higher cadmium binding activity (per cell) than either *Flavobacterium* sp. L-30 or *A. mysorens* 7. Therefore, the use of the first bacterial species in biopreparations is promising and may allow attaining the best rates of plant protection from the penetration of heavy metal ions.

Table 2. Migration and immobilization activity of bacteria during binding of various cadmium salts in soil

Bacteria	Y_{Cd}, $\left[\frac{\text{cells kg soil}}{\text{mm}^2 \text{ mg Cd}}\right]$	
	$Cd(NO_3)_2$	$CdCl_2$
Klebsiella mobilis 880	1.95×10^6	2.37×10^6
Flavobacterium sp. L-30	3.34×10^3	3.01×10^3
Arthrobacter mysorens 7	2.50×10^3	2.10×10^3

Thus, the reaction of a microbial–plant system to cadmium stress involves a succession of events associated with specific features of interactions between microorganisms and the plant in the rhizosphere. We assume that the scheme of events is as follows: the bacteria synthesize phytohormones (IAA and ethylene) ⟶ root excretory activity increases ⟶ the number of bacteria in the rhizoplane grows ⟶ the flux of bacteria migrating from the rhizoplane to the rhizosphere increases ⟶ the number of bacteria binding cadmium ions in the rhizosphere grows ⟶ the number of free ions entering the plant decreases.

Thus, mutualistic interactions between plants and bacteria are observed under conditions of cadmium stress: being in an association, each partner is more tolerant to the stress than in a free state. The method developed can be applied to selection of rhizobacterial strains possessing maximal phytoprotective activity under cadmium stress.

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