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EXPERIMENTAL ARTICLES

The Growth-promoting Effect of *Beijerinckia mobilis* and *Clostridium* sp. Cultures on Some Agricultural Crops

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Abstract—New strains of *Beijerinckia mobilis* and *Clostridium* sp. isolated from the pea rhizosphere were studied with respect to their promoting effect on the growth and development of some agricultural crops. Seed soaking in bacterial suspensions followed by the soil application of the suspensions or their application by means of foliar spraying was found to be the most efficient method of bacterization. The application of *B. mobilis* and *Clostridium* sp. cultures in combination with mineral fertilizers increased the crop production by 1.5–2.5 times. The study of the population dynamics of *B. mobilis* by the method of genetic marking showed that this bacterium quickly colonized the rhizoplane of plants and, therefore, had characteristics of an *r*-strategist. At the same time, *Clostridium* sp. was closer to *K*-strategists, since this bacterium slowly colonized the econiches studied. The introduction of the bacteria into soil did not affect the indigenous soil bacterial complex. The presence of *Clostridium* sp. slowed down the colonization of roots by the fungal mycelium. The possible mechanisms of the plant growth–promoting activity of *B. mobilis* and *Clostridium* sp. are discussed.

Key words: plant growth–promoting microorganisms, rhizosphere, soil, soil bacteria and fungi.

Microbial communities that reside on the rhizoplane and in the rhizosphere of plants considerably influence their growth and development [1–3]. The presence of phytopathogenic bacteria in the rhizosphere enhances risks of plant diseases, whereas their competition with nonphytopathogenic bacteria acts to diminish such risks. The reason why, under the same cultivation conditions, a crop may be damaged in some plots and not damaged in other plots is not yet understood. This reduces the efficiency of pest control measures and compels the doses of antimicrobial pesticides, which are often hazardous to the environment, to be increased. Recent trend in pest control strategy is to use specific microbial cultures that inhibit the development of phytopathogenic microorganisms by the mechanisms of competition and antagonism.

Some introduced microorganisms beneficially influence plants by producing plant growth–promoting metabolites (auxins, cytokinins, and gibberellins) [4, 5], which not only enhance the productivity of agricultural crops but also may improve crop quality, by increasing the content of proteins, essential amino acids, and vitamins [6]. Other mechanisms of the beneficial effect of microorganisms on plants are the fixation of molecular nitrogen and synthesis of substances that enhance the utilization of phosphorus- and iron-containing compounds [7] and other plant nutrients [8]. A microbial culture may possess one or several of these activities.

The most extensively studied microbial stimulants of plants are bacteria of the genera *Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia, Enterobacter,* and *Bacillus* [9–12]. Bacterial fertilizers (killed bacterial cultures) are widely used in developed countries. However, there is increasing evidence that live bacterial cultures are also very efficient in agriculture [13, 14].

The aim of the present work was to isolate new microbial strains from the plant rhizosphere and to study their plant growth–promoting ability and persistence in the rhizosphere and soil.

MATERIALS AND METHODS

Isolation and identification of bacterial strain. Bacterial strains were isolated from the pea (*Pisum sativum* L. cv. Viola) rhizosphere at different vegetative stages using plates with mineral Gauze 1 medium. Based on their morphological, cultural, physiological, and biochemical properties, two isolates were assigned to the genera *Beijerinckia* (Deex 1950) and *Clostridium* (Pzazmowskii 1980), the species *B. mobilis* and *Clostridium* sp. [15].

Genetic marking of *B. mobilis* and *Clostridium* sp. To facilitate the monitoring of bacteria introduced into the plant rhizosphere, they were genetically marked by imparting resistance to streptomycin at a concentration of 1 mg/ml medium [16].

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Table 1. The effect of *B. mobilis* and *Clostridium* sp. cultures on the germination of various seeds

Bacterization with	Seed germination rate, %					
	beet	barley	wheat	red radish	cucumber	tomato
Control (seed soaking in sterile water)	70	85	90	40	75	75
B. mobilis	90	90	100	60	85	100
Clostridium sp.	100	100	100	45	95	100

Table 2. The effect of bacterization on the seed germination rate and the growth of various cucumber cultivars

Bacterization with	Seed germination rate, %	Weight of one plant, g	Mean plant length (in cm) on day			
			12	21	40	50
Cultivar Vyaznikovskii						
Control	82	0.74 ± 0.05	7 ± 0.3	11 ± 0.4	12 ± 0.2	28 ± 2.8
B. mobilis	42	1.40 ± 0.08	7 ± 0.2	19 ± 1.1	33 ± 2.6	45 ± 2.3
Clostridium sp.	58	1.53 ± 0.07	8 ± 0.2	22 ± 2.0	38 ± 3.0	47 ± 3.3
Cultivar Parad						
Control	75	0.71 ± 0.12	10 ± 0.9	–	–	50 ± 2.5
B. mobilis	100	0.84 ± 0.15	13 ± 1.3	–	–	70 ± 5.6
Clostridium sp.	100	0.65 ± 0.08	12 ± 0.6	–	–	70 ± 8.3

Note: Plants were weighed and bacterized by means of foliar spraying on the 12th day of cultivation.

Bacterial biomass. Bacterial cells were grown in liquid mineral Gauze 1 medium and washed free of the growth medium by thrice centrifugation.

Test plants. Experiments were carried out with beet, barley, wheat, red radish, tomato, and cucumber cultivars Parad, Vyaznikovskii, and Libella plants.

Experiment design. Seed germination capacity was determined in laboratory experiments. To this end, seeds were soaked in bacterial suspensions containing 10^6 cells/ml for 10 h (control seeds were soaked in sterile distilled water for the same period of time), placed on wet filter paper in petri dishes, and allowed to germinate in a humid chamber at 20 to 22°C for 5–10 days.

Vegetative experiments were carried out in a greenhouse and involved (1) growing plants from bacterized seeds; (2) the foliar spraying of test plants with bacterial suspensions; (3) the introduction of the bacterial suspensions into the soil below the test plants; (4) the combined treatment of the test plants by the three aforementioned methods; (5) the treatment of the test plants with the two bacterial cultures taken either separately or together; and (6) the treatment of the test plants by each of the above five methods in combination with supplementary $P_{60}K_{120}$ and $N_{120}P_{60}K_{120}$ fertilization. The rhizosphere was inoculated with bacterial suspensions to give a density of 10^5 to 10^6 cells/g soil. Leaves were sprayed with bacterial suspensions containing 10^9 cells/ml. The test plants were grown in seedling pans containing 200 g or 4 kg of the greenhouse soil.

Monitoring of introduced bacterial cultures and study of their influence on indigenous microbial communities. The populations of introduced bacteria were analyzed using plates with a mineral Gauze 1 medium supplemented with 1 mg/ml streptomycin. Total bacterial count was performed by luminescence microscopy. Specimens for bacterial and fungal counts were stained with acridine orange and calcofluor white, respectively [17].

The rhizosphere and rhizoplane microbial communities were separated using the method proposed by Kirillova [18]. The rhizosphere microorganisms were first washed into a medium by shaking roots in this medium on a shaker, and the rhizoplane microorganisms were then detached from the roots by ultrasonic treatment.

Before microbiological analysis, soil and rhizosphere samples were sonicated using an UZDN-1 low-frequency ultrasonic disintegrator (22 kHz; 0.44 A; 2 min). The amount of soil washed off from the roots was determined by passing the washings through filter paper and weighing the air-dried control filters and filters with the residue cake. In all experiments, the mass of fresh roots was also determined.

RESULTS AND DISCUSSION

Table 1 summarizes data on the germination rate of bacterized seeds in a humid chamber. It can be seen that bacterization exerted a beneficial effect on seed germination in the chamber. The germination rate of bacterized cucumber cv. Vyaznikovskii seeds in the soil was slightly lower than that of the control seeds (Table 2). However, as early as on the 12th day of cultivation, the length and weight of cucumber plants grown from the bacterized seeds exceeded twofold the corresponding parameters of the control plants (Fig. 1). After the foliar bacterization of the experimental cucumber plants on the 12th day of cultivation, the experimental plants showed better growth and began flowering on the 30th day of cultivation, whereas the control plants began flowering only on the 40th day (Fig. 2).

Table 3. The effect of foliar spraying with a *Clostridium* sp. suspension on the growth of barley plants

Bacterization with	Mean plant length (in cm) on day	
	4	7
Control	4.5 ± 0.40	7 ± 0.85
Clostridium sp.	5.4 ± 0.45	18 ± 1.3

The bacterization of cucumber cv. Parad seeds enhanced their germination and promoted the growth of the seedlings. The bacterization of the experimental plants by means of side-dressing performed on the 12th day of cultivation led to an 1.5-fold increase in the plant height.

Fig. 1. The effect of seed bacterization with a *B. mobilis* culture on the growth of cucumber cv. Vyaznikovskii plants. Control 12-day-old plants were grown from seed soaked in sterile distilled water. Experimental plants of the same age were grown from bacterized seed. Scale, 1 : 5.

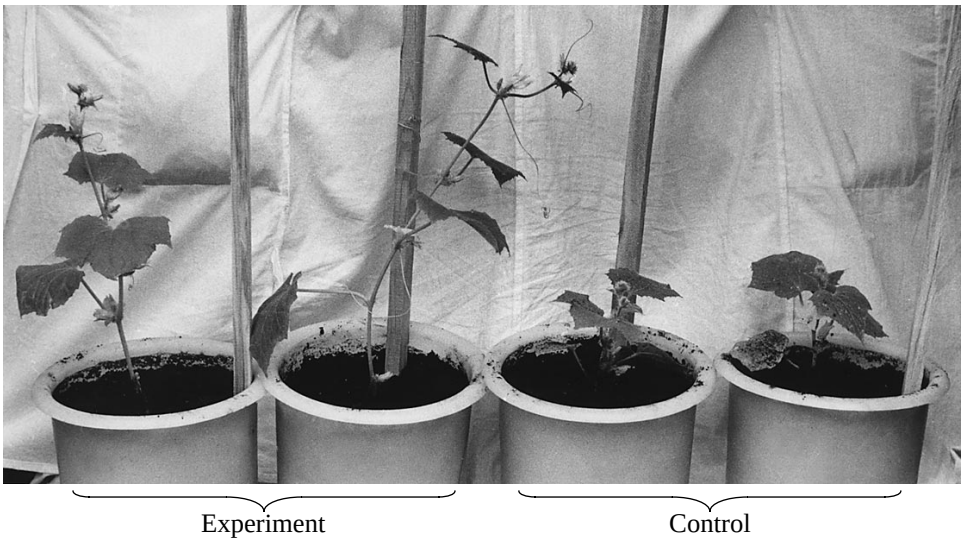
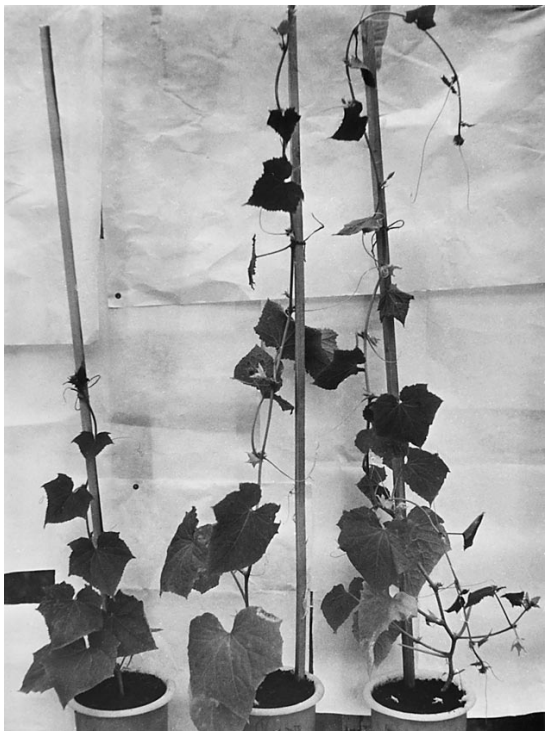


Fig. 2. Flowering of experimental cucumber cv. Vyaznikovskii plants after 1 month of cultivation. Control plants were grown from seed soaked in sterile distilled water. Experimental plants of the same age were grown from bacterized seed and additionally bacterized on the 12th day of cultivation by means of foliar spraying. Scale, 1 : 10.



Control Experiment B Experiment A

Fig. 3. Flowering of experimental cucumber cv. Libella plants after 1 month of cultivation. Control plants were grown from seed soaked in sterile distilled water. The ways of treatment of cucumber plants in experimental variants A and B are described in the text. Scale, 1 : 15.

The bacterization of barley seeds influenced little the growth of the seedlings (Table 3). However, the bacterization of the 4-day-old barley plants with a *Clostridium* sp. culture by means of foliar spraying promoted their growth by a factor of about 2.5 in comparison with the control.

Therefore, the bacterization of plants grown from bacterized seeds enhanced the beneficial effect of seed bacterization. The beneficial effect of plant bacterization depended on how the bacterial suspensions were applied (either foliar spraying or side-dressing).

The bacterization of cucumber cv. Libella plants enhanced the beneficial effect of mineral dressing with $N_{120}P_{60}K_{120}$ and $P_{60}K_{120}$ fertilizers. As early as the beginning of the vegetative period, the plants grown from bacterized seeds showed better growth than the control plants (Table 4). The combined bacterization of the experimental plants by foliar spraying and side-dressing (experiment A) was found to be the most efficient bacterization method. Side-dressing without foliar spraying (experiment B) was less efficient. Bacterial side-dressing and foliar spraying without seed bacterization (experiment C) were found to be completely inefficient.

When the $P_{60}K_{120}$ fertilizer was used, a statistically significant plant growth–promoting effect was observed in experiments A and B (Table 4). This effect was less pronounced than in the case of the $N_{120}P_{60}K_{120}$ fertilizer. Unlike the control plants, the experimental plants began flowering after 1 month of cultivation (Fig. 3). In experiment C, plant bacterization was efficient only when *B. mobilis* and *Clostridium* sp. cultures were applied together.

With the $N_{120}P_{60}K_{120}$ fertilizer, the mean weight of one plant and the production of cucumbers by one plant were maximal in experiment A, being higher than in the control by 30–60 and 50–140%, respectively (Table 5). In experiment B, side-dressing with a monoculture *Clostridium* sp. and a mixed culture *Clostridium* sp. +

Table 4. The effect of bacterization and mineral dressing on the growth of cucumber cv. Libella plants

Treatment	Experiment A		Experiment B		Experiment C	
	Mean plant length (in cm) on day					
	12	62	12	62	12	62
$N_{120}P_{60}K_{120}$	9 ± 0.8	172 ± 8.6	9 ± 0.8	182 ± 15	7 ± 0.5	153 ± 12
$N_{120}P_{60}K_{120}$ + *B. mobilis*	15 ± 1.5	220 ± 11	16 ± 1.6	195 ± 10	7 ± 0.4	153 ± 15
$N_{120}P_{60}K_{120}$ + *Clostridium* sp.	16 ± 1.6	230 ± 14	14 ± 1.4	220 ± 11	7 ± 0.6	147 ± 12
$N_{120}P_{60}K_{120}$ + *B. mobilis* + *Clostridium* sp.	14 ± 1	230 ± 23	15 ± 1.4	187 ± 10	7 ± 0.4	140 ± 14
$P_{60}K_{120}$	9 ± 0.9	163 ± 8.2	9 ± 0.9	163 ± 13	6 ± 0.4	150 ± 12
$P_{60}K_{120}$ + *B. mobilis*	19 ± 1.6	207 ± 12	18 ± 1.4	183 ± 15	7 ± 0.7	151 ± 18
$P_{60}K_{120}$ + *Clostridium* sp.	15 ± 1.5	180 ± 16	19 ± 1.9	203 ± 20	6 ± 0.4	110 ± 9
$P_{60}K_{120}$ + *B. mobilis* + *Clostridium* sp.	19 ± 1	230 ± 18	18 ± 1.4	204 ± 10	7 ± 0.4	207 ± 11

Note: Experiment A represents the bacterial side-dressing and foliar spraying of 12-day-old plants grown from bacterized seeds. Experiment B represents the bacterial foliar spraying of 12-day-old plants grown from bacterized seeds. Experiment C represents the bacterial side-dressing and foliar spraying of 12-day-old plants grown from untreated seeds.

Table 5. The effect of bacterization and mineral dressing on the growth of cultivar Libella plants and cucumber production

Treatment	Experiment A		Experiment B		Experiment C	
	1	2	1	2	1	2
$N_{120}P_{60}K_{120}$	200 ± 10	800 ± 56	210 ± 16	900 ± 48	180 ± 16	600 ± 80
$N_{120}P_{60}K_{120}$ + *B. mobilis*	260 ± 21	1200 ± 96	230 ± 10	1200 ± 45	290 ± 14	1400 ± 55
$N_{120}P_{60}K_{120}$ + *Clostridium* sp.	300 ± 16	1900 ± 95	280 ± 18	2300 ± 92	200 ± 16	800 ± 72
$N_{120}P_{60}K_{120}$ + *B. mobilis* + *Clostridium* sp.	320 ± 15	1500 ± 90	370 ± 21	1600 ± 62	240 ± 12	1300 ± 65
$P_{60}K_{120}$	190 ± 12	700 ± 48	180 ± 18	800 ± 56	170 ± 12	500 ± 72
$P_{60}K_{120}$ + *B. mobilis*	240 ± 30	1600 ± 64	240 ± 12	1400 ± 56	170 ± 18	1200 ± 60
$P_{60}K_{120}$ + *Clostridium* sp.	220 ± 13	1200 ± 60	260 ± 18	1900 ± 76	160 ± 10	700 ± 72
$P_{60}K_{120}$ + *B. mobilis* + *Clostridium* sp.	260 ± 10	1500 ± 50	280 ± 16	1600 ± 48	280 ± 18	1300 ± 78

Note: Experiments A, B, and C are described in the note to Table 4. Columns 1 show the weight (in g) of one fresh mature plant. Columns 2 show the yield of cucumbers (in g) from one plant.

B. mobilis were also efficient. In experiment C, side-dressing was efficient with a monoculture *B. mobilis* and the mixed culture.

With the $P_{60}K_{120}$ fertilizer, bacterization in experiment A increased the cucumber yield by 70 to 130%, the increase being almost independent of the bacterial culture used. In experiment B, the mean weight of plants and crop yield were, respectively, by 30–60% and 75–140% higher than in the control, and the results depended on the bacterial culture used. In experiment C, plant growth–promoting effects were observed for *B. mobilis* and the mixed culture.

The genetic marking of the bacteria allowed their distribution in the cucumber rhizosphere and the surrounding soil to be studied. The introduced bacteria could be detected in these habitats throughout the observation period (Fig. 4). The bacterium *B. mobilis* quickly colonized the plant rhizoplane and, therefore, had characteristics of an *r*-strategist. At the same time, *Clostridium* sp. was closer to *K*-strategists, since this bacterium slowly colonized the econiches studied. The analysis of prokaryotic and eukaryotic microorganisms by epifluorescence microscopy using specific dyes showed that the introduced bacterium *B. mobilis* did not influence the indigenous bacterial complex of the cucumber rhizosphere (Fig. 5), although, taking into account the results published by Mahaffe and Kloepper [19], some changes in this complex were to be expected. The introduction of *Clostridium* sp. promoted the development of indigenous bacterial populations in the cucumber rhizosphere and the surrounding soil but prevented the colonization of the cucumber roots by the fungal mycelium (Fig. 6). This could be due to the antifungal activity of *Clostridium* sp.

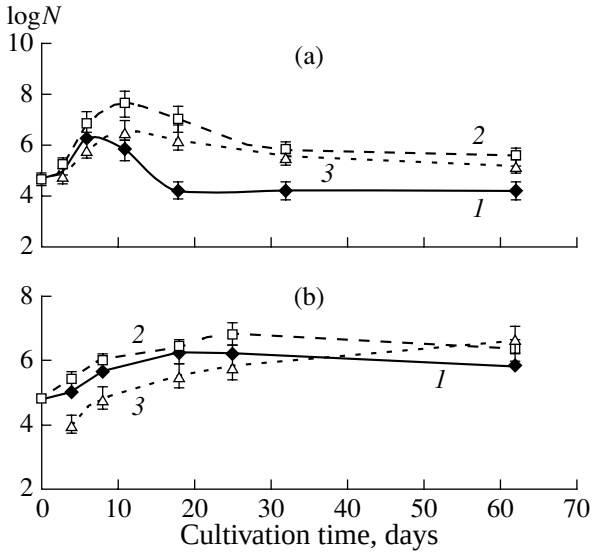


Fig. 4. The population dynamics of introduced (a) *B. mobilis* and (b) *Clostridium* sp. cultures in the (*1*) soil, (*2*) rhizosphere, and (*3*) rhizoplane of cucumber cv. Libella plants. The population density *N* is expressed in CFU/g soil or roots.

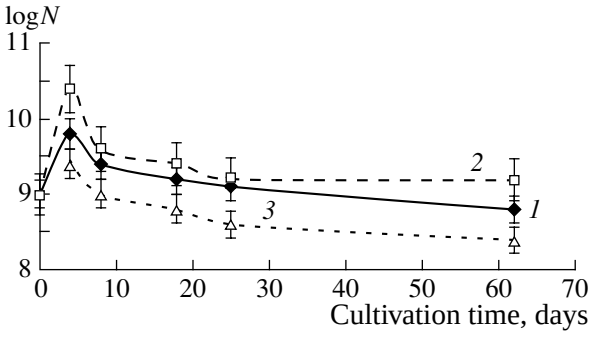


Fig. 5. The population dynamics of indigenous soil bacteria in the (*1*) soil, (*2*) rhizosphere, and (*3*) rhizoplane of cucumber cv. Libella plants in the presence of introduced *B. mobilis* culture. The population density *N* is expressed in cells/g soil or roots.

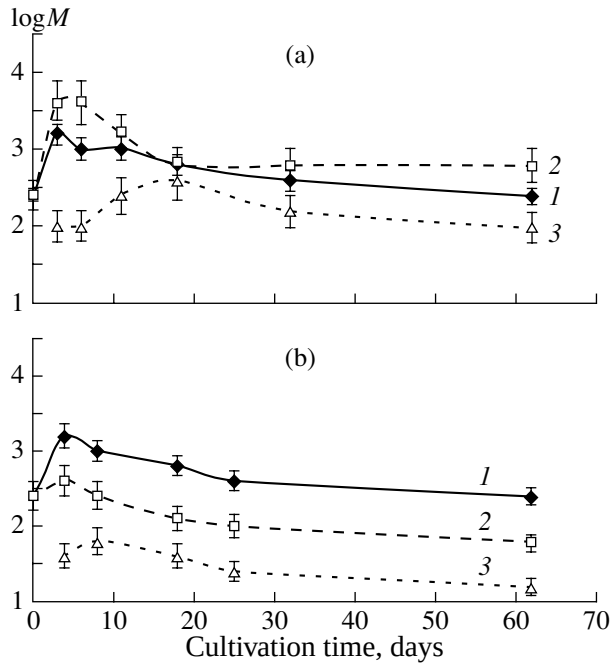


Fig. 6. The effect of introduced *Clostridium* sp. culture on the length of the fungal mycelium in the (*1*) soil, (*2*) rhizosphere, and (*3*) rhizoplane. The length of the fungal mycelium (*M*) is expressed in m/g soil or roots.

itself or the activation of antifungal indigenous soil bacteria [20].

In conclusion, the introduced bacteria *B. mobilis* and *Clostridium* sp. exerted a substantial promoting effect on the growth of cucumber and barley plants. The data obtained in experiments with the $P_{60}K_{120}$ and $N_{120}P_{60}K_{120}$ fertilizers can be interpreted as indicating that the nitrogen-fixing activity of these bacteria play a role in their beneficial effect on plants. Among other possible mechanisms, noteworthy are the synthesis of plant growth–promoting metabolites by the introduced bacteria or the inhibition of deleterious soil micromycetes by these bacteria. The combined effect of several mechanisms is also possible. The contribution of particular mechanisms to the overall effect of introduced bacteria may depend on the physiology of plants and the agronomic conditions of their cultivation.

It should be noted that the bacterization of plants significantly enhanced their response to mineral fertilization. As for the economic benefit of bacterization, it may appear beneficial not only in the greenhouse but also in open-air agriculture.

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