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REMEDIATION OF SOIL CONTAMINATED WITH OIL, LEAD AND CADMIUM BY INOCULATION WITH PLANT GROWTH PROMOTING RHIZOBACTERIA

Valery P. Shabayev
Institute of Physicochemical and Biological Problems in Soil Science,
Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia

ABSTRACT

Application of Pseudomonas rhizosphere bacteria promoting the growth of various agricultural plants was shown to eliminate to a significant extent the phytotoxicity of oil-contaminated soil for oat plants in greenhouse pot experiment and to have the same efficiency as a sorbent consisting of hydrolyzed lignin. A combined use of bacteria and sorbent did not enhance their positive effects. Inoculation with bacteria resulted in a significant decrease in the uptake of Pb by barley plants, their increased resistance to its toxic action and a decrease of Cd uptake in a series of greenhouse pot experiments on a soil contaminated with water-soluble compounds of these heavy metals.

Keywords: Oil, plant growth, Pb and Cd in plants and soil, Pseudomonas bacteria

INTRODUCTION

Remediation of oil-polluted soils is performed at present using a broad range of efficient commercial oil-oxidizing bacterial compositions. The developed compositions comprise bacteria of various systematic groups, of the genus *Pseudomonas* including [1, 2]. In the recent decade, phytoremediation – the use of plants and microbe–plant complexes – has been increasingly developed and applied [3]. Various organic and mineral sorbents with high contaminant absorption capacities [1], including those that contain hydrolyzed lignin [4], have also been proposed. Numerous research works show a high efficiency of oil-oxidizing microbial compositions and various sorbents in remediation. One of the promising remediation techniques for heavy metal-polluted soils is also considered to be the use of plant growth promoting rhizosphere bacteria [5, 6]. Research into the application of microbe–plant complexes, including those that comprise plant growth promoting rhizobacteria and introduction of hydrolized lignin, for remediation of oil-polluted soils, as well as into the effects of various bacteria on the behaviour of heavy metals in the soil–plant system, has been insufficient.

The aim of this work was to compare the effects of plant growth promoting *Pseudomonas* rhizosphere bacteria and a sorbent (hydrolyzed lignin) on plant growth at the contamination of soil with oil, as well as the action of bacteria at the contamination of soil with lead and cadmium.

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MATERIALS AND METHODS

Studies were conducted in greenhouse pot experiments during the growth of oat and barley plants on soil artificially contaminated with, respectively, oil and water-soluble Pb and Cd compounds against the background of optimal doses of NPK fertilizers and moisture conditions. Plants of barley (*Hordeum vulgare* L., cv. Moskovsky 2) and oat (*Avena sativa* L., cv. Suzdalets) were used; gray forest soil was from southern Moscow Region. The chemical characteristics of soil in the experiments varied: pH, within the range of 5.06–6.10; organic C, 0.96–1.15%; total N, 0.11–0.13%; P and K (0.2 N HCl), 42–87 and 86–220 mg kg^{-1} soil, respectively. The soil was fertilized with N, P and K in terms of 80 mg each element per kg soil as NH_4NO_3, KH_2PO_4 and K_2SO_4. Planted seeds were inoculated with *P. fluorescens* 20, *P. fluorescens* 21 and *P. putida* 23 bacteria promoting the growth of various agricultural crops [7]. Sterilized seeds were placed on the surface of soil in the pot, inoculated with aqueous suspensions of the bacteria at a rate of 10^8 colony-forming units per plant and covered with a 2-cm-thick layer of soil. Adequate amounts of autoclaved bacterial suspensions were used in variants without inoculation. Each pot was watered up to 60% WHC of soil. Treatments of variants were repeated four times. After cutting plants and taking soil samples, roots were separated from soil by tap water and washed with distilled water. Shoots and roots were dried at 70°C, weighed and ground to uniform consistency. The results of the studies were processed statistically by the analysis of variance, in which the treatment effects were evaluated. Significant differences between treatments were established by the least significant differences (LSD). Calculations were performed using the Statgraphics software package. All tests were considered significant at a 5% level ($P < 0.05$). Data for some parameters were also expressed as the mean ± standard deviation for each treatment.

Experiment 1: oil-contaminated soil

In pots (diameter, 6.5 cm; height, 7.5 cm) containing 200 mg of oil-contaminated soil each, five oat plants per each pot were grown up to the stooling stage for one month. Oil used in the experiment was from an Ufa oil deposit (Russia). Oil was applied on the surface of soil in a pot in terms of 5% of soil weight; then, the soil was held for one month for oil's light ends to evaporate. After that, the upper 2-cm layer of soil in the pot was loosened and sprouted seeds were planted. In one series of the experiment, seeds were inoculated with *P. fluorescens* 20, *P. fluorescens* 21 and *P. putida* 23 bacteria. In another, prior to the inoculation with these bacteria, a fine powder of polyphepan (Scientek, Russia) – a naturally occurring sorbent 90% of which is hydrolyzed lignin – was mixed with the upper 2-cm layer of oil-contaminated soil in terms of 5% soil weight in the pot. Besides, there were variants without bacterial inoculation for oil-contaminated soil, both without and with introduction of sorbent. The variant without oil contamination, without introduction of sorbent and without bacterial inoculation served as a control.

Experiments 2 and 3: Pb- and Cd-contaminated soils

In Experiment 2, in pots (diameter, 6.5 cm; height, 7.5 cm) containing 200 mg of oil each, five barley plants per each pot were grown up to the tillering stage for 14 days at the addition of $Pb(CH_3COO)_2$ (Reakhim, Russia) in terms of 300 mg Pb per kg soil. In the first variant, strain *P.*

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fluorescens 20 was added; in the second, strain *P. putida* 23. There was also a variant without bacterial inoculation.

In Experiment 3, in pots (diameter, 9.5 cm; height, 7.5 cm) filled with 800 mg of soil each, five barley plants per each pot were grown up to the heading stage for 45 days. In one series of experiment, $Pb(NO_3)_2$ (Merck, Germany) was added into soil in terms of 200 mg per kg soil; in the other, $Cd(NO_3)_2$ (Reakhim, Russia) in terms of 10 mg Cd per kg soil. Strain *P. fluorescens* 20 in one variant and strain *P. fluorescens* 21 in the other were introduced into the contaminated soil. Variants with and without contamination in the absence of bacterial inoculation were also used.

The heavy metal salts were thoroughly mixed with the soil volume in the pot 10 days before seed planting and inoculation. Treatments with $Pb(NO_3)_2$ or $Cd(NO_3)_2$ were adjusted to the same level of nitrogen as in the corresponding variants of uncontaminated and nitrogen-fertilized soil.

A 1-g plant sample was digested in a 20-ml mixture of concentrated HNO_3 and $HClO_4$ (2:1 v/v) and analyzed for the content of lead and cadmium. Air-dried soil samples were ground and passed through a 1-mm sieve. The contents of lead and cadmium in the fraction extracted from soil with 1 N CH_3COONH_4 (pH 4.8) after shaking 3 mg soil with 30 ml reagent were determined. An Optima 5900 DV inductively coupled plasma optical emission spectrometer (Perkin Elmer, USA) was used to measure the heavy metal concentrations in solutions. The pH of the soil suspension was assessed by a pH 211 pH meter (HANNA Instruments, Germany) after shaking 20 g soil with 50 ml of 1 N KCl.

RESULTS AND DISCUSSION

Experiment 1 found a significant inhibition of oat plant growth at the oil contamination of soil (Table 1, Figure). Inoculation with *Pseudomonas* bacteria and introduction of sorbent substantially reduced the negative effect of oil, thus contributing to a significant growth of plants under contamination conditions and, herewith, exerting approximately equal effects on them. The largest positive effect of bacterial inoculation was observed for *P. fluorescens* 21 and *P. putida* 23; at their application, the shoot weights of plants were, respectively, 54% and 62% with respect to the control without oil contamination, without sorbent introduction and without bacterial inoculation. *P. fluorescens* 20 had a smaller effect on plants. Introduction of sorbent into pots together with bacterial inoculation, as compared with their separate application, did not promote further plant growth under oil contamination conditions. The obtained regularities for the bacteria were also preserved at their application with sorbent.

Application of bacteria considerably decreased the uptake of Pb by barley plants in Experiments 2 and 3 (Table 2). In Experiment 2, the heavy metal content in shoots of plants in the tillering phase at the inoculation with *P. putida* 23 or *P. fluorescens* 20 was found to be, respectively, almost two and three times as low as in the uninoculated variant. In the early periods of plant growth in the above experiments, there was no noticeable difference in the weights of plants untreated and treated with bacteria. The most significant decrease in the concentration of lead in shoots under the influence of bacterial inoculations was observed in the heading stage in Experiment 3. The use of *P. fluorescens* 20 and *P. fluorescens* 21 reduced the Pb concentration in

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shoots 3.5- and 7-fold, respectively, in comparison with the elevated concentration of metal in the uninoculated variant. The presence of *P. fluorescens* 21 also reduced the Pb content in roots by 23%. Irrespective of bacterial inoculation, the root system contained an average of two orders of magnitude more metal than shoots.

Experiment 3 showed only a slight decrease in shoot weight during the heading stage in Pb-contaminated soil without bacterial inoculation (Table 2). Under Pb contamination conditions, the use of *P. fluorescens* 21 was found to have a positive effect on barley plant growth in the heading stage in this experiment. This was expressed in an increase of the plant shoot and root weight by, respectively, 17% and 19%. Besides, at the inoculation with both bacteria, we observed no toxic effect of metal on plants; at the inoculation with *P. fluorescens* 20, their biomass was on the same level; and with *P. fluorescens* 21, both the top part weight and the root weight were even greater as compared with the variant without Pb contamination and without inoculation. Growth response induced by inoculation with *P. fluorescens* 20 was less than that for *P. fluorescens* 21. A decrease in the concentration of Pb at the inoculation with bacteria corresponded to a 1.5-fold greater metal content in the soil fraction extracted with ammonium acetate and occurred without a change of soil pH (Table 2).

At the application of only *P. fluorescens* 21, Experiment 3 found a more than twofold decrease of Cd content in barley shoots in the heading stage; in roots, it decreased by 10% (Table 3). Herewith, the soil fraction extracted with acetate ammonium buffer solution contained four times as much Cd and no change of the soil medium reaction in it was registered. Inoculation with *P. fluorescens* 20 had no effect on the behaviour of metal in the soil–plant system. The root system of both uninoculated and inoculated plants contained approximately by an order of magnitude more Cd than in shoots. Contamination of soil with Cd was found not to inhibit plant growth; no positive effect of bacterial inoculation was observed in this case, either, which is, probably, due to the fact that at certain concentrations this metal has no negative effect on plants [8].

The key mechanism of restoring oil-polluted soils is degradation of hydrocarbons, which is enhanced at the introduction of oil-oxidizing bacteria, *Pseudomonas* including [1, 2]. Plant growth promoting bacteria can modify the growth and development of plants by producing antibiotics and suppressing phytopathogenic microorganisms [5], which develop in soil at oil and other pollutions [1, 3]. A positive effect from these bacteria can be also due to increased absorption of nutrients by plants owing to the formation of growth hormones [5, 9] and other mechanisms, including atmospheric nitrogen fixation [5, 7, 9] and protection of plants from heavy metals [5, 6]. The present observations indicated that the Pb concentration in inoculated plants, including roots, decreased without altering the reaction of the soil medium and that other mechanisms were responsible for the process. It is well known that the pH of soil has a direct impact on the mobility of metals. Concentrations of divalent cations in soil solution and their uptake by plants are usually reduced at high pH values. It is likely that the investigated *Pseudomonas* bacteria decreased the mobility of Pb and Cd in soil and their toxicity for plants as a consequence of the binding of heavy metals in soil by bacterial exometabolites siderophores [10] into relatively stable organic compounds extractable with ammonium acetate.

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The positive effect of introducing hydrolyzed lignin-based sorbent on plant growth is, undoubtedly due to its high ability to sorb oil, an improvement of the physico-chemical properties of soil and the activation of the aboriginal hydrocarbon-assimilating microflora [4]. The predominant accumulation of Pb and Cd in roots is, undoubtedly, determined by the barrier functions of the root system with respect to toxic heavy metals [8].

CONCLUSIONS

Application of *Pseudomonas* plant growth promoting rhizosphere bacteria substantially decreases the negative effect of oil pollution of soil on the growth of cultivated plants. On oil-contaminated soil, the largest effect during the growth of oat was found to be at the use of *P. fluorescens* 21 and *P. putida* 23. Application of bacteria on oil-contaminated soils is not inferior by efficiency as sorbent consisting of hydrolyzed lignin, and in this respect it has an advantage over introduced, as a rule, large amounts of sorbents. A combined application of bacteria and sorbent has no advantages over their separate use at oil contamination. Application of all investigated *Pseudomonas* bacteria significantly reduces the uptake of Pb by barley plants. Introduction of *P. fluorescens* 21 into the rhizosphere totally eliminates the toxic action of Pb on plants, to the highest degree reducing the metal uptake by plants, and decreases the accumulation of Cd in barley plants. A decrease in the uptake of heavy metals by inoculated plants is, probably, due to the binding of the metals in relatively stable complexes – a soil fraction extracted by ammonium acetate without changing the soil medium reaction. Considering that the composition of oil includes a broad range of heavy metals, including Pb and Cd, the positive effect of these bacteria on oil-contaminated soils can be due to, among other factors, a decrease in the phytotoxicity of these heavy metals.

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Table 1. Weight of oat plants at contamination of soil with oil, inoculation with bacteria and introduction of sorbent

Experiment number, plant development stage	Variant	Shoot dry weight	
		g/pot	% of control
Experiment 1, stooling	Control (no oil contamination, no sorbent, no inoculation)	0.61	100
	Oil	0.12	20
	Oil + *P. fluorescens* 20	0.24	39
	Oil + *P. fluorescens* 21	0.38	62
	Oil + *P. putida* 23	0.33	54
	Oil + sorbent	0.30	50
	Oil + sorbent + *P. fluorescens* 20	0.24	39
	Oil + sorbent + *P. fluorescens* 21	0.33	54
	Oil + sorbent + *P. putida* 23	0.33	54
	HCP_{05}	0.07	

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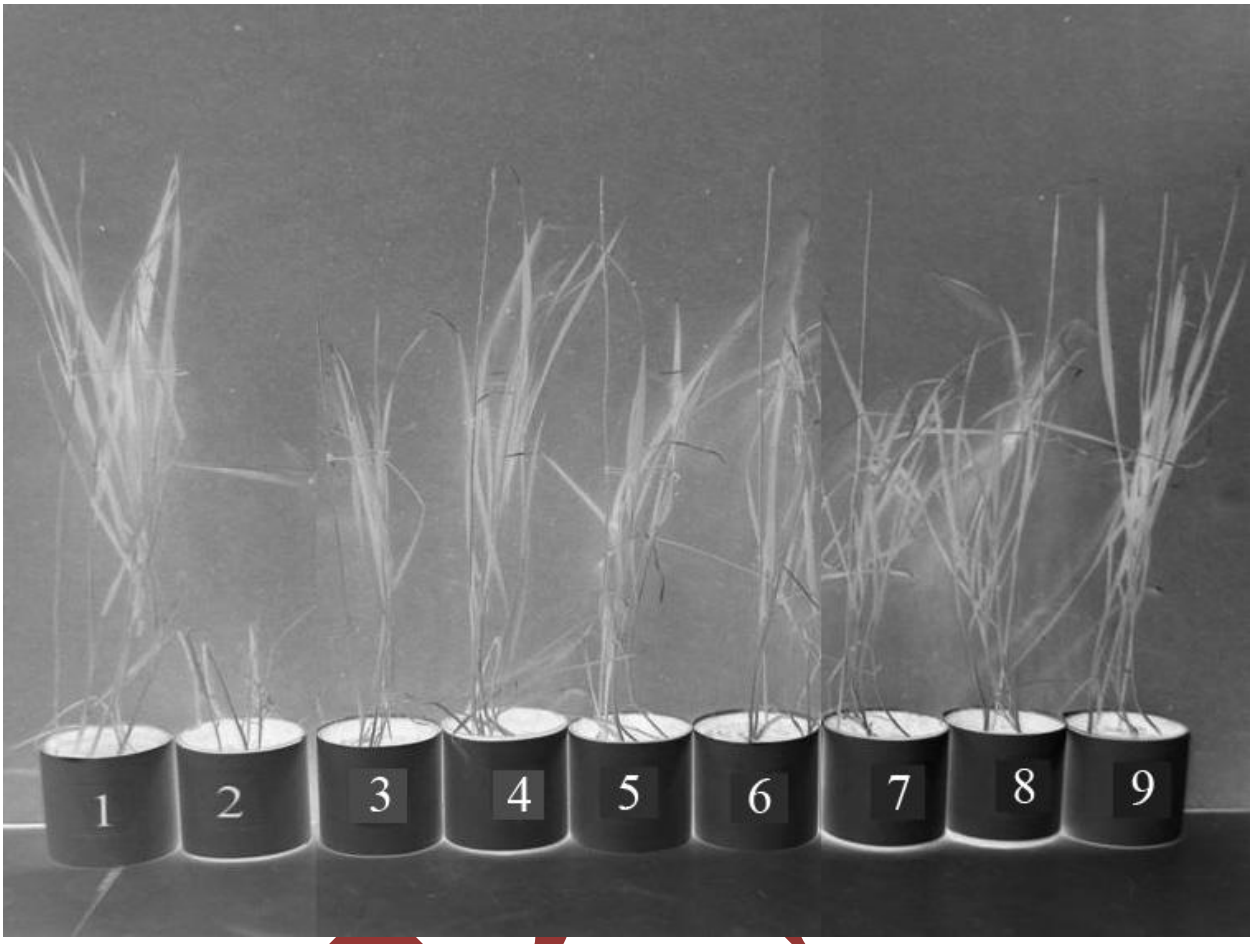

Figure. Oat plants (stooling stage), Experiment 1. 1, Control (no contamination with oil, no introduction of sorbent, no inoculation with bacteria); 2, oil; 3, oil + *P. fluorescens* 20; 4, oil + *P. fluorescens* 21; 5, oil + *P. putida* 23; 6, oil + sorbent; 7, oil + sorbent + *P. fluorescens* 20; 8, oil + sorbent + *P. fluorescens* 21; 9, oil + sorbent + *P. putida* 23.

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Table 2. Content of lead in barley plants, plant weight and soil indices at lead contamination and bacterial inoculation

Experiment number, plant development stage	Pb contamination, mg/kg soil	Variant	Indices at the end of experiment after plant growth	
Experiment 2, tillering	300	Pb without inoculation	Pb content in shoots, mg/kg	34
		Pb + *P. fluorescens* 20		11a
		Pb + *P. putida* 23		18a
		Pb without inoculation	Shoot dry weight, g/pot	0.14
		Pb + *P. fluorescens* 20		0.13
		Pb + *P. putida* 23		0.14
Experiment 3, heading	200	Without Pb without inoculation	Pb content in shoots, mg/kg	ND
		Pb without inoculation		7b
		Pb + *P. fluorescens* 20		2ab
		Pb + *P. fluorescens* 21		1ab
		Without Pb without inoculation	Pb content in roots, mg/kg	59a
		Pb without inoculation		188b
		Pb + *P. fluorescens* 20		173b
		Pb + *P. fluorescens* 21		144ab
		Without Pb without inoculation	Shoot dry weight, g/pot	2.16
		Pb without inoculation		1.99
		Pb + *P. fluorescens* 20		2.13
		Pb + *P. fluorescens* 21		2.33a
		Without Pb without inoculation	Root dry weight, g/pot	0.52
		Pb without inoculation		0.54
		Pb + *P. fluorescens* 20		0.53
		Pb + *P. fluorescens* 21		0.64ab
		Without Pb without inoculation	Pb content in AAB soil extract, mg/kg	ND
		Pb without inoculation		24b
		Pb + *P. fluorescens* 20		39ab
		Pb + *P. fluorescens* 21		35ab
		Without Pb without inoculation	pH of soil	5.18a
		Pb without inoculation		5.36b
		Pb + *P. fluorescens* 20		5.42b
		Pb + *P. fluorescens* 21		5.40b

Values are means ± standard deviations; ND, non-detectable.

[a] Significantly different from the corresponding variant with Pb without inoculation.

[b] Significantly different from the corresponding variant without Pb without inoculation.

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Table 3. Content of cadmium in barley plants, plant weight and soil indices at cadmium contamination and bacterial inoculation

Experiment number, plant development stage	Cd contamination, mg/kg soil	Variant	Indices at the end of experiment after plant growth	
Experiment 3, heading	10	Without Cd without inoculation	Cd content in shoots, mg/kg	ND
		Cd without inoculation		7b
		Cd + *P. fluorescens* 20		6b
		Cd + *P. fluorescens* 21		3ab
		Without Cd without inoculation	Cd content in roots, mg/kg	ND
		Cd without inoculation		81b
		Cd + *P. fluorescens* 20		84b
		Cd + *P. fluorescens* 21		71ab
		Without Cd without inoculation	Shoot dry weight, g/pot	2.16
		Cd without inoculation		2.21
		Cd + *P. fluorescens* 20		2.08
		Cd + *P. fluorescens* 21		2.14
		Without Cd without inoculation	Root dry weight, g/pot	0.52
		Cd without inoculation		0.54
		Cd + *P. fluorescens* 20		0.57
		Cd + *P. fluorescens* 21		0.62ab
		Without Cd without inoculation	Cd content in AAB soil extract, mg/kg	ND
		Cd without inoculation		4b
		Cd + *P. fluorescens* 20		3b
		Cd + *P. fluorescens* 21		18ab
		Without Cd without inoculation	pH of soil	5.18a
		Cd without inoculation		5.37b
		Cd + *P. fluorescens* 20		5.36b
		Cd + *P. fluorescens* 21		5.48b

Values are means ± standard deviations; ND, non-detectable.

[a] Significantly different from the corresponding variant with Cd without inoculation.

[b] Significantly different from the corresponding variant without Cd without inoculation.