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Promising Strains of Hydrocarbon-Oxidizing Pseudomonads with Herbicide Resistance and Plant Growth-Stimulating Properties for Bioremediation of Oil-Contaminated Agricultural Soils

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Abstract: Nowadays, large areas of agricultural land are contaminated with chemical plant-protection products. Agricultural soils are also susceptible to oil pollution as a result of accidents on oil pipelines. Bioremediation of such soils from oil with the help of hydrocarbon-oxidizing bacteria is hindered by the presence of additional pollutants such as herbicides. In this work, seven strains of *Pseudomonas* were isolated and identified, which showed differences in ability of oil biodegradation (32.7–77.3%). All strains showed resistance to herbicides based on 2,4-D and substances from the class of imidazolinones, possessed phosphate-solubilizing and nitrogen-fixing activity, and produced indolyl-3-acetic acid (305–1627 ng/mL culture liquid). They stimulated the growth of barley and clover in soil with oil, as well as the growth of clover in soil with herbicide. In a vegetative experiment of barley plants and *P. alcaligenes* UOM 10 or *P. frederiksbergensis* UOM 11, oil degradation was 48.1–52.7%, the same strains and clover plants, 37.9–38.6%. The studied bacteria have the potential to be used in the bioremediation of oil-contaminated agricultural soils, including in combination with phytomeliorant plants.

Keywords: *Pseudomonas*; oil; herbicides; bioremediation; plant growth-stimulating properties; growth-stimulating activity; barley; clover

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1. Introduction

In the near future, humanity is not ready to abandon the use of hydrocarbons as the main source of energy. Therefore, the pace of exploration, exploitation of deposits, and processing of these minerals will increase. Under such circumstances, the negative impact on the environment will also increase. Soil experiences the greatest impact, as it accumulates hydrocarbons, which change its physicochemical properties and suppress biological activity [1]. This, in turn, leads to inhibition of plant growth and development [2–4].

All over the world, significant areas of agricultural soil are contaminated with oil and oil products [5,6]. In most cases, arable soils were already initially contaminated with various chemical plant-protection products that are widely used in intensive crop production. Among them, the most numerous class (48% of the global consumption of pesticides [7]) are herbicides, which are used to kill weeds. Like petroleum products, these substances negatively affect soil microbiocenosis [8–11] and inhibit plant growth and physiological functions [12,13].

Being simultaneously present in the soil, pollutants can enhance the negative effects of each other, which greatly complicates soil purification and restoration [14,15]. Many studies of the separate effect of oil and herbicides on soil and soil biocenosis have been carried out, and various methods for the removal of these xenobiotics have been proposed [16–20]. However, there are very few publications devoted to the development of

methods for cleaning oil-contaminated soils in the presence of such additional toxicants as herbicides [21].

Bioremediation based on the ability of living organisms to decompose pollutants is considered a simple, safe, and cost-effective way to eliminate the consequences of anthropogenic pollution [22–25]. One of its directions is phytoremediation, which makes it possible to rehabilitate lands after various organic and inorganic pollutants have entered them [26,27]. The main limiting factor for its widespread use is the decrease in plant biomass as a result of stress caused by a combination of unfavorable conditions (pollutant toxicity, violation of the water–air regime of the soil, reduced availability of micronutrients, etc.). This, in turn, can lead to very slow recovery rates. It is known that bacteria can contribute to overcoming abiotic stresses in plants [28–30]. Therefore, in recent years, when developing approaches to soil bioremediation, special attention has been paid to bacterial strains that, on the one hand, are resistant and capable of biodegrading various pollutants, and, on the other hand, improve the growth of remediating plants [31].

From this point of view, bacteria of the genus *Pseudomonas* may be of great interest [32]. They are capable of biodegradation of various pollutants (including hydrocarbons and oil) due to the presence of enzymatic systems that catalyze the reactions of biotransformation of almost all classes of organic compounds in a wide range of concentrations and environmental conditions [33,34]. Many pseudomonads have additional genetic material in the form of biodegradation plasmids, which contain genes responsible for the degradation of various pollutants [35–37] and produce biosurfactants that facilitate the dispersion and solubilization of hydrophobic substances, hydrocarbons in particular [38,39].

Many bacteria of the genus Pseudomonas exhibit plant growth-stimulating properties (PGP properties, or Plant Growth-Promoting properties), that is, they can positively influence plant growth and development through the synthesis of phytohormones and siderophores and increase the availability of trace elements, suppress phytopathogens, and induce resistance to abiotic stress such as drought, mineral deficiency, and the presence of pesticides [40–43]. *Pseudomonas* spp. are widespread in the environment and are convenient to cultivate, since they maintain sufficient numbers on minimal nutrient media, have a high reproduction rate, and can be grown using cheap raw materials (for example, waste from other production, such as waste from the sunflower-oil process, waste frying oil, beet molasses) [44–50].

The aim of this study was to isolate new strains of hydrocarbon-oxidizing bacteria of the genus *Pseudomonas* resistant to herbicides from oil-contaminated agricultural soils and to study their PGP properties. We assumed that the isolated strains, in addition to their resistance to herbicides and ability to destroy oil, would also stimulate the growth and development of phytomeliorant plants. The results of these experiments can be used in studying the features of interaction between microorganisms and plants in cases of complex oil and herbicide pollution, and in developing methods for cleaning and restoring such anthropogenically disturbed areas.

2. Materials and Methods

2.1. Isolation of Hydrocarbon-Degrading Strains

Strains of hydrocarbon-oxidizing microorganisms were isolated from samples of oil-contaminated arable soils from the territory of the Republic of Bashkortostan, where various herbicides were used. We used the method of enrichment cultures [51]. A 100 mL measure of liquid Raymond mineral medium (composition (g/L): NH_4NO_3—2.0, $MgSO_4 \times 7H_2O$—0.2, KH_2PO_4—2.0, Na_2HPO_4—3.0, $CaCl_2 \times 6H_2O$—0.01, Na_2CO_3—0.1, pH—7.0) [52] was introduced into flasks, and 1% (*w*/*v*) oil (the only source of carbon and energy) and 2 g of soil were added. After that, cultivation was carried out on an orbital shaker-incubator ES-20/60 (SIA BIOSAN, Latvia) at 28 °C and 160 rpm for 7 days. Further seeding from enrichment cultures was carried out on nutrient agar (NA) (composition (g/L): peptone—5.0, yeast extract—3.0, glucose—1.0, NaCl—5.0, agar–agar—15.0), after which the microorganisms were cultivated for 5 days at 28 °C.

For further studies, the most actively growing isolates on agar and liquid Raymond medium with oil as the sole carbon source were selected. The intensity of growth in a liquid medium with oil (4% *w*/*v*) was estimated from the change in the appearance of the medium and the change in pH.

2.2. Identification of the Isolates

Cell morphology was studied using a Solver Pro-M scanning probe microscope (NT-MDT, Russia). Physiological and biochemical properties were studied according to generally accepted methods [53,54]. Identification of strains was carried out by MALDI-TOF mass spectrometry as described in [55]. Spectra were registered in the linear mode with delayed ion extraction using Autoflex Speed (Bruker Daltonics, Germany) with a time-of-flight analyzer (delay time was 350 ns, acceleration potential was 20 kV). The record of spectra was processed in positive-ion mode; the range of registered masses was 2–20 kDa. The external calibration of the analyzer was processed to a mixture of proteins of the Bruker Bacterial Test Standard (Bruker Daltonics); the resolution of the spectra was ±2 Da. The resulting spectra for each strain preparation were obtained by summing the spectra registered in 10–15 points of the analyzed preparations at 500 hits of the laser. The taxonomic classification decision of the strain was made using the Biotyper 3.0 program (Bruker Daltonics). MALDI-TOF mass spectrometry identification results were accepted at genus or species level according to Bruker's instructions. High-confidence identification indicates a score in the range of 2.00–3.00, which means reliable identification at species level. Low-confidence identification is accepted at genus level, with the score of 1.7–1.99. Scores below 1.7 are considered as non-reliable identifications at any level.

To clarify the species affiliation of microorganisms, 16S rRNA gene nucleotide sequences were determined. Total DNA from bacterial colonies was isolated according to the procedure described in [56]. Amplification of the 16S rRNA gene fragment was carried out with universal primers 27F (5′-AGAGTTTGATCTGGCTCAG-3′) and 1492R (5′-ACGGTACCTTGTTACGACTT-3′) [57] on a C1000 TouchTM Thermal Cycler amplifier (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were purified, and the subsequent sequencing reaction was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions, with the help of the ABI PRIZM 3730 automatic sequencer (Applied Biosystems, Waltham, MA, USA). The search for 16S rRNA nucleotide sequences similar to the corresponding sequences of the studied strains was carried out using the EzBioCloud server (https://www.ezbiocloud.net, accessed on 1 March 2023). The MEGA 6.06 software was used to construct a phylogenetic tree using the neighbor-joining method [58] with 1000 replicates of bootstrap values to ensure the robustness of the conclusions. The Jukes–Cantor method [59] was used to determine genetic distances and clustering.

2.3. Hydrocarbon-Oxidizing Activity of Strains

The hydrocarbon-oxidizing activity of the strains was assessed by the degree of biodegradation of the aliphatic fraction of oil using the gas chromatography method [60]. Bacteria were cultivated in liquid Raymond medium with oil (4% *w*/*v*) at 28 °C and 160 rpm for 5 days. The oil-degrading strain *Pseudomonas turukhanskensis* IB 1.1 was used as a reference [61]. After incubation, the paraffin–naphthenic oil fraction was extracted with hexane and analyzed on a gas chromatograph (Kristall Lux 4000, Russia) with a flame ionization detector and a Zebron™ ZB-1XT capillary column (30 m × 0.53 mm × 2.65 µm). The analysis mode was: initial column temperature 100 °C, heating rate 5 °C/min, final temperature 270 °C, carrier gas helium. The degree of oil biodegradation (%) was calculated on the basis of chromatographic data in accordance with the instructions for the device.

2.4. Resistance of Strains to Herbicides

The resistance of strains to herbicides was determined visually by the intensity of growth on NA medium with the addition of various concentrations of herbicides in com-

parison with the growth rates on the NA medium without herbicides after cultivation at 28 °C for 7 days. Russian-made selective herbicides were used (Table 1). The commercial products were chosen because surfactants and their other components can change the level of toxicity of herbicides [62]. The concentration of the products in the medium varied in the range 1–10 mL/L.

Table 1. Characteristics of herbicides.

Product	Manufacturer	Active Substance	Class of Chemical Compounds	Crops	Object of Influence (Weeds)
Octapon extra	AHK-AGRO, LLC	2,4-dichlorophenoxyacetic acid (2,4-D)	aryloxyalkanocarboxylic acids	cereals	annual and some perennial dicotyledons
Chistalan	AHK-AGRO, LLC	2,4-D (2-ethylhexyl ether) and dicamba (sodium salt)	aryloxyalkanocarboxylic acids	cereals corn	annual and perennial dicotyledons
Tapir	Agro Expert Group, LLC	imazetapir	imidazolinones	soy peas	dicotyledons and cereal
Hermes	Shchelkovo Agrokhim, CJSC	imazamox and quizalofop-p-ethyl	aryloxyphenoxypropionates and imidazolinones	sunflower peas	annual dicotyledons, annual and perennial cereals
Fenizan	Shchelkovo Agrokhim, CJSC	dicamba and chlorsulfuron	sulfonylurea	cereals fiber flax	annual dicotyledons, including 2,4-D-resistant and some perennial dicotyledons

2.5. Resistance of Strains to Heavy Metals

The resistance of strains to heavy metals (Zn, Co, Cd, Pb, Cu, Ni) was assessed visually by their growth on NA medium with salts of these metals ($ZnSO_4 \times 6H_2O$, $CoCl_2 \times 2H_2O$, $Cd(CH_3COO)_2 \times 2H_2O$, $Pb(CH_3COO)_2 \times 3H_2O$, $CuSO_4 \times 5H_2O$, $NiCl_2 \times 6H_2O$) in comparison with the growth rates on the NA medium without salts after incubation at 28 °C for 7 days. The concentration of metal ions was varied in the range 1–10 mmol/L.

2.6. Production of Hydrolytic Enzymes of Strains

The production of hydrolytic enzymes by the strains was determined by the following methods: protease—by liquefaction of gelatin, amylase—by the diameter of the starch hydrolysis zone, cellulase—by the presence of a zone of dissolution of carboxymethylcellulose, and lipases—by the presence of an opaque zone of calcium salts of fatty acids on a medium with Tween 80 [51].

2.7. PGP Properties of Strains

2.7.1. Phosphate Solubilization

The ability of strains to mobilize inorganic phosphates was determined on Pikovskaya medium (composition (g/L): glucose—10.0, $(NH_4)_2SO_4$—0.5, NaCl—0.2, KCl—0.2, $MgSO_4 \times 7H_2O$—0.1, $MnSO_4 \times 7H_2O$—0.5, $FeSO_4 \times 7H_2O$—0.5, yeast extract—0.5, agar-agar 15.0, pH—7.2) [63] with the addition of tricalcium phosphate. Bacteria were cultivated for 10 days at 28 °C. The solubilization halo (the translucent area surrounding the colony) diameter was measured. Based on these measurements, the solubilization index (SI) was calculated as follows: halo diameter (mm)/colony diameter (mm) [64]. If the value of the solubilization index (SI) was less than 2, then it was considered that the isolate had low

solubilization potential, if SI was 2–3, then the isolate had average solubilization potential, and if SI was greater than 3, then the isolate had high potential [65].

2.7.2. Nitrogen Fixation

The ability of the strains to fixate nitrogen was determined by growth rates on Ashby agar medium (composition (g/L): mannitol—20.0, K_2HPO_4—0.2, $MgSO_4 \times 7H_2O$—0.2, NaCl—0.2, K_2SO_4—0.1, $CaCO_3$—5.0, agar-agar—15.0) [51] after 5 days of cultivation at 28 °C. The strains were considered active if the number of their cells increased from 10^5 to 10^9 CFU/mL or more during 72 h of cultivation. A lower growth rate was interpreted as weak growth.

2.7.3. IAA Production

The content of indolyl-3-acetic acid (IAA) in the culture liquid was analyzed chromatographically as described [66]. Sampling to assess the content of IAA in the culture liquid was carried out after growing bacteria on the NA medium for 5 days. Culture liquid was subjected to centrifugation at 8000× *g* followed by ultrafiltration through cassettes with a pore diameter of 1 kDa (SARTOCOON Slice Cassete, Germany). Ultrafiltrates were analyzed on an LC-20 Prominence HPLC system with an SPD-M20A diode array detector (Shimadzu, Japan). For chromatographic separation, a Zorbax-ODS column (250 × 4.6 mm) (Shimadzu, Japan) was used in isocratic mode at a solvent ratio of 0.1% acetic acid in water: acetonitrile, 20:80. Absorption was recorded at a wavelength of 279 nm. The IAA concentration was determined from a calibration curve constructed using a standard (Sigma, St. Louis, MO, USA) in the concentration range of 10–10,000 ng/mL.

2.8. Growth Stimulating Activity

2.8.1. The Influence of Strains on the Growth and Development of Plants in Oil-Contaminated Soil

The ability of the strains to stimulate plant growth was tested on the seeds of barley (*Hordeum vulgare* L.) variety Chelyabinsky 99 and red clover (*Trifolium pratense* L.) variety Early 2. These plants were chosen because they belong to different families (*Poaceae* and *Fabaceae*), and barley and clover are resistant to oil pollution, responsive to bacterization, and have previously been used as phytoremediants [30,67–69]. Seeds were inoculated for 15 min. The required amount of liquid culture of bacteria was calculated so that the titer of cells per seed was 10^5 CFU for barley and 10^4 CFU for clover. Control seeds were similarly soaked in water. The seeds of barley and clover were placed on the moistened soil in 15 and 20 pieces per Petri dish and incubated for 3 and 4 days at 24–26 °C, respectively. Afterwards, the germination and length of the shoots and roots were measured. In barley, the total length of the roots was measured. We used non-sterile leached chernozem (Luvic Chernozem) from Ufa district of the Republic of Bashkortostan (Russia). The soil was characterized by the following parameters: pH_{KCl} 6.3, N_{total} 0.61%, the humus content 6.8%, and available (0.2 N KCl extract) P_2O_5 and K_2O 94.5 and 101.7 mg/kg soil, respectively. Coarse roots and other plant residues were preliminarily removed from the soil samples, then the soil was dried in air and sifted through a sieve (mesh size was 1 cm). Humidity was maintained at 60%.

We also tested the effect of bacteria on plant growth in the presence of oil. The soil was mixed with oil (2% *w*/*w*), then laid out in Petri dishes (40 g per dish) and left for 2 days to weather the toxic volatile components. The experiment was then carried out as described above.

2.8.2. Influence of Strains on Plant Growth and Development under Herbicide Contamination

The soil was placed in Petri dishes (40 g per dish) and moistened to 60% of soil moisture capacity. The characteristics of the soil used are given above. The soil was treated with a solution of Tapir herbicide (concentration 4 mL/L). A 180 µL measure of the working solution of the herbicide was added to one dish (the area of the soil plate was taken into account in the calculation). The amount of the herbicide was increased by 2 times compared to the consumption recommended by the manufacturer. After treatment, the soil was mixed

and clover seeds were laid out on its surface. Previously, we found that barley was resistant to this herbicide, so clover was used as a test object. Seed bacterization was carried out as described above. Control seeds were treated with tap water. The dishes were incubated at 24–26 °C for 4 days. After that, seed germination was calculated and the length of shoots and roots was measured.

2.9. Influence of Strains and Plants on the Content of Petroleum Hydrocarbons in the Soil

In the experiment on bioremediation of oil-contaminated soil (including that containing herbicide), barley and clover were used as phytomeliorants. Oil (2% *w*/*v*) was added to air-dry soil mixed with sand in a ratio of 9:1, and 450 g were placed in vegetation vessels (V = 500 mL). Herbicide Tapir was added in the amount of 2.0 mL of the working solution of the drug (see above) per 450 g of oil-contaminated soil. Soil moisture was maintained at the level of 60–80% of the total soil moisture capacity. The duration of the experiment was 30 days. The vessels were planted with six barley seeds or ten clover seeds. Throughout the experiment, the plants grew at a temperature of 22–26 °C and a 14-hour photoperiod on a light platform providing illumination of 240 μmol m^{-2} s^{-1} PAR. Before planting in the soil, the seeds were treated with the liquid culture of *P. alcaligenes* UOM 10 or *P. frederiksbergensis* UOM 11, as described above. These microorganisms were chosen due to their high hydrocarbon-oxidizing activity. In the variants of the experiment without the use of plants, the soil mixture was watered with the liquid culture of bacteria in such a way that the cell titer was at least 10^5 CFU/g of soil. Experimental variants without inoculation served as controls.

The content of residual hydrocarbons in soil samples was measured using EPA method 3540C (https://www.epa.gov/hw-sw846/sw-846-test-method-3540c-soxhlet-extraction (accessed on 1 March 2023)). Ten-gram soil samples were packed in filter paper and extracted in a Soxhlet extractor with 300 mL of hexane for 8 h at six extraction cycles per hour. The extraction product was transferred to a glass column filled with glass wool and Na_2SO_4 to remove any water it contained. The extract was collected in a flask for subsequent evaporation of the solvent using a rotary evaporator Rotavapor R-100 (Buchi Labortechnik AG, Flawil, Switzerland) until a final volume of 2 mL was reached. The concentrated solution was poured into a pre-weighed glass beaker and dried until a constant weight was reached. The total petroleum hydrocarbons present in the samples were then quantified by gravimetric analysis with a weighing accuracy of up to 0.1 mg.

The degree of hydrocarbon biodegradation (D) was calculated from the formula:

$$D(\%) = \frac{C_0 - C_1}{C_0} \cdot 100,$$

where C_0 is the initial concentration of oil hydrocarbons (20 g/kg soil), and C_1 is the concentration of oil hydrocarbons in the samples at the end of the experiment.

2.10. Statistical Analysis

The experiments were performed in triplicate. The data were processed using Statistica (Statsoft) software (version 10). In figures and tables, data are presented as mean ± standard error. The significance of differences was assessed by ANOVA followed by Duncan's test ($p \leq 0.05$).

3. Results

3.1. Isolation and Identification of Strains

Of the 54 isolates visually different when grown on NA, 18 isolates were selected for further studies. They were characterized by the most intensive growth on agar and liquid Raymond medium with oil. During the cultivation of each isolate in the liquid medium, the oil film disappeared on its surface and on the walls of the flasks, oil was dispersed, flakes were formed, and the pH shifted towards the acid side from between 6.70 and 6.74 at the beginning of incubation to between 5.56 and 6.38 at the end (Figure S1).

As a result of MALDI– -TOF mass spectral analysis of cellular proteins, each isolate was assigned a numerical identification rating (score). Based on these data, 7 isolates out of 18 were assigned to the genus *Pseudomonas* (UOM 9, UOM 10, UOM 11, UOM 13, UOM 14, UOM 15, UOM 16). The scores of isolate UOM 9 and UOM 15 were 1.903 and 1.989, which corresponds to a high degree of accuracy of generic identification. The scores of isolate UOM 10, UOM 11, UOM 13, UOM 14, and UOM 16 were 2.073–2.201. This corresponds to a high degree of species identification (Table 2).

Table 2. Differently identified isolates by MALDI-TOF MS and 16S rRNA gene sequencing.

Isolate	MALDI-TOF MS Identification (Score; Consistency Category)	16S rRNA Identification Closest Strain (% Similarity Score)	GenBank Accession Number
UOM 9	*Pseudomonas* spp. (1.903; B)	*P. silesiensis* A3^T (99.50)	OQ439800
UOM 10	*Pseudomonas alcaligenes* (2.122; A)	*P. alcaligenes* NBRC 14159^T (99.15)	OP692728
UOM 11	*Pseudomonas frederiksbergensis* (2.073; A)	*P. frederiksbergensis* JAJ28^T (99.72)	OP692729
UOM 13	*Pseudomonas arsenicoxydans* (2.186; A)	*P. arsenicoxydans* CECT 7543^T (99.44)	OQ439801
UOM 14	*Pseudomonas jessenii* (2.201; A)	*P. jessenii* DSM 17150^T (99.86)	OQ439802
UOM 15	*Pseudomonas* spp. (1.989; B)	*P. zhaodongensis* NEAU-ST5-21^T (99.01)	OQ439803
UOM 16	*Pseudomonas avellanae* (2.154; A)	*P. avellanae* BPIC 631^T (99.15)	OQ439804

In all isolates, a comparative analysis of the nucleotide sequence of the 16S rRNA gene was performed. Sequences were submitted to the GenBank genetic sequence database at the NCBI (Table 2). The phylogenetic position of the studied isolates compared to the closest and other known strains of genus *Pseudomonas* is shown in the dendrogram construct on the basis of the 16S rRNA gene sequences (Figure S2). Phenotypic and physiological–biochemical features of isolated *Pseudomonas* spp. are given in Table S1. Thus, it was found that isolate UOM 9 belongs to the species *P. silesiensis*, UOM 10 belongs to *P. alcaligenes*, UOM 11 to *P. frederiksbergensis*, UOM 13 to *P. arsenicoxydans*, UOM 14 to *P. jessenii*, UOM 15 to *P. zhaodongensis*, and UOM 16 to *P. avellanae*.

3.2. Hydrocarbon-Oxidizing Activity of Strains

Pseudomonas spp. had different abilities to decompose oil in the liquid medium (Figure 1). The degree of biodegradation in *P. silesiensis* UOM 9, *P. jessenii* UOM 14, *P. zhaodongensis* UOM 15, and *P. avellanae* UOM 16 was 32.7–42.4%, and in *P. arsenicoxydans* UOM 13 it was 50.8%. The highest level of oil degradation was recorded for the *P. alcaligenes* UOM 10 (74.8%) and *P. frederiksbergensis* UOM 11 (77.3%) strains. These parameters were equal to those of the reference oil-oxidizing strain *P. turukhanskensis* IB 1.1 (76.8%).

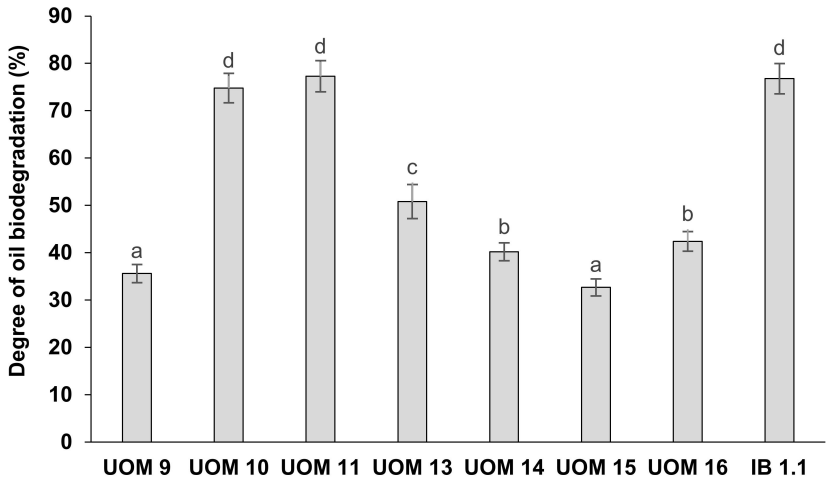

Figure 1. Degree of oil biodegradation by *Pseudomonas* spp. Statistically different means values are marked with different letters ($p \leq 0.05$).

3.3. Resistance of Strains to Herbicides

Herbicides Octapon extra, Tapir, and Germes contain different active ingredients (2,4-dichlorophenoxyacetic acid (2,4-D), imazethapyr, imazamox + khizalofop-P-ethyl, respectively). Despite this, all strains were resistant to them over the entire range of concentrations, which indicates the absence of toxicity of these drugs for the studied bacteria. The exception was *P. jessenii* UOM 14, which could not withstand the presence of Octapon extra in an amount of more than 5 mL/L (Table 3).

Table 3. Properties of the pseudomonad strains.

Property		Strain						
		UOM 9	**UOM 10**	**UOM 11**	**UOM 13**	**UOM 14**	**UOM 15**	**UOM 16**
Maximum concentration of herbicide, mL/L	Oktapon extra	10	10	10	10	5	10	10
	Chistalan	5	5	1	5	1	1	5
	Tapir	10	10	10	10	10	10	10
	Hermes	10	10	10	10	10	10	10
	Fenizan	5	5	5	5	5	5	5
Maximum concentration of heavy metals, mmol/L	Pb^{2+}	5	5	5	5	6	5	5
	Zn^{2+}	4	4	4	4	8	4	4
	Cd^{2+}	1	-	1	1	2	-	1
	Co^{2+}	3	3	4	3	3	2	4
	Cu^{2+}	2	3	3	2	4	3	2
	Ni^{2+}	4	4	4	4	4	4	4
Production of hydrolytic enzymes	lipase	−	+	+	+	+	+	−
	amylase	−	−	−	−	−	+	−
	protease	−	+	−	−	+	−	−
	cellulase	−	−	−	−	−	+	−
Solubilization index		2.2 ± 0.2	1.8 ± 0.1	3.2 ± 0.2	2.3 ± 0.2	2.0 ± 0.1	1.8 ± 0.2	3.0 ± 0.2
Nitrogen fixation		+	+	+	+	+	+	+
IAA production, ng/mL		539 ± 29	1627 ± 75	898 ± 40	305 ± 22	1615 ± 69	975 ± 48	940 ± 53

Note. + indicates the presence of the property, − the absence of the property. The maximum concentration of herbicides and heavy metals refers to the highest concentration at which bacterial growth is still possible.

Bacterial resistance to Chistalan and Phenazin was much lower (no more than 5 mL/L). The strains *P. frederiksbergensis* UOM 11, *P. jessenii* UOM 14, and *P. zhaodongensis* UOM 15 did not grow at the concentration of Chistalan in the medium above 1 mL/L (Table 3).

3.4. Resistance of Strains to Heavy Metals

Among all studied strains of *P. jessenii*, UOM 14 showed the highest resistance to four out of six metals (lead, cadmium, zinc, copper). Cadmium was the most toxic for microorganisms. It completely suppressed the growth of some strains (*P. alcaligenes* UOM 10 and *P. zhaodongensis* UOM 15). Lead, zinc, and nickel were the least toxic. The strains (with the exception of *P. jessenii* UOM 14) showed the same resistance to these metal ions (no more than 4–5 mmol/L) (Table 3).

3.5. Production of Hydrolytic Enzymes

The *P. silesiensis* UOM 9 and *P. avellanae* UOM 16 strains did not produce enzymes from the test set. The *P. zhaodongensis* UOM 15 strain produced lipase, amylase, and cellulase. Most of the bacteria (five out of seven) had lipase activity (Table 3).

3.6. PGP Properties of Strains

3.6.1. Phosphate Mobilization

All strains were capable of dissolving calcium phosphate, but to varying degrees. The strains *P. alcaligenes* UOM 10 and *P. zhaodongensis* UOM 15 had a low phosphate solubilization potential (SI less than 2), while the *P. frederiksbergensis* UOM 11 had a high potential (SI 3.2). Other bacteria had an average inorganic phosphate solubilization potential (SI 2-3) (Table 3).

3.6.2. Nitrogen-Fixing Ability

The strains showed good growth on Ashby's nitrogen-free medium (Table 3). The strain *P. silesiensis* UOM 9 produces mucus, which is an indirect indication that it synthesizes exopolysaccharide.

3.6.3. IAA Products

All studied strains synthesized IAA (Table 3). Its highest concentration was found in the culture liquid of *P. alcaligenes* UOM 10 and *P. jessenii* UOM 14 strains (1627 and 1615 ng/mL, respectively). Bacteria *P. silesiensis* UOM 9 and *P. frederiksbergensis* UOM 11 produce the least amount of this substance (539 and 305 ng/mL of culture liquid, respectively).

3.7. Growth-Stimulating Activity

3.7.1. Influence of Bacterization on the Growth and Development of Plants in Oil-Contaminated Soil

The germination of barley seeds, regardless of the variant of the experiment on pure soil, was 100%. However, treatment of barley seeds with strains *P. alcaligenes* UOM 10, *P. zhaodongensis* UOM 15, and *P. avellanae* UOM 16 led to shoot elongation by 12.1–21.9%, while the use of *P. alcaligenes* UOM 10, *P. frederiksbergensis* UOM 11, *P. jessenii* UOM 14, *P. zhaodongensis* UOM 15, and *P. avellanae* UOM 16 increased the total root length by 26.4–49.4% (Figure 2).

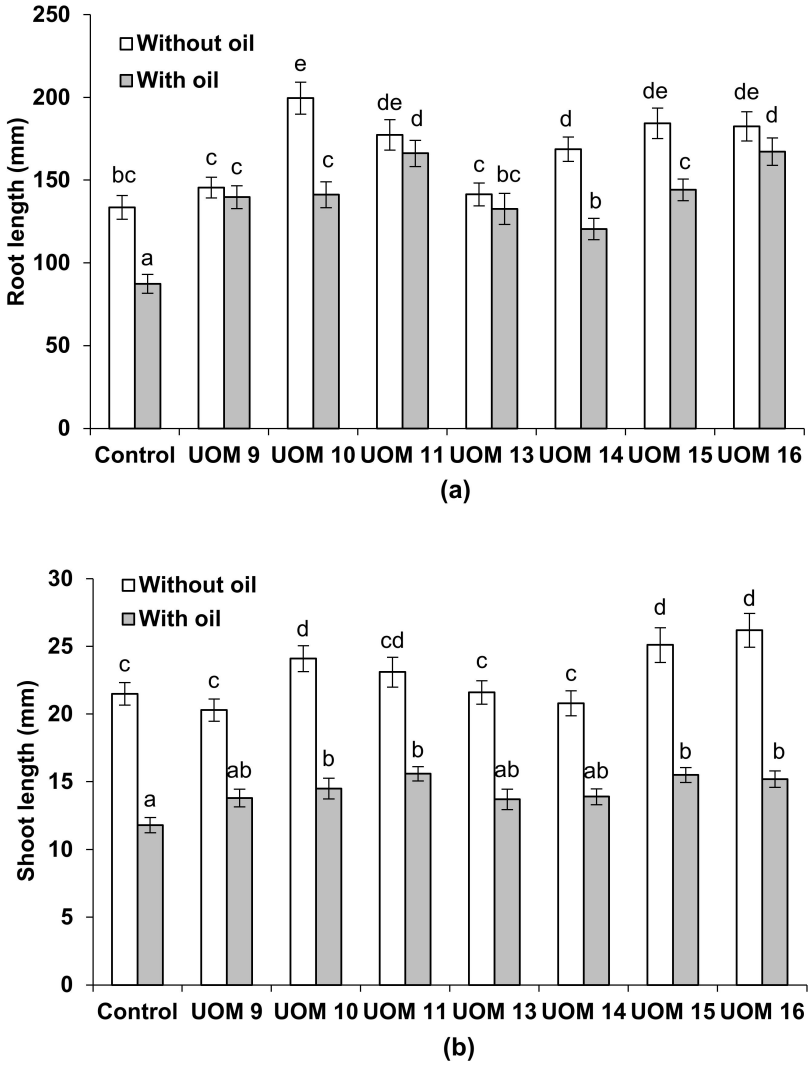

Figure 2. Effect of bacterization on the length of roots (**a**) and shoots (**b**) of barley plants. Statistically different means values are marked with different letters ($p \leq 0.05$).

The germination of clover seeds in the control was 72%. Bacterization of clover seeds with all strains contributed to the growth of shoots and roots of seedlings by 19.9–34.0% and 23.5–48.0% (Figure 3), and an increase in seed germination up to 77–86%.

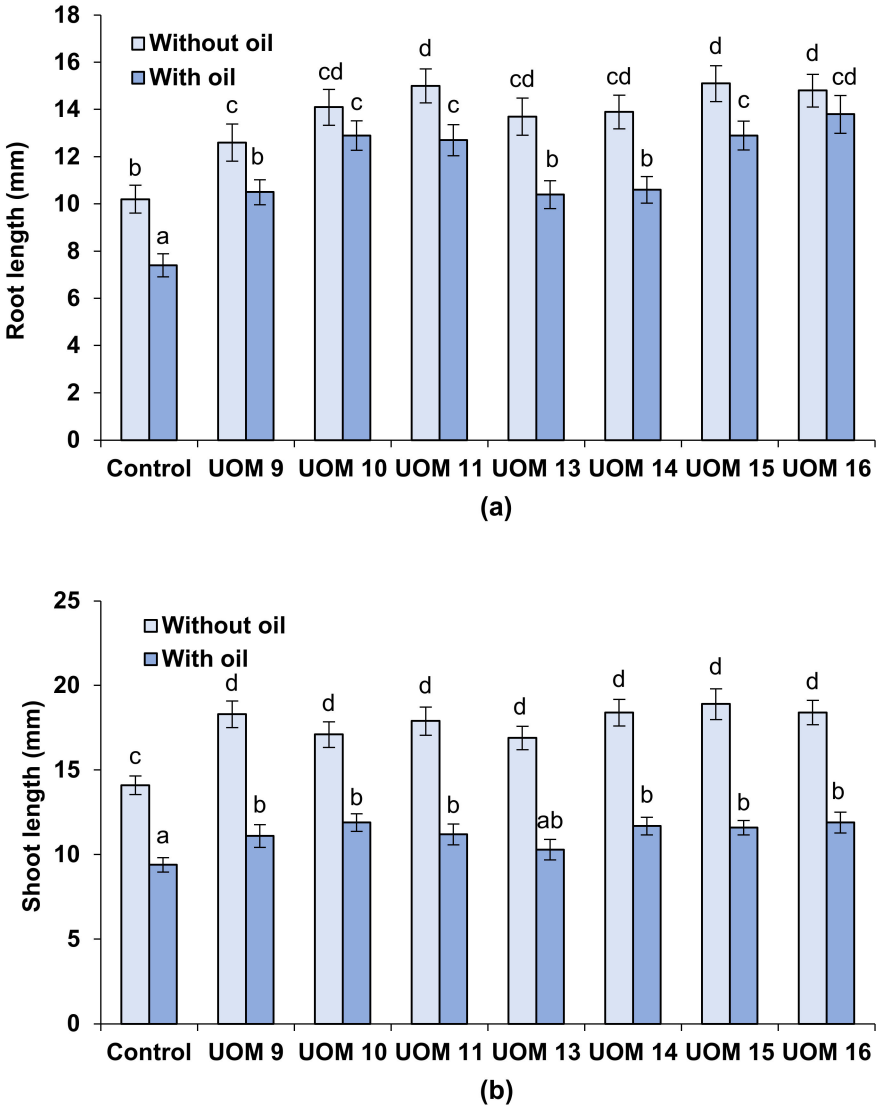

Figure 3. Effect of bacterization on the length of roots (**a**) and shoots (**b**) of clover plants. Statistically different mean values are marked with different letters ($p \leq 0.05$).

Seed germination on oil-contaminated soil generally remained the same as on clean soil, although oil had a noticeable negative effect on the development of both plants. The length of the roots and shoots of barley in the control decreased by 34.6% and 45.1%, clover—by 27.5% and 33.3%, respectively (Figures 2 and 3). The root/shoot ratio of both plants increased compared to that in pure soil (Table 4).

Under the influence of bacterization, the plants were able to compensate for the lag in the growth of roots and partially shoots. Roots of inoculated barley plants in oil-contaminated soil were comparable in length to those of control plants in clean soil. In the presence of oil, the length of roots of clover seedlings treated with strains *P. alcaligenes* UOM 10, *P. frederiksbergensis* UOM 11, *P. zhaodongensis* UOM 15, and *P. avellanae* UOM 16 was 24.5–35.3% higher than in the pure control. In the oil-contaminated soil, the inoculated barley plants had longer shoots than the untreated plants in the control (by 22.9–32.2% when treated with *P. alcaligenes* UOM 10, *P. frederiksbergensis* UOM 11, *P. zhaodongensis* UOM 15, and *P. avellanae* UOM 16). Under the same conditions, the length of clover shoots after

inoculation increased by 18.1–26.6%. Thus, the use of bacteria had a positive effect on the morphometric parameters of plants in clean and oil-contaminated soil (Figure 4).

Table 4. Root/shoot ratio in barley and clover plants.

Plant	Soil	Variant							
		Control	UOM 9	UOM 10	UOM 11	UOM 13	UOM 14	UOM 15	UOM 16
Barley	pure	6.2	7.2	8.3	7.7	6.5	8.1	7.3	6.9
	with oil	7.4	10.1	9.7	10.7	9.7	8.7	9.3	11.0
Clover	pure	0.72	0.69	0.82	0.84	0.81	0.76	0.79	0.80
	with oil	0.79	0.95	1.08	1.13	1.01	0.91	1.11	1.16
	with Tapir	0.86	0.75	0.78	0.77	0.75	0.72	0.69	0.76

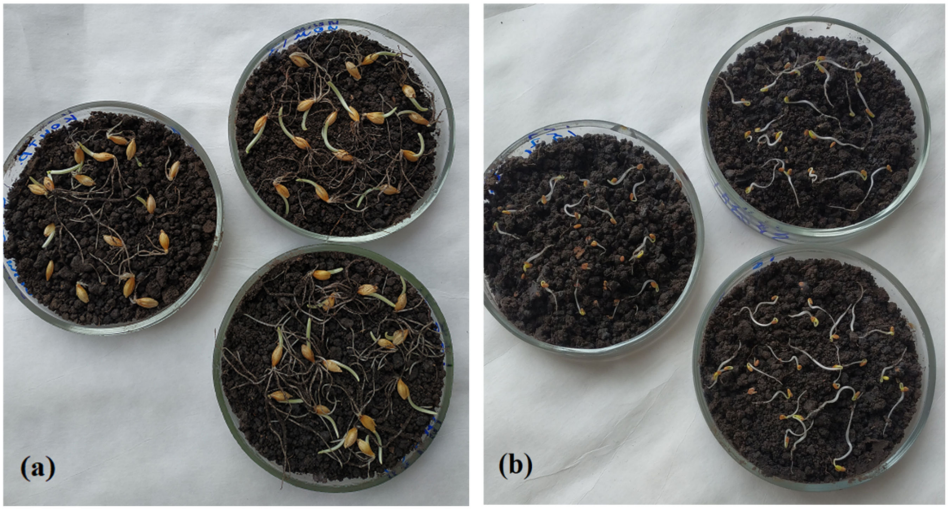

Figure 4. Effect of bacterization on barley (**a**) and clover (**b**) plants in oil-contaminated soil. In figures (**a**,**b**), on the left is the Petri dish with a control, on the top right is the dish with plants treated with *P. alcaligenes* UOM 10 strain, and on the bottom right is the Petri dish with plants treated with *P. frederiksbergensis* UOM 11 strain.

As a result of bacterization, the ratio of the length of plant roots to the length of shoots increased on clean and oil-contaminated soil, but its maximum values were recorded in inoculated plants on soil with oil (Table 4).

3.7.2. Influence of Strains on Plant Growth and Development under Herbicide Contamination

To assess the growth-stimulating activity of the strains, the herbicide Tapir was used, since this drug is resistant to degradation and can accumulate in the soil. Tapir is intended for the destruction of dicotyledons and cereal weeds in soybean and pea crops. Previously, we found that barley was resistant to this herbicide, so clover was used as a test object. Seed germination and morphometric parameters of its seedlings on soil treated with the herbicide turned out to be significantly lower than on pure soil. Germination decreased from 72% to 52%, root and shoot length decreased by 12.7% and 26.9%, respectively (Figures 5 and 6). The ratio of root length to shoot length increased from 0.72 to 0.86 (Table 4).

Bacterization of clover seeds before their germination on soil with the herbicide improved all three analyzed parameters. Germination of inoculated seeds was 76–84%. The length of shoots after the application of microorganisms increased by 28.2–47.6%. The length of the roots also increased, but significant differences from the control (by 22.5–29.2%) were found only in the variants with strains *P. alcaligenes* UOM 10, *P. frederiksbergensis* UOM

11, and *P. avellanae* UOM 16. The ratio of root length to shoot length in treated plants approached the value of this indicator on pure soil and amounted to 0.69–0.78 (Table 4).

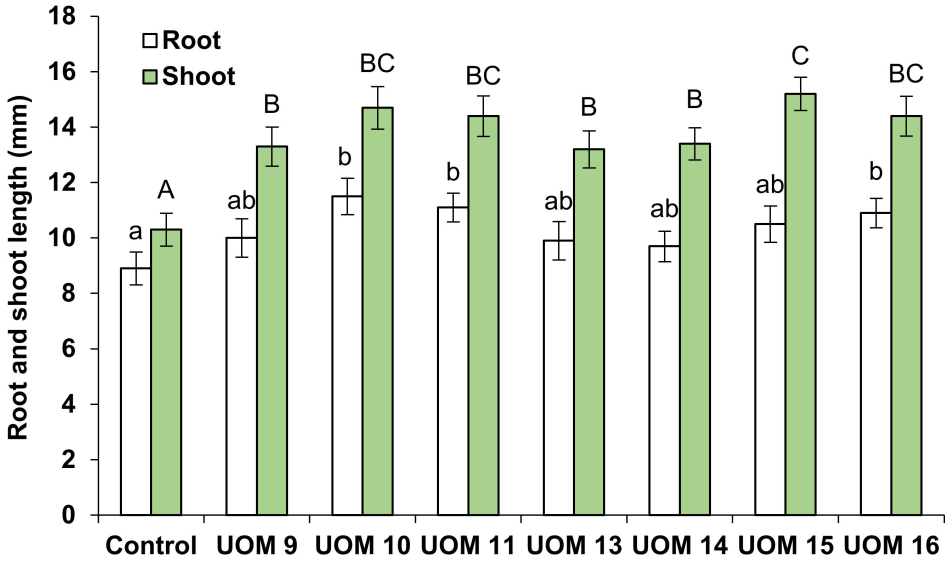

Figure 5. Effect of bacterization on the length of roots and shoots of clover plants in the presence of herbicide. Statistically different mean values are marked with different letters ($p \leq 0.05$).

Figure 6. Effect of bacterization on clover plants in herbicide-treated soil. On the left is the Petri dish with a control, on the top right is the dish with plants treated with *P. alcaligenes* UOM 10 strain, and on the bottom right is the Petri dish with plants treated with *P. frederiksbergensis* UOM 11 strain.

3.8. Biodegradation of Hydrocarbons in the Soil

In the soil experiment, the strains *P. alcaligenes* UOM 10 and *P. frederiksbergensis* UOM 11 were used, since they also had the highest activity in the decomposition of oil in vitro. The degree of oil biodegradation for four weeks in the variants with the use of these strains was 37.5–38.2%. When using barley and clover plants without the introduction of bacteria, this indicator was 33.4% and 26.4%, respectively (Figure 7). The combined use of the bacteria and plants increased the efficiency of oil decomposition. In the presence of microorganisms, the hydrocarbon concentration decreased at approximately the same rate: in the variants with barley, the degree of destruction was 49.3–51.2%, and with clover, 40.6–42.7%.

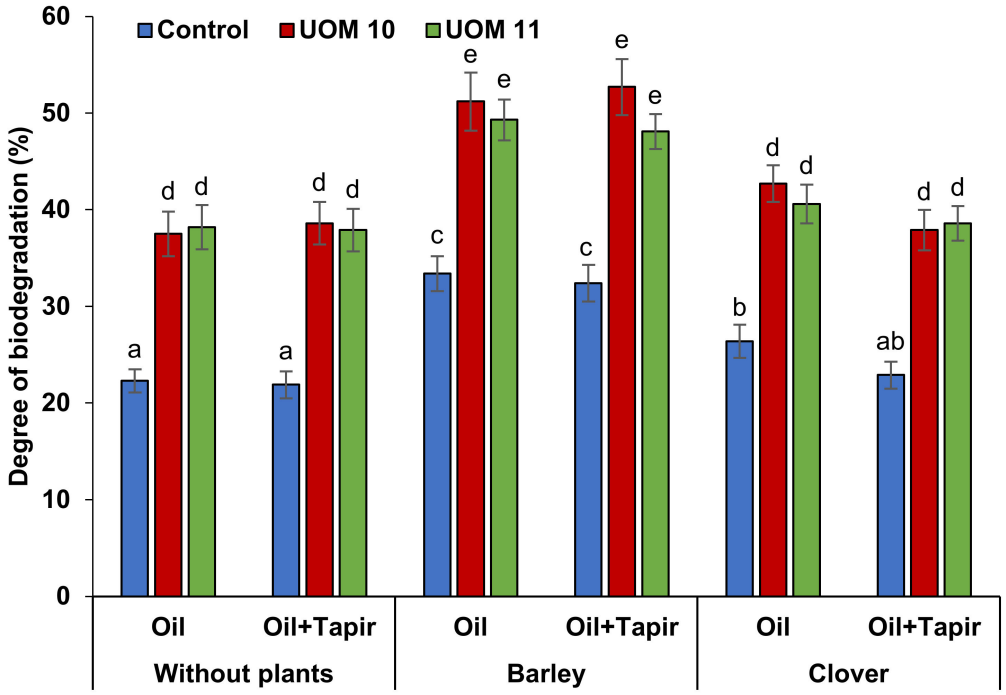

Figure 7. The degree of hydrocarbons biodegradation in various reclamation options. UOM 10 and UOM 11 are variants of experiments with the introduction of *Pseudomonas alcaligenes* UOM 10 and *P. frederiksbergensis* UOM 11 strains, respectively. Statistically different mean values are marked with different letters ($p \leq 0.05$).

The addition of herbicide Tapir to oil-contaminated soil did not have a significant effect on the process of hydrocarbon decomposition. When the soil was simultaneously contaminated with oil and Tapir, destruction also occurred more intensively in those variants where barley and clover plants were subjected to bacterial treatment.

4. Discussion

At present, much scientific and practical experience has been accumulated in the elimination of the consequences of oil pollution [18,70]. However, given the important role of hydrocarbons in the world economy, as well as the diversity of soil and climatic conditions on our planet and the variability in the qualitative and quantitative composition of oil and oil products, the task of developing and implementing effective methods for reclamation of hydrocarbon-contaminated areas is still very relevant. In this study, seven strains of hydrocarbon-oxidizing pseudomonads were isolated and identified from oil-contaminated arable soils. Our interest was focused specifically on pseudomonads, since they have a wide range of properties useful for environmental biotechnology [71].

Undoubtedly, the most important quality of hydrocarbon-oxidizing bacteria is their ability to degrade oil. The isolated microorganisms showed different degrees of oil degradation (32.7–77.3%) (Figure 1). *P. alcaligenes* UOM 10 and *P. frederiksbergensis* UOM 11 proved to be the most effective. They are representatives of species that are well-known for their ability to degrade oil, petroleum products, and polycyclic aromatic hydrocarbons (PAHs) [72–77]. An additional advantage of the isolated bacteria is their ability to synthesize lipase (Table 3). This enzyme can serve as an effective tool for the decomposition of hydrocarbons [78,79], and the lipase activity of microorganisms is used to monitor the biodegradation of oil and oil products during bioremediation [80].

Today, anthropogenic soil pollution most often has a complex character. For example, oil and pesticide contamination can occur as a result of accidents on oil pipelines passing through agricultural land on which chemical plant protection products were used [5,6].

The presence of additional pollutants inhibits the vital activity of the native hydrocarbon-oxidizing microorganisms and leads to a decrease in the efficiency of self-purification of oil-contaminated soil [81]. Therefore, for its bioremediation, it is necessary to use oil-degrading microorganisms that are resistant to the presence of other pollutants. All *Pseudomonas* spp. isolated by us had equally high resistance to herbicides Octapon extra, Tapir, and Hermes, despite the fact that their active substances belong to different classes of chemical compounds. Resistance to Chistalan and Phenizan was at least two times lower (Table 3). The composition of these two herbicides includes an additional component of dicamba (3,6-dichloro-2-methoxybenzoic acid). Perhaps it could have a negative effect on bacterial growth.

In addition to herbicides, heavy metals can be found in oil-contaminated agricultural soils. They accumulate during crude-oil spills and as a result of violation of the regulations for the use of pesticides and fertilizers [82–85]. Heavy metals have their own toxicity, which can have a negative effect on microorganisms [86,87]. To date, there are no clear criteria for dividing strains into resistant and unresistant to the effects of heavy metals. For example, it was proposed that pseudomonads, for which growth is not inhibited by $NiCl_2$, $ZnSO_4$, or $Pb(CH_3COO)_2$ at a concentration of 1 mmol/L, are resistant to nickel, zinc, and lead [88]. In [89], bacteria of the genus *Pseudomonas* isolated from water containing metal salts (circulating cooling water of iron and steel plant) were considered resistant if they grew in the presence of nickel, zinc, and lead in amounts of 3.5–4.0, 2.5–3.0, and 2.5–3.0 mmol/L, respectively. The bacteria isolated in this study showed resistance to lead, zinc, and nickel ions in the amount of 4.0–5.0 mmol/L (except for *P. jessenii* UOM 14) (Table 3). This feature can serve as an additional advantage of the strains in the selection of agents for bioremediation of oil-contaminated agricultural soils.

To improve the efficiency of soil bioremediation, microbiological destruction and phytoremediation are often combined. Microorganisms used for this should not only destroy pollutants, but also stimulate plant growth, i.e., have PGP properties [90]. Therefore, it seemed important to find out whether the studied hydrocarbon-oxidizing pseudomonads belong to the PGPB group. In particular, they can increase the availability of nitrogen and phosphorus for plants, which are key elements in the mineral nutrition of plants [91]. Nitrogen is part of the amino acids from which proteins are synthesized. It plays an important role in almost all metabolic processes in plant cells. Phosphorus affects the formation of the rudiments of the reproductive parts of plants and the branching of the roots. At the same time, phosphorus contained in the soil is practically inaccessible to plants due to poor solubility and the formation of complexes with metals [92]. Therefore, strains with nitrogen fixing and phosphate solubilizing activity are of great practical interest for environmental and agricultural biotechnology. Many *Pseudomonas* spp. have these properties [45,93], including the bacteria studied in this work, which had the potential to fix atmospheric nitrogen. They actively grew on a nitrogen-free medium (Table 3), i.e., are at least oligonitrophils. In addition, the strains were capable of phosphate solubilization. Particularly promising from this point of view was the strain *P. frederiksbergensis* UOM 11, whose ability to dissolve phosphates was high (SI 3.2) (Table 3). This is a characteristic feature of many members of this species [94,95].

Bacterization positively affected the length of roots and shoots of barley and clover in clean and oil-contaminated soil, as well as the growth and development of clover seedlings in herbicide-treated soil (Figures 2–6). The ability of bacteria to increase the growth rates of plants (the length of roots, in particular) is very important when bioremediation is carried out using microbial–plant complexes. The presence of hydrocarbons in the soil reduces the water-holding capacity and air exchange, and also leads to a change in the physical and chemical properties of the soil, and the availability of mineral nutrients [96,97]. Therefore, increased growth of the underground part is an important plant response to oil stress. In general, roots provide surfaces for attachment of microorganisms and secrete exudates, which contribute to an increase in their abundance in the rhizosphere [98,99], and also produce enzymes that destroy organic pollutants in the soil [100].

As is known, the production of phytohormones by bacteria plays an important role in their stimulating effect on plants [29,101]. Auxins are the main regulators of plant growth and development, and IAA is the most common indole compound of this group. It enhances plant cell division, their elongation and differentiation, and promotes colonization of roots by bacteria and protection against pathogens [102–106]. It is known that exogenous auxins help plants overcome abiotic stress caused by drought, salinity, and pesticides [40,107]. All the studied strains synthesized IAA, but according to its content in the culture liquid, they can be divided into two groups: microorganisms with a low level of IAA production (*P. silesiensis* UOM 9 and *P. arsenicoxydans* UOM 13) and with an average level (Table 3). It was found that IAA enhances root growth [29]. Probably, the different ability of the studied microorganisms to increase the length of the roots in both plants in contaminated soil depends on the amount of IAA produced (Figures 2a and 3a). Thus, *P. silesiensis* UOM 9 and *P. arsenicoxydans* UOM 13, which secrete the least of this phytohormone (539 and 305 ng/mL), had a weaker stimulating effect than other strains. The *P. alcaligenes* UOM 10 and *P. jessenii* UOM 14 strains producing the same amount of auxin (1627 and 1615 ng/mL) differed from each other in their ability to enhance plant growth. The obtained results are consistent with the literature data, which show that stimulation of plant growth is due to the complex effect of microorganisms and a high concentration of IAA in the culture liquid does not guarantee the presence of a growth stimulating effect from bacterization [108].

The root/shoot ratio characterizes the growing conditions of plants. The worse the provision of plants with nutrients and water, the higher this ratio. On the example of both plants, we saw that the ratio increased when the soil was contaminated with oil and herbicide. For barley in clean and oil-contaminated soil, it was 6.2 and 7.4 (Table 4). When treated with *Pseudomonas* spp., the root/shoot ratio in barley plants on oil-contaminated soil turned out to be 1.2–1.5 times higher than in the variant without inoculation. This indicates that, under the influence of bacterization, plants in the early stages of their development were able to form a more powerful root system, which in the future should ensure enhanced growth of the aerial part.

When studying clover plants, we were able to compare how the introduction of the bacteria affected their development in the presence of different xenobiotics. As with barley, clover inoculation led to an even greater increase in root formation in oily soil. The root/shoot ratio also increased by 1.2–1.5 times compared to the control. A completely different relationship was found in soil contaminated with Tapir herbicide. The introduction of bacteria led to a decrease in the root/shoot ratio compared to untreated plants (0.86 in the control and 0.69–0.78 in the variants using pseudomonads) (Table 4). This means that the bacterized plants successfully overcame stress and maintained growth rates at the level of plants that developed on clean soil.

Thus, presowing seed treatment contributed to better adaptation of plants to abiotic stress conditions caused by various pollutants. Under the influence of bacterization, plants were able to almost completely neutralize the consequences of the presence of the herbicide in the soil and partially compensate for the negative effect of oil on growth characteristics. Apparently, this is due to the fact that the obstacle to the development of plants was not the toxic effect of pollutants, but the ability of oil to have a negative effect on the physicochemical and structural properties of the soil.

The results obtained were used in the preparation of a vegetation experiment for cleaning up oil-contaminated soil (including in the presence of imazethapyr-based herbicide) using the most active oil-degrading microorganisms *P. alcaligenes* UOM 10 or *P. frederiksbergensis* UOM 11. It was previously noted (see review by [109] and references) that the use of degrading bacteria with PGP properties makes phytoremediation more effective by stimulating plant growth and enhancing the biodegradation of pollutants. In the present study, it was found that the combined use of oil-degrading strains *P. alcaligenes* UOM 10 or *P. frederiksbergensis* UOM 11 and phytomeliorant plants had a positive effect on the rate of removal of hydrocarbons from the soil. The best result in soil cleaning was obtained when using the bacteria in conjunction with barley. The degree of biodegradation for the month

of the experiment was 49.3–51.2%. The use of barley without bioaugmentation led to lower results, but was more effective than the use of unbacterized clover. The low efficiency of clover (the degree of oil biodegradation is 26.4%), in our opinion, is associated with a weak root system of plants and a slow growth rate of the aerial part. When clover seeds were inoculated with strains *P. alcaligenes* UOM 10 or *P. frederiksbergensis* UOM 11, the degree of soil purification increased by 14.2–16.3%.

The presence of the herbicide Tapir in the soil against the background of oil pollution (20 g/kg of soil) had almost no effect on the rate of biodegradation. It is known that Tapir (imazethapyr) can persist in the soil for up to two years and has significant phytotoxicity [110]. Despite this, apparently, both barley and clover can be used in the reclamation of areas containing, among other things, residual amounts of this herbicide.

During the analysis of the obtained data, the authors faced the problem of comparative evaluation of the results of the experiment. There is a sufficient number of studies on this topic [111–113], but it is almost impossible to compare our results with data in the literature. This is due to significant differences in the experimental conditions. The differences relate to the qualitative and quantitative composition of oil and oil products, soil characteristics, the method of reclamation, the duration of the experiments, methods for assessing the residual amounts of hydrocarbons in the soil, etc. Nevertheless, the analysis of publications allows the authors to assert that the level of hydrocarbon biodegradation established in this work (in the range of 40–50% per month) is sufficient to consider the *P. alcaligenes* UOM 10 and *P. frederiksbergensis* UOM 11 strains as promising agents for bioremediation of oil-contaminated soils, including in the presence of herbicides.

According to the available literature, this study is one of the few in which oil-degrading bacteria resistant to additional pollutants and enhancing plant growth under conditions of oil and herbicide pollution were isolated. Our future efforts will be aimed at studying how these microorganisms (including together with plants) will affect the content of hydrocarbons in the soil in the presence of herbicides and heavy metals. In addition, we plan to study whether these pseudomonads can degrade herbicides and inactivate heavy metals in the soil. It will also be interesting to test the effectiveness of using various consortiums of isolated strains for these purposes. The obtained results can become the basis for the development of effective bioremediation technologies for complexly contaminated soils.

5. Conclusions

In the present study, seven strains of hydrocarbon-oxidizing *Pseudomonas* were isolated and identified which showed resistance to herbicides based on various active substances (2,4-D, imazethapyr, a mixture of imazamox and chizalofop-P-ethyl). All studied bacteria possessed PGP properties and stimulated the growth of roots and shoots of barley and clover in pure and oil-contaminated soil to varying degrees, and also had a positive effect on clover seedlings in soil with herbicide. Thus, the microorganisms helped the plants overcome the stress caused by the presence of pollutants. The strains *P. alcaligenes* UOM 10 and *P. frederiksbergensis* UOM 11 had the greatest potential as the basis of microbial–plant complexes for bioremediation of oil-contaminated agricultural soils due to their high ability to degrade oil and stimulate the growth and development of plants. However, other strains also have prospects for use in the process of cleaning and restoring soils. They can be used to accelerate the growth of remediating plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13061111/s1, Figure S1: the appearance of Raymond's medium with oil without inoculation (left flask) and with the cultivation of UOM 11 isolate (right flask); Figure S2: neighbor-joining tree illustrating the phylogenetic position of isolated *Pseudomonas* spp. and other species in the genus *Pseudomonas* based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) over 50% are shown at branching nodes. Bar, 0.005 substitutions per nucleotide position; Table S1: phenotypic and physiological–biochemical features of isolated *Pseudomonas* spp.

Author Contributions: Conceptualization, T.K. and E.K.; methodology, T.K. and E.K.; validation, T.K. and E.K.; formal analysis, S.M.; investigation, S.M., Y.S. and M.I.; data curation, T.K., S.M. and E.K.; writing—original draft preparation, T.K.; writing—review and editing, T.K. and E.K..; visualization, S.M., Y.S. and M.I. All authors have read and agreed to the published version of the manuscript.

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