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Biodiversity of Plants Rhizosphere and Rhizoplane Bacteria in the Presence of Petroleum Hydrocarbons

Togzhan D. Mukasheva, Anel A. Omirbekova, Raikhan S. Sydykbekova, Ramza Zh. Berzhanova, Lyudmila V. Ignatova

Abstract—Following plants-barley (*Hordeum sativum*), alfalfa (*Medicago sativa*), grass mixture (red fescue-75%, long-term ryegrass - 20% Kentucky bluegrass - 10%), oilseed rape (*Brassica napus biennis*), resistant to growth in the contaminated soil with oil content of 15.8 g / kg 25.9 g / kg soil were used. Analysis of the population showed that the oil pollution reduces the number of bacteria in the rhizosphere and rhizoplane of plants and enhances the amount of spore-forming bacteria and saprotrophic micromycetes. It was shown that regardless of the plant, dominance of *Pseudomonas* and *Bacillus* genera bacteria was typical for the rhizosphere and rhizoplane of plants. The frequency of bacteria of these genera was more than 60%. Oil pollution changes the ratio of occurrence of various types of bacteria in the rhizosphere and rhizoplane of plants. Besides the *Pseudomonas and Bacillus* genera, in the presence of hydrocarbons in the root zone of plants dominant and most typical were the representatives of the *Mycobacterium* and *Rhodococcus* genera. Together the number was between 62% to 72%.

Keywords—Identification, micromycetes, pollution, root system.

I. Introduction

SOIL contamination by oil and oil products is an urgent problem of modern time and this raises the question of restoration of soil fertility. Currently various methods of soil remediation is developing. Phytoremediation has a special role among them, namely rhizodegradation-destruction of pollutants by microorganisms of root area of plants [1]. The principle of this method is that the destruction of hydrocarbons is not by the plant itself, but using microorganisms habiting the roots, ie in the rhizosphere. Plants affect the abundance, diversity and activity of microorganisms by bioactive root exudates [2], [3]. Rhizosphere microorganisms are more numerous than soil microorganisms. Because of more favorable habitat conditions in the rhizosphere, microorganisms having the enzymes necessary for the degradation of pollutants are developing more often.

It is known that root exudates of higher plants, being the main source of nutrients for the prevailing number of soil microbial population - heterotrophs - have a significant impact on the microbial cenoses [4], [5].

Currently, most researchers believe that the main part of microbial community of the root zone make negative bacteria of *Pseudomonas, Klebsiella, Enterobakter, Alcaligenes* genera and little data is known about rhizosphere and rhizoplane microbial complexes during oil pollution [6], [7]. The purpose of this study was to investigate the structure of rhizosphere and rhizoplane microbial complexes of plants grown on oil-contaminated soils.

T. Mukasheva, A. Omirbekova, R. Sydykbekova, R. Berzhanova, L. Ignatova are with the Al-Farabi Kazakh National University, Almaty, Kazakhstan (e-mail: togzhan.mukasheva @kaznu.kz, anel.83 @mail.ru, raihan.sydykbekova @kaznu.kz, ramza.berzhanova @kaznu.kz, Lyudmila.Ignatova @kaznu.kz).

II. Materials and Methods

Barley (*Hordeum sativum*), alfalfa (*Medicago sativa*), grass mixture (red fescue- 75%, long-term ryegrass - 20% Kentucky bluegrass-10%), oilseed rape (*Brassica napus biennis*) resistant to growth in the contaminated soil with oil content of 15.8 g / kg 25.9 g / kg soil were used [8]. Soil from Uzen deposit of Mangistau region containing 21.2 - 32.3 g of oil / kg soil was exploited. Plants were grown in pots in volume of 5 kg; filled with oil-polluted soil, as a control we used native soil. Duration of experiment was 50 days.

Determining the number of rhizosphere and rhizoplane microorganisms carried out by the method of plating on solid media [9]: Nutrient agar medium, Saburaud, Starch-Amonia medium and the e 8E medium.

The plants were removed from the pots; the bulk soil was shaken off the roots. The sample of root with adhering soil (10 g) was washed in 90 ml of sterile water by shaking during 30 min. Suspension after range of dilutions was used to isolation of rhizosphere microorganisms. The root sample taken from first flask was washed again in 90 ml of sterile water by shaking 30 min; the suspension was used to isolation of microorganisms from rhizoplane of plants [9].

To determine the diversity of bacteria in the rhizosphere and rhizoplane of plants the total number of colonies grown on Nutrient agar medium was counted. Differentiated count of bacterial colonies of different taxonomic groups was carried out. For this purpose, initially on each plate colonies with macro morphological types were isolated. Isolated rhizosphere and rhizoplane microorganisms were identified by morphological, physiological and biochemical characteristics using conventional management [10], [11].

Molecular-genetic analysis of identification of isolated strains was performed by National Center for Biotechnology, National Scientific Shared Laboratory of Biotechnology, Kazakhstan. Identification of yeast was performed by determining the nucleotide sequence of the direct ITS region (intergenic transcribed region) and strain of bacteria by direct determination of the nucleotide sequence of 16S rRNA gene fragment, followed by determination of the nucleotide identity with the sequences deposited in the international Gene Bank database, as well as the construction of phylogenetic trees from nucleotide sequences of the reference strains. The

chromosomal DNA of strains was isolated by Kate Wilson method.

The PCR reaction was performed with universal primers 8F 5'-AgAgTTTgATCCTggCTCAg-806R-3 and the 5' ggACTACCAgggTATCTAAT a total volume of 20 microliters. PCR mixture contained 150 ng of DNA, 1 unit of Maxima Hot Start Taq DNA Polymerase (Fermentas), 0,2 mM of each dNTP, 1 x PCR buffer (Fermentas), 2,5 mM $MgCl_2$, 10 pmol of each primer. The PCR amplification program included long denaturation 95° C for 7 minutes; 35 cycles: 95° C - 15 seconds; 52° C-30 sec; 72° C - 30 seconds; final elongation at 72° C-7 minutes. The PCR program was executed using the thermocycler GeneAmp PCR System 9700 (Applied Biosystems).

Purification of PCR products from unrelated primers was performed by enzymatic method using the Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas).

Sequencing reactions were performed using BigDye ® Terminator v3.1 Cycle Sequencing Kit (Applide Biosystems) according to the manufacturer's instructions followed by separation of the fragments on an automated Genetic Analyzer 3730xl DNA Analyzer (Applide Biosystems).

The nucleotide sequences of ITS region of identified bacteria and yeast were analyzed and combined into one sequence in SeqScape 2.6.0 software (Applide Biosystems). Nucleotide sequence with the length of 530 bp was identified in the GeneBank using BLAST algorithm.

TABLE I
NUMBER OF POPULATION OF ECOLOGICAL AND PHYSIOLOGICAL GROUPS OF MICROORGANISMS IN THE RHIZOSPHERE AND RHIZOPLANE OF PLANTS

Plants	Ammonified bacteria, 10^6 CFU/g of soil	Bacteria using mineral forms of nitrogen, 10^6 CFU/g of soil	Spore-forming bacteria, 10^3 CFU/g of soil	Saptortrophic micromycetes, 10^3 CFU/g of soil
		Rhizosphere		
Alfaalfa	23,6±1,18	20,9±1,04	8,4±0,42	14,5±0,725
Oilseed rape	65,5±2,60	28,9±1,15	11,1±0,54	19,5±0,85
Barley	13,1±0,45	26,5±1,24	2,1±0,14	12,6±0,90
Grass-mixture	16,5±1,28	29,8±1,25	4,9±0,35	11,1±0,85
		Rhizoplane		
Alfaalfa	25,6±1,16	17,3±1,05	17,6 ±1,09	5,6±0,24
Plants	Ammonified bacteria, 10^6 CFU/g of soil	Bacteria using mineral forms of nitrogen, 10^6 CFU/g of soil	Spore-forming bacteria, 10^3 CFU/g of soil	Saptortrophic micromycetes, 10^3 CFU/g of soil
Oilseed rape	33,5±1,94	28,5±1,52	4,9±0,09	13,4±0,92
Barley	13,3±0,75	18,2±1,02	5,3±0,12	10,1±1,24
Grass-mixture	15,8±0,82	58,3±2,85	5,8±0,09	4,8±0,06

III. RESULTS

Effect of oil pollution on population of physiological groups of microorganisms in the rhizosphere rhizoplane of plant: One of the most important characteristics of soil microbiota is to determine the number and ratio of the main physiological groups of microorganisms. Analysis of the number of microorganisms by seeding of them on solid nutrient media revealed some features. Because of oil contamination, in soil and rhizosphere and rhizoplane of barley, alfalfa, grass mixture and oilseed rape the number of bacteria decreased, probably due to the toxic effect of pollutant (Tables I and II).

For example, the number of bacteria on the nutrient agar medium and starch-ammonia medium from rhizosphere and rhizoplane of oilseed rape in the presence of oil was slightly lower by almost in 2 times. The most significant decrease in the number of bacteria of rhizosphere and rhizoplane was typical for barley and grass mixture, the amount of bacteria was one order of magnitude lower comparing with uncontaminated soil. The studies of the effect of oil pollution on the number of spore-forming bacteria attract big interest, because the oil pollution helped to increase the number of this group of bacteria in the rhizosphere and rhizoplane of all plants. Also, it is well established that the number of saprotrophic micromycetes increased in the presence of oil.

TABLE II
EFFECT OF OIL POLLUTION ON POPULATION OF ECOLOGICAL AND PHYSIOLOGICAL GROUPS OF MICROORGANISMS IN THE RHIZOSPHERE AND RHIZOPLANE OF PLANTS

Plants	Ammonified bacteria, 10^6 CFU/g of soil	Bacteria using mineral forms of nitrogen, 10^6 CFU/g of soil	Spore-forming bacteria, 10^3 CFU/g of soil	Saptortrophic micromycetes, 10^3 CFU/g of soil
		Rhizosphere		
Alfaalfa	10,7±0,52	18,8±0,94	23,7±1,19	27,1±1,34
Oilseed rape	37,3±1,86	13,3±0,67	13,3±0,52	31,4±1,71
Barley	5,3±0,06	11,5±0,78	6,5±0,4	23,3±1,23
Grass-mixture	6,4±0,14	12,3±0,51	14,5±0,65	21,1±1,5
		Rhizoplane		
Alfaalfa	13,6±1,6	11,2±0,56	21,5±1,03	14,3±0,58
Oilseed rape	17,8±0.62	13,3±0,4	14,3±0,65	18,5±1,1
Barley	5,3±1,6	11,3±0,5	8,8±1,6	16,3±0,9
Grass-mixture	6,1±0,23	22,6±1,05	13,3±0,65	14,3±0,7

Soil contamination with oil has stimulated the development of the population of microorganisms - oil destructors in one to two orders of magnitude (Fig. 1). The most significant increase in their numbers noted in the rhizosphere and rhizoplane of grass mixture. It is known that in the period of intensive development of plants exudative substances that attract a variety of microorganisms is releasing back and at this time microbial population of the rhizosphere reaches the maximum level [12] and it is likely that the increase in the number of hydrocarbon-oxidizing microorganisms in the

studied plants associated with the ability of plants to form these substances.

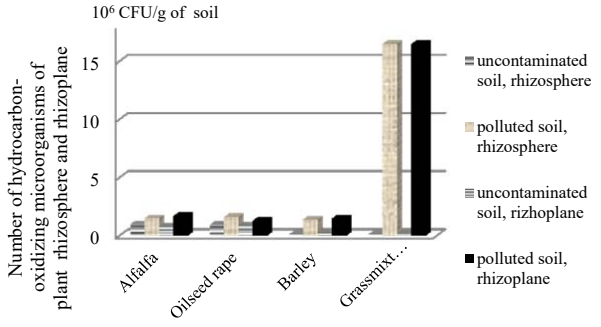

Fig. 1 Number of hydrocarbon-oxidizing microorganisms in the rhizosphere and rhizoplane of plants cultivated on oil-polluted soil during recultivation

Distribution of plant rhizosphere and rhizoplane bacteria genera: Significant number of reviews is devoted to the analysis methods and approaches for assessing microbial diversity of soils [13]-[15], which emphasizes the role of soil as a source of immense microbial diversity. Thus, the method for the quantification of crop diversity microbial communities is commonly used. Dish methods have certain advantages over other methods; they are reliable and allow isolating and identifying these microorganisms that perform a specific function in the community. Therefore, the analysis of bacterial diversity of rhizosphere and rhizoplane of plants was carried out by analyzing community of bacteria grown on solid nutrient agar medium. Bacterial communities in the rhizosphere and rhizoplane of plants in the presence of hydrocarbons was evaluated by the frequency of occurrence of representatives of various sorts. Frequency of occurrence (%) was defined as the percentage of samples in which the particular genus is found from the total number of analyzed samples. As the frequency of occurrence, bacteria in the samples were ranked into 3 groups: the dominant (the frequency of occurrence is more than 60 %), typical (30% to 60 %) and rare (less than 30 %). Furthermore, along with the identification of microorganisms by conventional methods using the culture and the morphological characteristics, as well as chemical and biochemical reactions, methods for the determination of microorganisms based on the comparison of nucleotide sequences of genes of various microorganisms are widely used. Accordingly, generic identification of bacteria belonging to the "dominant" group was carried out using molecular biological techniques based on sequencing of the amplified fragments of the 16S rRNA gene.

The data (Table II) shows that regardless of the bacterial community of plants and allocation space dominance of bacteria of the Pseudomonas and Bacillus genera is typical. The frequency of bacteria was more than 60% and therefore t these genuses were related to dominant genera in the rhizosphere and rhizoplane of the studied plants.

Obtained data (Table III) show that the genera of gram-positive bacteria are most ornate epresented in the rhizosphere and rhizosphere of plants. By the frequency of occurrence the dominant genera were *Mycobacterium, Arthrobacter, Rhodococcus* and *Streptomyces*.

TABLE III
STRUCTURE OF THE BACTERIAL COMMUNITY ON THE FREQUENCY OF OCCURRENCE IN THE RHIZOSPHERE AND RHIZOPLANE OF PLANTS

Plants	Dominant	Typical	Rare
		Rhizosphere	
Alfaalfa	*Bacillus* *Pseudomonas*	*Mycobacterium* *Arthrobacter* *Rhodococcus* *Streptomyces*	*Gordonia* *Micromonospora*
Oilseed rape	*Bacillus* *Pseudomonas*	*Mycobacterium* *Arthrobacter* *Rhodococcus* *Streptomyces*	*Gordonia* *Micromonospora*
Barley	*Bacillus* *Pseudomonas*	*Mycobacterium* *Arthrobacter* *Rhodococcus* *Streptomyces*	*Gordonia* *Micromonospora*
Grass-mixture	*Bacillus* *Pseudomonas*	*Mycobacterium* *Arthrobacter* *Rhodococcus* *Streptomyces*	*Gordonia* *Micromonospora*
		Rhizoplane	
Alfaalfa	*Bacillus* *Pseudomonas*	*Arthrobacter* *Streptomyces*	*Gordonia* *Micromonospora*
Oilseed rape	*Bacillus* *Pseudomonas*	*Arthrobacter* *Streptomyces*	*Gordonia* *Micromonospora*
Barley	*Bacillus* *Pseudomonas*	*Arthrobacter* *Streptomyces*	*Gordonia* *Micromonospora*
Grass-mixture	*Bacillus* *Pseudomonas*	*Arthrobacter* *Mycobacterium* *Streptomyces*	*Gordonia* *Micromonospora*

The share of the various species of this genus was between 35 % to 58 %. Representatives of the *Microbacterium, Gordonia* and *Micromonospora* genera also were dominant and typical in the rhizosphere of all plants, but their incidence ranged from 5 to 20% and they were referred to rare genera.

Generic diversity of bacteria did not differ from the plant rhizosphere and rhizoplane samples grown on contaminated soil. However, oil pollution changes the ratio of occurrence of various types of bacteria in the rhizosphere and rhizoplane of plants. Thus, the population of bacteria of the *Bacillus* and *Pseudomonas* genus also got a benefit in the rhizosphere and rhizoplane so that more than half of the isolated bacteria of these genera possessed the hydrocarbon activity [16]. Furthermore, most dominant and typical representatives were bacteria of the *Mycobacterium* and *Rhodococcus* genera. Together these accounted for between 62% to 72%.

From the Tables III-IV, it is seen that the bacterial community in the rhizosphere and rhizoplane of plants grown in the presence of hydrocarbons range of "typical" genera have been changed. In the native soil typical soil genera were represented by bacteria *Mycobacterium, Arthrobacter, Rhodococcus* and *Streptomyces* genera. In contaminated soils typical genera were represented by bacteria of the *Arthrobacter, Microbacterium* and *Gordonia* genera.

The spectrum of bacteria rare genera of in the rhizosphere and rhizoplane of plants in the presence of hydrocarbons also changed. Thus, the incidence of Derxia, Streptomyces and Micromonospora occurrence ranged from 5 to 20%, these genera are related to rare, in the native soil Streptomycegenus s was related to typical and the Gordonia genus to rare.

TABLE IV
STRUCTURE OF THE BACTERIAL COMMUNITY ON THE FREQUENCY OF OCCURRENCE IN THE RHIZOSPHERE AND RHIZOPLANE OF PLANTS IN PRESENCE OF OIL HYDROCARBONS

Plants	Dominant	Typical	Rare
		Rhizosphere	
Alfaalfa	*Bacillus*	*Arthrobacter*	*Derxia*
	Pseudomonas	*Microbacterium*	*Streptomyces*
	Mycobacterium	*Gordonia*	*Micromonospora*
	Rhodococcus		
Oilseed rape	*Bacillus*	*Arthrobacter*	*Derxia*
	Pseudomonas	*Microbacterium*	*Streptomyces*
	Mycobacterium	*Gordonia*	*Micromonospora*
	Rhodococcus		
Barley	*Bacillus*	*Arthrobacter*	*Microbacterium*
	Pseudomonas	*Gordonia*	*Derxia*
	Mycobacterium		*Streptomyces*
	Rhodococcus		*Micromonospora*
Grassmixture	*Bacillus*	*Arthrobacter*	*Microbacterium*
	Pseudomonas	*Gordonia*	*Derxia*
	Mycobacterium		*Streptomyces*
	Rhodococcus		*Micromonospora*
		Rhizoplane	
Alfaalfa	*Bacillus*	*Pseudomonas*	*Microbacterium*
	Mycobacterium	*Arthrobacter*	*Derxia*
	Rhodococcus	*Gordonia*	*Streptomyces*
			Micromonospora
Oilseed rape	*Bacillus*	*Pseudomonas*	*Gordonia*
	Mycobacterium	*Arthrobacter*	*Derxia*
	Rhodococcus	*Microbacterium*	*Streptomyces*
			Micromonospora
Barley	*Bacillus*	*Pseudomonas*	*Derxia*
	Mycobacterium	*Arthrobacter*	*Streptomyces*
	Rhodococcus	*Microbacterium*	*Micromonospora*
		Gordonia	

Microorganisms by frequency of occurrence in the rhizosphere and rhizoplane of plants were selected for further identification using molecular genetic techniques. Identification of the strains was performed using a direct nucleotide sequence determination of 16S rRNA gene fragments, followed by determination of nucleotide identity with the sequences deposited in the international database Gene Bank.

As shown at Fig. 2, strains identified as 1 and 2 during identification in the Gene Bank are located in one clade with *Bacillus subtilis* and *Bacillus amyloliquefaciens*, as well as the straint 3 is in branch with *Bacillus thuringiensis*.

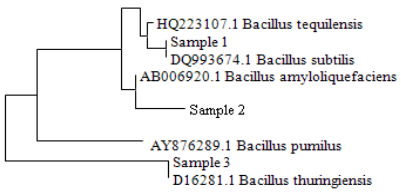

Fig. 2 Phylogenetic tree constructed on the basis of 16S rRNA gene fragment analysis of *Bacillus* group

Moreover, the percent identity of Sample 1 was 100 %. Sample 4 after identification in Gene Bank is located on one clade with *Pseudomonas plecoglossicida*, as well as strain 5 is on one clade with *Arthrobacter nitroguajacolicus* (Fig. 3).

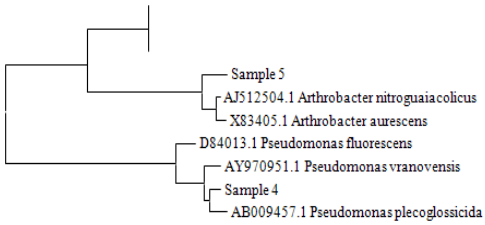

Fig. 3 Phylogenetic tree constructed on the basis of 16S rRNA gene fragment analysis of *Pseudomonas* and *Arthrobacters* groups

Samples 6-10 were also analyzed; it was shown that the strains of microorganisms are in the same clade with *Rhodococcus fascianis*, *Rhodococcus erythropolis*, *Microbacterium oxydans* and *Microbacterium paraoxydans* (Figs. 4 and 5).

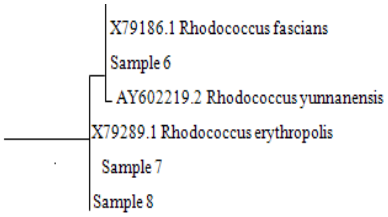

Fig. 4 Phylogenetic tree constructed on the basis of 16S rRNA gene fragment analysis of *Rhodococcus* group

IV. CONCLUSION

Positive role of plants in soil purification from impurities associated with their ability to absorb and transform toxicants, revitalize the microbial community, and, as a consequence to intensify chemical processes and biochemical transformation of foreign compounds in the soil [17], [18]. Survival of plants in contaminated soil contributes by the rhizosphere microbial community through his stimulating plant growth activity, as well as by reducing the phyttoxicity of pollutant due to its biodegradation. So, the using of eastern galega (*Galega orientalis Lam.*) in bioremediation of oil contributed to the recovery and activation of microbocenosis. Acceleration of physiological and biochemical processes involved and sharing of stable groups of microorganisms in the process of degradation of contaminants in the soil provided plants with additional sources of supply [19]. Numerous studies have shown a positive effect of plant-microbe systems for cleaning the surrounding environment from polycyclic aromatic hydrocarbons [20], [21]. Rhizosphere and rhizoplane microorganisms during phytoremediation may participate in transformation of pollutants and their uptake by plants or plants directly can be involved in the degradation of hydrocarbons. However, many researchers have noted the role of microorganisms in the degradation of hydrocarbons, which

is closely associated with the root zone of plants. Perhaps, the study of diversity of microorganisms of root zone of plants grown in the presence of hydrocarbons will make representations about the functions of individual genera of microorganisms associated with their participation in the processes of decomposition of hydrocarbons in contaminated biogeocenoses. Comparison of the microbial community in the rhizosphere and rhizoplane of plants grown under different conditions showed that they differ little from each other in the number of microorganisms, however, as part of microbocenosis there are noticeable differences. Thus, a slight decrease in the number of heterotrophic bacterial community in contaminated soils was revealed. At the same time there was a slight increase in the number of spore-forming bacteria and saprotrophic micromycetes. Micromycetes increasing in polluted ecosystems noted by various researchers [22]. So, in the rhizosphere and rhizoplane of plants grown in the presence of oil, in bacterial communities bacteria of *Gordonia, Microbacterium* and *Derxia* genera were identified, which is found in all studied samples in the amount of 30.8% , 39.4% and 25.1 %, respectively. From "useful" genera *Gordonia* and *Derxia* can be mentioned. According to the literature, among the representatives of these genera some species able to degrade hydrocarbons can be detected [23], [24]. Nucleotide sequence analysis showed that the dominant and typical bacteria from rhizoplane and rhizosphere of contaminated soils belong to the *Bacillus, Pseudomonas, Arthrobacter* and *Microbacterium* genera.

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