Influence of oil on the grain culture of *S.cereale (L)*

Kuzmin A.[1], Bondareva L.[2], Kalyakina O.[3], Pakharkova N.[4], Fedorova N.[5], Rakitsky V.[6]

[1,2,3,4]Siberian Federal University, Krasnoyarsk, Russia
[2,5,6]Federal Scientific Center of Hygiene. F.F.Erismana, Moscow, Russia

***Abstract*—** *The toxic effect of oil on a gramineae species – rye S. cereale L. has been studied. When introducing oil into soils it has been found that the soil fraction with the particle size being 0.05-0.45 mm absorbs the oil least of all. Here, the distribution of the estimated polyaromatic hydrocarbons is directly dependent on the portion of the size fractions of the soils under study. During the experiments the oil loss from the soils amounts to ~17 %, which is due to the possible oil decomposition by the plant roots and partial evaporation of oil from the soil surface. With the oil content being 9 %, the young rye shoots are suppressed, but the biomass is quite viable, which is evidenced by IR spectroscopy and morphological indicators. However, chromosome disturbances have been observed as a result of cytogenetic studies of the root system of rye, which are associated, among other things, with the effect of the soil particle agglutination around the root system. This may disturb the chloroplast motionin the cells, giving rise to anomalies in cell structures. Thus, the conducted experiments can indicate that rye can be applied for the phytoremediation of soils polluted by oil spills.*

Keywords*— oil, rye *S. cereale L., chtomatography, distribution, toxicity.

I. INTRODUCTION

Three groups of environmental factors are known to interact under the conditions of oil pollution, namely: 1) the unique complexity of oil composition, with the latter undergoing constant changes; 2) the heterogeneity of the composition and structure of any ecosystem which is in the process of constant development; 3) the diversity and variability of external factors influencing the ecosystem: temperature, pressure, humidity, conditions of the atmosphere and hydrosphere etc. (Pikovsky, 1993). Apparently, the consequences of the oil pollution of the ecosystem must be estimated and the methods of eliminating the consequences must be chosen taking into account a specific combination of these three groups of factors.

A vast majority of emergency oil spills caused considerable and, in many respects, irreversible damages to ecosystems. The oil pollution results in various changes in the functioning of natural ecosystems and in land degradation. This, in its turn, hinders the growth and development of plants (Adam, Duncan, 1999), which is, first of all, related to the phytotoxic action of the pollutant (Ammosov, Trofimov, Sukhanov, 1999).

Using the cytogenetic characteristics of plants as the biomarkers of oil pollutions allows one to obtain the information which cannot, essentially, be obtained by analyzing the levels of the pollutant content in the environment or in an organism. The sensitivity of biological test-systems is recognized to be higher in most cases than that of chemico-analytical methods (Skrobek et al., 2006, Kitano, 2002). There is a long list of plant test-organisms (Inckot, Santos, De Souza, Bona, 2011, US Environmental Protection Agency, 2002.).

When estimating the mutagenic danger of the environment with the long history of chemical pollution, allochtonic species are more preferably taken to be the controls. Most of the investigations on estimating the mutagenic effect of oil pollution are carried out using terrigenous plants, mainly, the family Gramineae (McGill and Cole, 1981, Golikov, Sanotsky, Tiunov, 1986, SanMiguel et al., 1996, Tikhonov et al., 1996).

The aim of the present work is to study the toxic action of oil on one of the grain crop species, rye *S. cereale L.* using a simulation experiment.

II. MATERIALS AND METHODS

A plant of the family *Gramineae*, rye, was used as the object of study. Rye (*Secale cereale*) is a grass grown extensively as a grain, a cover crop and as a forage crop. It is a member of the wheat tribe (*Triticeae*) and is closely related to barley (*Hordeum*) and wheat (*Triticum*). Rye grain is used for flour, rye bread, rye beer, crisp bread, some whiskeys, some vodkas, and animal fodder. It can also be eaten whole, either as boiled rye berries, or by being rolled, similar torolled oats. Rye is a cereal grain and should not be confused with ryegrass, which is used for lawns, pasture, and hay for livestock (Zohary, Hopf, 2000).

For the experiments use was made of oil from the deposit Kumkol, the Republic of Kazakhstan: the oil is characterized by the low content of sulfur (0.47%), it is paraffin-based (14.3% of paraffin, the solidification temperature is minus 6.5°C), with the content of asphaltic resinous components varying from 2.85 to 5.45%, and coking being 1.64%.

To ensure reproducibility, the experiments were carried out using a universal soil "Garant" (Russia). The agrochemical soil characteristics are the following: the content of nitrogen is 30-50 mg/kg, phosphorus – 70 mg/kg, potassium – 80-100 mg/kg, pH = 6.5 – 7.4, with the presence of microelements, such as Mg, Cu, Fe, Mo, B, Mn etc.

2.1 Experimental technique.

The plants were sowed into polyethylene containers with the size of 20x 15 x 15 cm, each of them filled with 500 g. of soil. In the simulation experiment the following systems were set: S_0 – the control (soils without introducing the pollutant), S_{oil}–soils with the introduction of oil in the amount of 3 wt.%. (S_{oil}-1), 6 wt.%. (S_{oil}-2)and 9 wt.%. (S_{oil}-3).

After the introduction of oil the systems were uniformly moistened every 48 hours. The amount of water added into the systems was 70-100 ml. The duration of the first experimental stage was 20 days.

One part of the soils was subjected to analysis; the other part was used for sowing preliminary germinated rye seeds. The increase in the seed growth was observed, as well as further plant development. The duration of the whole experiment was 50 days. After the experiment the soils and plants were studied.

2.2 Method of Soxhlet

The initial soil sample S_0 and the soils after carrying out the first stage of the experiment (S_0, S_{oil}-1, S_{oil}-2, S_{oil}-3) were successively fractioned using the method of Soxhlet (Crompton, 2013). 20 grams of the soil were mixed with 160 ml of the mixture hexane/acetone/toluene in the ratio 10:5:1 and extracted for 12 h. Then, the organic extract was separated from the soil and washed twice with 300 ml of distilled water. The organic phase was dried using anhydrous salt Na_2SO_4 (10 g) and further evaporated in the nitrogen atmosphere. To specify the distribution of the oil components between the soil particles of different sizes the soil samples S_{oil} and S_0 were divided into three main fractions according to the soil classification system ASTMD-422. These fractions were designated as F_{1-3}. The method of Soxhlet was also applied for studying the obtained soil fractions and for the soils after carrying out the experiments with the plants.

2.3 Hydrocarbon separation method

The concentrates obtained as a result of the extraction from the soil samples (S_{oil}-1, S_{oil}-2, S_{oil}-3), were divided into component groups by the method of open column chromatography (Nadirov, Kotov, Kamyanov et al. 1984).First, as phaltenes were excluded from the extracts using 35 ml of n-heptane. The asphaltenes were precipitated from the oils using n-heptane according to the «hot» Golde method (Petrov, 1971).Resins were extracted from the deasphalted oil according to State All-Union Standard (GOST 11858—66) with the removal of oil using n-heptane with the Soxhlet apparatus (Nadirov, Kotov, Kamyanov et al. 1984.). For this purpose, the deaspalted organic extracts were introduced into a chromatographic column (500 mmx 8,5mm), filled with a mixture of silica gel and alumina (2:1). Saturated hydrocarbons were eluted with the mixture of 35 ml n-pentane and 40 ml dichloromethane. Polar compounds were eluted with the mixture of methanol : chloroform (1:1).

2.4 Cytogenetic method – determination of chromosome aberrations

Mutagenic activity was determined using ana-telophase analysis (Barberrio, Voltolini, Mello, 2011), which allows one to study the mutation frequency by taking into account the sum of chromosome aberrations (CA) and lagging of chromosomes (lag.) at anaphase and telophase stages (Elag+CA, %) (Barberrio, Voltolini, Mello, 2011). Chromosome aberrations, i.e. chromosome structured isorders, include bridging and fragments which are due to deletions and translocations. Chromosome lagging is associated with the mitotic spindle damages or chromosome disorders in the mitotic spindle (Barberrio, Voltolini, Mello, 2011). The degree of the mutagenic impact was estimated according to the intensity of the mutagenic impact (Barberrio, Voltolini, Mello, 2011). The latter is determined as multiplicity of the percent exceedance of the induced mutations over the control value (spontaneous level) and expressed in points. The points of the mutagenic impact intensity were ranged according to the levels of the mutagenic impact and classified as strong, medium, weak or the absence of the impact. The statistical processing of the results was carried out by the software “Statistica” (t-test and ANOVA). The values at $p<0,05$ were taken to be the significance point (Barberrio, Voltolini, Mello, 2011).

2.5 Sample preparation for IR-spectroscopy

The air-dry weight samples of the plants were finely ground. Afinely-ground sample obtained was mixed with KBr used as a matrix and tablets were formed. All the samples were prepared under similar conditions (the time of mixing with potassium bromide, forming pressure, vacuum treatment time). The dry plant biomass (6 mg) was mixed with 1g of KBr.

2.6 Sample preparation for the cytogenetic investigation of the root meristems

Root fragments with the length of 0.8–1.5 cm were fixed in ethyl aldehyde (3:1) for 24 hours, then they were paced into an ethanol solution (70%), and stored at + 5^0C until the preparation formation. The cytogenetic analysis of the squash preparations was carried out using the method of Z.P. Pausheva (Pausheva, 1980). The rye roots were dyed using acetocarmine including boiling in water for 10 min, then, the material was placed into an acetic acid solution (45%) for 5 min. Maceration was carried out in chloral hydrate. The preparations were studied with a microscope MIKMED-6 with the magnification of 40×15×10. Differences between the control and the treated systems were observed. The chromosome aberrations were considered, recorded and their images were taken. The test was run four times. The obtained experimental data were statistically processed using the applied software package "Microsoft Excel 2000". The reliability of the data discrepancy was determined using the t-criterion of Student (Plohinsky, 1970).

2.7 Investigation of the changes in the biological structure of the plants by the method of IR-Fourier spectroscopy.

To investigate possible changes in the structural (stable) cell fragments the method of IR-Fourier spectroscopy was used (IR-spectroscope Fourier Nicolet 380).

The measurement results were processed using the software Varian Resolutions Pro, the characteristic frequency values corresponding to the plant chemical composition were identified using tabular spectral data. The functional groups caused by anthropogenic pollution in the sample of the plant material, which were not characteristic for the plant chemical composition were identified using the standard samples as well as according to the built-in library (in the spectrometer). The averaging of the characteristics was achieved owing to the 20-fold scanning of the samples, followed by the statistical processing of the results (Al-Holy et al., 2006, Kemsley, 1998).

2.8 Determination of the hydrocarbon content using gas chromatography

Some polyaromatic components were determined by the method of gas chromatography using the mass-spectrometry detector (GC-MS). The organic oil extracts Soil-1, Soil-2, Soil-3 (F1-3) were analyzed using Clarus 500 (PerkinElmer, USA). The gas chromatograph was equipped with a Chrompack WCOT column, CP-Sil 5 CB / MC (30 м, 0.32 ммID) column, the elution rate being equal to 1,1 ml/min. Due to the wide range of the molecular masses of the components of interest the technique of introducing the samples into the column was strictly fixed. The heating temperature was increased from 50 °C to 65 ^{o}C at a rate of 15 ^{o}C /min, from 65 ^{o}C to 150 ^{o}C at a rate of 8 °C/min and from 150 ^{o}C to 300 ^{o}C at a rate of 3 °C/min. The samples were introduced in a hexane solution (100 parts per million) with *n*-dodecane as the internal standard (Drugov, Rodin, 2007).

III. RESULTS AND DISCUSSION

3.1 Soil analysis of the experimental systems

When carrying out the simulation experiments on the oil influence on the soil-plant system a detailed study of the introduced oil distribution in the soil size fractions was carried out both before planting and at the end of the experiment. All the factions of the soils under study were weighted after the solvent evaporation and the obtained results are presented in Table 1.

As is shown in Table 1, before planting the total oil content amounted to ~ 3, 6 and 9 %, which corresponded to the added pollutant content. The obtained discrepancies in the content of the size fractions of the analyzed soils are quite logical and connected with the soil particle aggregation due to sticking under the influence of the added oil. The oil distribution according to the size fractions is predominantly associated with the quantitative ratio of the fractions. The results of determining the oil content in the soil fractions of different sizes indicate that the fraction with finer particles contains a smaller concentration of the oil components.

TABLE 1
THE CHARACTERISTICS OF THE SOIL FRACTIONS AND THE CONTENT OF THE ORGANIC POLLUTANT

Soil Fraction	Grain size (mm)	Soil Fraction yield (% w)		Content (% from total)	
		before plants	after plants	before plants	after plants
S_0F_1	>2.00	25.7	23.2	0	0
S_0F_2	>0.45	51.4	54.9	0	0
S_0F_3	>0.05	22.9	21.9	0	0
S_{oil}-$1F_1$	>2.00	26.7	23.1	1.0	1.0
S_{oil}-$1F_2$	>0.45	54.9	57.0	2.0	1.5
S_{oil}-$1F_3$	>0.05	18.4	19.9	0	0
S_{oil}-2 F_1	>2.00	28.2	24.1	2.0	1.5
S_{oil}-2 F_2	>0.45	55.4	56.6	3.0	2.5
S_{oil}-$2F_3$	>0.05	16.4	19.3	1.0	1.0
S_{oil}-$3F_1$	>2.00	30.9	28.2	3.0	2.3
S_{oil}-$3F_2$	>0.45	60.2	57.7	5.0	4.2
S_{oil}-$3F_3$	>0.05	8.9	14.1	1.0	1.0

This is likely to be due to the minimal availability of the fine fraction to the introduced pollutant owing to the earlier process associated with the adhesion of the soil fragments located in the surface layers of the studied soils.

At the end of the whole experiment a decrease of the oil content in all the soil samples studied was observed which was equal to ~17 % of the introduced amount (from 3 to 2.5 %, from 6 to 5 % and from 9 to 7.5 %). This is likely to be due to the oil decomposition by the plant root system as well as to the partial evaporation of oil from the soil surface.

The extracts obtained from the soil samples F1-3 using the Soxhlet method were also divided into groups (saturated, aromatic and polar components) by extraction using simplified open column chromatography: the extracts were put into a glass column (340 mmx 6 mm) loaded with 2 g of wet silica gel and 1 g of wet aluminum oxide. Aliphatic hydrocarbons were eluted with a mixture of hexane (10 ml) and benzene (25 ml). The hydrocarbons were separated and identified by gas chromatography using mass-spectrometry detection. The results are presented in Table 2.

TABLE 2
THE COMPONENT CONTENT IN THE EXTRACTS, %

Sample	Saturates	Aromatics	Resins	Asphaltenes
S_{oil}-$1F_1$	34.0	34.7	17.3	14.0
S_{oil}-$1F_2$	40.3	35.4	13.9	10.4
S_{oil}-$1F_3$	25.7	29.9	12.3	12.7
S_{oil}-$2F_1$	32.0	31.0	27.0	10.0
S_{oil}-$2F_2$	38.7	35.3	13.0	13.0
S_{oil}-$2F_3$	30.3	36.0	23.2	10.6
S_{oil}-$3F_1$	29.8	37.1	20.1	13.0
S_{oil}-$3F_2$	41.0	36.5	12.5	10.0
S_{oil}-$3F_3$	31.3	40.5	15.0	13.2

The evidence obtained proves the regularities in the distribution of the oil content in the soil fractions.

Table 3 presents the results of identifying individual components in the soil extracts obtained before planting.

The resulting contents of certain polyaromatic hydrocarbons are distributed in full agreement with the ratio of the size fractions of the studied soils. Taking into account the fact that the larger portion is provided by the 0.45-2 mm fraction, it is this fraction which is likely to be the most sensitive to organic pollutants such as oil.

TABLE 3
THE RESULTS OF IDENTIFYING INDIVIDUAL COMPONENTS BY GC-MS

	Name	Monitored Ions M/z			Concentration, ppm								
					S_{oil}-1			S_{oil}-2			S_{oil}-3		
					F-1	F-2	F-3	F-1	F-2	F-3	F-1	F-2	F-3
1	Acenaphthene	154	153	152	3			2	3		3	5	1
2	Fluorene	166	165	167	2			2	2		2	3	2
3	Phenanthrene	178	179	176	33	25		42	37	5	49	32	6
4	Anthracene	178	176	179	13	10		15	11	2	21	17	2
5	Fluoranthene	202	101	203	53	44		59	54	8	78	69	12
6	Pyrene	202	200	203	118	113		124	115	21	207	176	26
7	Chrysene	228	226	229	117	127		143	152	34	254	296	60
8	Benzo(b)fluoranthene	252	253	125	87	90		74	105	13	138	159	20
9	Benzo(a)pyrene	252	253	125	70	96		92	102	11	154	198	21
10	Benzo(ghi)perylene	276	138	277	64	55		71	67	17	102	84	25

3.2 Analysis of the plants

The results for the morphological indications of the plants are given in Table. 4.

TABLE 4
MORPHOLOGICAL CHARACTERISTICS OF *S. CEREAL L.* IN OIL EXPERIMENTS (n-10, P-0.95)

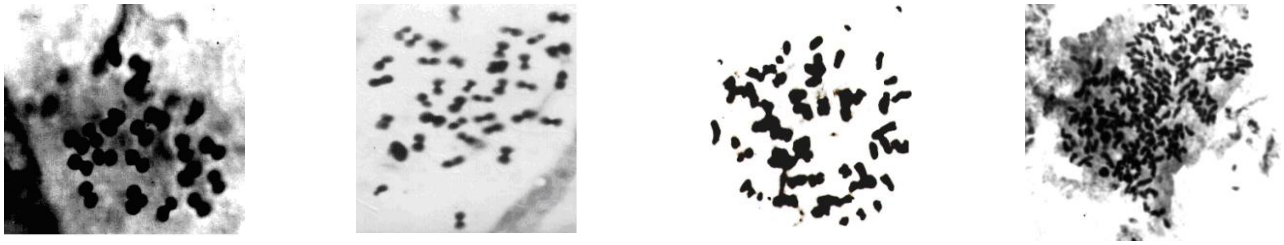
Experiment		Size of the plant parts, length, cm		Plant weight, $m_{cp.} \pm \Delta$	Survival, %
Contaminant, wt. %	The number of plants, pcs	Above-ground part $L_{cp.} \pm \Delta$	Root part $L_{cp.} \pm \Delta$		
Control – 0 %	15	22 ± 1	5.7 ± 0.4	1.3 ±0.2	100
3%	15	21.2±0.7	5.7±0.3	1.2±0.5	100
6%	15	22.5±0.5	6.3±0.5	1.1±0.4	100
9%	15	21.6±0.3	7.2±0.6	0.6±0.3	100

The results obtained indicate the inhibition of the plant development, but despite this, all the physiological functions persist. However, in the plants subjected to pollution the growth of the underground parts is suppressed and the capability to accumulate biomass is reduced.

The IR-Fourier spectroscopy method was used to reveal the origin of the possible effect of oil on the top part of the studied plants – vegetative components of the plant solid residue after drying out (cell walls, membranes, intracellular organelles etc.) both in the control (without the oil addition) and in the experimental system. As a result of the performed studies of the vegetative residue sit is revealed that the structure of the solid cell fragments of the rye shoots grown in the soils containing up to 3 % of oil hardly differs from that of the control plants. However, with the oil content increasing from 6% and higher, there arise new groups and their content increases with increasing the oil content. For example, the analysis of the bands with increasing absorption intensity with in the spectral range of 1000-1200 cm^{-1} along with the absorption at 1735 cm^{-1} indicate the increase of ester compounds in the structural component of a cell (Coates, 2000). The intensity of other groups in the spectrum is also different from that in the control spectrum, for example, the intensity increase of the negative band 1746 cm^{-1} which is associated with the pectin group and that of the bands 1605 and 1419 cm^{-1} which are associated with carboxylic groups (Marchessault, 1983, Chen, Wilson, McCann, 1997). This is likely to be due to the fact that entering living organism he oil hydrocarbons destroy cell membranes and easily penetrate through the lypoprotein barriers of plants, resulting in metabolic and morphological disturbances. Large molecules of polycyclic aromatic hydrocarbons of oil slowly penetrate into a cell and dissolve in the lipids of cell membrane, which can cause the cell death (Smith, Lethbridge and Burns, 1999).

3.3 Cytogenetic study of the *S. cereal (L)* root system

Chromosomes of the given gramineae type are small, 1-2 μm and smaller, which does not allow one to reliably measure them. By morphology the chromosomes are bi-armed, meta- or submetacentric. Metaphase plates with different amount of chromosomes *S. cereal L.* are presented in Fig. 1 a-d.



A) **B)** **C)** **D)**

FIG. 1. METAPHASE PLATES OF *S. CEREALE L.* WITH DIFFERENT CHROMOSOME NUMBER: a – 2n=24; b – 2n=40; c – 2n=56; d – 2n=96.

In the cells of the *S. cereal L.* roots, chromosome disturbances were detected in the studied systems. The total frequency of the chromosome disturbances in ana-telophases turned out to be 3-4 times higher in the experimental samples as compared to the control (Table 5). The differences in the occurrence and spectrum of chromosome disturbances in the metaphases of *S. cereal L.* between the experimental systems and the control were even higher. As for the character of the disturbances, the agglutination and fragmentation of chromosomes were observed as well as ring chromosomes, non-uniform chromosome disjunction in anaphase, lagging chromosomes, multiple disturbances of the chromosome disjunction etc.

The abnormal chromosome disjunction in mitosis can be associated with the functional disturbances of the contractile proteins of the mitotic spindle. The agglutination results from the chromatin agglutination in metaphase or anaphase. The emergence of the ring structures evidences the division of the chromosome's arms, resulting in a chromosome segment with two "sticky" ends, the latter forming a ring. Consequently, two fragments are formed. The presence of bridging indicates the emergence of the asymmetric translocation.

Numerous cases of the cytoplasm vacuolization were observed – almost in 100% cells *S. cereal L.* in the systems with the introduced 9 % of oil, in 50% and 30% plant cells, correspondingly, in the systems with the introduced 6 and 3 % of oil. In the cells of the root meristems of *S. cereal L.* grown in the system with the introduced 9 % amount of oil, micronuclei were found which formed from the intact chromosomes resulting from the disturbances in the normal segregation in mitosis. They could also be formed from acentric fragments due to the chromosome aberrations. Yet another type of anomalies was observed in the system with the introduced 9 % of oil – a residual nucleolus in the early metaphase while, normally, it must disappear in prophase of mitosis. The given peculiarity evidences the abnormally-extended processes of the ribosome gene transcription and rRNA synthesis.

Mitoses are the most common anomalies which can arise due to the microtubes damaged by oil and, this, in its turn, results in C-mitosis, aneuploidia (Fiskesjo,Wang, Gorsuch and Hughes, 1997). Other anomalies, such as disturbancesin ana- and telophases, can be due to the oil effect on the microtubes of the formations (Alkio, Tabuchi, Wang et al., 2005),as they may well be due to the chromosomes loosing their ability to move owing to the presence of oil (Ajay, Sarbhoy, 1988). This can also be associated with physical adhesion of proteins in the chromosome (Patil, Bhat, 1992).

Stickness is also considered to be the indicator of toxicity resulting in the cell death (Fiskesjo, Wang, Gorsuch and Hughes, 1997, El-Ghamery, EI-Nahas, 2000). The anomalies on the interface nuclei can be caused by the toxic effect of oil. The micronuclei destroy the microtubes and give rise to acentric fragments of chromosomes (Chauhan, Dikshith and Sundararaman, 1986, Chauhan and Sundararamab, 1990). Binuclear cells take it to be the inhibition of cytocinesis in any control points of the cell cycle (Ateeq, AbulFerah et al., 2002). The anaphase bridges were observed, mainly, on mono-bridges while on di- and tri-bridges they were also observed but to a smaller extent. This, in its turn, could be caused by chromosome mutations (El-Ghamery, EI-Nahas, 2000).

The main result of the performed investigations consists in finding that the studied culture *S. cerealeL.* can be used for the soils more or less polluted by oil spills. Here, planting the studied gramineae species would result in remediation of polluted

soils. Though the top part of the plants develops with a visible delay as compared to the control, the vegetative mass can be used, for example, in animal husbandry as food. Thus, using the complex chemical and biological technology of bioremediation of the soils polluted by oil products would allow one to increase the intensity of this process and to achieve a more complete removal of the oil components.

As a result of the performed investigations the following conclusions were made:

1. The results of determining the oil content in soil fractions of different size show that the fraction with finer particles has the lowest concentration of the oil components (from 0, 05 to 0.45 mm).
2. Inallthecasesitisfoundthatattheendoftheexperimentsongrowingplants, the total decrease of the oil content is equal to ~17 %, which is attributed both to the possible decomposition of oil by plant roots and to the partial evaporation of oil from the soil surface.
3. The distribution of some detectable polyaromatic hydrocarbons is directly dependent on the portion of the size fraction of the studied soils.
4. The morphological studies of the plant top part show that despite the complete survival of the rye shoots, when introducing 9 % of oil, the shoot inhibition is observed while, when introducing 3 and 6 % of oil, almost no inhibition is found and the samples are hardly different from the control.
5. The IR-spectroscopy studies of the plant material show that the structure of the solid fragments of biomass, when introducing 3% oil, hardly differs from the control samples, with slight changes in the spectral lines being observed with the 6 and 9 % oil content. Here, new vibration lines are found to appear and the intensity of the present spectral lines is changed.

In cytogenetic studies of the root system of rye shoots, chromosome disturbances are observed which are also associated with the effect of soil particle agglutination around the root system. This can disturb the motion of chloroplasts in the cells, resulting in the development of anomalies in the cell structures.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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