Terek, O, Lapshyna, O, Velychko, O, Bunyo, L, Dovgaiuk-Semeniuk, M: Crude oil contamination and plants - Kőolajszennyeződés és a növények

Abstract

The objective of this study is to investigate the physiological mechanisms of plant adaptation under crude oil contamination of soil. Different plant species used in the study sedge (Carex hirta L.), bean (Faba bona Medic.), alfalfa (Medicago lupulina L.) and clover (Trifolium pratense L.) showed various biochemical and morphological reactions under oil pollution. The effect of crude oil on root elongation, shoot growth and dry matter accumulation of the four species was evaluated. All investigated plant species under oil contamination formed powerful root system, however the growth of plant aboveground part was diminished.

Results of phytohormones content investigation in C. hirta confirmed that the morphological growth reactions occurred due to changes in phytohormonal balance of plants. Crude oil reduced amount of main growth activator auxin in sedge leaves and stimulated the increase of abscisic acid amount. In sedge roots auxin accumulation occurred under oil contamination.

We studied antioxidant system of C. hirta and F. bona plants under oil pollution. Results of our investigations show that for adaptation under oil pollution sedge plants accumulate low molecular weight antioxidants, e.g. polyphenols and maintain reduced cell status due to high reduced/oxidized glutathione ratio in root system. In roots of F.bona activation of antioxidant enzymes catalase and peroxidase under oil effect occured.

It was shown that crude oil effects nitrogen metabolism in alfalfa and clover plants. The amount of non-protein and amine nitrogen in plants increased, while the proportion of protein nitrogen decreased under oil contamination. To improve soil nitrogen supply we inoculated the seeds of alfalfa and clover with 348a, BN9 and A91 strains of Rhizobium leguminosarum bv. trifolii for intensification of nodulation in oil contaminated soil. Although the number of nodules on plant roots in oil contaminated soil decreased, their weight was greater than in the control. Improvement of plants nitrogen nutrition in oil contaminated soil requires further investigations.

Introduction

The scale of oil spills all around the world increases each year. A terrestrial oil spill perturbs both the abiotic and biotic portions of the environment. Soils may become hydrophobic, exhibit altered albedo and possess high carbon to nitrogen ratios. Plant, animal, bacterial and fungal abundance and diversity are generally reduced in polluted soil [Amadi et al., 1996, Freedman, 1996]. One of the most prospect techniques for restoration of oil contaminated soils is environmentally friendly, cost effective phytoremediation [Frick et al., 1999, Banks et al., 2003]. Long-rootage grasses, known with their high adaptive potential under oil pollution, are often used for phytoremediation of oil contaminated sites [Xia, 2004]. During the monitoring of plant communities of Boryslav oil region in Western Ukraine sedge (*Carex hirta* L.) plants showed substantial recovery from crude oil spills. Sedge plants penetrate to oil spills due to strong rhizomes and form pioneering plant communities on first stages of oil contaminated sites overgrowth. Legumes adapt to oil contamination because of an advantage in nitrogen poor oil polluted soil due to nitrogen symbiotic fixation with nodule bacteria [Merkl et al., 2005]. We studied *F. bona*, *M. lupulina* and clover *T. pratense* for their ability to adapt under oil pollution. Physiological basis of plant adaptation mechanisms under oil influence remain to be elucidated. A better understanding of these mechanisms will lead to improve remediation traits of resistant plants.

We investigated main growth parameters of 30-days-old *C. hirta*, *F. bona, M. lupulina, T. pratense* plants grown in soil containing 5 % crude oil. Plant growth depends on phytohormonal balance. The hormones are part of an intrinsic genetic network controlling organ development and growth [Kende, Zeevaarth, 1997]. Phytohormones mediate the responses to variable extrinsic factors and play a crucial role in the response to biotic and abiotic stressors [Khan et al., 2012, Fraire-Velazquez et al., 2011]. The effect of crude oil on main phytohormones – auxine (IAA), cytokinins (CKs) and abscisic acid (ABA) amount in *C. hirta* plants was studied in this work.

The equilibrium between the production and the scavenging of ROS may be perturbed by various biotic and abiotic stress factors such as salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks [Gill, Tuteja, 2010].These disturbances in equilibrium lead to sudden increase in intracellular levels of ROS which can cause significant damage to cell structures. Stress-induced ROS accumulation is counteracted by antioxidant systems that include a variety of scavengers, such as superoxide dismutase, catalase, peroxidase et al. and non-enzymatic low molecular metabolites, such as ascorbic acid, glutathione, carotenoids flavonoids et al. [Haliwell, 2006]. InIn this work we also investigated physiology-biochemical parameters of oxidative stress and activity of antioxidant defense system of sedge and bean plants under oil contamination of soil.

An important condition of the oil polluted soil qualities restoration is an improvement of its nitrogen regime. The nitrogen lack in oil polluted soil arises as a result of dramatic changes of the microbiological processes forced by acquired anoxity of the soil. In such soil conditions, the vital functions of aerobic ammonificators and nitrificators are depressed, while the anaerobic denitrificators activate [Ismajlov, Dulherova, Hilyazov, 1980]. At the same time the microorganisms which use hydrocarbons as the source of nutrition intensively propagate in the soil, also requiring significant amounts of nitrogen [Zelenko, 2003; Miroshnichenko, 2000]. A valuable tool for oil polluted soil's nitrogen regime improvement could be the cultivation in such soil of legume plants, which are able to fix molecular nitrogen in the symbiosis with nitrogen-fixing bacteria. In this paper we provide the results of investigation of the nodulation ability of clover plants in oil polluted soil after seeds inoculation with active stains *Rhizobium leguminosarum bv. trifolii.*

Materials and methods

All plants were grown under concentration of crude oil in soil 5%. Sod-podzolic soil samples were artificially contaminated with crude oil obtained from Boryslav region of Lviv area (Ukraine) to yield a concentration of 50 g/kg of soil (5%). After 30 days period necessary for evaporation of the most toxic volatile hydrocarbons sedge plants were transplanted from uncontaminated sites, bean, clover and alfalfa plants were grown from seed.

At 30 days the plants were harvested, their length and that of their roots were measured, the plants were dried at 70°C for 3 days, and the plant biomass dry weight was recorded [Dorn and Salanitro, 2000].

Determination of total antioxidant activity was carried out in reaction of methanol plant extracts with 1,1-diphenyl-2-picrylhydrazyl (DPPH) [Cervato G. et al., 2000]. Glutatione was measured in reaction with Ellman's reagent [Lay M., Casida J., 1976]. Determination of polyphenols was carried out by a modified method of Folin-Ciocalteu [Shetty K., 2004]. The activity of catalase was evaluated by a decrease in color intensity of the complex of hydrogen peroxide with ammonium molybdate [Goth, 1991]. Peroxidase activity was determined by the intensity of oxidation of benzidine by hydrogen peroxide [Мусієнко М., 2001].

Analytical determination of phytohormones was performed by HPLC on Agillent 1200 LC with diode-array detector [Musatenko et al, 2003].

Alfalfa and clover seeds bacterization was conducted with the standard stain 348a and active stains BN9 and A91 from the *Rhizobium* collection of the Institute of Plant Physiology and Genetics, National Academy of Science, Ukraine. These stains are known for their resistance under unfavorable ecological conditions, in particular lack of moisture [Kots, 2011]. The nodulation ability was measured by the quantity and weight of the root nodules. The content of protein, non-protein and amine nitrogen in the plants was determined by the chloramine method [Pochynok, 1988].

Standard error of the means were calculated for the replicate readings. Data were analyzed to detect significant differences between the treated and control groups, followed by Student's t-test (a *p value of* ≤ 0.05 was considered to be statistically significant).

Results and discussion

Table 1 shows the effect of oil components on the seedling growth (stem and root elongation), and dry weight of the sedge *(Carex hirta L.),* bean *(Faba bona Medic.),* alfalfa *(Medicago lupulina L.)* and clover *(Trifolium pratense L.)* plants species. In relation to the stem elongation, the most significant toxic effect of oil components on this parameter was observed in *C. hirta* L. (40%) and *T. pratense* L. (58%) plants. The plant height decreased relative to the controls for each plant species. The length of *C.hirta* roots increased 17% and volume of *F.bona* roots increased 64% compared with the control. The roots of *M. lupulina* and *T. pratense* plants in oil polluted soil were shorter then control.

Dry matter production in leaves was reduced in contaminated soil in all examined plants after 30 days. (tab. 1). Crude oil caused the greatest reduction (25%) of dry matter accumulation in sedge leaves compared with the control. Crude oil effected dry mater accumulation in leaves and roots of alfalfa and clover plants. However dry matter yield in roots of sedge and bean plants under oil pollution significantly increased.

	Plant height, cm		**Root length, cm**		**Leaves dry matter, %**		**Roots dry matter, %**	
	Control	**5% oil**	**Control**	**5% oil**	**Control**	**5% oil**	**Control**	**5% oil**
Carex hirta L.	28,5 ± 2,7	17,1 ±2,4	15,7 ± 2,1	18,9 ± 1,9	31,5± 0,8	23,5 ± 0,6	20,0± 1,3	38,4± 1,8
Faba bona Medic.	24,1 ± 0,8	22,7 ± 0,8	1,7 ± 0,4 *	4,8 ± 0,2*	10,23± 0,5	9,21± 0,3	9,8± 0,4	16,5± 0,6
Medicago lupulina L.	12,5 ± 1,1	9,5 ± 1,1	10,5 ± 8,7	8,8 ± 6,6	25,1±2,1	24,1±3,0	29,2±2,5	21,6±1,6
Trifolium pratense L.	8,5±0,7	3,6±0,4	7,3±0,8	5,8±0,9	13,3±1,1	10,9±1,4	14,1±0,7	13,1±1,2

Table 1 Growth parameters of plants under crude oil contamination (M ± m; n=30)

*- volume of roots, cm^3

In general, crude oil had a significant toxic effect on dry weight and also on stem elongation of all examined plants. For adaptation under deterioration of air, water and mineral feed conditions in oil contaminated soil plants bean and sedge plants formed the powerful root system.

Growth reactions occur due to accumulation, redistribution and activation of phytohormones. Under oil contamination significant changes in phytohormonal balance occurred in both above and underground organs of *C. hirta* plants. The character of this changes depended strongly on oil effect on different plant organs. In sedge leaves, which are not subjected to the direct impact of oil (at the time of sampling the most toxic volatile hydrocarbons evaporated from the soil) total content of auxin was 7-fold lower compared with control (tab. 2). The content of other phytohormones stimulators of plant growth - cytokinins in sedge leaves under oil pollution increased 3 times compared with the control (tab. 2) Results of our work show that in *C. hirta* leaves under oil stress accumulation of abscisic acid occurred.

Underground organs (root and rhizomes) of *C. hirta* plants, affected directly by crude oil showed another character of hormonal changes than leaves. Total amount of cytokinins and abscisic acid (ABA) in *C. hirta* roots under oil contamination remained on a control level. However auxin content in sedge roots increased more than 6 times compared with plants from not contaminated sites (tab. 2). In *C. hirta* rhizomes content of ABA and cytokinins decreases relative to control. And as well as in the roots IAA content in rhizomes increased more than 2-fold relative to control (tab. 2).

Carex hirta L. organs		IAA	ABA	CKs
Leaves	Control	39,8 ± 1,80	2,3± 0,07	6,2 ± 0,06
	5% oil	5,8 ± 0,05	3,5 ± 0,06	19,2 ± 0,30
Roots	Control	0,3 ± 0,08	1,6 ±0,02	4,7 ± 0,07
	5% oil	1,9 ± 0,09	1,9± 0,08	4,9 ± 0,06
Rhizomes	Control	9,2 ± 0,30	8,5 ± 0,01	14,4 ± 0,60
	5% oil	19,8 ± 0,20	5,8 ± 0,06	89,6 ± 0,30

Table 2. IAA, ABA and CKs amount (ng/g RW) in C. hirta plants under 5% oil contamination

Such phytohormones amount changes can explain inhibition of aboveground part growth. Auxin is the main stimulator of root growth, thus its accumulation in rhizomes can serve for support of growth activity under oil stress.

Generation of reactive oxygen species (ROS) is a universal non-specific answer reaction to many stressors. Now hydrogen peroxide and other ROS are increasingly viewed as a signal molecule rather then the cell damaging agents. Under 5% of crude oil in soil the concentration of hydrogen peroxide and thiobarbituric acid (TBA) reactive substances did not change and even decreased in some organs of bean and sedge plants [Karpyn, 2012]. Presumably under 5% of crude oil in soil hydrogen peroxide in plants can be considered as a signal molecule that runs antioxidant defense reactions and lead to adaptation of plants.

Parameters of antioxidant system considered in *C. hirta* and *F. bona* plants under oil pollution were concentration of glutathione, polyphenols and total antioxidant activity as well as activity of catalase and peroxidase.

Polyphenols are one of the most important antioxidants in plant organism. Under oil pollution of soil polyphenols content in bean and sedge plants increased several times (fig. 1)

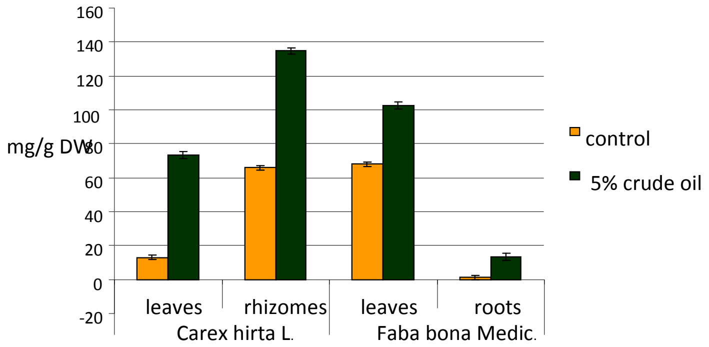

Figure 1 Polyphenols content in F.bona and C.hirta plants under oil pollution of soil (mg/g DW)

It is known that reduced/oxidized (GSH/GSSG) glutathione ratio may have a greater impact on the metabolism of cells rather than the individual effect of each. High GSH/GSSG ratio (9-10) increases adaptation potential of plants [Tausz et al., 2004]. In roots of *F. bona* plants under 5% of crude oil in soil this ratio was 23,16. Reduced state of cells medium causes ROS scavenging and als stimulates mitotic activity. Bean roots under 5% of crude oil in soil accumulate high biomass and have the largest volume.

Integral parameter of total antioxidant activity of *C. hirta* plants under oil contamination increased while in *F. bona* plants this parameter remained on a control level. Activity of main antioxidant enzymes catalase and peroxidase increased in *F. bona* roots under oil contamination 5%.

Thus in *C. hirta* and *F. bona* plants for support of oxidation processes at stable level under 5% of crude oil in soil different mechanisms function. In *C. hirta* rootage the increase of general antioxidant activity and considerable growth of polyphenols content is marked, while in *F. bona* a pro/antioxidant equilibrium is related to the activity of catalase and peroxidase and increase in reduced/oxidized glutathione ratio.

It was found that alfalfa and clover plants adaptation in oil polluted soil resulted in phenotype and metabolic changes. In particular, changes in alfalfa sprouts structure were in shortening of internodes length. Modifications of roots morphological structure were expressed by reduction of the main root diameter (3,4 ± 0,2 mm in control vs 1,9 ± 0,2 mm under 5% oil in soil) and by appearance of numerous lateral roots which increase the suction roots surface in conditions of oxygen, nutrition and moisture deficiency in oil polluted soil.

Results of our investigations show that oil pollution caused the accumulation of nonprotein nitrogen compounds in particular free amino acids in the sprouts clover plants At the same time the protein nitrogen content decreased under oil contamination (tab.3). Similar changes of nitrogen metabolism were observed under crude oil action in alfalfa plants [Velychko, 2011].

	Control		5% oil in soil	
	Root	leaves	root	leaves
Content of protein nitrogen, *% by weight of dry matter*	2,4±0,10	3,8±0,50	2,5±0,20	3,5±0,20
Content of nonprotein nitrogen, *% by weight of dry matter*	0,4±0,01	0,5±0,01	0,6±0,02	1,1±0,02
Content of amine nitrogen, *% by weight of dry matter*	-	12,6±1,20	-	19,1±2,30

Table 3 Contents of protein and nonprotein nitrogen in T. pretense plants organs on the stage of first trifoliate leaf under oil contamination of soil

Low-molecular weight nitrogen compounds play important role in the cell osmotic potential formation, have protective and regulatory functions [Jiang, Huang, 2002]. Among the studied non-protein nitrogen compounds (amino acids, amides, ammonia, nitrates) in clover leaves under oil pollution most significant was increasing the content of amine nitrogen (tab. 3). Increase in the amine nitrogen amount may indicate the inhibition of protein synthesis or activation of proteolysis processes in plants. The role of free amine acids in the plant's adaptation to unfavorable conditions in oil polluted soil is an object of our current investigations.

It was shown that alfalfa and red clover plants form symbiosis with nodule bacteria in oil polluted soil. However the number of nodules on plant roots significantly decreased under oil contamination. At the same time nodules mass in oil treated soil was greater than mass of nodules in control (tab. 4). In oil polluted soil the vast majority of nodules formed on lateral roots while nodules in the soil without oil concentrated mainly closer to main root and laid closer to soil surface.

In this study we biostimulated the native process of nitrogen fixation in soil contaminated with crude oil by inoculation with nodules bacteria *R. leguminosarum bv. trifolii*. It was defined that seed inoculation *T. pratense* seeds with highly active strains of nodules bacteria significantly increased nodulation ability (tab. 4).

	Stain *R. legumino-sarum bv. trifolii*	Amount of nodules, items / plant	Mass of 10 nodules, Mg
Control	–	79 ⍰⍰⍰⍰⍰	1,7⍰⍰0,23
	348a	98 ⍰⍰⍰⍰⍰	1,4 ⍰⍰0,12
	BN9	194 ⍰⍰⍰⍰⍰⍰	1,6 ⍰⍰0,14
	A91	138 ⍰⍰⍰⍰⍰⍰	2,4 ⍰⍰0,25
5 % oil	–	43 ⍰⍰⍰⍰⍰	1,8 ⍰⍰0,19
	348a	80 ⍰⍰⍰⍰⍰	3,4 ⍰⍰0,27
	BN9	88 ⍰⍰⍰⍰⍰	2,8 ⍰⍰0,31
	A91	112 ⍰⍰⍰⍰⍰⍰	3,6 ⍰⍰0,41

Table 4: Bacterization effect on legume - rhizobial symbiosis of T. pratense plants with Rh. leguminosarum bv. trifolii in the oil polluted soil

Clover seed bacterization with *Rh. leguminosarum bv. trifolii* stains 348a and BN9 increased the nodulation ability index of clover plants in oil polluted 2-fold (tab. 4). The seed inoculation with the stain A91 was proved to be the most effective.

Inoculated plants have advantage in the oil polluted soil over not inoculated ones due to their own additional source of nitrogen. The following mineralization of their remains (roots and nodules together with aboveground part) will provide guaranteed and stable enrichment of polluted soil with organic nitrogen.

Conclusions

Thus studied physiological parameters of *sedge (Carex hirta L.), bean (Faba bona Medic.), alfalfa (Medicago lupulina L.) and clover (Trifolium pratense L.)* plants show their high adaptive capacity under oil pollution. For adaptation in oil contaminated soil *C. hirta* and *F. bona* plants inhibited the growth of aboveground organs. At the same time developed root system, which accumulates antioxidants contribute to plant adaptation under oil stress. *C. hirta* and *F. bona* posses high adaptive potential under crude oil pollution and can be recommended for phytoremediation of oil contaminated sites.

The adaptive reconstructions on the phenotype level of *M. lupulina* and *T. pratense* consisted in the morphological root structure modification. Under oil contamination nitrogen metabolism of clover plants displayed in accumulation of the amine nitrogen. Positive influence of artificial bacterization of alfalfa and clover seeds on the formation of symbiotic partnership with *Rh. leguminosarum bv. trifolii* strains in oil polluted soil was showed. For improvement of nitrogen nutritional conditions in oil contaminated soil additional bacterization of *M. lupulina* and *T. pratense* plants can be applied.

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