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Adaptative Responses of Two *Fabaceae* Species to Heavy Crude Oil of Polluted and Remediated Soils

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Abstract **– Petroleum is a complex mixture of thousands of hydrocarbons with toxic and carcinogenic potential. Industrial accidents, oil leaks from pipes or tanks, inadequate manipulation lead to the contamination of soil and underground waters. The purpose of this study is to identify plant species that possess the potential to phytoremediate petroleum contaminated soils from the area of Suplacu de Barcău, Bihor County (Remediation Station 1). The efficiency of the remediation process of the two species (*Medicago sativa* 'Magnat' and *Trifolium repens* 'Lirepa') has been tracked through physico-chemical analysis (total petroleum hydrocarbons - TPH) and enzyme analysis (catalase, acid and alkaline phosphatase, dehydrogense). The germination rate, growth and evolution of the plantlets were monitored for 21 days since the moment of dissemination. Have been noted the moment when the first plantlets sprout, the medium length of the main root, and the medium length of the hypocotyl while taking into consideration the total number of viable plantlets. The two species have different responses to conditions offered by the soils contaminated with petroleum and can be considered species with phytoremediation potential for this kind of soil.**

***Keywords* – *Medicago Sativa* 'Magnat', *Trifolium Repens* 'Lirepa' Remediation, Suplacu de Barcău.**

I. INTRODUCTION

Petroleum, excellent source of fuel and plastic materials, it's a complex mixture of thousands of hydrocarbons with toxic and carcinogenic potential (Doak et al, 1985), (Yasin et al, 2013). Industrial accidents, oil leaks from pipes or tanks, inadequate manipulation lead to the contamination of soil and underground waters. In consequence, most of the highly affected soils with this kind of pollution are often missing vegetation and the activity of microorganisms is deeply disturbed (Maranho et al, 2009; Eze et al, 2013).

In Romania, European country known for petroleum production, there is a series of contaminated areas (e.g. Ploiești, Suplacu de Barcău) and as a consequence it is important for technologies that contribute to their remediation to be developed. Phytoremediation is the use of plants to partially or completely remediate selected contaminants in contaminated soil, sludge, sediment, ground water, surface water and waste water (Zand et al, 2010). It utilizes a variety of plant biological processes and the physical characteristics of plants to aid in site remediation (Interstate Technology & Regulatory Council 2009). Phytoremediation is a continuous process, with different developments occurring to different degrees for diverse conditions, media, contaminants and plants. Is a technology based on synergistic cooperation of plant roots and soil microorganisms for decomposition, transfer, deactivate and inverting contaminant compounds of soil (Vaziri et al, 2013). Thus, phytoremediation is an alternative to excavating and removing all contaminated soil, covering the site with uncontaminated soil and installing a runoff control and drainage system to limit the potential contamination of surface water or groundwater or other in-situ treatment methods that involve complex physical and/or chemical neutralization. The process is realized through phytoextraction, phytostabilization, phytotransformation, phytostimulation (rhizoremediation), phytovolatilization or rhizofiltration (Lan, 2004). Phytoremediation of contaminated soil is utilized in association with different remediation techniques (physico-chemical, microbiological). It needs a relatively long time to act and has variable efficiency of waste removal depending on the targeted zone (Cunningham et al, 1995). Out of the species from the family of *Fabaceae* that were studied for the phytoremediation capacity on soils contaminated with petroleum we mention: *Medicago sativa* L. (Muratova et al, 2008; Liu et al, 2012; Shan et al, 2014), *Trifolium repens* L. (Barrutia et al, 2011), *T. pratense* L., *Onobrychis antasiatica* Khin. (Muratova et al, 2008).

A series of studies concerning the effects of petroleum fractions on *Panicum miliaceum* L. crops (Colibaş et al, 1995; Şandor and Sabău, 2007) and phytoremediation process of soils affected by pollution (Sabău et al, 2002) from Suplacu de Barcău (Bihor County) were conducted highlighting correlations between growth and the concentration of petroleum found in soil (Sabău et al, 2010 a, b). However, studies regarding the adaptive responses and remediation capacity of certain species of *Fabaceae* have not been approached yet. For this reason, the purpose of our study was assessing the adaptive responses underlining some morphological adaptations and phytoremediation capacity of the two *Fabaceae* species as a consequence to their exposure to asphaltic contaminated soils, after chemical remediation and bioremediation.

II. MATERIALS AND METHODS

Materials

Soil samples were collected in March 2014 from Remediation station 1 (Suplacu de Barcău) kindly offered by Mr. Claudiu Faur from S.C. STILO EVORA S.R.L, company, Marghita, Bihor County, Romania.

Bacterial consortium BFL 6000HC (Biofuture Ltd., Dublin, Ireland), commercial product standardized for bioremediating soils contaminated with petroleum fractions utilized in the process of remediating the tested soils was received due to Mr. Pietrangelo De Luca, Waste Management Expert good will.

The Seeds of the two plant species selected for this study were *M. sativa* ‘Magnat’ (MSM) and *T. repens* ‘Lirepa’ (TRL) provided by the Department of Biotechnologies, Faculty of Biotechnologies (University of Agricultural and Veterinary Medicine from Bucharest).

Briefly, this study consisted in 3 experimental variants (Table 1): in the first variant, each plastic planter contained 450 g contaminated soil or control soil has been sown with the 100 seeds from selected plant species (1.1-1.4) and monitored for 21 days. In the second variant, the contaminated soil and control soil have been exposed to bioremediation with bacterial consortium BFL 6000HC during a period of 21 days and then it have been phytoremediated for 21 days (2.1-2.4); in the third variant it was introduced chemical remediation, a process in which the soil is oxidized with ammonium persulfate, 1% solution during a 21 days period (Huang et al, 2002), bioremediated using bacterial consortium BFL 6000HC (21 days) and then subjected to phytoremediation for another 21 days (3.1-3.4). The obtained results were compared with the control that was processed the same as the contaminated samples. As control we has been utilized collected soil from Systematic Aria of Botanic Garden “D. Brândză” Bucharest, from a spot there where dominant T. repens L. subsp. repens (x.4).

Table 1. The Experimental variants.

Experimental variant	Soil sample origin	Plant species	Experimental variant code
Experimental variant 1 (phytoremediation for 21 days)	1 – Remediation station from Suplacu de Barcău	*M. sativa* ‘Magnat’	1.1 MSM
		T. repens ‘Lirepa’	1.1 TRL
	4 – Control soil from Systematic Sector Botanic Garden	*M. sativa* ‘Magnat’	1.4 MSM
		T. repens ‘Lirepa’	1.4 TRL
Experimental variant 2 (bioremediation with bacterial consortium BFL 6000HC for 21 days and phytoremediation for 21 days)	1 – Remediation station from Suplacu de Barcău	*M. sativa* ‘Magnat’	2.1 MSM
		T. repens ‘Lirepa’	2.1 TRL
	4 – Control soil from Systematic Sector Botanic Garden	*M. sativa* ‘Magnat’	2.4 MSM
		T. repens ‘Lirepa’	2.4 TRL
Experimental variant 3 (chemical remediation for 21 days, bioremediation with bacterial consortium BFL 6000HC for 21 days and phytoremediation for 21 days)	1 – Remediation station from Suplacu de Barcău	*M. sativa* ‘Magnat’	3.1 MSM
		T. repens ‘Lirepa’	3.1 TRL
	4 – Control soil from Systematic Sector Botanic Garden	*M. sativa* ‘Magnat’	3.4 MSM
		T. repens ‘Lirepa’	3.4 TRL

Plantlets Adaptive Capacity Evaluation Methods

To study the adaptive responses and capacity of phytoremediation, samples consisting of 100 seeds were prepared for each soil sample, for each experimental variant. The seeds were sown in 0.75 L plastic planters sterilized with ethanol 70% with 450g soil. They were kept in laboratory for 21 days at a temperature of 22-25°C with natural light, being watered twice a day with 25 mL distilled water. The samples were monitored daily. To determine the germination rate the germinated seeds were counted (starting with the stage when the radix penetrated the seeds coating). At the end of the 21 days the viable plantlets were harvested and measured. The length of the main root was considered the zone in between the tip of the root and the end of the white colored zone and the hypocotyl is considered as being the level where the green color can be spotted and continuing to the cotyledons insertion level. After measuring the dimensions of the two organs for each viable plantlet the medium length (cm) has been calculated (Boldor et al, 1983).

Analytical Methods

Total petroleum hydrocarbons (TPH) was determined using spectrophotometricaly measurements at $\lambda = 420$ nm, the value obtained being plotted on a calibration curve obtained through solubilization of 1.75 g of crude oil from Suplacu de Barcău in 100 mL of toluene (Yeung et al, 1997).

The efficiency of the chemical remediation and bioremediation process, respectively the phytoremediation one was demonstrated through biochemical analysis, biological activity especially the presence of microorganisms in soil. The tests used to quantify the biological activity were acid and alkaline phosphatase (EC 3.1.3.2.-ACP/ EC 3.1.3.1-ALP) (Tabatabai and Bremner 1969), catalase (Wang et al, 2012) and total dehydrogenase activity (DHA) (Casida et al, 1964). A specific activity (SA) is the amount of enzyme that catalyses the transformation of 1 µmol of substrate/hour/gram of soil/30°C. The values obtained are the average values out of three different experiments and the results were statistically analyzed using T test. All chemicals were of analytical grade.

III. RESULTS AND DISCUSSION

In this study it was used ammonium persulfate for chemical oxidation because of its underground stability compared to H_2O_2 or O_3 and can last up to a few weeks suggesting a reduced demand for the natural oxidant. The persulfate anion ($S_2O_8^{2-}$) it is not directly involved in absorption reactions. These characteristics make persulfate an attractive oxidant because of its persistence underground. It can be injected in high concentrations, can be transported through spongy mediums, will be subjected to diffusion and will be directed by high density in materials with low permeability (Huang et al, 2002).

TPH concentration and initial enzymatic marker values for each experimental variant are shown in Table 2. Compared to the initial concentration, the process of phytoremediation without chemical and microbiological treatment conducted in a substantial decrease of TPH by approximately 50%. In case of a combined microbiological and vegetal treatment, the values recorded are slightly lower than the values when phytoremediation only was used. The experimental variant here a chemical treatment is used as well results in a significant decrease in the value of TPH by approximately 75% compared to the initial soil. In correspondence with the results obtained by other authors, the value of TPH decreases with 78% compared to the minimum alert level according to Romanian legislation, for industrial areas (Pavelescu et al, 2008).

Table 2. Total Petroleum Hydrocarbons Values of soil extract (mg/mL). -Phyto – without phytoremediation; +Phyto – with phytoremediation.

	-Phyto	+Phyto
1.1 MSM	5.553±0.28	3.25±0.16
1.1 TRL		2.77±0.14
1.4 MSM	none	0.01±0.0005
1.4 TRL		0.04±0.002
2.1 MSM	3.27±0.16	2.59±0.13
2.1 TRL		1.91±0.1
2.4 MSM	none	0.01±0.0005
2.4 TRL		0.03±0.0015
3.1 MSM	2.4±0.12	2.22±0.11
3.1 TRL		1.06±0.05
3.4 MSM	none	0.01±0.0005
3.4 TRL		0.03±0.001

In Table 3 the biochemical values presented are obtained after phytoremediation with the two *Fabaceae* species. It can be observed that the treatment that includes all three stages enhances the plants remediation capacities.

The activity of ACP is different for *M. sativa* 'Magnat' (MSM) in comparison to *T. repens* 'Lirepa' (TRL). As it can be observed, for MSM the activity has decreased aprox. a unit compared to TRL where it grows up to 11 units. This can be a proof that ACP has the purpose of controlling the mineralization of organic phosphate present in soils. The studies conducted by Huang et al, (2011) indicated that the changes in ACP are dependent of humidity, not substrate.

Table 3. Specific enzyme activities after remediation processes. ACP –Acid phosphatase; ALP –Alkaline phosphatase; CAT- Catalase Activity; DHA –Total Dehydrogenase Activity.

Experiment	ACP (U/g soil)	ALP (U/g soil)	CAT (mg H_2O_2/g soil)	DHA (µmoli/h/30°C/g soil)
1.1 MSM	29,67±0,89	25,05±0,75	1,84±0,05	0,11±0,003
1.1 TRL	16,29±0,48	35,75±1,07	1,95±0,05	0,81±0,024
1.4 MSM	35,75±1,07	64,45±1,93	0,69±0,02	0,16±0,004
1.4 TRL	37,45±1,12	84,4±2,53	0,81±0,02	0,81±0,024
2.1 MSM	28,45±0,85	23,35±0,7	2,2±0,06	0,14±0,004
2.1 TRL	22,01±0,66	26,63±0,79	1,99±0,05	0,16±0,004
2.4 MSM	97,47±2,92	64,21±1,92	0,83±0,02	0,15±0,004
2.4 TRL	92,79±2,78	61,45±1,84	0,74±0,02	0,22±0,006
3.1 MSM	27,24±0,81	21,64±0,64	2,57±0,07	0,16±0,004
3.1 TRL	27,72±0,83	17,51±0,52	2,03±0,06	0,23±0,006
3.4 MSM	153,24±4,59	63,97±1,91	0,97±0,02	0,13±0,003
3.4 TRL	148,13±4,44	38,5±1,15	0,67±0,02	0,09±0,002

Even though the function of microbial ALP is not fully determined this can serve as a mean of generating free phosphate used in absorbing and utilizing it afterwards. This simple hypothesis is backed by the fact that only the microorganisms deprived of a phosphate source can produce ALP (Ohiri et al, 2013). The results show that both in case of MSM and TRL the activity of ALP decreases between 4 and 20 units, fact that is a good indicator that the microorganisms present in soil adapt to that source of phosphate.

Catalase activity increases for soils that pass through more stages of treatment. This being a validation to the fact that an increase catalase activity determinate a great role in improving soil quality (Wang et al, 2012). The soil enzyme can be strongly correlated with carbon content, total microbial biomass, O_2 consumption, CO_2 depletion or DHA activity (Nwaogu et al, 2012). We can assume that this enzyme is a great antioxidant that reduces soil H_2O_2 in a passive manner (without energy consumption) and with high speed.

Regarding DHA activity, all tests represent the main indicator of enzyme activity in soil, the enzymes catalyzing this reaction ensuring the correct order of all biochemical paths from the biogeochemical cycles. The DHA activity can be considered an equivalent of all direct microorganisms metabolically reactions in the moment of the test. Accordingly the results, an increase in DHA activity with up to 50% can be observed compared to phytoremediated soil. The increase is highly affected by climate changes and soil type.

According the synthetized data from Fig. 1 *M. sativa* ‘Magnat’ has been the species that presented the highest values in germination rate compared with *T. repens* ‘Lirepa’. In the first two variants of the experiment the germination percent obtained from *M. sativa* ‘Magnat’ for the soil taken from Remediation Station 1 presented low values (1%) in comparison to control sample (Fig. 1). The third variant of the experiment concluded a value of 19% fact that confirms that phytoremediation must be applied at the end of a complex remediation process (Huang et al, 2005). For *T. repens* ‘Lirepa’ the values obtained are very low (1-2%) and the most interesting fact is the recording of the value „0“ in case of two control samples (1.4 TRL and 3.4 TRL) and a contaminated soil sample (3.1 TRL).

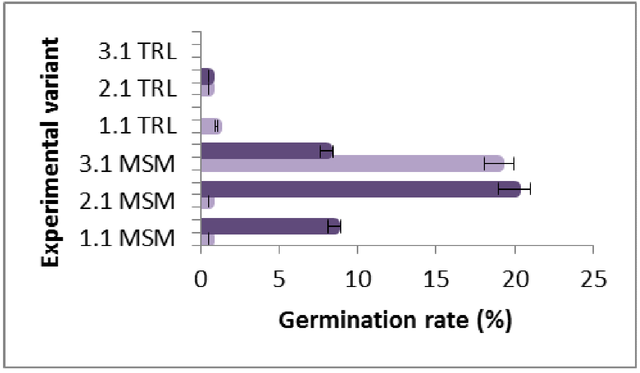

Fig. 1. Germination rate after 21 days (Legend: MSM – *Medicago sativa* ’Magnat’; TRL – *Trifolium repens* ’Lirepa’; 1.1 – Experimental variant 1, Soil sample 1; 1.4 – Experimental variant 1, Soil sample 4; 2.1 – Experimental variant 2, Soil sample 1; 2.4 – Experimental variant 2, Soil sample 4; 3.1 – Experimental variant 3, Soil sample 1; 3.4 – Experimental variant 3, Soil sample 4).

The results can be a consequence of the presence of certain substances that can reduce the level of water imbibition of the seed. The moist seed coating should allow the emergence of the radix. This factor led to the arresting of the meristematic cells from the root tip of the plantlet (Boldor et al, 1981), (Taiz and Zeiger 2006).

Fig. 2. *Medicago sativa* ‘Magnat’ plantlets on soil from Remediation Station 1, experimental variant 1 (1.1 MSM)

Fig. 3. *Medicago sativa* ‘Magnat’ plantlets on soil from Remediation Station 1, experimental variant 2 (2.1 MSM).

The data is confirmed as well by the aspects presented in the next figures where there are shown plantlets of *M. sativa* ‘Magnat’ grown on untreated petroleum contaminated soil (Fig. 2), only microbiologically treated (Fig. 3) and chemically and microbiologically treated (Fig. 4). It is determined that for the plantlets that grow on untreated contaminated soil (Fig. 2) the number of viable plantlets (vertical position with green cotyledons) is relatively low compared to the number of plantlets from stage 2 (Fig. 3). These plantlets present an obvious growth of the hypocotyl to better display the cotyledons. Through assimilation accomplished in cotyledons in the first stages of evolution it is ensured an optimal source of organic substances most needed to continue the process of growth. The existence in both cases of devitalized plantlets (Fig. 2, Fig. 3) prove that in that soil there are still present substances that can induce inhibition of cellular division, both in main root and future stem. A special situation can be observed for the plantlets grown on soil treated chemically and microbiologically (Fig. 4). Even though the number of germinated seeds is large, only a low number of plantlets remained viable. Most plantlets present a long hypocotyl but they can’t maintain a vertical position most needed for cotyledon display. It is very possible for the cellular divisions to maintain a normal rate, being followed by the lagging of the cellular differentiation. This could have direct effects on the mechanical resistance, the stem being an organ with negative geotropic growth (Grințescu, 1985).

Fig. 4. *Medicago sativa* ‘Magnat’ plantlets on soil from Remediation Station 1, experimental variant 3 (3.1 MSM).

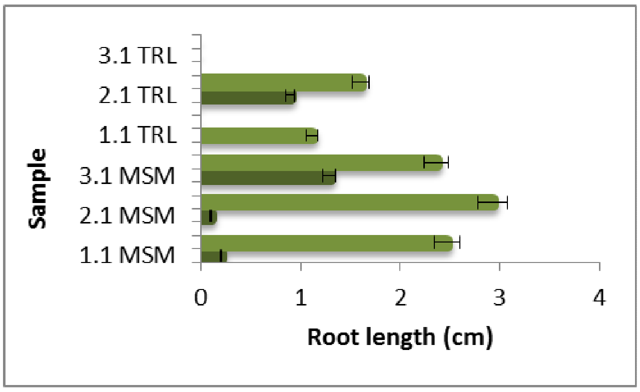

Fig. 5. Root medium length after 21 days (Legend: MSM – *Medicago sativa* ’Magnat’; TRL – *Trifolium repens* ’Lirepa’; 1.1 – Experimental variant 1, Soil sample 1; 1.4 – Experimental variant 1, Soil sample 4; 2.1 – Experimental variant 2, Soil sample 1; 2.4 – Experimental variant 2, Soil sample 4; 3.1 – Experimental variant 3, Soil sample 1; 3.4 – Experimental variant 3, Soil sample 4).

The highest values of the medium main root length calculated on viable plantlets have been obtained on *M. sativa* ‘Magnat’ compared to *T. repens* ‘Lirepa’ (Fig. 5). In case of all controls excepting 1.4 TRL and 3.4 TRL that have recorded the value „0“, the medium length has been over 1.5 cm and for the plantlets that grew on contaminated soil the value was below 1.5 cm. This proves that in the contaminated soil there probably is a series of substances that hinder the division rate of meristematic root cells, followed by devitalization of most plantlets appeared after 10 days since sowing. Another possible restrictive factor can also be the soil compaction that won't allow root growth (Boldor et al, 1981).

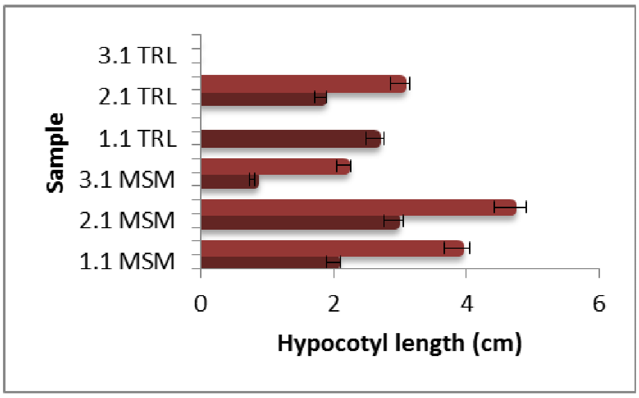

Fig. 6. Hypocotyl medium length after 21 days (Legend: MSM – *Medicago sativa* ’Magnat’; TRL – Trifolium repens ’Lirepa’; 1.1 – Experimental variant 1, Soil sample 1; 1.4 – Experimental variant 1, Soil sample 4; 2.1 – Experimental variant 2, Soil sample 1; 2.4 – Experimental variant 2, Soil sample 4; 3.1 – Experimental variant 3, Soil sample 1; 3.4 – Experimental variant 3, Soil sample 4).

The medium hypocotyl length presented higher values compared to the medium root length with only one exception: sample 3.1 MSM. Similar to the above parameter all the samples from the control soil presented values higher than the ones attributed to the plant that grow on the contaminated soil (Fig. 6). The obvious growth of the hypocotyl compared to the main root represents another adaptation strategy of studied species to the conditions they are forced to endure.

IV. CONCLUSION

The study of enzymatic activities as biochemical marker confirms relatively high degradation capacity of the two species of *Fabaceae* so: by decreasing phosphatase activity between 8.19-51% lower, reflecting that requirement against phosphate decreased by rebalancing potential mineralization phosphate; an increase by 28.4% dehydrogenase activity as an equivalent direct metabolic activities of microorganisms in the soil in the process of bioremediation; a decrease of 31.25% of catalase activity, reflecting catalase antioxidant capacity, reflected by increasing microbial mass, and reducing the carbon content of fractions asfaltence.

Differences registered between the evolution of the main root and hypocotyl concerning a third of the analyzed plantlets show an increased adaptation level to the pollution existent in soil (Ayolagha and Peter, 2013). The obvious growth of the hypocotyl, followed by the fast display of the cotyledons is probably a compensation of the slow growth rate of the main root related to the low absorption rate of the nutrients from hydrocarbon contaminated soils. Intensifying photosynthesis ensures an additional share of the nutrients needed for plantlet growth and development, also it enlarges the surface on which water vapors can condense and be utilized as a second source of moisture.

Rhizosphere in the phytoremediation technology has a stimulant effect on microbial activities which provide a suitable environment for growth and proliferation leading to decreasing petroleum contamination of the soil (Zhong et al, 2012).

The two species of *Fabaceae* selected: *T. repens* ‘Lirepa’ and *M. sativa* ‘Magnat’ respond differently to given conditions on soils contaminated with asphaltenic petroleum fractions. The two species show a significant growth of the remediation of the soil in last stages of this complex process of removing the pollutants. Besides, the enzyme activities confirmed the derivative capacity of the two *Fabaceae* species to make phytoremediation process.

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