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Effect of Siberian Light Crude Oil on Alfalfa (*Medicago sativa* L.) Cultivars

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The environmental petroleum contamination problem is getting increasingly important as the need for fertile land increases. Alfalfa (*Medicago sativa* L.) is a forage legume, which has uses in petroleum phytoremediation. In this study, three local cultivars of alfalfa from Eastern Anatolia were screened for their potential with respect to their for petroleum phytoremediation capacity and the effect of symbiotic nitrogen fixation of a symbiotic bacteria, *Rhizobium meliloti*, was evaluated. Local alfalfa cultivars; Savas, 1313 and 1312 were germinated and grown at soil mixed contaminated with different Siberian Light (SBL) crude oil concentrations for one month. Paralel studies were conducted with the application of *R. meliloti*. It could be said that the alfalfa cultivars are not very tolerant to the higher concentration of petroleum hydrocarbon contamination, but can only manage to grow on slightly contaminated areas.

Key Words: Petroleum, Oil contamination, Phytoremediation, Siberian light crude oil, Alfalfa, *Medicago sativa*, *Rhizobium meliloti*.

INTRODUCTION

Oil, which is one of the most widely used energy sources, bring up environmental problems together with its advantages. Petroleum is a complex mixture of hydrocarbons composed of saturated (aliphatic) hydrocarbons, aromatic hydrocarbons and an asphaltic or polar fraction[1]. Pollutants such as petroleum hydrocarbons are spread on the surface of the earth and threaten the environmental and public health[2]. Many aromatic hydrocarbons, including nitroaromatic compounds, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, diauxins and their derivatives are highly toxic, mutagenic and/or carcinogenic to natural microflora as well as to higher systems including humans[3]. Phytoremediation refers to the use of the plants together with their associated microbes for environmental cleanup[3-5], which is a cost effective technology to extract and detoxify organic or inorganic pollutants with the use of plants[1,6]. In specific, the utilization of plants for the removal of several environmental contaminants such as petroleum hydrocarbons has been also considered as an alternative technology for the remediation of oil contaminated areas. There has been an increasing interest on phytoremediation studies mainly due to its ease of implementation and cost-effectiveness both in terms of set-up, sustainability and maintenance[1,6-10]. On the other hand, the usability of plants for the removal of petroleum hydrocarbons is limited by the suitability of edaphic and climatic factors for plant growth.

Even though many plant species can be considered at some degree tolerant to hydrocarbon contamination, either growth or germination is adversely affected by the toxic components in oil. For instance, polyaromatic hydrocarbons (PAHs) may not reduce germination, but can stunt the early stages of growth[2]. Furthermore, there has been reports about the facilitating effect of vegetation in the degradation of certain petroleum hydrocarbons in soil; *e.g.*, *Medicago sativa* L. (Alfalfa), *Thinopyrum ponticum* (Podp.) Z.-W. Liu and R.-C. Wang (tall wheatgrass) and *Trifolium pratense* L. (red clover)[11].

For example, Schwab *et al.*[12] shown that naphthalene was adsorbed to alfalfa and tall fescue roots and the amount of hydrocarbon adsorbed to the roots increased as plants get older. Although some plants have been suggested as effective phytoremediators, the complex interactions between plant roots and soil is critical in determination of overall condition of the plant and the remediation potential of terrestrial vegetation[2]. Plants with larger rhizosphere encouraging microbial activity and growth are more suitable for phytoremediation[13]. An advantage of these plants is their ability to fix, nitrogen by forming symbiotic association with *Rhizobium* spp. which is particularly important in the soils contaminated with petroleum hydrocarbons; that is because, increase in the carbon/nitrogen ratio leads to an accentuated nitrogen deficiency.

No data is available on the effects of crude oil on local cultivars of alfalfa. The main purpose of this study is to provide insights about the molecular mechanism of alfalfa (*M. sativa* Savas, 1313 and 1312) and the role of symbiont bacteria, *R. meliloti* in the process. In more specific, the potential of three local alfalfa cultivars of Erzurum province of Turkey for the cleaning up the soils contaminated with petroleum hydrocarbons has been studied.

EXPERIMENTAL

Three local cultivars of alfalfa (*M. sativa* Savas, 1313 and 1312) were obtained from Eastern Anatolian Agricultural Research Institute, Erzurum, Turkey. Alfalfa symbiont, *R. meliloti*, was purchased from Soil and Manure Research Institute, Ankara, Turkey.

Alfalfa (*Medicago sativa* L.) is a deep-rooted perennial plant that belongs to the Leguminosae (Pea) family. Alfalfa is more drought tolerant than grasses. However, it is not well adapted to acidic or poorly drained soils[14].

Specificity among strains of *R. meliloti*, particularly with *Medicago* sp. also plays an important role in establishing effective nitrogen-fixing symbiosis.

For the plant growths, soil was taken from a site near Sekerpinar (Adapazari, Turkey). The experimental soil was chosen according to the official guidelines on the examination of petroleum hydrocarbons[16]. Due to tendency of the soil to silt up, it was mixed with river sand in 2:1 soil:sand ratio. The pH of the soil was confirmed to be suitable at the beginning of the study. Soil temperature and humidity were monitored throughout the study. All experiments were carried out using SBL (classified as light oil) obtained from Turkish Petroleum Rafineries Co. (TUPRAS) which is one of the most widely used oils in Anatolian region (Table-1). For measuring more precisely observe the physiological impact of petroleum hydrocarbons on plants, the plants are grown on pots containing 2 L of soils mixed with different concentrations of crude oil at final petroleum percentages of 0 % (control), 1, 2, 5 and 7 % V_{oil}/V_{soil} (volume/volume). Seeds were sowed one day after soil contamination.

TABLE-1
PROPERTIES OF THE SIBERIAN LIGHT CRUDE OIL

Oil properties		Oil properties	
API Gravity	37.8	C1-C4	1
Sulfur (weight %)	0.42	Gas (62-180 ºC)	22
Density (g/mL)	0.8358	Kerosene (120-240 ºC)	25
Pour point (ºC)	-17	Diesel (180-350 ºC)	32

Physico-chemical tests: 0 % (control), 1, 2, 5 and 7 % vol. SBL containing soil mixtures with *Rhizobium* application and control was prepared in pots and left to settle overnight. Twenty-five seeds from each alfalfa cultivars were surface sterilized and imbibed overnight and then were sown in pots in replicates of two for each experimental factors; *i.e.*, Rhizobium (+) and Rhizobium (-). Experimental and control plants were grown in the greenhouse for a month under the conditions of 25 ºC during the day and 19 ºC at night and soil ranging between 4 and 9 %. The results of particle size distribution analysis of the soil used in this experiment. Soil samples evaluated consists of gravelly-sandy units (Table-2).

TABLE-2
PARTICLE SIZE OF THE SOIL USED IN THE STUDY (%)

Grain size type	(+)	(–)	Control
Coarse sand (%)	16.96	17.63	18.34
Medium sand (%)	78.16	78.51	75.52
Fine sand (%)	4.88	3.86	6.14
Clay (%)	0.00	0.00	0.00

(+): Rhizobium supplemented soil, (–): Rhizobium free soil.

To gauge the effect of petroleum hydrocarbon contamination to alfalfa plants, the several physiological parameters including germination rate, stem length, number of leaves and stem lengths are measured. The germination rate is calculated as the total number of germinated seeds until the time when a stable number of germination is reached. Stem lengths and the leaf widths from the widest section of the lower leaves were also measured at the end of 30th of germination. To better assess the overall fitness of the plants, average number of leaves per plant is calculated by dividing overall number of leaves (*i.e.*, from all the plants) to the number of plants.

RESULTS AND DISCUSSION

All cultivars of *M. sativa* germinated at all percentages of SBL (control, 1, 2, 5 and 7 % v/v), however, germination rate, as expected, decreased with the increase in percentage of SBL Rhizobium supplementation and was different between alfalfa cultivars (Fig. 1). It has been previously reported about the impeding effects of polyaromatic hydrocarbons (PAHs) on germination. However, it needs to be noted that freshly contaminated soils were used in many of these or the seeds were treated with solutions containing different amount of PAH compounds[16]. Smith *et al.*[16] also reported similar results where indicated treatments did not adversely affect germination of the plants and indicates that germination tests alone would not predict the growth of plants on PAH contaminated soil. The germination success differs between cultivars (Fig. 1). The level of tolerance to petroleum contamination was different between three different alfalfa cultivars and in general Rhizobium had positive impact on the plant growth. For examples, the seedlings grown on soil contaminated with 7 % oil (v/v) failed to survive at the end of 4 weeks, regardless of Rhizobium application. At the end of the first week of growth, Savas variety on Rhizobium (-) pots of 7 % SBL application failed to survive. At the second week stable growth was observed for all applications. In the third week of the experiment, "1312" variety growing in the 7 % SBL applied pots failed to survive regardless

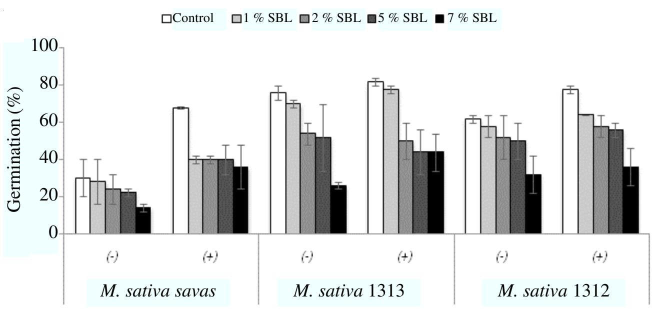

Fig. 1. Germination of *M. sativa* cultivars containing in control (0 %) and contaminated soil (1, 2, 5 and 7 %), (-): Rhizobium free soil, (+): Rhizobium supplemented soil

of Rhizobium application. At the end of the experiment, *i.e.*, 4th week of growth, all cultivars at 7 % SBL application and rhizobium treated 5 % SBL applied "1313" variety failed to survive.

Although leaf width decreased with the increasing concentration of SBL, there was difference in the rate between alfalfa cultivars hinting a possible association between Rhizobium application and leaf morphology (Fig. 2). The stem lengths of all alfalfa cultivars grown on petroleum containing mixtures were are less than the ones observed in control plants. In contrast to leaf width, Rhizobium application didn't have significant impact on this physiological parameter. (Fig. 3). As for the number of leaves, in parallel to the increase in petroleum percentage, the average number of leaves per plant decreased. However, petroleum contamination had different impact on this parameter in different cultivars. Similar to the stem length, Rhizobium (+) or Rhizobium (-) plants did not show significant difference with respect to this physiological parameter (Fig. 4). Last, the fresh biomass of the plants also diminished with the hike in petroleum percentage regardless of the cultivar and like stem length, leaf number, this characteristic is not influenced by the Rhizobium application.

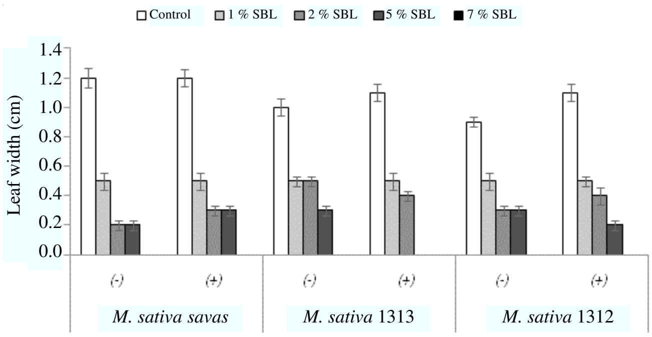

Fig. 2. Leaf width of *M. sativa* cultivars grown at control (0 %) and contaminated soil (1, 2, 5 and 7 %), (-): Rhizobium free soil, (+): Rhizobium supplemented soil.

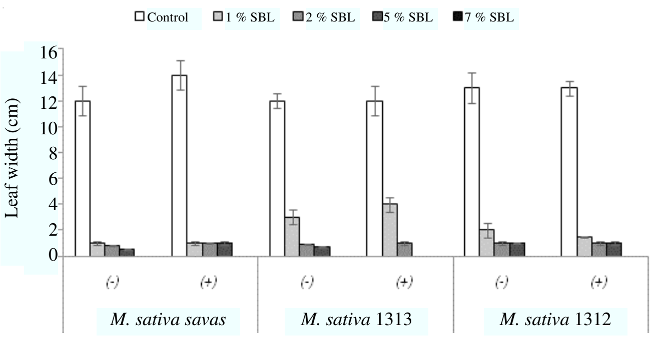

Fig. 3. Shoot lengths of *M. sativa* cultivars grown at control (0 %) and contaminated soil (1, 2, 5 and 7 %). (-): Rhizobium free soil, (+): Rhizobium supplemented soil

The most successful cultivar in terms of germination rate in petroleum contaminated soils is likely to be was *M. sativa* 1312. Plant-microbe interactions have been proposed to increase the bioremediation capacity of plants[17]. The association of different bacterial species; *e.g.*, *Rhizobium* spp. and *Burkholderia cepacia* are receiving ever increasing attention for the use in bioremediation of both, metals[18-21] and organics[22]. In present

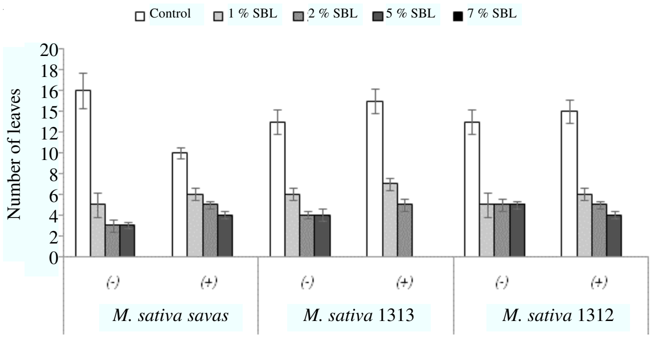

Fig. 4. Number of leaves of *M. sativa* cultivars grown at control (0 %) and contaminated soil (1, 2, 5 and 7 %), (-): Rhizobium free soil, (+): Rhizobium supplemented soil

study, it is also observed that the positive effect of *R. meliloti* in the germination success for all of the alfalfa cultivars, thus corroborating the previous finding about the role of micro-organism in rhizosphere. This study provided further evidences about the better suitability of plants that promote microorganismal growth in rhizosphere for bioremediation.

Conclusion

All of *Medicago sativa* cultivars tried in this study failed to survive in mixtures containing 7 % petroleum. However, *M. sativa* 1312 was the most tolerant cultivar according to stem lengths, leaf width and number and fresh biomass. *Rhizobium meliloti* was observed to be intolerant to the oil stress when we consider the parameters indicated above. Finally, it could be said that the alfalfa cultivars are not very tolerant to the higher concentration of petroleum hydrocarbon contamination, but can only manage to grow on slightly contaminated areas. Among alfalfa cultivars used in this study, 1312 cultivar is the most promising for the possible use of them in petroleum bioremediation.

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