

Phytoremediation of oil contaminated soil by some arid legume tree species

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Abstract. Petroleum contamination of soil is a serious problem throughout the world. Phytoremediation is a promising technology for the clean up of petroleum contaminated soil. In the present work the rhizosphere of *Prosopis cineraria* (L.) Druce, *Acacia senegal* (L.) Willd. and *Acacia nilotica* (L.) Willd. Ex. Del. plants were tested for their abilities to stimulate the microbial degradation of soil pollutants in desert soil contaminated with 2.5-2.6% crude petroleum oil. The results showed that the roots of the three different plants were density associated with total bacteria, fungi and oil-degrading microorganisms, this is confirmed from the (R+/S+) ratios which ranged from 55.2-250.8 (for total bacteria), 20-131.3 (for fungi) and 95.7-296.1 (for oil degraders). Percentages of oil-degraders were higher in the rhizosphere soil of *P. cineraria* (65.5%) as compared to the rhizosphere soil of *A. senegal* and *A. nilotica* plants (22.5 % and 20.2 % respectively). The results of the biodegradation of oil and its fractions showed that great reduction (26 %) of total petroleum hydrocarbons (TPHs) was observed in the rhizosphere soil of *P. cineraria* as compared to 15.6 % and 12.8 % reduction in rhizosphere soil of *A. senegal* and *A. nilotica* respectively. It was observed also that in the polluted non-cultivated soil the TPHs were reduced by 8.2 - 10.5 % as a result of biostimulation process only (addition of nutrients). The results also showed that *P. cineraria* rhizosphere was able to reduce more of the saturated (43.0 %) and more of the aromatics (25.7 %) fractions, compared to (35.2 % and 7.9 %) for *A. senegal* and (31.2 % and 4.1 %) for *A. nilotica* rhizospheres. It is of interest to find that 5.3 % of the hardly degradable fraction resins were degraded in rhizosphere soil of *P. cineraria*. The present results clearly demonstrated that *P. cineraria* provided successful phytoremediation process of a contaminated desert soil as compared to the other two legume trees.

Key Words: phytoremediation, desert soil contaminated, petroleum hydrocarbons, rhizosphere.

Introduction. Oil contamination is one of the most dangerous pollution factors known today. It can cause a threat to the environment. It is very feared by environmentalists and it's very hard to control if it gets out of hand. Oil contamination in soil results in an imbalance in the carbon-nitrogen ratio at the spill site, because crude oil is essentially a mixture of carbon and hydrogen. This causes a nitrogen deficiency in oil soaked soil, which retards the growth of bacteria and the utilization of carbon sources. Furthermore, large concentrations of biodegradable organics in the top layer deplete oxygen reserves in the soil and slow down the rates of oxygen diffusion into deeper layers.

Contaminated soils pose a major environmental and human health problem. Microorganisms and plants can have complementary roles in phytoremediation of the polluted soil. Phytoremediation refers to the use of plants to clean contaminated soil (Joner et al 2004). Increased biodegradation of organic contaminants occurs in the rhizosphere, the zone of soil directly adjacent to and under the influence of plant roots (White et al 2006).

The application of plants for remediation of soil contaminated with petroleum hydrocarbons is one of the promising cost and environmental effective approach. Rock & Sayre (1998) estimated phytoremediation clean up costs of \$ 162/m³ compare to \$ 810/m³ for excavation and incineration.

For successful phytoremediation both plants and microorganisms must survive and grow in crude oil contaminated soil. Phytoremediation involves growing or encouraging the growth of plants in the contaminated soil either artificially constructed (using cultivated plants) or naturally (using the already existing plants) for a required growth period, to remove contaminants from the site. The plants can be subsequently harvested processed and disposed.

In petroleum contaminated sites, phytoremediation can be applied at moderate contamination levels or after the application of other remediation measures as a polishing step to further degrade residual hydrocarbons and to improve soil quality (Vangronsveld et al 2009). Yateem et al (2000) investigated the degradation of total petroleum hydrocarbons (TPH) in the rhizosphere and non-rhizosphere soil of three domestic plants namely, alfalfa (*Medicago sativa*), broad bean (*Vicia faba*) and rayegrass (*Lolium perenne*). Although the three domestic plants exhibited normal growth in the presence of 1% TPH, the degradation was more profound in the case of leguminous plants. They found that the soil cultivated with broad bean and alfalfa was 36.6% and 35.8% respectively, compared with 24% degradation in case of rayegrass. Adams & Duncan (2003) found that the legume plant (*Vicia sativa*) was able to grow in soil contaminated with diesel fuel, and the total numbers of nodules were significantly reduced in contaminated plants as compared to control plants, but nodules on contaminated plants were more developed than corresponding nodules on control plants. These authors found that the amount of diesel fuel remaining after 4 months in the legume plant *Vicia sativa* was slightly less than in the rayegrass planted soil.

Rosado & Pichtel (2004) studied the decomposition of used motor oil in soil as influenced by plant treatment. Soil contaminated with used motor oil (1.5% w/w) was seeded with soybean (*Glycine max*), green bean (*Phaseolus vulgaris*), sunflower (*Helianthus annuus*), Indian mustard (*Brassica juncea*), mixed grasses/maize (*Zea mays*) and mixed clover (*Trifolium partense*, *Trifolium repens*). After 150 days in the clover treatment the added oil was no longer detected. A total of 67% of the oil was removed in sunflower/mustard, and with addition of NPK fertilizer, the oil was completely removed. The grass/maize treatment resulted in a 38% oil reduction, which increased to 67% with fertilizer application. Based on oil residue and biomass results, the clover and sunflower/mustard treatments are considered superior to other plant treatments in terms of overall phytodegradation of used oil hydrocarbons.

Merkl et al (2005) tested three legume plants and three grasses for their ability to stimulate microbial degradation in a sandy soil contaminated with 5% (w/w) crude oil. They showed that the overall advantage of the chosen grass species is their extensive, widely branched root system providing a large root surface for the growth of microbial population. Legumes are considered to be specially promising because of their ability to fix atmospheric nitrogen. Their experiment evaluates the ability of selected species to grow in oil-contaminated soil and enhance oil degradation. Radwan et al (2000) reported that *Vicia faba* plant can tolerate up to 10% (w/w) crude oil in sandy desert soil, therefore their potential was assessed for cleaning oily desert soil using rhizosphere technology. They found that the amount of hydrocarbons recovered from oily desert samples supporting *Vicia faba* were lower than in uncultivated oily sand samples.

The objective of the present research is to study the effects of a three legume tree species namely *P. cineraria*, *A. senegal* and *A. nilotica* on the changes of the rhizosphere microflora and its degradation potential in response to hydrocarbon-contamination of soil. The advantage of the chosen legume plant is their ability to fix atmospheric nitrogen this is in addition to the ability of these legume tree species to tolerate up to 10% (w/w) crude oil.

Material and Method

Field Experiments. Four plots each of 5×5 m² were delimited in an area (Botanical garden, Department of Botany, J. N. V. University, Jodhpur, India) without any history of pollution. The soil in each plot at 0-50 cm depth were ploughed and thoroughly mixed with weathered crude oil so as to give initial concentration of 2.2-2.3% w/w soil. Each

plot received the suitable nitrogen and phosphorus (NP) concentrations (500 mg ammonium nitrate and 50 mg K_2HPO_4 /kg soil). Plot No. 1 was planted with 25 seedlings of *P. cineraria*; Plot No. 2 was planted with 25 seedlings of *A. senegal*; Plot No. 3 was planted with 25 seedlings of (*A. nilotica*) and Plot 4 was left without seeding.

Another 4 plots (plots 4-8) received only nutrients (i.e. left unpolluted) to behave as control. The plots were separated by 5m from each other. After 90 days growth period of each plant, samples were taken from the rhizosphere and non-rhizosphere soil of each plant (both polluted and non-polluted). Samples also were collected from the non-cultivated plots. At the beginning of the experiments soil samples were also collected. Samples were analyzed microbiologically and chemically for the determination of residual hydrocarbons. Each of the developed plant shoot system was carefully removed, dried at 60°C and kept for further studies to detect if hydrocarbons are accumulated in plant tissues or not.

The needed moisture was added (50% of the water holding capacity, as described by Vecchioli et al (1990) at the beginning of the experiment and periodically to each plot. The soil in each plot was ploughed weekly for aeration.

Determination of the Residual Oil and its Fractions. Ten grams of the air-dried soil samples were mixed with 10 grams of anhydrous sodium sulphate to remove moisture. The hydrocarbons were Soxhlet extracted with chloroform for 8h. The chloroform extract was evaporated in a preweighed dish, and the amount of total petroleum hydrocarbons (TPHs) was determined, and the loss (%) of TPH was then calculated.

The extracted residual oil was suspended in hexane and filtered through tared filter paper to remove and to determine the insoluble fraction (asphaltene). The hexane-soluble fraction was fractionated by liquid-solid chromatography into saturates, aromatics and resins. The amount of each fraction was determined according to Chaîneau et al (1996).

Microbiological Analysis. For counting colony forming units (CFU) of bacteria and fungi, the usual dilution plate method was used. Nutrient agar (Oxoid) medium supplemented with 0.4% (w/w) soluble starch was used for counting bacteria. For counting fungi malt-yeast extract agar was used. The colonies appeared on the different plates were counted and expressed as CFU/g soil. Plates for counting bacteria were incubated 5-7 days at 30°C, and for fungi the incubated temperature was 25°C for a period of 10-12 days.

For counting hydrocarbon-degrading microorganisms the three tubes mean probable number (MPN) method was used as described by Chaîneau et al (1996).

Results and Discussion. The soil sample used in the present study is sandy soil, with pH 7.6 - 7.8. This soil was poor in phosphorus (0.17 ppm) and nitrogen (0.02%) contents. Results of the microbial contents of the polluted and non-polluted plots of *P. cineraria*, *A. senegal* and *A. nilotica* plants are found in Tables 1-3.

The results show that the CFU/g of total bacteria, fungi and oil-degraders are higher in rhizosphere soil (both polluted and non-polluted) than in the non-rhizosphere soil of the above three plants. These results reflect the positive rhizosphere effects of the three plants on the microbial communities as indicated from the results of (R/S) ratios (Tables 1-3) (counts in the rhizosphere / counts in the non-rhizosphere) of more than one. The (R+/S+) values were more pronounced in the polluted plots than in the non-polluted one (control). Murotova et al (2003) explained that the success of phytoremediation of hydrocarbon contaminated soil is connected with the plants capacity to enhance microbial activity in the rhizosphere.

In the polluted *P. cineraria* plots (Table 1) (R+/S+) values were in the range of 18 (for fungi) to 244.1 (for oil-degraders). In *A. senegal* plots (R+/S+) values (Table 2) were 123.6 (for fungi) to 223.6 (for total bacteria), while in (*A. nilotica*) plots (Table 3) values of 51.4 (for fungi) to 96.4 (for oil-degraders) were recorded.

In non-polluted plots (R-/S-) values were significantly lower than those of the polluted plots. Generally, addition of 2.2-2.3% (w/w) of crude oil to this type of soil stimulated the development of more microorganisms as compared to the control sample. Kuiper et al (2003) reported that when the mean population densities of bacteria in

samples from contaminated soil are significantly greater than in background samples, the pollutants are being utilized; they suggested that microbial enumeration is a screening level tool which can be used to evaluate the response of microorganisms to hydrocarbons.

Table 1

Microbial contents of rhizosphere soil (R) and non-rhizosphere soil (S) of *P. cineraria* plant after 90 days growth period

Microorganisms	0-time CFU/g soil	90 Days growth period					
		R ⁺ FU /g soil	S ⁺ CFU/g soil	R ⁺ /S ⁺	R ⁻ CFU /g soil	S ⁻ CFU/g soil	R ⁻ /S ⁻
Fungi	18.2 × 10 ²	12.0 × 10 ³	4 × 10 ²	30.0	8.1 ± 0.3 × 10 ⁴	4.9 ± 0.6 × 10 ³	16.5
Total bacteria	31.0 × 10 ⁴	181.4 ± 7.2 × 10 ⁸	30.2 ± 2.1 × 10 ⁷	60.0	42.7 ± 1.8 × 10 ⁷	2.1 ± 0.3 × 10 ⁷	20.3
Oil-degraders	23.0 × 10 ²	195.9 ± 8.9 × 10 ⁸	5.6 ± 0.16 × 10 ⁷	349.8	32.3 ± 2.5 × 10 ⁵	6.1 ± 0.5 × 10 ⁵	5.2
Oil-degraders (%)	0.79	68.8	15.2	-	0.8	2.7	-

R⁺=polluted rhizosphere soil, S⁺=polluted non-rhizosphere soil, R⁻=non-polluted rhizosphere soil, S⁻=non-polluted non-rhizosphere soil.

Table 2

Microbial contents of rhizosphere soil (R) and non-rhizosphere soil (S) of *A. senegal* plant after 90 days growth period

Microorganisms	0-time CFU/g soil	90 Days growth period					
		R ⁺ FU /g soil	S ⁺ CFU/g soil	R ⁺ /S ⁺	R ⁻ CFU /g soil	S ⁻ CFU/g soil	R ⁻ /S ⁻
Fungi	18.2 × 10 ²	20.1 ± 2.4 × 10 ⁷	16.1 ± 0.7 × 10 ⁵	124.8	68.0 ± 4.2 × 10 ⁵	7.2 ± 1.1 × 10 ⁴	94.4
Total bacteria	31.0 × 10 ⁴	829.8 ± 20.7 × 10 ⁷	32.1 ± 1.8 × 10 ⁶	258.5	178.1 ± 4.7 × 10 ⁷	2.1 ± 0.7 × 10 ⁷	84.8
Oil-degraders	23.0 × 10 ²	166.4 ± 5.2 × 10 ⁸	12.8 ± 0.2 × 10 ⁷	130.0	27.4 ± 2.8 × 10 ⁵	5.9 ± 0.7 × 10 ⁵	4.64
Oil-degraders (%)	0.79	20.2	9.1	-	0.3	2.8	-

R⁺=polluted rhizosphere soil, S⁺=polluted non-rhizosphere soil, R⁻=non-polluted rhizosphere soil, S⁻=non-polluted non-rhizosphere soil.

Table 3

Microbial contents of rhizosphere soil (R) and non-rhizosphere soil (S) of *A. nilotica* plant after 90 days growth period

Microorganisms	0-time CFU/g soil	90 Days growth period					
		R ⁺ FU /g soil	S ⁺ CFU/g soil	R ⁺ /S ⁺	R ⁻ CFU /g soil	S ⁻ CFU/g soil	R ⁻ /S ⁻
Fungi	18.2 × 10 ²	76.0 ± 1.8 × 10 ⁶	15.2 ± 0.8 × 10 ⁴	50.0	13.1 ± 0.8 × 10 ⁶	7.2 ± 0.6 × 10 ⁵	18.1
Total bacteria	31.0 × 10 ⁴	170.8 ± 4.9 × 10 ⁷	30.1 ± 1.7 × 10 ⁶	56.7	76.7 ± 3.2 × 10 ⁵	1.9 ± 0.2 × 10 ⁵	40.3
Oil-degraders	23.0 × 10 ²	29.6 ± 0.6 × 10 ⁷	2.9 ± 0.18 × 10 ⁶	102.0	3.7 ± 0.8 × 10 ⁵	5.6 ± 0.3 × 10 ⁴	6.6
Oil-degraders (%)	0.79	18.7	10.4	-	0.5	3.1	-

R⁺=polluted rhizosphere soil, S⁺=polluted non-rhizosphere soil, R⁻=non-polluted rhizosphere soil, S⁻=non-polluted non-rhizosphere soil.

Narino et al (2004) reported positive rhizosphere effects of maize and oat on microorganisms of the only contaminated soil in comparison with uncontaminated planted soil. The maize has provided a more stimulatory influence on the microbial community of the polluted soil in comparison to oat plant. Results of the distribution of oil-degrading microorganisms in the polluted rhizosphere and nonrhizosphere soil of *P. cineraria*, *A. senegal* and *A. nilotica* plots show that the polluted rhizosphere soil of the three plants stimulated the development of higher counts (CFU/g soil) of such organisms as compared to the non-rhizosphere soil (Table 1-3).

The percentages of oil degraders also were higher in the rhizosphere soil than in the nonrhizosphere one. *P. cineraria* rhizosphere contained the highest values (65.5%) as compared to *A. senegal* (22.5%) and *A. nilotica* (20.2%) rhizosphere soil. As a comparison the percentages of oil-degraders in the polluted non-rhizosphere soil are in the range of 9.1-11.9%. On the other hand the non-polluted plots contained significantly lower counts and lower percentages (0.3-3.1%). The above results confirmed the ability of plant roots to neutralize and/or to remove the toxic effects of the oil pollutants; this is through the exudates, nutrient and other materials.

Murotova et al (2003) explained that the success of phytoremediation of hydrocarbon contaminated soil is connected with the plant's capacity to enhance microbial activity in the rhizosphere. The efficiency of this process is often connected with high number of degrader microorganisms and their degradative activities in the rhizosphere of plants. Murotova et al (2003) also suggested that additional studies are necessary to determine whether the population of hydrocarbon-degrading microorganisms protects the plant from toxic effects of pollutants or whether the plant provides the favorable conditions of this population activity.

Merkl et al (2005) tested three legume plants and three grasses for their ability to stimulate microbial degradation in sandy soil contaminated with 5% (w/w) crude oil. They considered legumes to be specifically promising because of their ability to fix atmospheric nitrogen. Radwan et al (2005) found that total number of oil-degrading bacteria increased in the rhizosphere of *P. cineraria* plant and more hydrocarbons were eliminated in sand close to the root. The effects of plant roots on the dissipation of organic pollutants has been attributed mainly to increased microbial numbers and selection of specialized microbial communities in the rhizosphere (Reilley et al 1996; Banks et al 1999), but also to improved physical and chemical soil conditions, supply of root exudates for cometabolic processes (Yoshitomi & Shann 2001) and increased humidification and absorption of pollutants increasing their bioavailability (Joelle et al 2002).

Table 4

Biodegradation of oil and its fractions in the rhizosphere of *P. cineraria* (RPC) plant as compared with the non-rhizosphere soil (S) after 90 days growth period

Fractions	0-time mg/100g soil	90 Days growth period			
		S mg/100g soil	Loss (%)	RPC mg/100g soil	Loss (%)
Saturates	780.0 ± 1.2	636.0 ± 4.9	21.7	440.2 ± 1.7	41.0
Aromatics	1102 ± 26.2	989 ± 20.7	7.9	816 ± 4.7	20.8
Resins	210.0 ± 3.7	201 ± 5.7	-	192.6 ± 1.8	6.4
Asphaltenes	190.0 ± 3.2	190 ± 3.4	-	180.4 ± 4.4	2.1
Total	2282	2016 ± 11.9	10.9	1629.2 ± 8.9	29.4

Table 5

Biodegradation of oil and its fractions in the rhizosphere of *A. senegal* (RAS) plant as compared with the non-rhizosphere soil (S) after 90 days growth period

Fractions	0-time mg/100g soil	90 Days growth period			
		S mg/100g soil	Loss (%)	RAS mg/100g soil	Loss (%)
Saturates	910 ± 9.2	686 ± 3.2	25.4	530.8 ± 2.4	29.4
Aromatics	1090.0 ± 24.3	978 ± 5.2	7.2	1026.4 ± 3.6	7.2
Resins	189.0 ± 9.8	199 ± 2.4	-	193.2 ± 4.2	-
Asphaltenes	236.0 ± 4.2	240 ± 4.2	-	282.8 ± 1.8	-
Total	2425 0	2103 ± 8.4	11.1	2033.2 ± 4.2	15.8

Table 6

Biodegradation of oil and its fractions in the rhizosphere of *A. nilotica* (RAN) plant as compared with the non-rhizosphere soil (S) after 90 days growth period

Fractions	0-time mg/100g soil	90 Days growth period			
		S mg/100g soil	Loss (%)	RAN mg/100g soil	Loss (%)
Saturates	780 ± 26.2	650 ± 4.1	20.9	552.4 ± 1.9	25.8
Aromatics	1040 ± 37.8	971 ± 3.2	3.4	999.8 ± 7.2	3.9
Resins	160 ± 7.8	184 ± 1.7	-	198.3 ± 8.9	-
Asphaltenes	280 ± 9.2	252 ± 7.8	-	256.4 ± 21.4	3.8
Total	2260	2057 ± 6.4	9.2	2006.9 ± 7.4	14.1

Results of the effects of plant roots on the biodegradation of oil and its fractions are found in Tables (4-6). From these results it can be seen that crude oil (Total petroleum hydrocarbons, TPH) was reduced by 30% in the rhizosphere soil of *P. cineraria* plant and by 16.8% and 13.7% in the rhizosphere soil of *A. senegal* and *A. nilotica* plants respectively. This is in contrast to reduction of 11.2%, 11.5% and 9.2% of the non-rhizosphere soil of the above three plants respectively. This shows that TPH biodegradation was enhanced in the rhizosphere soil of the *P. cineraria* as compared to the other two plants (*A. senegal* and *A. nilotica*). Yateem et al (2000) investigated the degradation of TPH in the rhizosphere and nonrhizosphere soil of three domestic plants mainly, alfalfa (*Medicago sativa*), *V. faba* and raye grass (*Lolium perenne*). They found that TPH degradation in soil cultivated with broad bean and alfalfa was 36.6% and 35.8% respectively, compared with 24% degradation in case of rayegrass.

Results of the effects of the roots of *P. cineraria*, *A. senegal* and *A. nilotica* plants on the degradation of the different oil fractions (Tables 4-6) show that the most degradable fraction was the saturates followed the aromatics while the recalcitrant fractions were resins and asphaltene. *P. cineraria* roots were able to degrade more of the saturates (48.2%) and the aromatics (28.4%) as compared to the roots of *A. senegal* (39.1% for saturates and 8.3% for the aromatics) and *A. nilotica* (34.1% and 3.8% for saturates and aromatics respectively). It is of interest to observe from this work that 5.4% of the hardly degradable fraction resin was degraded in the rhizosphere of *P. cineraria*.

On the other hand the recalcitrant fraction asphaltenes was reduced by 3.7% in the rhizosphere of *A. nilotica* and by 1.8% in the rhizosphere of *P. cineraria*. The above results lead to the conclusion that the plant *P. cineraria* as compared to the other two plants demonstrates successful phytoremediation of the polluted desert soil.

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