ISSN : 0974 - 7451 Volume 9 Issue 12

Environmental Science
An Indian Journal
Current Research Paper

ESAIJ, 9(12), 2014 [438-444]

Study of bioremediation possibility of engine-oil polluted soils by *ranunculus arvensis* L. and its root associated fungi for

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ABSTRACT

Pollution of soils with engine oil is a common disaster in both oil-bearing and industrial countries. Bioremediation of oil contamination in soils is based on the stimulation of petroleum hydrocarbon-degrading fungal and microbial communities. Prior researches showed that there are some petroleum-resistant plants and their root associated fungal strains which grow in petroleum polluted soils. *Ranunculus arvensis* L. (Rannunculaceae) is one of these, that was collected from an industrial garage in Tabriz. The root-associated fungi of the plant were determined and results showed the presence of 8 species which were associated with the roots of the plants growing in the polluted areas but only five of them were found in non-polluted soils. Culturing of the fungi in oil-contaminated media showed that all the studied fungi were resistant to low engine oil pollution (1% w/w) and a few species, especially *Fusarium* species, showed higher resistance to petroleum pollution (10% w/w) and it seems that they may be suitable for bioremediation in highly polluted areas. Bioremediation tests with *R. arvensis*, with and without fungal strains, showed that application of both plant and its root associated fungal strains was more effective than plant and fungi separately. Results indicated that fungal strains had the main role in bioremediation of engine oil-polluted soils but plant roots enhance the process.

KEYWORDS

Engin oil pollution;
Rhizospheral fungi;
Bioremediation;
Ranunculus arvensis.

INTRODUCTION

Pollution of soil with petroleum and its derivate chemicals is a global problem that a common phenomenon in most countries[19]. There are several soil cleaning methods including burning, washing, application of chemicals and bioremediation[14]. Bioremediation is use of plants and microorganisms to remove or detoxify environmental contaminants. It has been intensively studied over the past two decades, driven by the need for a low-cost, *in-situ* alternative to more expensive engineering-based remediation technologies[19,5,8]. In petroleum and its derivate polluted conditions, plants or plant associated microflora can convert hydrocarbons (HCs) to non-toxic forms[8].Bioremediation has been applied to remove crude oil [21,28,31,36], motor oil[9] and diesel fuel[4] from soil but the removal efficiency is highly variable[2]. Since bioremediation of petroleum-contami-

nated soils is mainly based on biodegradation by the fungal strains that are present in the rhizosphere of plants[23] or are associated and attached with roots[12], the root system of the plant is one of the most important factors. Plants can indirectly influence degradation by altering the physical and chemical conditions of the soil[8,17]. Many organic and inorganic substances are exuding from the plant roots during normal metabolism. These root exudates act as substrates for soil microorganisms, thereby enhancing the degradation of toxic organic chemicals[1].

Merkel et al.[21] have shown that some tropical grasses and legumes are resistant to petroleum pollution and root surface showed an increase in the graminoids namely *Brachiaria brizantha*, *Cyperus aggregatus* and *Eleusine indica* in petroleum polluted soils.

There are different economically and environmentally important uses of microorganisms, one of these being remediation and rehabilitation of petroleum contaminated soils[10,11,13,25.26,37]. Some prior researches have shown that some fungal species are resistant to petroleum and oil derivate pollutants and they are capable of cleaning soil pollution. The results of Ulfig et al[32] depict that keratinolytic fungi, specially *Trichophyton ajelloi*, are a potential tool for assessment of soil petroleum hydrocarbon contamination and associated bioremediation process. Fungal strains namely *Alternaria alternate*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium solani*, *Mucor racemosum*, *Penicillium notatum* and *Ulocladium atrum* have been isolated from the soils in the petroleum polluted areas in Saudi Arabia[16]. Eggen and Majcherczykh[11] showed that white rot fungus, *Pleurotus ostreatus* could remove polycyclic aromatic hydrocarbons (PAH) in contaminated soil. According to Seker and Ozturk[30] white rot fungi can very well be utilized for the decolorization of highly contaminated waste waters. Little attention has been paid to the role of plant root associated fungal species in the environmental biotechnology and bioremediation of petroleum pollution, especially in Middle Eastern region[16,34].

There are many agricultural and non-agricultural areas that are contaminated considerably with crude, refined oil and oil derivate chemicals in different countries. Iran as a developmental and also one of the major oil producing countries faces the same situation. For bioremediation purposes we have to think first about the ecological features of the area which differ from country to country depending upon the climate and other geographical conditions. Due to different geological and ecological conditions, in using of plants for bioremediation, we need to apply native plants or microorganisms for each area. Our aim here is to evaluate the ability of *R. arvensis* and its root associated fungal species for remediation of engine oil polluted soils.

MATERIAL AND METHODS

Study site

The study area was is located in Tabriz city (East Azerbaijan) in the west of Iran. It is an old industrial garage for truck and bus repairing that funded in 1964. Soils around the area were sandy loam, containing about 75% sand, 18% loam, 6% sludge and 1% organic matter with 6.7 pH. Regarding the activities in this region, a high degree of used engine-oil pollution was observed in this garage and also in the soils out of garage. The identification of soil contamination was based on a visual examination of the soil and also experimental assays. *R. arvensis* was collected from the engine-oil polluted soils in the area.

Isolation of fungi associated with the roots

Plant root samples with 1 cm length were harvested, washed and dried. The samples were kept in sodium hypochloride (1% -3 min) and then ethanol (70% -3 min) for removing the peripherally attached microorganisms, washed with distilled water, dried and kept in PDA media containing lactic acid. The petri dishes were incubated in 25 ± 2 æ%C for 4 days. Different fungal colonies were isolated and cultured separately in PDA. Fungal specimens were examined under light microscope and identified using morphological and other taxonomical features[15,24,35]. The root associated fungi for each plant collected from the petroleum polluted area were compared with the non-polluted ones in order to find out oil resistant species.

Determination of the fungal growth ability under engine oil pollution

Growth assay was used to find out fungal species

resistant to soil engine-oil contamination. The assays were conducted by comparing the growth rates of fungal strains, as colony diameter, on the engine-oil contaminated and control petri dishes. Test dishes were prepared by adding engine-oil to warm PDA solution. In order to have a uniform concentration of oil in all plates, the solution was thoroughly mixed with a magnetic stirrer, right before it was added to the plates. Three concentrations of Oil/PDA mixture (1%, 4%, and 10% w/w) were prepared. Pure PDA was used in control plates. All dishes were inoculated with 2 mm diameter plugs of fungal mycelia taken from agar inoculum plates. The dishes were incubated at 25±2 °C in an incubator. Fungal mycelia extension on the plates (colony diameter) was measured using a measuring tape after 4 days and compared with the control plates.

Evaluation of engine-oil removal

R. arvensis is a engine-oil resistant plant, common and native in the engine-oil polluted area. It is a dominating species in the area, especially in the humid region of the engine-oil polluted sites. 82 pots were prepared in June 2012 and filled with 4 kg of sterilized soil collected from an agricultural area. These were divided in to 16 groups; each group with 5 pots. The groups were divided as follows; 1) Sterile soil, 2) Sterile soil + Plant, 3) Sterile soil + *Altenaria*, 4) Sterile soil + *Altenaria* + Plant, 50 Sterile soil + *Cladosporium* Sp., 6) Sterile soil + plant + *Cladosporium* Sp. 7) Sterile soil + *Fusarium acuminatum*, 8) Sterile soil + plant + *F. acuminatum*, 9) Sterile soil + *F. equiseti*., 10) Sterile soil +plant + *F. equiseti*, 11) Sterile soil + *F. reticulatum*, 12) Sterile soil + Plant + *F. reticulatum*, 13) Sterile soil + *Penicillium*, 14) Sterile soil + Plant + *Penicillium*, 15) Sterile soil + *Rhizoctonia*, 16) Sterile soil + Plant +*Rhizoctonia*, 17) Sterile soil + *Trichoderma harzianum*, 18) Sterile soil + plant + *Trichoderma harzianum*, 19) Sterile soil + all the fungi, 20) Sterile soil + Plant + all the fungi.

In the groups which contained the plant, each pot had 3 seedlings of *R. arvensis*. All pots were polluted with engine-oil (5% w/w). They were left under the same conditions in greenhouse at Bu-Ali Sina University. *R. arvensis* plants were removed and deposited at the end of the growing period, after 3 months. The soil of experimental and control pots was homogenized separately and kept at 4ºC in a refrigerator until further process. Concentrations of crude oil were determined in the soil of experimental and control pots.

Determination of total oil and grease (TOG)

Soil samples from the experimental and control pots were collected separately. Each soil sample, without root segments, was homogenized and stored at 4°C until further processing. TOG belonging to used engine-oil was analyzed according to the EPA method 9071 A and EPA Method 3540 B (U.S. EPA, 1994). Fifteen gram of the soil in two replicates were acidified with hydrochloric acid to pH=2 and dehydrated with magnesium sulphate monohydrate. After 15 min, samples were transferred into paper extraction thimbles and placed in a soxhlet apparatus. TOG was extracted with dichloromethane for 8 h. The extract was filtered through filter paper (Whatman No. 4) with 1g sodium sulphate. The solvent was evaporated with a rotary evaporator and the weight of dry extract was determined. Percentage of remained oil was calculated based on soil dry weight and compared between control and experimental pots.

For statistical evaluation between the experimental groups and control ones, analysis of variance (ANOVA) followed by the least significant difference test (LSD) were performed between studied groups (Chehregani et al. 2005). Each data was represented as mean ± SD of 5 samples for experimental groups and also 5 for control.

RESULTS

Isolation of rizospheric fungi

The rhizospheric fungi of *R. arvensis* were collected, isolated and identified by morphological characters and taxonomical keys (TABLE1). The results of the identification of plants root associated fungi showed the presence of 8 fungal species in the roots of this plant collected from the engine-oil polluted soils. These were; *Alternaria sp., Cladosporium* Sp., *Fusarium acuminatum, F. equisetti, F.reticulatum, Penicillinium sp., Rhizoctonia sp.* and *Trichoderma harzianum.* Only four of these were found to be associated with the roots of the plants in non-polluted soils namely; *Alternaria sp., Penicillinium sp., Rhizocto-*

TABLE 1 : Comparison of fungal species in the roots of Ranunculus arvensis plant in polluted and non-polluted areas

Fungi in non-polluted area	Fungi in petroleum polluted area
Alternaria, Penicillium, Rhizoctonia	Alternaria, Fusarium acuminatum, F. equisetti, F. reticulatum, Penicillium, Rhizoctonia

nia sp. and *Trichoderma harzianum.* The studied plant had different fungal population as their root association and only four fungal species were common in the roots of all plants in both polluted and non-polluted areas (TABLE1).

Results of growth assay

The growth activity of eight fungal strains was carried out under different concentrations of crude oil and was expressed as the diameter of the colony (TABLE 2). The results showed that all the studied fungi were more or less resistant to petroleum pollution and they made a sufficient colony in 1% crude oil concentration; but only some of them save their growth activity in 10 % petroleum pollution. Among the studied fungi, *Fusarium equiseti*, *F. reticulatum* and *F. acuminatum* had the highest resistance to petroleum (with 85, 55 and 48 mm diameter of colony, respectively) and *Penicillium sp.* was the most sensitive one (with 11 mm diameter of colony) in the 10 % engine-oil polluted PDA.

Bioremediation by root associated fungi

R. arvensis is one of the common plants in the polluted areas of the engine-oil polluted area and could grow effectively on such soils. It propagates by means of seeds and underground gemma. After three months bioremediation using plants and their root associated fungal strains, concentrations of engine-oil pollution were determined in the soil of controls and contaminated soils. The data showed that concentration of engine-oil pollution decreased considerably in the all pots but was constant in control ones (Figure 1). It also showed that decrease in the experimental pots containing plant together with all fungal strains was more than other groups (up to 87%). Meanwhile, decrease of petroleum pollution was also considerable in the pots containing plant added *Fusarium equiseti* and *F. reticulatum* (up to 67% and 79%, respectively). The data showed that all fungal species were capable to decrease petroleum pollution solitary (Figure 1), but they were more effective when applied with the plant. *Altenaria sp.* singly result in a decrease up to 16% but when applied with plant, decrease was 47% and also *Rhizoctonia* reduced soil pollution up to 14% solely, when applied with plant decrease was raised up to 56%.

DISCUSSION

Petroleum and its derivate pollution of soils is a major environmental pollution in many countries[18]. Serious risks can occur to the public health and environment when the soil is polluted by crude oil[25]. Results of this work showed that engine oil, in the concentrations presented here (up to 10%) did not kill the studied plant and fungal species. This is in accordance with the results of earlier studies by Nicolotti and Egli[8,17], who showed several legumes and graminoids can flourish

TABLE 2 : Growth ability of rhizospheral fungi in PDA containing engine-oil. (Data expressed as diameter of colony-mm)

Oil treatments Microorganism	Non-contaminated (control)	1% Oil	4% Oil	10% Oil
Alternaria sp.	49±6	*28±8	*18±6	*14±4
Cladosporium sp.	22±4	21±3	19±4	16±2
Fusarium acuminatum	34±5	42±10.3	50±10	48±12
F. equiseti	12±2	*46±4	*63±4	*85±8
F. reticulatum	33±9	45±6	61±7	55±5
Penicillium	40±5	30±4	24±4	11±2
Rhizoctonia sp.	88±1.2	*67±9	*42±3	*18±5
Trichoderma harzianum	24±3	22±3	19±2	18±3

Each data represents the mean±SE of 3-5 samples; *Data significantly different from the control (p≤0.05)

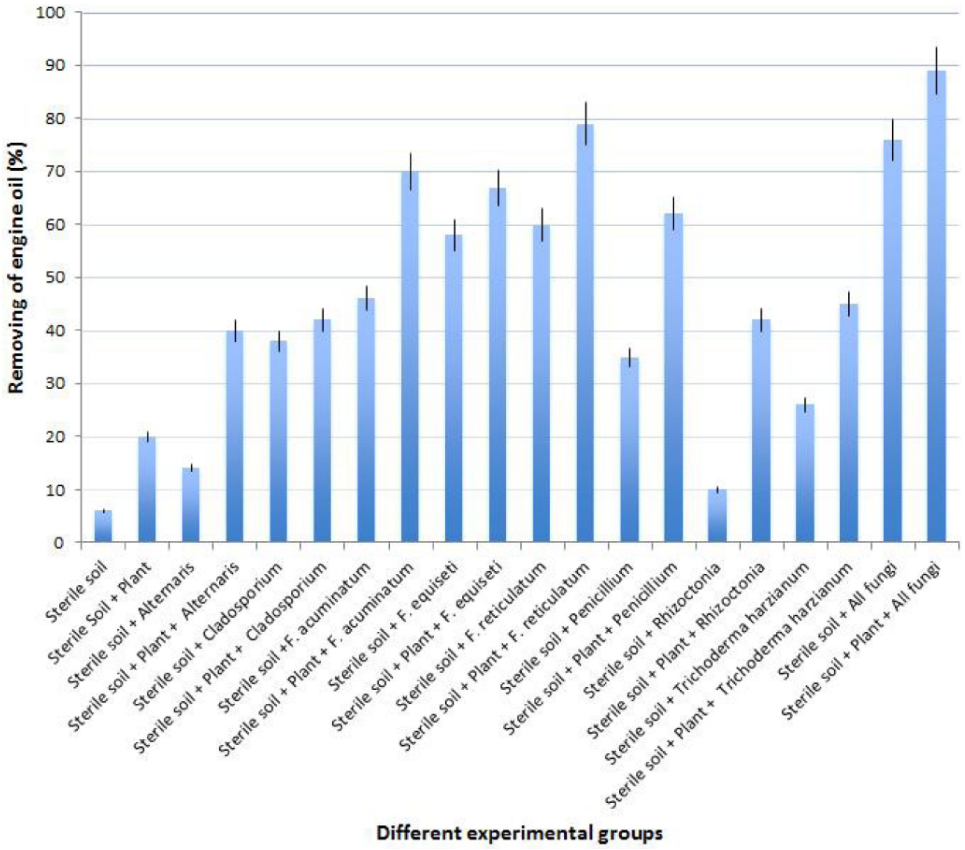

Figure 1 : Decrease of engine-oil pollution concentration (%) in the polluted soils after bioremediation by *Ranunculus arvensis* and its rhizospheric fungal strains. All pots contained 5% w/w engine-oil pollution before the beginning of experiment. Data indicates amount of engine-oil pollution decreased due to bioremediation. Decrease of engine-oil in control pots is the result of evaporation. Decrease of pollution between experimental and control groups are significant (P<0.01). Each data represents the mean ± SE of 5 samples

on petroleum polluted soils with about 5% pollution. The direct effects of oil derivate on the soil are resulted in more or less marked reduction in plant growth and biomass[19]. Similar findings have been reported for other plant species: *Festuca rubra* and *Puccinellia maritime*[3], *Trifolium rubra*[18] and different legumes and grasses[21].

Study on fungal species showed that *Alternaria*, *Penicillium* and *Rhizoctonia* were the common fungi that have been observed in the roots of studied plant, both in polluted and non-polluted soils. Based on our data, fungal variation in engine-oil polluted area was more than non-polluted one (TABLE1). This means that roots of the plants had more fungi yielded in polluted areas than non-polluted ones, which is in accordance with the findings of some prior workers[1,16,34]. It seems that the fungal species use oil compounds as nutrients, because engine-oil pollution increases fungal growth. Similar results have been reported by some other researchers [10,11,25,26,37].

In vitro growth test of fungi showed a species-specific response. Most of studied fungal strains were capable of growth in 1% w/w oil pollution and therefore could be useful for the remediation of light soil pollution. Some fungal species were inhibited by high oil concentrations (10% w/w). These species were *Alternaria* sp., *Cladosporium* sp., *Penicillium sp., Trichoderma harzianum* and *Rhizoctonia* sp., while others actually grew well in oil-contaminated media, even at very high concentrations. These are *Fusarium acuminatum, F. equiseti,* and *F. reticulatum*. It seems that engine-oil could supply some nutrients for these fungi and they could prove more effective for oil degradation. Our findings are in accordance with those of other researchers about other fungal species [10,11,25,26,37].

Bioremediation of a petroleum and its derivates-contaminated soil is mainly based on biodegradation in the rhizosphere[12,27], root-associated fungi are one of

the most important factors. The results of this study propose that above-mentioned fungi can be evaluated for the further remediation tests and this is the first report about their remediation capacity. The data of this study indicates that isolated strains of *Fusarium acuminatum*, *F. equseti* and *F. reticulatum* may have the potential for bioremediation of rngine oil in highly polluted soils especially in semi--dry regions.

R. arvensis abundantly found in the polluted areas when chosen for bioremediation test together with its root associated fungi show that the concentrations of crude oil decreased in the pots containing plant with all fungal strains added. The pots containing the plant added *Fusarium equiseti*, *F. acuminatum* and *F. reticulatum* also showed the highest decrease in the engine-oil pollution (Figure 1). Results show that although all the subjected fungal strains cause to decrease in the engine-oil concentration in soils but application of plant together with associated fungal strains proves more effective (Figure 1). It means that plant root exhausts result in an increase of engine-oil biodegradation driven by fungal strains as proposed by few prior reports[14,22,23]. Phytoremediation of petroleum and its derivates pollution is a cost-effective green technology; there are more advantages, when it comes to the use of native plants and fungi[29]. This is the first report on the ability of *R. arvensis* and its rhizospheric fungi, especially *Fusarium acuminatum*, *F. equseti* and *F. reticulatum*, for remediation of engine-oil polluted soils.

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