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Identification of Petroleum Resistant Plants and Rhizospheral Fungi for Phytoremediation of Petroleum Contaminated Soils

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Petroleum pollution is a major and global disaster. Phytoremediation of petroleum polluted soils was based on degradation of oil using plants and its dependent fungi. A field study was conducted in a petroleum contaminated site at Arak refinery (Iran) to find the petroleum-resistant plant species and the rhizospheral fungi for being used in phytoremediation. Results showed that eight plant species were growing on the contaminated sites: *Polygonum aviculare*, *Centureae virgata*, *Carthamus onyacantha*, *Alhaji cameleron*, *Glycyrrhiza glabra*, *Poa* sp., *Lactuca serula* and *Hordeum bulbosum*. The germination assay showed that all studied plants are capable to survive in oil contaminated soils but they have different germination ability under petroleum pollution. 22 fungal species were found in the rhizosphere of the plants growing in the polluted areas; four of these species were common in all the plants and the others have species-specific distribution within the plants. The variation of fungi in petroleum-polluted areas was more than non-polluted zones. Culture of fungi in oil-contaminated media showed that although all the studied fungi were resistant to low petroleum pollution (1%) but a few species, especially *Fusarium* species, are resistant to higher petroleum pollution (10%).

Keywords
Petroleum pollution, Phytoremediation, Petroleum resistant plant, Rhizospheral fungi

1. Introduction

Phytoremediation 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[1),2)]. In phytoremediation, plants are used to remove or detoxify environmental contaminants. Since each area has special ecological properties with different climates, in phytoremediation we need to apply native or naturalized plants in newly subjected areas. In petroleum polluted conditions, plants or plant associated microflora can convert hydrocarbons (HCs) to non-toxic forms[3)]. Phytoremediation has been applied to remove crude oil[4)~6)], motor oil[7)], and diesel fuel[8)] from soil. Phytoremediation can also remove weathered HCs from soil[9),10)], but the removal efficiency is highly variable[11)]. Since phytoremediation of petroleum-contaminated soils is mainly based on biodegradation in the rhizosphere of plants[12)], the root system of the plant is one of the most important factors. Plants and their rhizospheres can indirectly influence degradation by altering the physical and chemical conditions of the soil[3)]. Plant roots exude organic and inorganic substances to the rhizosphere during normal metabolism. Root exudates act as substrates for soil microorganisms, thereby enhancing the degradation of toxic organic chemicals[13)].

Numerous sites are contaminated worldwide with crude or refined oil in different countries. Hence, there is a vast need of various plants and microorganisms, especially native ones, for the phytoremediation of petroleum-polluted soils. The aim of this research was to find suitable and effective plants and their rhizospheral fungi for the remediation of petroleum polluted soils in the Middle Eastern region, specially in Iran.

2. Material and Methods

2. 1. Selection of Samples

The Arak refinery, located in west of Iran, was selected in this study. Regarding the oil refining activities, in this region a high degree of petroleum pollution is illustrated in some areas. The identification of soil con-

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tamination was based on a visual examination of the soil to which auger was added and the verification of the plant roots that were in contact with an obviously contaminated, dark-discolored soil. Plants were collected for a spatial identification from September to November 2007 and the root associated soils were collected in October and November 2007. Only herbs were collected in this study, trees and shrubs were not considered since they require higher efforts for breeding and maintenance than herbs and graminoides[1),14)]. Collected specimens were identified in the herbarium of Bu-Ali Sina University, Hamedan, Iran.

2. 2. Species Occurrence

The population density was rated high, medium and low, respectively, by subjective visual inspection[1)]. A high population density was defined as coherent growth of individuals of a species, whereas a medium density referred to individuals occurring frequently on a site without forming extensive coherent populations. Low population density referred to a scattered growth of individuals on a site.

2. 3. Determination of Total Oil and Grease (TOG)

The soil of vegetated and non-vegetated areas was collected separately. Each sample was homogenized and stored at 4℃ until further processing. TOG was analyzed according to the EPA method 9071 A and EPA Method 3540 B (U.S. EPA, 1994). Fifteen grams soil in two replicates were acidified with hydrochloric acid to pH 2 and dehydrated with magnesium sulphate monohydrate. After a 15 min, samples were transferred into paper extraction thimbles and placed into a Soxhlet apparatus. TOG was extracted with dichloromethane for 8 h. The extract was filtered through filter paper (Whatman No. 4) with 1 g sodium sulphate. The solvent was evaporated with a rotary evaporator and the weight of dry extract was determined. Percentage of TOG was calculated based on soil dry weight and compared in the vegetated and non-vegetated areas.

2. 4. Propagation Experiments under Oil Pollution

The seeds of the studied plants were subjected to a germination test in the presence of crude oil. To break the seeds physical dormancy, seeds were immersed in concentrated sulphuric acid for 5 to 15 min[15)]. In some cases, sandpaper was used to detach the seeds from other propagated structures, simultaneously resulting in the scarification of the seed coat. After coating them with a fungicide (Vitavax^{TM} 200F), one hundred seeds in three replicates were germinated in Petri dishes. These dishes contained several layers of filter paper moistened with distilled water to which 5% crude oil was added. This procedure was undertaken as the most polluted soils contain the same amount of pollution. The dishes were kept in the greenhouse under natural daylight/night conditions (approx. 12/12 h) and the emergence of the radicule was recorded until no further germination could be observed for five days.

2. 5. Isolation of Rhizospheral Fungi

Small parts of the roots, collected from both polluted and non-polluted areas, with a length of about 1 cm were mixed with distilled water and shacked for 20 min. 100 μl of resulting liquid were spread on the surface of a PDA (Potato Dextrose Agar) medium in Petri dishes, for fungal culturing. The meda contained lactic acid for inhibition of bacterial growth. Cultures were incubated in 25 ± 2℃ for 4 days. Then, different fungal strains were isolated and cultured separately in PDA. Fungal specimens were examined under light microscope after preparations and were identified using morphological characters. The rhizospheral fungal communities of plants collected from the petroleum polluted area were compared with the non-polluted ones for finding oil resistant fungal species.

2. 6. Study of the Fungal Growth Ability under Petroleum Pollution

The growth assay was used to determine the resistance of the selected fungal species to petroleum contamination of the soil. The assay was conducted by comparing the differences between the fungal growth rates on the test and control Petri dishes. Test dishes were prepared by adding weathered crude 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%) 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's plates. The dishes were incubated at 25 ± 2℃ 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.

3. Results

3. 1. Species Occurrence

Only eight species from different families were found growing on the contaminated sites in Arak refinery area in the west of Iran (**Table 1**). This indicated that environmental factors are not sufficient for most of the plants. The species were all naturally growing; no cultivated species was found. *Polygonum aviculare* grew in the highest population densities and it was also found in all the studied polluted areas.

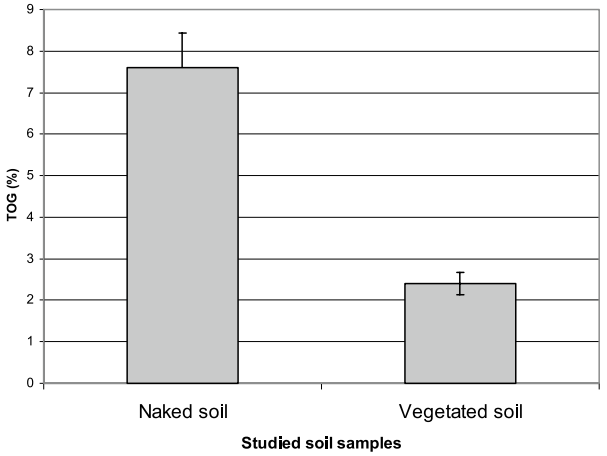
3. 2. TOG Analysis in the Naked and Vegetated Soils

Determination of TOG levels (**Fig. 1**) showed that a significant influence of plants and rhizospheral fungi on oil degradation in the vegetated areas ($p \leq 0.01$). Our results showed that percentage of TOG in the non-vegetated areas is 7.6 ± 1.2% but in the vegetated areas is 2.4 ± 0.6%. Results indicated that plants and their

Table 1 Plant Species Collected from the Petroleum-contaminated Sites of Arak Refinery, Iran

Species name	Family	Density	Life cycle	Germination[a)]
Alhaji cameleron	Fabaceae	L	Perennial	45 ± 6.4
Carthamus onyacantha	Asteraceae	M	Perennial	86 ± 10.3
Centureae virgata	Asteraceae	M	Perennial	72 ± 4.5
Hordeum bulbosum	Geramineae	M	Annual	56 ± 12.4
Glycyrrhiza glabra	Fabaceae	M	Perennial	58 ± 9.5
Lactuca serula	Asteraceae	L	Annual	42 ± 8.7
Poa sp.	Geramineae	L	Perennial	35 ± 10.3
Polygonum aviculare	Polygonaceae	H	Annual	69.5 ± 15.0

H = high density population, M = medium density population, L = low population density.
a) Each data represents the mean of the germination rate % ± St Dev of at least 100 seeds.



Data showed that the amounts of total oil and greases in naked soils are more than vegetated ones. Each date represented the means ± SE 12 samples in naked soil and 15 samples in vegetated soil. Differences between subjected soils are significant ($p \leq 0.01$).

Fig. 1 Amounts of Petroleum Pollution in the Naked and Vegetated Soils

rhizospheral fungi are capable to degrade oil and greases and also cause to decrease petroleum pollution in the planted soils.

3. 3. Seed Germination

The seeds germination of the studied species was evaluated under 5% petroleum polluted condition (**Table 1**). Results showed that *Carthamus onyacantha* had the highest seed germination under petroleum pollution (86% germination). *Centureae virgata* (with 72% germination) and *Polygonum aviculare* (with 69.9% germination) have also a high percent of seed germination. The lowest germination was determined for *Poa* sp. (35%), but it produced numerous seeds and survived in petroleum polluted conditions.

3. 4. Rhizospheral Fungi

The rizospheral fungi of the subjected plants were collected, isolated and determined by morphological characters. The results showed that there are 22 fungal species in the rhizoshpere of the plants in petroleum polluted soils namely; *Alternaria* sp., *Aspergillus flavus*, *Aspergillus terreus*, *Bipolaris* sp., *Botrytis* sp., *Cladosporium* sp., *Colletrichum* sp., *Fusarium aquaeductuum*, *F. acuminatum*, *F. equiseti*, *F. oxysporum*, *F. sambucinum*, *F. solani*, *F. reticulatum*, *Helminthosporium* sp., *Macrofomina* sp., *Mucur* sp., *Oloclodium* sp., *Paecilomyces* sp., *Penicilinium* sp., *Rhizoctonia* sp., and *Scolecubasidium.*

It also came to the fact that the studied plants had different fungal population in their rhizosphere and only four species including *Alternaria* sp., *Aspergillus* sp., *Bipolaris* sp. and *Penicilinium* sp. were common in rhizosphere of all the plants in both polluted and non-polluted areas (**Table 2**). The largest amount of fungal species was collected in the rhizosphere of *Polygonum aviculare* (10 species in polluted and 8 in non-polluted areas) and the least were collected from the rhizosphere of *Lactuca serula*, and *Alhaji cameleron* (3 species in controls and 5 in polluted areas).

3. 5. Results of the Growth Ability under Petroleum Pollution

The growth activity of fungi carried out under different concentrations of petroleum and was expressed as the diameter of the colony (**Fig. 2**). The results showed that all the collected fungi were more or less resistant to petroleum pollution and they made a sufficient colony in 1% petroleum pollution; meanwhile, only some of them save their growth activity in a 10% petroleum pollution. Among the studied fungi, *Fusarium sambucinum* had the highest resistance to petroleum (with 90 mm diameter of colony) and *Helminthosporium* was the most sensitive one (with 0.5 mm diameter of colony) in the 10% petroleum polluted PDA.

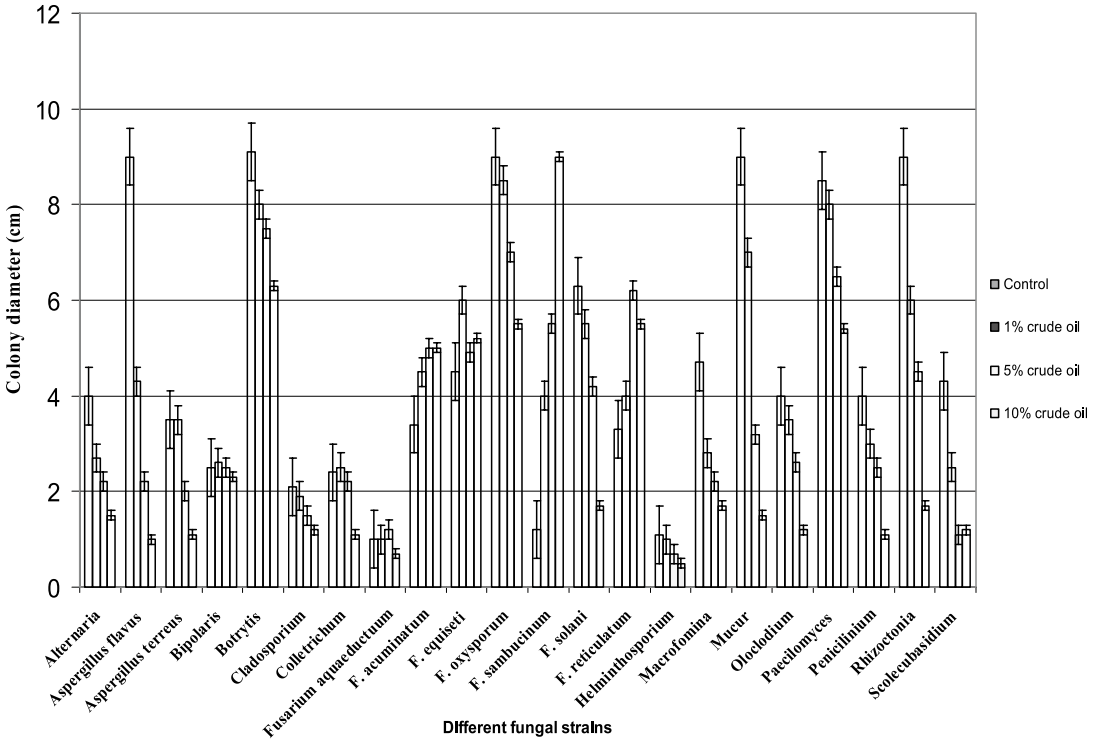
4. Discussion

Environmental pollution with crude oil is a common phenomenon in oiled countries. Serious risks can occur to the public health and environment when the soil is polluted by crude oil. Plants or plant associated microflora can convert hydrocarbons (HCs) to non-toxic forms and are useful for bioremediation of polluted soils[3)]. Since oil pollution is a global disaster, we need to find vast variety of plants and microorganisms to re-

Table 2 The Comparison of Fungal Species in the Rhizosphere of the Studied Plants in Polluted and Non-polluted Areas

Plants	Non-polluted	Polluted
Polygonum aviculare	*Alternaria**, *Aspergillus**, *Bipolaris**, *Botrytis*, *Cladosporium*, *Fusarium oxysporum*, *F. solani*, *Penicilinium**	*Alternaria**, *Aspergillus**, *Bipolaris**, *Botrytis*, *Colletrichum*, *Penicilinium**, *Fusarium acuminatum*#, *F. reticulatum*#, *Rhizoctonia*#, *Scolecubasidium*#
Centureae virgata	*Alternaria**, *Bipolaris**, *Penicilinium**, *Rhizoctonia*	*Alternaria**, *Fusarium acuminatum*#, *F. equiseti*#, *F. reticulatum*#, *Penicilinium**, *Rhizoctonia*
Poa sp.	*Alternaria**, *Bipolaris**, *Fusarium reticulatum*	*Alternaria**, *Aspergillus**, *Fusarium acuminatum*#, *F. reticulatum*, *Mucur*#, *Penicilinium**, *Helminthosporium*#
Carthamus onyacantha	*Alternaria**, *Bipolaris**, *Cladosporium*, *Fusarium oxysporum*, *Penicilinium**	*Alternaria**, *Aspergillus**, *Macrofomina*#, *Oloclodium*#, *Penicilinium**, *Rhizoctonia*#
Alhaji cameleron	*Alternaria**, *Bipolaris**, *Fusarium oxysporum*, *Penicilinium**	*Alternaria**, *Aspergillus**, *Fusarium reticulatum*#, *Paecilomyces*#, *Penicilinium**
Glycyrrhiza glabra	*Alternaria**, *Aspergillus**, *Bipolaris*, *Cladosporium*, *Fusarium oxysporum*, *F. sambucinum*, *Mucur*, *Penicilinium**	*Alternaria**, *Aspergillus**, *Bipolaris**, *Fusarium reticulatum*#, *Penicilinium**
Lactuca serula	*Alternaria**, *Penicilinium**, *Mucur*	*Alternaria**, *Aspergillus*#, *Fusarium acuminatum*#, *Mucur*, *Penicilinium**
Hordeum bulbosum	*Alternaria**, *Aspergillus**, *Bipolaris**, *Fusarium solani*	*Acromonium*#, *Alternaria**, *Aspergillus**, *Bipolaris**, *Fusarium acuminatum*#, *F. aquaeductuum*#, *F. reticulatum*#, *Penicilinium**#

*: Fungal strains those are common in the rhizosphere of the studied plants.
#: Fungal species that are present in the polluted areas and absent in the non-polluted areas.



Data expressed as diameter of colony (cm). Each data represented the means ± SE of 3-5 samples.

Fig. 2 The Growth Ability of Different Studied Fungi in PDA Containing Crude Oil

mediate petroleum pollution in different areas with various climates.

Results and observations of this research work showed that crude oil, in the concentrations presented in subjected areas neither affects seed germination nor kills seedlings of the collected plant species. The seeds of the all eight species were found in quantities that were sufficient for the germination tests. The species were different in the amount of the seeds that they produced under petroleum pollution. The highest

number of obtained seeds belonged to *Centureae virgata* but its seeds were extremely small; therefore both handling and counting the seeds and as a result the evaluation of the germination test faced a great complication. The seeds of *Poa* sp. and *Polygonum aviculare* are firmly attached to their propagules making it difficult to separate them. The lowest seed number was observed in *Alhaji cameleron* because each legume contains only 2-4 seeds. Seeds of other species including *Carthamus onyacantha*, *Hordeum bulbosum*, *Glycyrrhiza glabra* and *Lactuca serula* were found to be produced in large numbers and the collection and handling of seeds was easy.

Based on germination assay, the studied plant species could survive and continue to growth under petroleum pollution. Regarding the results of an earlier study[15], it seems that crude oil has a caustic or lethal effect only when it comes into direct contact with the tissues of a plant. Crude oil's indirect effect on the soil is confined to a more or less marked reduction in plant growth and biomass[4], possibly attributable to the changes brought about in the soil microbial populations. On the other hand, the growth of certain plants, such as *Alhaji cameleron*, *Carthamus onyacantha*, *Centureae virgata*, *Hordeum bulbosum*, *Glycyrrhiza glabra*, *Lactuca serula*, *Poa* sp. and *Polygonum aviculare* can not be inhibited by crude oil that is in accordance with the prior reports about other species: *Festuca rubra* and *Puccinellia maritime*[16], *Trifolium rubra*[18], and different legumes and grasses[7].

The findings in this study indicated that amounts of petroleum pollution in the non-vegetated soils are about three times more than vegetated soils. Our results are in accordance with some prior researches on other plant species in tropical regions[4),5),19)] and desert regions[5),20),21)].

Since phytoremediation of a petroleum-contaminated soil is mainly based on biodegradation in the rhizosphere[12], rhizospheral fungi are one of the most important factors. Plant and their roots enhance microbial activity by supplying oxygen and the root exudates for the degradation of contaminates[22)~24)], leading to microbial populations that are 5 to 100 times greater in the rhizosphere than in bulk soil[13]. The results of this study reveal a very high variability in the data, especially in the rhizospheral fungi data. Our data showed that *Alternaria* sp., *Aspergillus* sp., *Bipolaris* sp. and *Penicilinium* sp. are common fungi that were seen in rhizosphere of all the studied plants. Based on our data in all the studied cases, there are more fungal species in the rhizosphere of the plants in polluted areas than in non-polluted areas (**Table 2**). It means that the rhizosphere of the plants have more fungi yielded in polluted areas than the non-polluted ones. For example, in the rhizosphere of *Polygonum aviculare*, the additional fungi that were found in the polluted soils and were absent in the control soil included; *Aspergillus* sp., *Fusarium acuminatum*, *F. reticulatum* and *Rhizoctonia* sp., and also other extra yield for *Carthamus onyacantha* were *Oloclodium*, *Rhizoctonia* sp. and *Macrofomina* sp. As illustrated in **Table 2**, we can conclude that petroleum pollution causes an increased variation of fungal population in petroleum polluted areas. It seems that the fungi used oil compounds as nutrients.

The *in vitro* growth test showed a species-specific response. All studied fungal strains are capable of growth in 1% oil pollution and are therefore useful for the remediation of light soil pollution (**Fig. 2**). Some fungal species are inhibited by high concentration of oil (10%). These species include *Alternaria*, *Aspergillus flavus*, *Aspergillus terreus*, *Botrytis*, *Cladosporium*, *Colletrichum*, *Fusarium aquaeductuum*, *F. oxysporum*, *F. solani*, *Helminthosporium*, *Macrofomina*, *Mucur*, *Oloclodium*, *Paecilomyces*, *Penicilinium*, *Rhizoctonia* and *Scolecubasidium* while others actually grow better in oil-contaminated soil, even at very high concentrations (*Fusarium acuminatum*, *F. equiseti*, *F. sambucinum*, *F. reticulatum*). In this specific case, it seems that oil supply major nutrients for these fungi and that they are more effective for oil degradation. The result of this study proposes the above mentioned fungi for the future remediation tests. The only fungus that displays no definite reaction to oil pollution is *Bipolaris* sp.

The application of microorganisms activities is prominent in current biotechnology. There are different economically and environmentally important uses for microorganisms, such as remediation and rehabilitation of petroleum contaminated soils[25)~29)]. Little attention has been paid to the role of microorganisms in the environmental biotechnology and bioremediation of petroleum pollution, specially in the Middle Eastern region. The findings of this study suggest that crude oil pollution in vegetated area does not cause long-term environmental damage and amounts of petroleum pollution in vegetated soils was less that non-vegetated ones. This could be due to bioremediation on the part of the plants root function and microflora in the soil, as already reported for certain fungi[30)~33)]. Moreover, results show that some of the rhizospheral fungi that survive in contaminated soil may actually use crude oil as a nutrient and thus make their own contribution to the process of bioremediation, and the data of this study indicates that all the studied fungi specially *Fusarium acuminatum*, *F. equiseti*, *F. sambucinum* and *F. reticulatum* have the potential for biodegradation of crude oil in high polluted condition and they are also proposed for the remediation of highly polluted areas in semi-dry regions. This is the first report of their ability.

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要　　旨

石油汚染土壌のファイトレメディエーションのための石油耐性植物および根菌真菌の同定

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石油による環境汚染は主要な世界的問題である。石油汚染土壌のファイトレメディエーションは，植物やその真菌による石油の分解によるものである。ファイトレメディエーションに用いる耐石油植物種および根菌類の探索のためのフィールドスタディを Arak 製油所（イラン）の石油汚染地区で行った。その結果，汚染地区において *Polygonum aviculare*，*Centureae virgata*，*Carthamus onyacantha*，*Alhaji cameleron*，*Glycyrrhiza glabra*，*Poa* sp.，*Lactuca serula*，*Hordeum bulbosum* の8種類の植物で成長が確認された。発芽アッセイではすべての植物が汚染土壌中で生存したが，成長力には差が見られた。22種の真菌を汚染地区で生育している植物の根菌から発見した。そのうち4種が全ての植物に共通であり，他は特異的に分布していた。石油汚染地区の根菌真菌は非汚染地域に比べ多様であった。石油汚染培地中で根菌を培養した結果，すべての根菌真菌が低濃度汚染（1%）に対して抵抗を持ち，数種，特に *Fusarium* 種は高濃度汚染（10%）に対しても抵抗を有していることが分かった。

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