Advanced Research in Microbial Metabolites and Technology
ARMMT

Rhodococcus ruber KE1 augmented phytoremediation of crude oil contamination using *Lolium perenne* and *Festuca rubra rubra*

Monire Abolhasani Sooraki[1], Vahid Poozesh[1]*, Fatemeh Salimi[2], Ahmad Reza Mehrabian[3]

[1]*Department of Plant Sciences, School of Biology, Damghan University, Damghan 36716-41167, Iran.*
[2] *Department of Cellular and Molecular Biology, School of Biology, Damghan University, Damghan 36716-41167, Iran.*
[3]*Department of Plant Sciences and Technology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, GC, Tehran, Iran.*

Article Info

Received 15/11/2020
Received in revised form 06/05/2021
Accepted 17/05/2021

Keywords:
Bioremediation
Crude oil
Festuca rubra rubra
Lolium perenne
R.ruber KE1

Abstract

Phytoremediation is an eco-friendly technique for hydrocarbon bioremoval. Phytoremediation efficiency can be enhanced through the cooperation of plants and crude oil degrading bacteria. This study was aimed to select crude oil tolerant grasses and clarify the bioremoval efficiency of *R.ruber* KE1-augmented phytoremediation. For this purpose, the resistance of *Festuca rubra rubra, Festuca rubra commutate, Lolium perenne, and Poa pratensis* to crude oil was evaluated. Further, the supportive and augmenting role of *R.ruber* KE1 treatment on the morphological and biochemical properties of these grasses and crude oil phytoremediation was assessed. According to those results, *Festuca rubra rubra* and *L. perenne* were selected as more crude oil resistant grasses. *R.ruber* KE1 was able to significantly enhance its growth parameters (radicle, root, and shoot length) in the presence of crude oil. Results showed the most applied concentration of crude oil (5% w/w) inhibited *Festuca rubra rubra* growth while *R.ruber* KE1 treatment improved *Festuca rubra rubra* growth ($P<0.05$). A combination of *R.ruber* KE1 with *L. perenne* or *Festuca rubra rubra* resulted in a higher degradation rate of >70% in all applied concentrations of crude oil after 40 days. Also, *R.ruber* KE1 treatment enhanced biodegrading of insoluble (36%→1.82%) and soluble (53.86%→14.52%) compounds of crude oil. *R.ruber* KE1-augmented phytoremediation could be a promising approach to degrade recalcitrant hydrocarbon pollutants and remediate contaminated soils.

1. Introduction

The contamination of terrestrial and aquatic ecosystems by petroleum hydrocarbons due to high toxic, carcinogenic, mutagenic, and deleterious effects on the environment, plant, animal, and human health is considered a life-threatening problem (Fatima et al., 2018). It has been estimated that high amounts of these pollutants (600,000 metric tons/year) accidentally (e.g., the disaster in Mexico) or through human activities or wars (e.g., the armed conflict between Iraq and Kuwait) enter ecosystems (Bashir et al., 2020). These events have led to the distribution of millions of barrels of crude oil into the environment and compromised wildlife as a persistent pollutant. For example, animals are

* Corresponding author. Tel: +982335220223
E-mail address: poozesh@du.ac.ir
DOI: 10.22104/ARMMT.2021.4526.1049

exposed through the ingestion, absorption, and inhalation of this pollutant. The pollutant influences plants by reducing the availability of water, oxygen, and nutrients, thus creating a high level of oxidative stress, degradation of chlorophyll, and consequently diminishing seed germination (Guvvala et al., 2020; Manisalidis et al., 2020)

Following-up the adverse effects of petroleum hydrocarbons, various mechanical (such as boomers), physical (like skimmers and adsorbent materials to control spreading oil), chemical (such as dispersants and solidifiers that limit oil spill spread) and thermal (which act through burning of the oil) strategies have been applied to control, limit, and degrade these dangerous pollutants (Dave & Ghaly, 2011; Tewari & Sirvaiya, 2015).

Because of the high cost and limitations of mechanical, physical, and chemical methods, a noninvasive, relatively affordable, and eco-friendly strategy "bioremediation" has raised much interest. In this bio-based clean-up strategy, various prokaryotes like bacteria (bioremediation) and eukaryotes-like plants (phytoremediation) have been applied to detoxify, degrade, remove, or accumulate pollutants owing to their diverse metabolic capabilities (Shirdam et al., 2008). Bioremediation can occur through natural attenuation, biostimulation, or bioaugmentation mechanisms. Natural attenuation acts by applying the degradation activity of indigenous microorganisms (Okoh et al., 2020).

In this method, no change or damage is imposed on the ecosystem, but its time-consuming feature is a limiting factor for this strategy. The detoxification rate of biological methods can be accelerated through the introduction of specific degraders to the contaminated ecosystem; these degraders successfully survive by multiply in that polluted environment or using a combination of a pollutant degrading plant and bacteria (Varjani & Upasani, 2019).

In this regard, potent crude oil degrader bacteria that can compete with indigenous microbial communities are needed. *Actinobacteria* are one of the most prolific bacteria with suitable properties like surviving in extreme conditions, producing cell-bound surfactants, and showing high cell hydrophobicity. These properties make them appropriate candidates for bioremediation purposes (Kügler et al., 2015).

It has been shown that better bioremediation efficiency can be achieved using known pollutant degrading bacteria instead of indigenous microorganisms. Microorganisms that are isolated from extreme environments can provide a potential benefit for phytoremediation in contaminated soils under adverse conditions. According to previous studies, oil-degrading bacteria belong to *Rhodococcus*, *Nocardia*, *Micrococcus*, *Bacillus*, *Pseudomonas*, *Acinetobacter*, and *Flavobacterium* genera (Das & Chandran, 2011).

Rhodococcus is a promising genus of *Actinobacteria* for the biodegradation of recalcitrant pollutants such as petroleum hydrocarbons. Through its physiological and ecological adaptations to harsh environmental conditions, successful competition with other bacterial populations, extensive catabolic versatility, and unique enzymatic capabilities, *Rhodococcus* bacteria could be efficiently used as a bioaugmentation agent in bioremediation programs (Kuyukina & Ivshina, 2010). Due to producing cell-associated biosurfactants, *Rhodococcus* can adhere to liquid hydrocarbons as well as hydrophobic solid surfaces. Hence, they can efficiently colonize in hydrocarbon contaminated soils (Neu, 1996; Whyte et al., 1999).

Rhodococcus strains, such as *R. ruber,* are frequently isolated from hydrocarbons contaminated ecosystems. *Rhodococcus* strains uptake large oil drops via direct bacterial contact as carbon and energy sources. In this regard, successful and efficient bio-based remediation will be achieved by introducing *Rhodococcus* to a contaminated environment (Kuyukina & Ivshina, 2010).

Moreover, more promising bioremediation can be achieved by simultaneously utilizing the degradation abilities of *Rhodococcus* strains and plants. Whenever a strong and sustained plant–bacteria interaction is established, the synergistic effect can significantly enhance the efficiency of bioremediation. In this tactic, plants should have a high growth rate and significant resistance to the pollutant of interest.

Therefore, we aimed to assess the synergistic effect of a combination of *R.ruber* KE1, a known oil degrading bacterium, and four grasses, *Festuca rubra rubra, Festuca rubra commutate, Lolium perenne, and Poa pratensis,* on the enhancement of bioremediation of petroleum contaminated soil. This is the first study evaluating the synergism of *R.ruber* KE1 and grasses in the bioremediation of crude oil contaminated soil.

The success of the proposed approach will be assessed for biological remediation of some crude oil contaminated soil by evaluating the crude oil resistance of these grasses, determining their potential in phytoremediation of crude oil, and finally, assessing their synergism with microbial cells in the bioremediation of hydrocarbons.

2. Materials and methods

2.1. Plant seeds and microbial strain

Seeds of *Festuca rubra rubra, Festuca rubra commutate, Lolium perenne, and Poa pratensis* were purchased from Diten Tadbir. *Rhodococcus ruber* strain KE1, an oil-degrading bacterium isolated from drilling oil-based mud in Khuzestan, Iran with the accession number of JQ963338.1, was received from the petroleum microbiology department of the Research Institute of Applied Sciences, ACECR, Evin, Tehran, and heavy crude oil was kindly obtained from the Ahvaz oil refinery.

2.2. Soil analysis and preparing

Cultivated soil was randomly collected from a farm in Amiriyeh village, Damghan, Iran (36°29′ 32″N 54°54′56″E). The collected soil samples were mixed uniformly and sieved. The mixed soil used throughout the experiments was composed of cultivable soil (70%), sandy soil (20%), and a mixture of peat moss soil (10%). A sample of the prepared soil was analyzed to determine its physicochemical properties (Sparks et al., 2020).

2.3. Preparing crude oil contaminated soil for experiment

The prepared soil was contaminated by heavy oil (0.5% w/w). These soil samples were individually transferred to trays.

2.4. Selection of more heavy oil-resistant plants

Twenty seeds of four grasses, including *Festuca rubra rubra*, *Festuca rubra commutate*, *Lolium perenne*, *and Poa pratensis*, were treated with *R.ruber* (10^6 cell/ml, test group) or physiological serum (blank). For this purpose, a suspension of *R.ruber* (OD_{625}=0.5) was prepared in saline serum. Subsequently, seeds sterilized with hypochlorite sodium (1% v/v) were inoculated by the bacterial suspension and transferred to crude oil contaminated (0.5% w/w) and uncontaminated soil containing trays with five replicates. Untreated seeds were regarded as the control. Irrigation was done twice a day for 10 days. At the end of the experiment, more crude oil-resistant plants were selected for further studies.

2.5. Seed germination of *R.ruber* treated seeds with various concentrations of crude oil

To determine the range of a tolerable concentration of crude oil for selected seeds, the effect of various concentrations of crude oil was evaluated on seed germination and radicle length of *R.ruber* treated seeds. For this purpose, seeds of more crude oil-resistant plants were soaked in hypochlorite sodium (1% v/v) for 5 min, rinsed with water 5 times, and inoculated by *R.ruber*. Then, ten seeds were placed between two crude oil-soaked Wattman papers [5 mL, 2, 4, and 6 % (w/v) with three replicates] in 10 cm petri dishes. The plates were incubated for 10 days at 27 ºC. Untreated seeds were considered as the control. At the end of the experiment, the percentage of seed germination and length of radicles were calculated.

2.6. Phytoremediation potential of *R. ruber* treated plants

Twenty seeds of more crude oil-resistant grasses were inoculated by *R. ruber* (10^6 cell/ml, test group) or physiological serum (blank) and transferred to crude oil contaminated (0.5, 1, 3. 5% w/w) and uncontaminated soil containing pots with three replicates (the experimental groups are

explained in Table 1). The experimental design was a randomized complete block. Irrigation was applied thrice and twice daily for the initial ten days (from seeding to germination) and remained thirty days, respectively. The temperature range was 25± 2 ºC to 38± 2 ºC from the initiation to the end of the experiment. After plant growth, they were harvest, washed with tap water to remove soil particles, and then preserved for morphologic and biochemical analysis.

Table 1 Experimental groups in the present study.

Experimental groups	Applied grass	Applied bacteria	Crude oil	Regarded as	Aim
1	*Lolium perenne*	*R.ruber* KE1	0.5-5%	Treated group in stress condition	To assess role of *R.ruber* KE1 in amelioration of crude oil stress on morphological and biochemical properties of *Lolium perenne* and their interaction in crude oil bioremediation.
2		Lack of bacteria		Untreated group in stress condition	To assess the role of crude oil stress on morphological and biochemical properties of *Lolium perenne.*
3		*R.ruber* KE1	Lack of crude oil	Treated group in normal condition	To assess the effect of *R.ruber* KE1 inoculation on morphological and biochemical properties of *Lolium perenne.*
4		Lack of bacteria		Untreated group in normal condition	To ensure that *Lolium perenne* grow properly in normal condition.
5	*Festuca rubra rubra*	*R.ruber* KE1	0.5-5%	Treated group in stress condition	To assess role of *R.ruber* KE1 in amelioration of crude oil stress on morphological and biochemical properties of *Festuca rubra rubra* and their interaction in crude oil bioremediation.
6		Lack of bacteria		Untreated group in stress condition	To assess the role of crude oil stress on morphological and biochemical properties of *Festuca rubra rubra*.
7		R.ruber KE1	Lack of crude oil	Treated group in normal condition	To assess the effect of *R.ruber* KE1 inoculation on morphological and biochemical properties of *Festuca rubra rubra*.
8		Lack of bacteria		Untreated group in normal condition	To ensure that *Festuca rubra rubra* grow properly in normal condition.
9	No grass	Lack of bacteria	0.5-5%	Untreated groups	To measure crude oil in contaminated soil.
10	No grass	Lack of bacteria	3%	Untreated group	To measure quantity of hydrocarbon compounds of crude oil in contaminated soil.

2.7. Morphologic analysis of plants

The shoot and root length and dry weight of plants in various experimental groups were measured and compared with each other. To measure the dry

weight of plants, they were washed twice in sterile distilled water and dried at 70 ºC till they attained a constant weight.

2.8. Biochemical analysis of plants

The quantity of photosynthetic pigment, sugar, and protein were measured in experimental groups as biochemical properties of plants.

2.9. Determination of photosynthetic pigment content

Photosynthetic pigment content was evaluated quantitatively as previously described in De Kok and Graham (1989). Briefly, 0.05 g of fresh leaves was homogenized in 80 % acetone (1.0 ml). Then, its supernatant was obtained by centrifugation at 1600 *g* for 5 min. The absorbance value of the supernatant was read according to Lichtenthaler (LICHTENTHALER & Wellburn, 1983) at 663, 646, and 470 nm. Pigment content (mg g^{-1}) was calculated as follows:

Chlorophyll a= $(12.25A_{663}-2.79A_{646})$

Chlorophyll b= $(21.21A_{646}-5.1A_{663})$

Carotenoid= $(1000A_{470} – 1.8Chla – 85.02Chlb)/198$

2.10. Sugar content determination

Fresh leaves of the plants (0.05 g) were homogenized in phosphate buffer, and its supernatant was obtained using centrifugation at 1600 *g* for 5 min. Then, phenol solution (5% w/v, 1mL) and sulfuric acid (98%, 3 mL) was added to the supernatant (2 mL). This mixture was shaken vigorously, and its absorbance was read at 485 nm after 1 hour. The absorbance of the characteristic yellow-orange color was measured at 485 nm. Glucose was used to make a standard curve (Bi et al., 2016; Somogyi, 1952).

2.11. Protein content determination

The soluble protein content of plants was determined as previously described in Bradford (1976). Briefly, the leaves of a plant (0.5 g) were homogenized in phosphate buffer in freezing conditions. This mixture was centrifuged at 10,000 g in 4 ºC for 25 min. The samples (50 µl) were mixed with Bradford reagent (1.5 ml) and incubated at an ambient temperature for 2 min. The absorbance was measured at 595 nm (Stoscheck, 1990). Bovine serum albumin was used to make a standard curve.

2.12. Bacterial enumeration after the experiment

At the end of the experiment, one gram of the oil-contaminated soils, in which *R.ruber* KE1 treated seeds of *Festuca rubra rubra* or *Lolium perenne*, had been cultivated was dissolved in 1mL physiological serum. This microbial suspension was serially diluted (10^{-1}-10^{-10}), and then 100 µL was spread on an MS medium containing crude oil instead of sucrose to enumerate the viable *R .ruber* KE1. Colonies were counted after an incubation period (37 ºC for 3-4 days).

2.13. Hydrocarbon (%) measurement in R.ruber KE1 treated and untreated oil-contaminated soil samples

Soil samples were taken from each pot before and after the experiment. The amount of crude oil in the soil samples was determined by the gravimetric method, according to Latha and Kalaivani et al. (2012). The sample with the most weight difference before and after the experiment was analyzed by gas chromatography.

2.14. Statistical analysis

The normality of data was evaluated by the Shapiro-Wilk test, and because there was a normal distribution of data and more than two groups, the mean difference between various groups was analyzed by ANOVA test in SPSS 20.0. An asterisk (*) denotes a significant difference at a 95% level of confidence interval.

3. Results and Discussion

Petroleum hydrocarbons are potential sources of ecosystem contamination. Phytoremediation is a cost-effective, eco-friendly, and effective approach that can easily operate *in situ* in large areas of polluted sites using tolerant plant species. *Mirabilis Jalapa*, *Impatiens balsamina*, *Canna indica*, *Chromolaena odorata*, *Biden pilosa*,

Gmelina arborea, *Azadirachta indica*, *Michelia champaca*, *Sebastiania commersoniana*, *Zea mays*, *Lolium multiflorum*, *Astragalus membranaceus*, and *Medicago sativa* are potential terrestrial plants that have been studied for phytoremediation of petroleum hydrocarbon (in a wide range [400-50000 mg Kg^{-1}] of petroleum concentration) and have shown various efficiencies (9-80%) in different treatment times (21 days-one year) (Yavari et al., 2015). In this regard, the soil was artificially polluted with crude oil (Table 2). The results of agrology experiments revealed the physicochemical properties of the soil used in the present study (Table 3).

Table 2 Characteristics of the used heavy crude oil

Substance	Quantity	Substance	Quantity
Clay	7%	Organic matter	0.53%
Silt	42%	N	0.068%
Sand	51%	K	276 ppm
T.N.V	38%	P	5.8 ppm
EC	6.6 Dsm^{-1}	Cu	0.3 ppm
pH	7.6	Mn	2.15 ppm
ESP	14.4	Zn	1.1 ppm
SAR	9.6	Fe	0.89 ppm
Na^{+}	35.1 Meq^{-1}	Cl^{-}	54 Meq^{-1}
$Ca2^{+}Mg2^{+}$	26.5 Meq^{-1}	$HCO3^{-}$	9 Meq^{-1}

Table 3 Physicochemical properties of the cultivable soil, sandy soil, and a mixture of peat moss soil.

Property		Iranian Heavy Crude Oil
Specific gravity (kg/m^3)		0.8814
API gravity (°API)		29.0
Sulfur (wt%)		1.96
Nitrogen (wt%)		0.21
Vanadium (ppm)		88.0
Nickel (ppm)		24.0
Pour point (°C)		-14
Viscosity (cSt)	At 20 °C	21.52
	At 40 °C	10.43

3.1. Selection of more crude oil resistant plants

The results showed that *R.ruber* KE1 treated *Festuca rubra rubra* and *Lolium perenne* are more resistant than *R.ruber* KE1 treated *Poa pratensis* and *Festuca rubra commutate* to crude oil contamination. Therefore, the phytoremediation ability of *Festuca rubra rubra* and *Lolium perenne* in association with *R.ruber* KE1 were further evaluated. *Lolium perenne* is a perennial herbaceous plant and one of the most commonly employed forage and turf grasses. It is known as an oil-tolerant grass (Mâsu, Morariu, & Dragomir, 2013). It can grow well in high pH soil containing NaCl. *Lolium perenne* has been reported as an efficient grass in bioremediation of various pollutants, such as petroleum products (with 31% efficiency in phytoremediation), polyaromatic hydrocarbons, and heavy metal (Cu, Cd, Pb, and Zn) (Cao et al., 2016; Gołda & Korzeniowska, 2016; Yarahmadi et al., 2017). This may be due to its comparatively rapid growth, extensive and strong root system, and possessing various strategies to cope with high concentrations of various pollutants. Although its phytoremediation efficiency may be limited due to the adverse effect of recalcitrant pollutants, such as crude oil, on growth, morphological, and the biochemical features of *Lolium perenne* (Zhang et al., 2012; Zhu et al., 2018).

This limitation can be ameliorated, and the phytoremediation approach can be augmented by appropriate microorganisms that are tolerant to high concentrations of pollutants of interest and possess a high survival rate in a wide range of environmental conditions. Bacterial augmented phytoremediation can give significantly better efficiency in comparison to phytoremediation. This complex interaction is mutually beneficial to both organisms. By degrading pollutants and alleviating their corresponding stress, bacteria improve plant growth and root systems. In turn, through its exudates and aeration activity, the plant root enhances the activity of the microorganism, which consequently can lead to more efficient degradation.

A few promising results have been reported on the efficiency of a combinational approach using specific bacterial species with plants, e.g., Xie et

al. (2018) showed the enhanced efficiency of a combinatorial strategy using strain DXZ9 of *Stenotrophomonas* sp. with ryegrass to biologically removal of DDT (81%) and DDE (55%) in comparison to phytoremediation of untreated ryegrass for DDT (72%) and DDE (48%) in a pot experiment. Cao et al. (2016) showed that *Streptomyces pactum* Act12 treated *Lolium perenne* L. had higher biomass with a greater height and root tiller number in comparison with the uninoculated *Lolium perenne* L. under Pb stress. Tang et al. (2010) reported the amplified ability of bacterial treated *Lolium perenne* in phytoremediation of petroleum contaminated soil. According to their results, a combination of ryegrass with mixed microbial strains including *Bacillus subtilis*, *Sphingobacterium multivolume*, *Acinetobacter radioresistens*, *Rhodococcus erythropolis*, and *Pseudomonas fluorescens* gave the best result with a degradation rate of 58% after 162 days.

Therefore, remediation of crude oil polluted soil with limited damages to its morphological and biochemical properties can be achieved by *Lolium perenne* treatment with crude oil degrading bacteria.

3.2. Seed germination potential of *R. ruber* KE1 treated *Festuca rubra rubra* and *Lolium perenne*

The results showed that bacterial treatment decreased seed germination (%) of *Festuca rubra rubra* and *Lolium perenne* in contaminated and uncontaminated soils (Fig. 1). According to the results, seed germination (%) of *R.ruber* KE1 treated and untreated *Festuca rubra rubra* was higher than *Lolium perenne* (Fig.1).

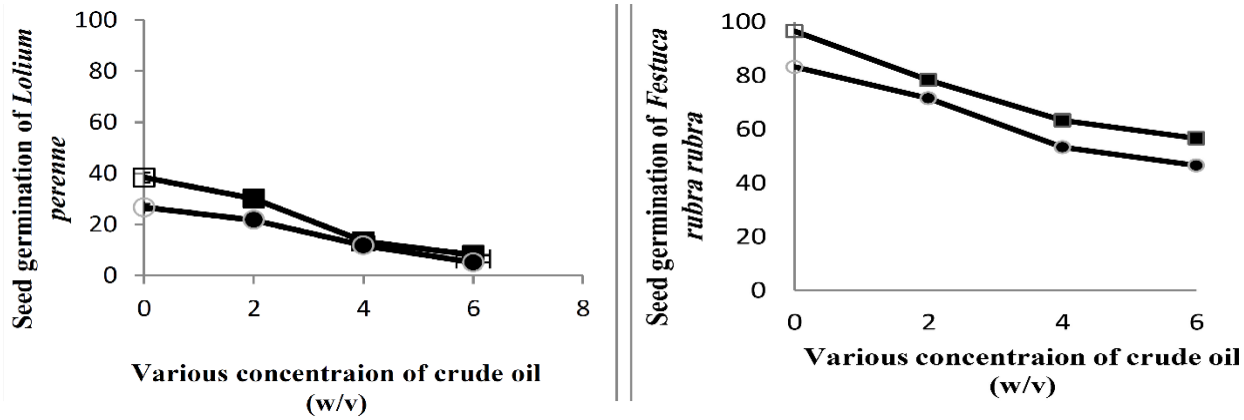

Fig. 1 The effect of various concentrations of crude oil on germination rate (%) of *R.ruber* KE1 treated (●) and untreated (■) seeds after 10 days of growth at 27 ºC. ○ and □ indicate seed germination rate (%) of *R.ruber* KE1 treated and untreated seeds after growth during 10 days at 27 ºC in uncontaminated soil, respectively.

3.3. Radicle length of *R.ruber* KE1 treated *Festuca rubra rubra* and *Lolium perenne*

The radicle length of untreated *Lolium perenne* (37 mm) was more than that of untreated *Festuca rubra rubra* (23 mm) in uncontaminated soil. *R.ruber* KE1 inoculation did not lead to a significant increase in radicle length of *Lolium perenne* (38 mm) or *Festuca rubra rubra* (25 mm) in uncontaminated soil ($P>0.05$). Also, the radicle length of *Lolium perenne* was higher than *Festuca rubra rubra* in all applied concentrations of crude oil. The bacterial treatment prevented a dramatically reduction of radicle length of both treated grasses, e.g., the radial length of untreated *Lolium perenne* and *Festuca rubra rubra* showed a 29 cm and 16 cm reduction when increasing the crude oil concentration from 0 to 5% v/v, respectively, while *R.ruber* KE1 treatment

ameliorated this dramatic reduction to 14 cm and 8 cm (P<0.05), respectively (Fig .2). The *R.ruber* strain KE1 was isolated from a unique ecosystem (drilling oil-based polluted soil of Khuzestan, Iran). According to the results, the *R.ruber* strain KE1 with biodegradation ability assisted grasses in coping with the adverse effects of crude oil, especially in high concentrations.

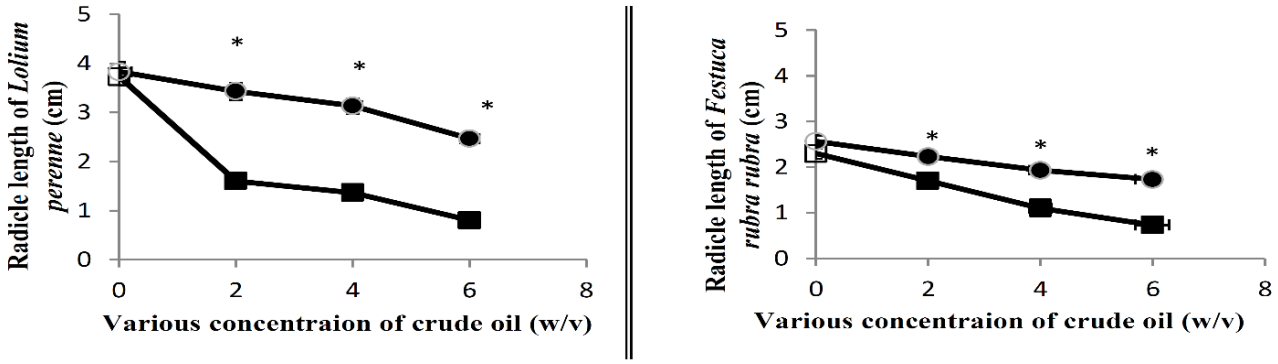

Fig. 2 The effect of various concentrations of crude oil on the radicle length (cm) of *R.ruber* KE1 treated (●) and untreated (■) seeds in crude oil contaminated soil after 10 days of growth at 27 ºC. ○ and □ indicate the radicle length (cm) of *R.ruber* KE1 treated and untreated seeds after 10 days of growth at 27 ºC in uncontaminated soil, respectively. The * shows that there is a significant difference at confidence interval of 95%.

3.4. *Festuca rubra r3ubra* and *Lolium perenne* root and shoot length changes in the presence of crude oil

Per the previous experiment, crude oil concentrations of 0.5, 1, 3, and 5% (w/w) were selected for further experiments. The shoot length of *R.ruber* KE1 treated and untreated *Festuca rubra rubra* and *Lolium perenne* was affected by crude oil stress. As crude oil concentration increased, shoot length decreased in both inoculated and uninoculated treatments (Fig. 3). Also, shoots of *Lolium perenne* were higher than *Festuca rubra rubra* in all applied crude oil concentrations. The highest applied concentration of crude oil (5%) completely inhibited shoot and root growth of untreated *Festuca rubra rubra*, but this did not occur in *R.ruber* KE1 treated *Festuca rubra rubra.* The bacterial treatment prevented the dramatic reduction of shoot growth in both treated grasses (P<0.05). This effect was exacerbated in higher concentrations of crude oil. Shoot lengths of *R .ruber* KE1 treated and untreated *Lolium perenne* did not reach zero in any concentrations of crude oil.

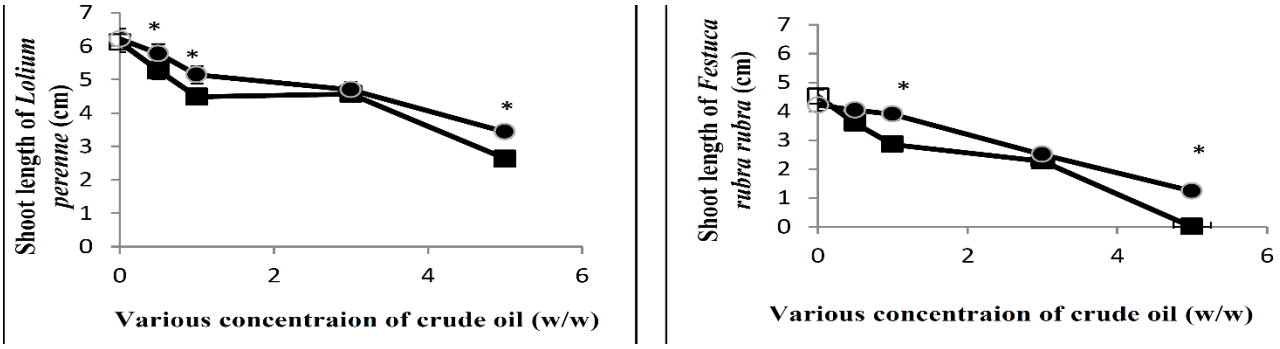

Fig. 3 The effect of various concentrations of crude oil on the shoot length (cm) of *R.ruber* KE1 treated (●) and untreated (■) seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in crude oil contaminated soil. ○ and □ indicate the shoot length (cm) of *R.ruber* KE1 treated and untreated seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in uncontaminated soil, respectively. The * shows that there is a significant difference at a confidence interval of 95%.

The root length of both investigated grasses showed a completely different response to increases in crude oil concentration. In this way, the root length of *Lolium perenne* (*R.ruber* KE1 treated and untreated) and *Festuca rubra rubra* (*R.ruber* KE1 treated and untreated) increased with higher crude oil concentrations until it reached 1% and 3%, respectively. Applying higher concentrations of crude oil (3 and 5% of *Lolium perenne* and 5% of *Festuca rubra rubra*) resulted in a root length reduction. The highest concentration (5%) led to a lack of root growth in untreated *Festuca rubra rubra*, while the *R.ruber* KE1 treatment hampered this effect (P<0.05) (Fig. 4).

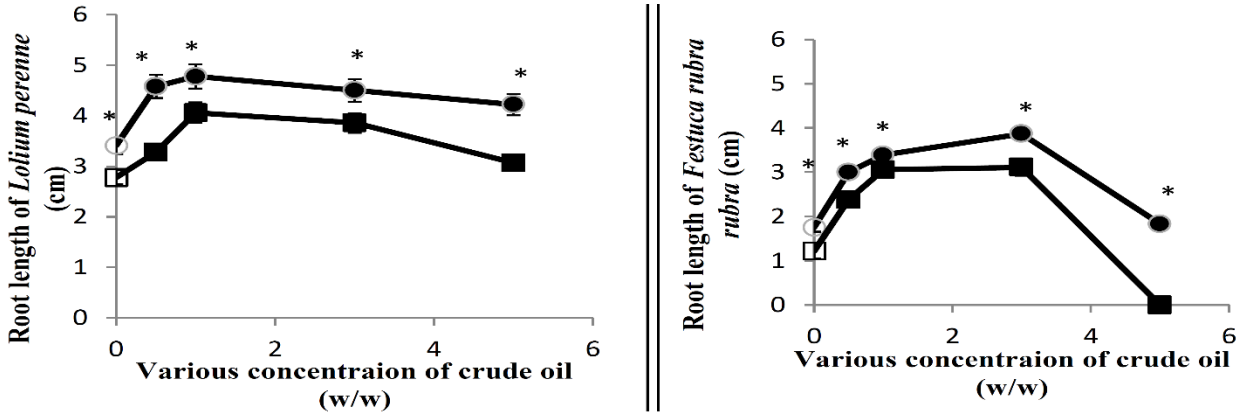

Fig. 4 The effect of various concentrations of crude oil on the root length (cm) of *R.ruber* KE1 treated (●) and untreated (■) seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in crude oil contaminated soil. ○ and □ indicate the root length (cm) of *R.ruber* KE1 treated and untreated seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in uncontaminated soil, respectively. The * shows that there is a significant difference at a confidence interval of 95%.

3.5. Dry weight of *Festuca rubra rubra* and *Lolium perenne* shoots and roots in the presence of crude oil

The dry weight of *Lolium perenne* and *Festuca rubra rubra* shoots and roots (*R.ruber* KE1 treated and untreated) severely decreased by increasing the crude oil concentration. The *R.ruber* KE1 treatment had an obvious positive effect on the dry weight of *Festuca rubra rubra* and *Lolium perenne* shoots and roots in the presence of the highest applied concentration of crude oil (5%). This was due to the smaller reduction of the shoot and root dry weight of *R.ruber* KE1 treated *Lolium perenne* (~40% shoot, ~29.5% root) and *Festuca rubra rubra* (~68% shoot, 77.5% root) in comparison with the dry weight of untreated *Lolium perenne* (~65% shoot, ~44.8% root) and *Festuca rubra rubra* (100% shoot, 100% root) shoots (P<0.05) (Figs. 5, 6).

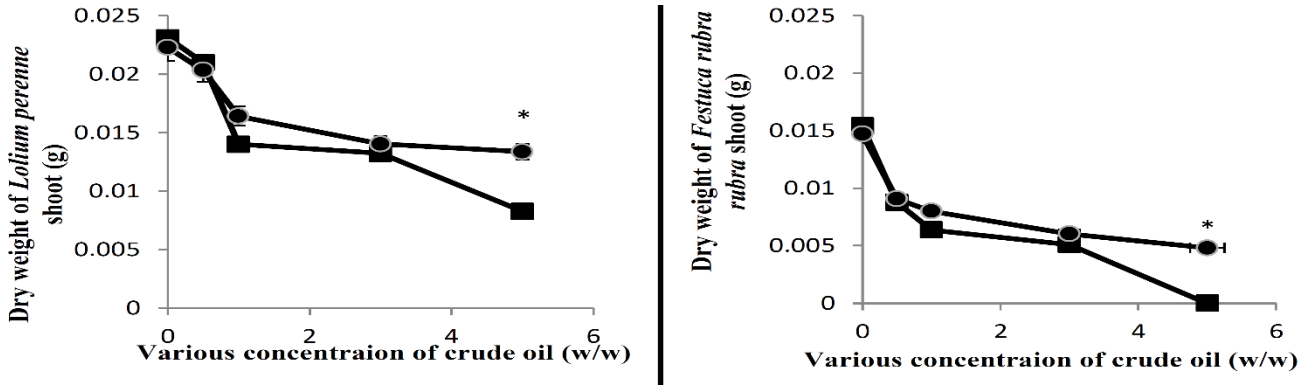

Fig. 5 The effect of various concentrations of crude oil on the shoot dry weight (g) of *R.ruber* KE1 treated (●)and untreated (■) seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in crude oil contaminated soil. ○ and □ indicate the shoot dry weight (g) of *R.ruber* KE1 treated and untreated seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in uncontaminated soil, respectively. The * shows that there is a significant difference at a confidence interval of 95%.

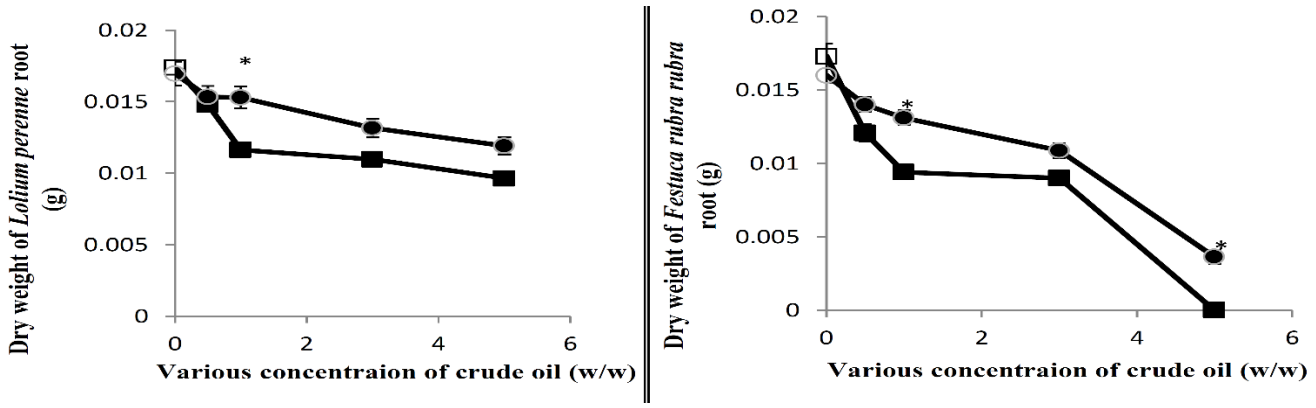

Fig. 6 The effect of various concentrations of crude oil on the root dry weight (g) of *R.ruber* KE1 treated (●) and untreated (■) seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in crude oil contaminated soil. ○ and □ indicate the root dry weight (g) of *R.ruber* KE1 treated and untreated seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in uncontaminated soil, respectively. The * shows that there is a significant difference at a confidence interval of 95%.

3.6. Chlorophyll and carotenoid content of *Festuca rubra rubra* and *Lolium perenne* in the presence of crude oil contamination

The results showed that *R.ruber* KE1 treatment did not increase chlorophyll a and b content of treated *Festuca rubra rubra* and *Lolium perenne* in uncontaminated soil. According to the results, *R.ruber* KE1 treated *Festuca rubra rubra* could preserve its chlorophyll a and b in comparison with untreated contaminated soil. The role of *R.ruber* KE1 treatment was further revealed in the highest applied concentration of crude oil (5% v/w). In this situation, *R.ruber* KE1 alleviated crude oil stress (P<0.05). As seen in Figs. 7 and 8, the chlorophyll a and b content of untreated *Festuca rubra rubra* fell to zero, while the *Rruber* KE1 treatment was able to maintain chlorophyll a content at 5.31 and 2.1 mg g^{-1}, respectively (P<0.05). *Rruber* KE1 treatment had no significant effect on the chlorophyll a content of treated *Lolium perenne* (Fig. 8) (P>0.05).

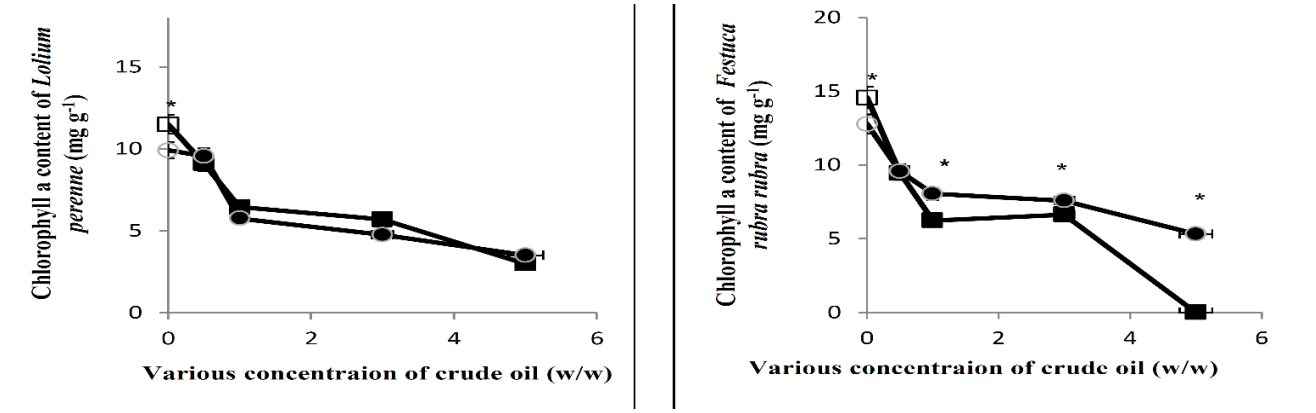

Fig. 7 The effect of various concentrations of crude oil on the chlorophyll a content (mg g^{-1}) of *R.ruber* KE1 treated (●) and untreated (■) seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in crude oil contaminated soil. ○ and □ indicate the chlorophyll a content (mg g^{-1}) of *R. ruber* KE1 treated and untreated seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in uncontaminated soil, respectively. The * shows that there is a significant difference at a confidence interval of 95%.

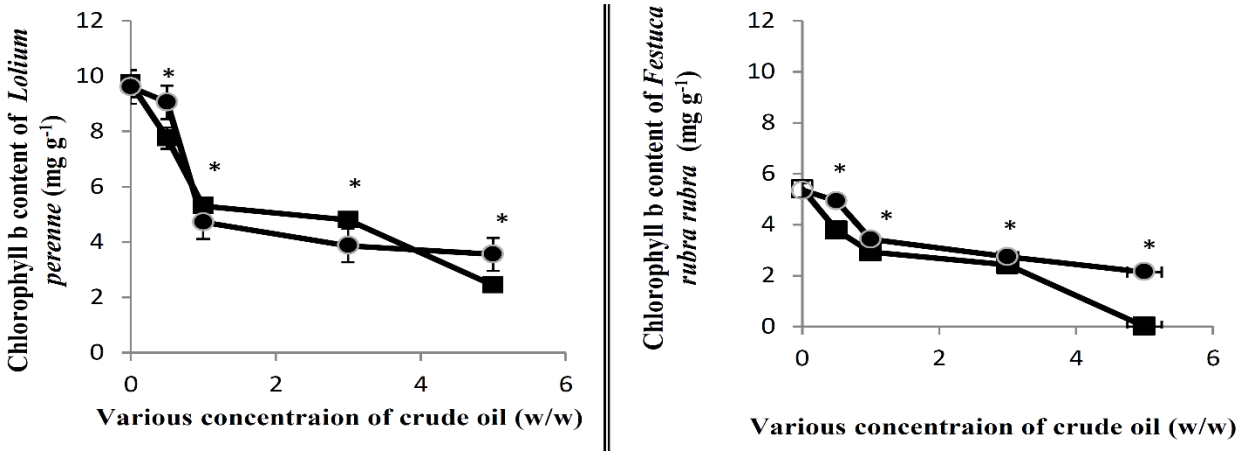

Fig. 8 The effect of various concentrations of crude oil on the chlorophyll b content (mg g^{-1}) of *R.ruber* KE1 treated (●) and untreated (■) seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in crude oil contaminated soil. ○ and □ indicate the chlorophyll b content (mg g^{-1}) of *R.ruber* KE1 treated and untreated seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in uncontaminated soil, respectively. The * shows that there is a significant difference at a confidence interval of 95%.

Also, the results showed that the carotenoid content of *R.ruber* KE1 treated and untreated *Lolium perenne* and *Festuca rubra rubra* was reduced when the crude oil concentration increased, except at the 0.5% concentration, which did not lead to a decrease in carotenoid content in *R.ruber* KE1 treated *Festuca rubra rubra*. The carotenoid content of *R.ruber* KE1 treated and untreated *Lolium perenne* and *Festuca rubra rubra* showed no significant difference in concentrations of 0.5 and 1% of crude oil. *R.ruber* KE1 treatment prevented a reduction of carotenoid content in grasses in comparison with untreated grasses at higher concentrations of crude oil (P<0.05) (Fig. 9).

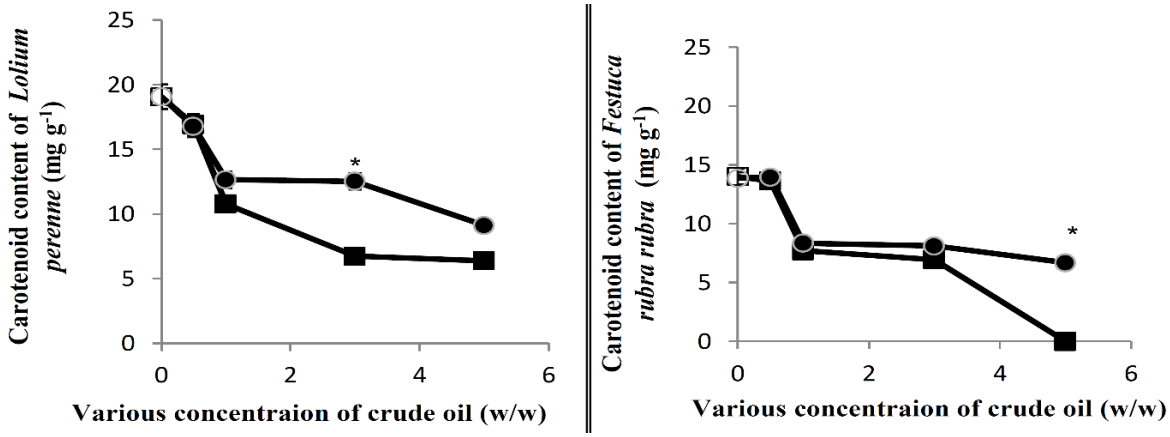

Fig. 9 The effect of various concentrations of crude oil on the carotenoid content (mg g^{-1}) of *R.ruber* KE1 treated (●) and untreated (■) seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in crude oil contaminated soil. ○ and □ indicate the carotenoid content (mg g^{-1}) of *R.ruber* KE1 treated and untreated seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in uncontaminated soil, respectively. The * shows that there is a significant difference at a confidence interval of 95%.

3.6 The sugar content of *Lolium perenne* and *Festuca rubra rubra* in the presence of crude oil contamination

R.ruber KE1 did not make a significant difference in the soluble sugar content of *R.ruber* KE1 treated and untreated *Lolium perenne* and *Festuca rubra rubra* in uncontaminated soil. The soluble sugar content of *R.ruber* KE1 treated and untreated *Lolium perenne* and *Festuca rubra rubra* increased by increasing the crude oil concentration (except at the 0.5% concentration), which lead to a dramatic decrease in the soluble sugar content of

untreated *Festuca rubra rubra,* while the *R.ruber* KE1 treatment increased the soluble sugar content of *Festuca rubra rubra* in the 5% concentration of crude oil (Fig. 10).

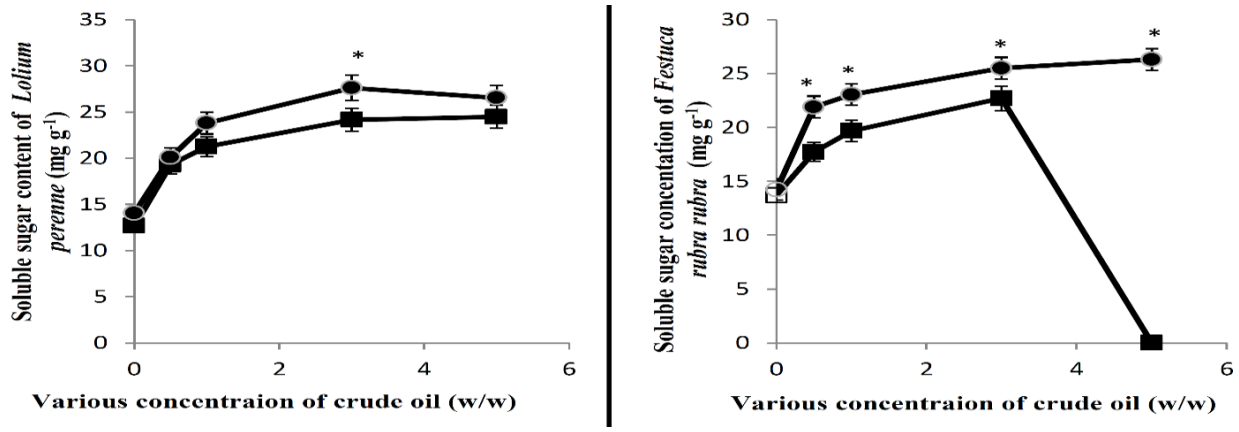

Fig. 10 The effect of various concentrations of crude oil on the soluble sugar content (mg g^{-1}) of *R.ruber* KE1 treated (●) and untreated (■) seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in crude oil contaminated soil. ○ and □ indicate the soluble sugar content (mg g^{-1}) of *R.ruber* KE1 treated and untreated seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in uncontaminated soil, respectively. The * shows that there is a significant difference at a confidence interval of 95%.

3.7. The sugar content of *Lolium perenne* and *Festuca rubra rubra* in the presence of crude oil contamination

The results showed that minimum protein concentrations (0 and 1.06 mg g^{-1}) were observed in the wet tissue of untreated *Festuca rubra rubra* in the presence of crude oil stress (5% concentration) and *Lolium perenne* in normal conditions, respectively. The results revealed that maximum protein concentrations (1.69 and 2.04 mg g^{-1}) were observed in the wet tissue of *R.ruber* KE1 treated *Festuca rubra rubra* and *Lolium perenne* in the presence of crude oil stress (1% and 3% concentration, respectively) (Fig.11). The hydrophobic surface of *R.ruber* KE1 allows its adherence to hydrocarbons and results in easier biodegradation of hydrocarbonic pollutants. In the present study, *R.ruber* KE1 improved the effect of *Lolium perenne* and *Festuca rubra rubra* on plant growth under crude oil stress, as indicated by higher pigment, sugar, and protein content as well as shoot and root length and dry weight.

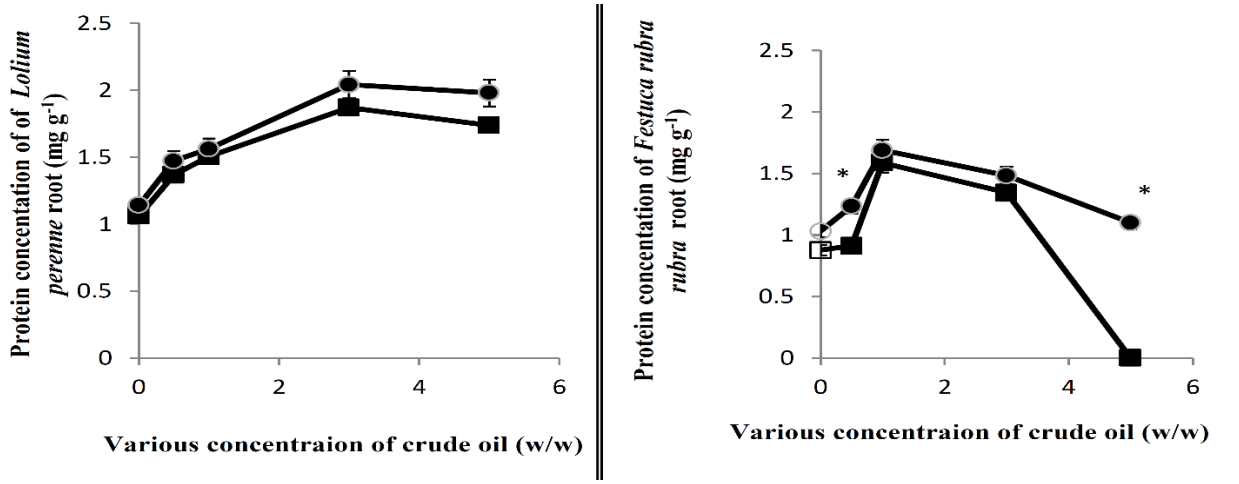

Fig. 11 The effect of various concentrations of crude oil on the protein content (mg g^{-1}) of *R.ruber* KE1 treated (●) and untreated (■) seeds after 40 days of growth at 25± 2 ºC to 38± 2 ºC in crude oil contaminated soil. ○ and □ indicate the protein content (mg g-1) of R.ruber KE1 treated and untreated seeds after 40 days of growth at 25± 2 oC to 38± 2 oC in uncontaminated soil, respectively. The * shows that there is a significant difference at a confidence interval of 95%.

Rhodococcus bacteria have received great attention because of their large chromosome, large linear plasmids, their capability to catabolize a wide range of compounds, their ability to produce bioactive compounds like steroids, acrylamide, acrylic acid (Füchtenbuschet al., 1998), and biosurfactant (Ivshina et al., 2013; Lee et al., 2018), their involvement in fossil fuel biodesulfurization, and their capability to biodegrade persistence compounds like pyridine (Yoon et al., 2000), azo dyes, pesticides (e.g., dichlorodiphenyltrichloroethanes and hexachlorocyclohexanes) (G.-D. Sun et al., 2014; G. Sun et al., 2015), as well as polychlorinated biphenyls and bioconversion of toluene, naphthalene, di-(2-ethylehxyl) phthalate (Liet al., 2006; T. Yang et al., 2018), aniline, phenol (Rehfuss & Urban, 2005), vinyl chloride (Malachowskyet al., 1994), dichlorobenzene (Rehfuss & Urban, 2005), chlorobenzene (Rehfuss & Urban, 2005), herbicides, and PCBs (Bock et al., 1996; Goodfellow et al., 2004; Larkin et al., 1998; van der Geize & Dijkhuizen, 2004; Zheng et al., 2012). Their outstanding properties such as having metabolic, enzymatic (e.g., dioxygenases), and nutritional versatility, as well as aerobic and microaerophilic respiration makes them suitable for bioremediation under a wide range of environmental conditions, especially phytoremediation of recalcitrant pollutants (van der Geize & Dijkhuizen, 2004).

3.8. Bacterial multiplication in the presence of various concentrations of crude oil

The results showed that *R.ruber* KE1 has had a higher rate of proliferation in the crude oil contaminated soil in comparison with the uncontaminated soil. The *R.ruber* KE1 proliferation rate increased along with higher concentrations of crude oil in the presence of *Festuca rubra rubra,* while a constant proliferation rate was observed in 1, 3, and 5% (w/w) concentrations of crude oil in the presence of *Lolium pernne* (Fig. 12). It has been reported that the bacteria belonging to the *Rhodococcus* genus have a high ability to grow in an extended range of recalcitrant contaminants, like aliphatic and aromatic hydrocarbons (Kotake et al., 2016; H.-Y. Yang et al., 2014), phenols (Szőköl et al., 2014), chlorophenols (Hou et al., 2016), benzotrifluoride (Yano et al., 2015), and xenobiotic components (Khairy et al., 2015) and can degrade and transform them.

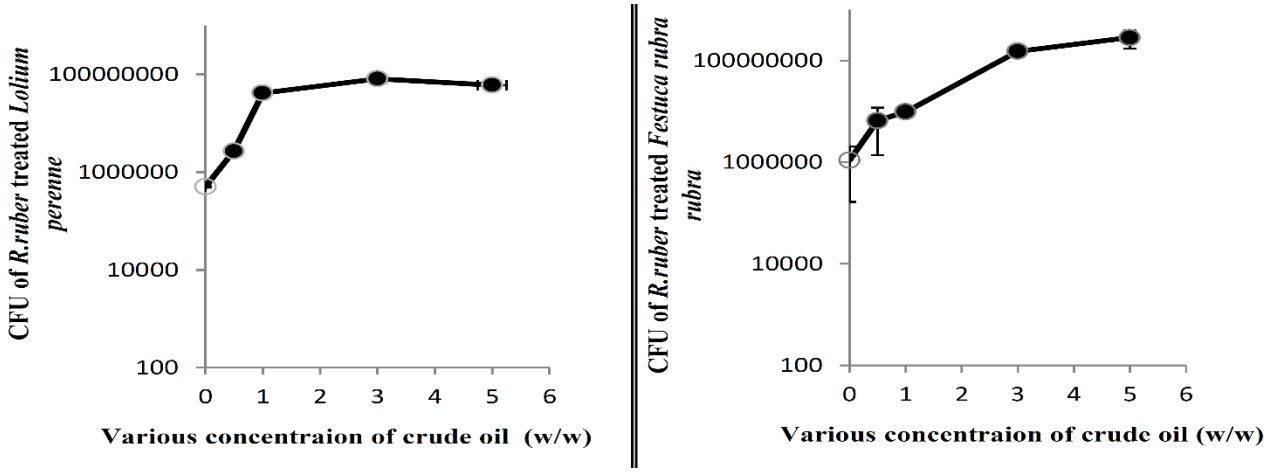

Fig. 12 Numeration of bacteria in contaminated soils at various concentrations of crude oil (●) and uncontaminated soils (○) at the end of the experiment.

3.9. Enhanced ability of *R.ruber* KE1 treated *Lolium perenne* and *Festuca rubra rubra* in phytoremediation of crude oil contamination

The results showed that both investigated grasses, *Lolium perenne* and *Festuca rubra rubra*, have significant phytoremediation ability. Because the crude oil remaining in soil with *Lolium perenne* and *Festuca rubra rubra* along with *R.ruber* KE1 was lower than that of soil with *Lolium perenne* and *Festuca rubra rubra* in the absence of *R.ruber* KE1 (Fig.13). We conclude that the phytoremediation ability of *Lolium perenne* and *Festuca rubra rubra* increased in the presence of *R.ruber* KE1. Although *Lolium perenne* can significantly remediate crude oil itself (>57%), employing *R.ruber* KE1 with *Lolium perenne* increased its bioremediation efficiency. This enhanced phytoremediation may be due to *Rhodococcus ruber* KE1's ability to produce biosurfactant, which can decrease surface tension through emulsification of crude oil and also enhance hydrocarbon bioavailability for degradation (Parach et al., 2017). However, more research should be done to determine the best strategy for *R.ruber* KE1 enhanced degradation of crude oil in the presence of *Lolium perenne* and *Festuca rubra rubra.*

Also, previous studies showed the beneficial cooperation of microbial cells with plants decreased the phytoremediation duration for long-lasting contaminants and increased its efficiency. Microorganism-augmented phytoremediation is efficient and environment-friendly compared to standard phytoremediation. Nevertheless, an extensive investigation must be conducted to understand the underlying mechanisms needed to boost the yield of the decontamination procedure (Yang et al., 2020).

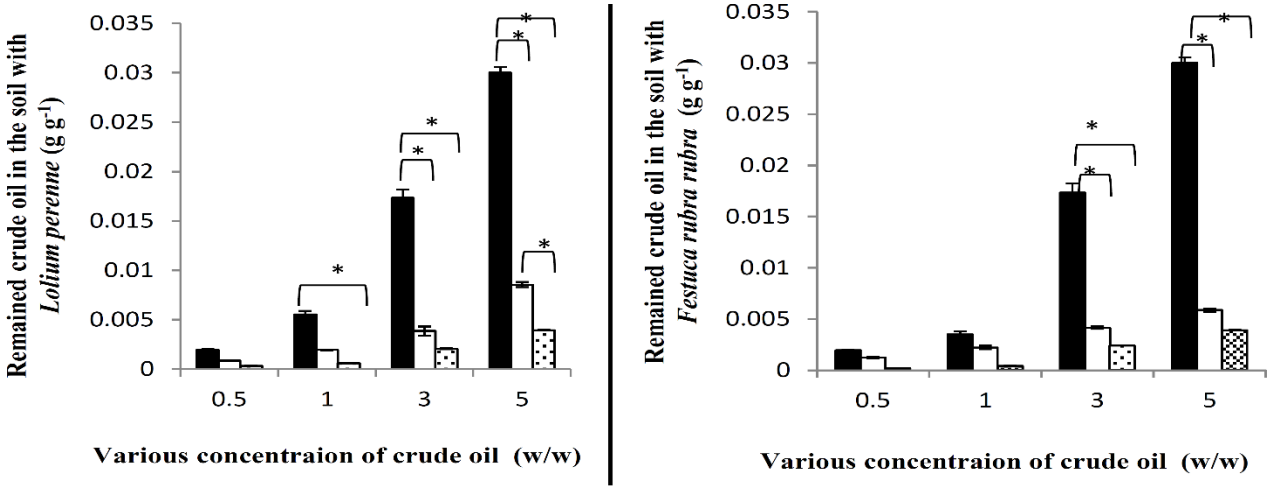

Fig. 13 The crude oil remaining in soil without *Festuca rubra rubra* or *Lolium perenne*,and *R.ruber* KE1 (black column), in soil with *Festuca rubra rubra* or *Lolium perenne* and without *R.ruber* KE1 (white column), and in soil with *Festuca rubra rubra* or *Lolium perenne* and *R.ruber* KE1 (dotted column), after 40 days of growth at 25± 2 ºC to 38± 2 ºC. The * shows that there is a significant difference at a confidence interval of 95%.

3.10. Validation by gas chromatography-mass spectrophotometry of *R. ruber* KE1's role in enhancing bioremediation activity of treated *Lolium perenne*

Gas chromatography-mass spectrophotometry validated the role of *R.ruber* KE1 in enhancing the biodegradation activity of *Lolium perenne*. *R.ruber* KE1 treatment enhanced the biodegrading efficiency. The gas chromatography analysis showed the used crude oil consisted of soluble (saturates, resins, and aromatic substances) [53.86%], insoluble (asphaltenes) [36.05%], and volatile compounds [10.09%]. According to the GC results, the amounts of soluble compounds (53.86%→22.31%) and insoluble substances (36.05%→6.99%) decreased in the soil treated

with *Lolium perenne*. It has been reported that plants accelerate complex formation via pollutants and reduce their toxicity via their exudates, like hydrogen ions, organic acid anions, phytochelators, enzymes, and carbon-containing primary and secondary metabolites (Y. Yang et al., 2020). *R.ruber* KE1 treatment considerably augments phytoremediation efficiency in such a way that the amounts of soluble (22.31%→14.52%) and insoluble (6.99%→1.82%) compounds declined compared to the soil treated with *Lolium perenne*. Overall, the results showed that biological agents, including *Lolium perenne* and *R.ruber* KE1, efficiently degraded insoluble compounds, like asphaltenes, of crude oil. These compounds are recalcitrant to degradation in the environment and highly toxic to organisms. A small number of microorganisms like *Neosartorya fischeri* and *Pestalotiopsis* sp. are able to degrade asphaltenes (the most resistant crude oil fraction) (Pourfakhraei et al., 2018). Our results suggest that it is likely that *R.ruber* KE1 plays an improving role in the decomposition of crude oil. According to Parach et al.'s study on *Rhodococcus ruber* KE1, the biodegradation of crude oil was improved at 40 ºC, so it can be applied in a high-temperature ecosystem. According to this study, *Rhodococcus ruber* KE1 shows maximum microbial growth in the presence of 1% (v/v) crude oil in comparison with 3, 5, and 10% (v/v) concentrations of crude oil. It is possible that by restricting oxygen, these concentrations of crude oil impose an inhibitory effect on *Rhodococcus ruber* KE1 growth and its corresponding biodegradation (Parach et al., 2017). In contrast, our results indicated that none of the applied concentrations inhibited *Rhodococcus ruber* KE1 growth, but this may be due to the supportive role of grasses in their aeration activity and root exudates.

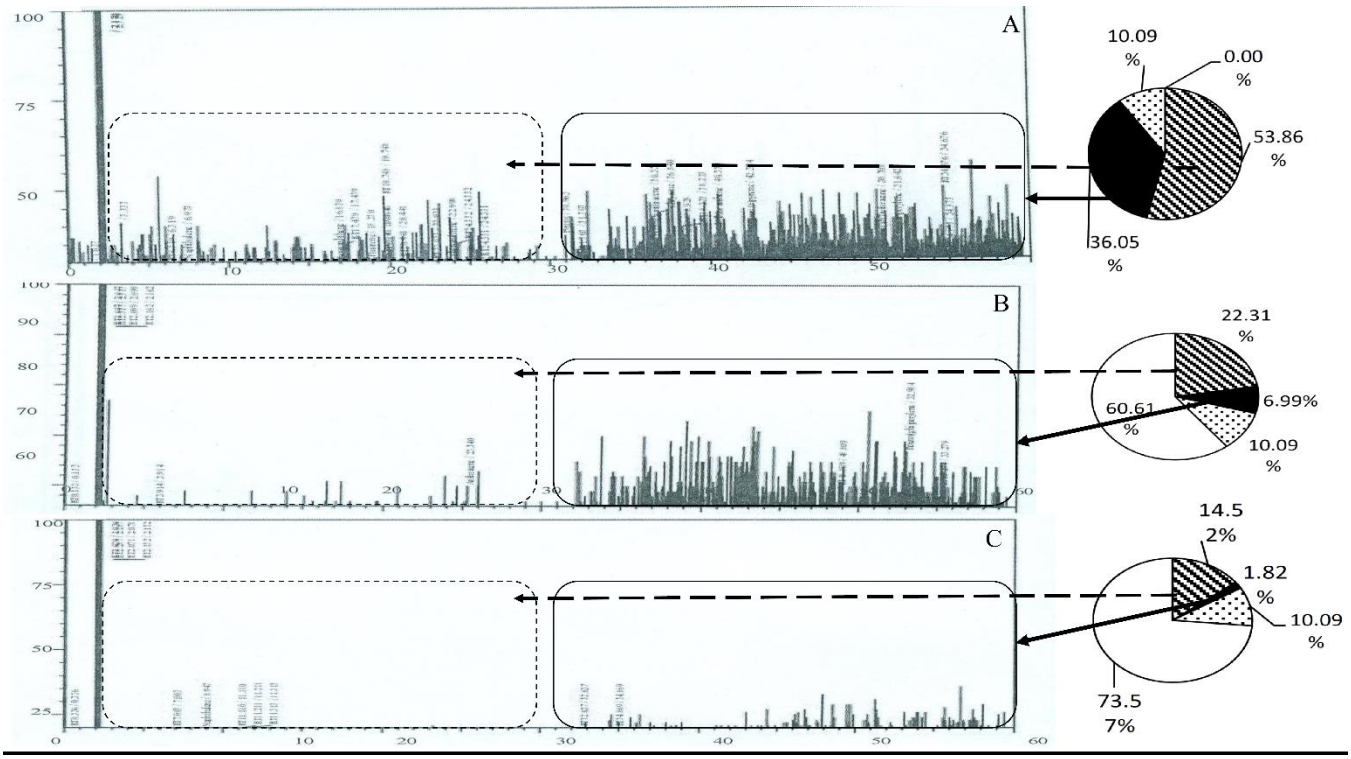

Fig. 14 Chromatogram and amounts of insoluble (black part), soluble (dashed part), evaporated (dotted part), and biodegraded (Wight part) compounds in crude oil contaminated soil (A), crude oil contaminated soil treated by *Lolium perenne* (B), crude oil contaminated soil treated by *Lolium perenne* and *R.ruber* KE1 (C).

4. Conclusions

According to the present study, applying *R.ruber* KE1 significantly improved the growth parameters of *Lolium perenne* and *Festuca rubra rubra*, i.e., their radicle, shoot and root length, the amount of chlorophyll b, and soluble sugar in the presence of

crude oil. In addition, *R.ruber* KE1 considerably decreased the remaining crude oil in soil treated with *Lolium perenne*. Also, the amounts of soluble and insoluble compounds in *R.ruber* KE1-assisted phytoremediation were more reduced in comparison with soil treated with *Lolium perenne*. Therefore, it can be concluded that phytoremediation efficiency was increased by applying *R.ruber* KE1.

Conflict of Interest

The authors declare that there is no conflict of interests.

Acknowledgements

The authors would like to thank the Damghan University for the financial supports of this research.

Ethical approval

This article does/does not contain any studies with human participants or animals performed by any of the authors. This article does/does not contain any studies with human participants or animals performed by any of the authors.

Open access

References

[1] Bashir, I., Lone, F., Bhat, R. A., Mir, S. A., Dar, Z. A., & Dar, S. A. (2020). Concerns and threats of contamination on aquatic ecosystems *Bioremediation and Biotechnology* (pp. 1-26): Springer. Doi:10.1007/978-3-030-35691-0_1

[2] Bock, C., Kroppenstedt, R., & Diekmann, H. (1996). Degradation and bioconversion of aliphatic and aromatic hydrocarbons by Rhodococcus ruber 219. *Applied microbiology and biotechnology, 45*(3), 408-410. Doi.org/10.1007/s002530050704.

[3] Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry, 72*(1-2), 248-254. Doi: 10.1006/abio.1976.9999.

[4] Cao, S., Wang, W., Wang, F., Zhang, J., Wang, Z., Yang, S., & Xue, Q. (2016). Drought-tolerant Streptomyces pactum Act12 assist phytoremediation of cadmium-contaminated soil by Amaranthus hypochondriacus: great potential application in arid/semi-arid areas. *Environmental Science and Pollution Research, 23*(15), 14898-14907. Doi: 10.1007/s11356-016-6636-y.

[5] Das, N., & Chandran, P. (2011). Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology research international, 2011*. Doi: 10.4061/2011/941810.

[6] Dave, D., & Ghaly, A. E. (2011). Remediation technologies for marine oil spills: A critical review and comparative analysis. *American Journal of Environmental Sciences, 7*(5), 423-440. Doi: 10.3844/ajessp.

[7] De Kok, L., & Graham, M. (1989). Levels of pigments, soluble proteins, amino acids and sulhydryl compounds in foliar tissue of Arabidopsis thaliana during dark-induced and natural senescence. *Plant Physiology and Biochemistry, 27*(2), 203-209.

[8] Fatima, K., Imran, A., Amin, I., Khan, Q. M., & Afzal, M. (2018). Successful phytoremediation of crude-oil contaminated soil at an oil exploration and production company by plants-bacterial synergism. *International journal of phytoremediation, 20*(7), 675-681. Doi: 10.1080/15226514.2017.1413331.

[9] Füchtenbusch, B., Fabritius, D., Wältermann, M., & Steinbüchel, A. (1998). Biosynthesis of novel copolyesters containing 3-hydroxypivalic acid by Rhodococcus ruber NCIMB 40126 and related bacteria. *FEMS microbiology letters, 159*(1), 85-92. Doi: 10.1111/j.1574-6968.1998.tb12845.x

[10] Gołda, S., & Korzeniowska, J. (2016). Comparison of phytoremediation potential of three grass species in soil contaminated with cadmium. *Ochrona Srodowiska i Zasobów Naturalnych, 27*(1), 8-14. Doi: 10.1515/oszn-2016-0003.

[11] Goodfellow, M., Jones, A. L., Maldonado, L. A., & Salanitro, J. (2004). Rhodococcus aetherivorans sp. nov., a new species that contains methyl t-butyl ether-degrading actinomycetes. *Systematic and applied microbiology, 27*(1), 61-5. Doi: 10.1078/0723-2020-00254.

[12] Guvvala, P. R., Ravindra, J. P., & Selvaraju, S. (2020). Impact of environmental contaminants on reproductive health of male domestic ruminants: a review. *Environmental Science and Pollution Research, 27*(4), 3819-3836. Doi: 10.1007/s11356-019-06980-4.

[13] Hou, J., Liu, F., Wu, N., Ju, J., & Yu, B. (2016). Efficient biodegradation of chlorophenols in aqueous phase by magnetically immobilized aniline-degrading

Rhodococcus rhodochrous strain. *Journal of nanobiotechnology, 14*(1), 1-8. Doi: 10.1186/s12951-016-0158-0.

[14] Ivshina, I. B., Kuyukina, M. S., Krivoruchko, A. V., Plekhov, O. A., Naimark, O. B., Podorozhko, E. A., & Lozinsky, V. I. (2013). Biosurfactant-enhanced immobilization of hydrocarbon-oxidizing Rhodococcus ruber on sawdust. *Applied microbiology and biotechnology, 97*(12), 5315-5327. Doi: 10.1007/s00253-013-4869-y.

[15] Khairy, H., Wübbeler, J. H., & Steinbüchel, A. (2015). Biodegradation of the organic disulfide 4, 4′-dithiodibutyric acid by Rhodococcus spp. *Applied and environmental microbiology, 81*(24), 8294-8306. Doi: 10.1128/AEM.02059-15.

[16] Kotake, T., Matsuzawa, J., Suzuki-Minakuchi, C., Okada, K., Nojiri, H., & Iwata, K. (2016). Purification and partial characterization of the extradiol dioxygenase, 2′-carboxy-2, 3-dihydroxybiphenyl 1, 2-dioxygenase, in the fluorene degradation pathway from Rhodococcus sp. strain DFA3. *Bioscience, biotechnology, and biochemistry, 80*(4), 719-725. Doi: 10.1080/09168451.2015.1123605.

[17] Kügler, J. H., Roes-Hill, L., Syldatk, C., & Hausmann, R. (2015). Surfactants tailored by the class Actinobacteria. *Frontiers in microbiology, 6*, 212. Doi: 10.3389/fmicb.2015.00212.

[18] Kuyukina, M. S., & Ivshina, I. B. (2010). Application of Rhodococcus in bioremediation of contaminated environments. *Biology of Rhodococcus* (pp. 231-262): Springer. Doi: 10.1007/978-3-642-12937-7_9

[19] Larkin, M. J., De Mot, R., Kulakov, L. A., & Nagy, I. (1998). Applied aspects of Rhodococcus genetics. *Antonie van Leeuwenhoek, 74*(1-3), 133-153. Doi: 10.1023/a:1001776500413.

[20] Latha, R., & Kalaivani, R. (2012). Bacterial degradation of crude oil by gravimetric analysis. *Advances in Applied Science Research, 3*(5), 2789-2795.

[21] Lee, D. W., Lee, H., Kwon, B.-O., Khim, J. S., Yim, U. H., Kim, B. S., & Kim, J.-J. (2018). Biosurfactant-assisted bioremediation of crude oil by indigenous bacteria isolated from Taean beach sediment. *Environmental Pollution, 241*, 254-264. Doi: 10.1016/j.envpol.2018.05.070.

[22] Li, J., Chen, J.-a., Zhao, Q., Li, X., & Shu, W. (2006). Bioremediation of environmental endocrine disruptor di-n-butyl phthalate ester by Rhodococcus ruber. *Chemosphere, 65*(9), 1627-1633. Doi: 10.1016/j.chemosphere.2006.03.005.

[23] Lichtenthaler, H. K., & Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemical Society Transaction, 11 (5): 591–592. Doi:10.1042/bst0110591

[24] Malachowsky, K., Phelps, T., Teboli, A., Minnikin, D., & White, D. (1994). Aerobic mineralization of trichloroethylene, vinyl chloride, and aromatic compounds by Rhodococcus species. *Applied and environmental microbiology, 60*(2), 542-548. Doi: 10.1128/aem.60.2.542-548.1994.

[25] Manisalidis, I., Stavropoulou, E., Stavropoulos, A., & Bezirtzoglou, E. (2020). Environmental and health impacts of air pollution: a review. *Frontiers in public health, 20, 8-14*. Doi: 10.3389/fpubh.2020.00014.

[26] Mâsu, S., Morariu, F., & Dragomir, N. (2013). Using different tolerant plant for phytoremediation of contaminated soils with total petroleum hydrocarbons. *Scientific Papers Animal Science and Biotechnologies, 46*(2), 175-179.

[27] Neu, T. R. (1996). Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiological reviews, 60*(1), 151. Doi: 10.1128/mr.60.1.151-166.1996.

[28] Okoh, E., Yelebe, Z., Oruabena, B., Nelson, E., & Indiamaowei, O. (2020). Clean-up of crude oil-contaminated soils: bioremediation option. *International Journal of Environmental Science and Technology, 17*(2), 1185-1198. Doi: 10.1007/s13762-019-02605-y.

[29] Parach, A., Rezvani, A., Assadi, M. M., & Akbari-Adergani, B. (2017). Biodegradation of heavy crude oil using Persian Gulf autochthonous bacterium. *International Journal of Environmental Research, 11*(5-6), 667-675. Doi: 10.1007/s41742-017-0059-6.

[30] Pourfakhraei, E., Badraghi, J., Mamashli, F., Nazari, M., & Saboury, A. A. (2018). Biodegradation of asphaltene and petroleum compounds by a highly potent Daedaleopsis sp. *Journal of basic microbiology, 58*(7), 609-622. Doi: 10.1002/jobm.201800080.

[31] Rehfuss, M., & Urban, J. (2005). Rhodococcus phenolicus sp. nov., a novel bioprocessor isolated actinomycete with the ability to degrade chlorobenzene, dichlorobenzene and phenol as sole carbon sources. *Systematic and applied microbiology, 28*(8), 695-701. Doi: 10.1016/j.syapm.2005.05.011.

[32] Shirdam, R., Zand, A., Bidhendi, G., & Mehrdadi, N. (2008). Phytoremediation of hydrocarbon-contaminated soils with emphasis on the effect of petroleum hydrocarbons on the growth of plant species. *Phytoprotection, 89*(1), 21-29. Doi: 10.7202/000379AR.

[33] Sparks, D. L., Page, A., Helmke, P., & Loeppert, R. H. (2020). *Methods of soil analysis, part 3: Chemical methods* (Vol. 14): John Wiley & Sons. Doi: 10.2136/sssabookser5.3.

[34] Stoscheck, C. M. (1990). [6] Quantitation of protein *Methods in enzymology* (Vol. 182, pp. 50-68): Elsevier. Doi: 10.1016/0076-6879(90)82008-P.

[35] Sun, G.-D., Xu, Y., Liu, Y., & Liu, Z.-P. (2014). Microbial community dynamics of soil mesocosms using Orychophragmus violaceus combined with Rhodococcus ruber Em1 for bioremediation of highly PAH-contaminated

soil. *Applied microbiology and biotechnology, 98*(24), 10243-10253. Doi: 10.1007/s00253-014-5971-5.

[36] Sun, G., Zhang, X., Hu, Q., Zhang, H., Zhang, D., & Li, G. (2015). Biodegradation of dichlorodiphenyltrichloroethanes (DDTs) and hexachlorocyclohexanes (HCHs) with plant and nutrients and their effects on the microbial ecological kinetics. *Microbial ecology, 69*(2), 281-292. Doi: 10.1007/s00248-014-0489-z.

[37] Szőköl, J., Rucká, L., Šimčíková, M., Halada, P., Nešvera, J., & Pátek, M. (2014). Induction and carbon catabolite repression of phenol degradation genes in Rhodococcus erythropolis and Rhodococcus jostii. *Applied microbiology and biotechnology, 98*(19), 8267-8279. Doi: 10.1007/s00253-014-5881-6.

[38] Tang, J., Wang, R., Niu, X., & Zhou, Q. (2010). Enhancement of soil petroleum remediation by using a combination of ryegrass (Lolium perenne) and different microorganisms. *Soil and Tillage Research, 110*(1), 87-93. Doi: 10.1016/j.still.2010.06.010.

[39] Tewari, S., & Sirvaiya, A. (2015). Oil spill remediation and its regulation. *International Journal of Engineering Research and General Science, 1*(6), 1-7.

[40] van der Geize, R., & Dijkhuizen, L. (2004). Harnessing the catabolic diversity of rhodococci for environmental and biotechnological applications. *Current opinion in microbiology, 7*(3), 255-261. Doi: 10.1016/j.mib.2004.04.001.

[41] Varjani, S., & Upasani, V. N. (2019). Influence of abiotic factors, natural attenuation, bioaugmentation and nutrient supplementation on bioremediation of petroleum crude contaminated agricultural soil. *Journal of environmental management, 245*, 358-366. Doi: 10.1016/j.jenvman.2019.05.070.

[42] Whyte, L., Slagman, S., Pietrantonio, F., Bourbonniere, L., Koval, S. F., Lawrence, J. R., Inniss, W. E., & Greer, C. (1999). Physiological adaptations involved in alkane assimilation at a low temperature by Rhodococcus sp. strain Q15. *Applied and environmental microbiology, 65*(7), 2961-2968. Doi: 10.1128/AEM.65.7.2961-2968.1999.

[43] Xie, H., Zhu, L., & Wang, J. (2018). Combined treatment of contaminated soil with a bacterial Stenotrophomonas strain DXZ9 and ryegrass (Lolium perenne) enhances DDT and DDE remediation. *Environmental Science and Pollution Research*, 1-11. Doi: 10.1007/s11356-018-1236-7.

[44] Yang, H.-Y., Jia, R.-B., Chen, B., & Li, L. (2014). Degradation of recalcitrant aliphatic and aromatic hydrocarbons by a dioxin-degrader Rhodococcus sp. strain p52. *Environmental Science and Pollution Research, 21*(18), 11086-11093. Doi: 10.1007/s11356-014-3027-0.

[45] Yang, T., Ren, L., Jia, Y., Fan, S., Wang, J., Wang, J., Nahurira, R., Wang, H., & Yan, Y. (2018). Biodegradation of Di-(2-ethylhexyl) Phthalate by Rhodococcus ruber YC-YT1 in Contaminated Water and Soil. *International journal of environmental research and public health, 15*(5), 964. Doi: 10.3390/ijerph15050964.

[46] Yang, Y., Liu, Y., Li, Z., Wang, Z., Li, C., & Wei, H. (2020). Significance of soil microbe in microbial-assisted phytoremediation: an effective way to enhance phytoremediation of contaminated soil. *International Journal of Environmental Science and Technology, 17*(4), 2477-2484. Doi: 10.1007/s13762-020-02668-2.

[47] Wachi, M., Tsuchida, S., Kitazume, T., & Iwai, N. (2015). Degradation of benzotrifluoride via the dioxygenase pathway in Rhodococcus sp. 065240. *Bioscience, biotechnology, and biochemistry, 79*(3), 496-504. Doi: 10.1080/09168451.2014.982502.

[48] Yarahmadi, Z., Baharlouei, J., Shokoohi, R., Alikhani, M. Y., & Shirmohammadi-Khorram, N. (2017). The efficiency of Lolium perenne for phytoremediation of anthracene in polluted soils in the presence of Bacillus aerophilus. *Petroleum Science and Technology, 35*(7), 647-652. Doi: 10.1080/10916466.2016.1252771.

[49] Yavari, S., Malakahmad, A., & Sapari, N. B. (2015). A review on phytoremediation of crude oil spills. *Water, Air, & Soil Pollution, 226*(8), 279. Doi: 10.1007/s11270-015-2550-z.

[50] Yoon, J.-H., Kang, S.-S., Cho, Y.-G., Lee, S. T., Kho, Y. H., Kim, C.-J., & Park, Y.-H. (2000). Rhodococcus pyridinivorans sp. nov., a pyridine-degrading bacterium. *International journal of systematic and evolutionary microbiology, 50*(6), 2173-2180. Doi: 10.1099/00207713-50-6-2173.

[51] Zhang, X. S., Kang, Y. J., & Xu, D. J. (2012). Physiological response of *Lolium perenne* to petroleum pollution and removal efficiency in petroleum-polluted soil. Paper presented at the Advanced Materials Research. Doi: 10.4028/www.scientific.net/AMR.518-523.2665.

[52] Zheng, C., Yu, L., Huang, L., Xiu, J., & Huang, Z. (2012). Investigation of a hydrocarbon-degrading strain, Rhodococcus ruber Z25, for the potential of microbial enhanced oil recovery. *Journal of Petroleum Science and Engineering, 81*, 49-56. Doi: 10.1016/j.petrol.2011.12.019.

[53] Zhu, H., Gao, Y., & Li, D. (2018). Germination of grass species in soil affected by crude oil contamination. *International journal of phytoremediation, 20*(6), 567-573. Doi: 10.1080/15226514.2017.1405376.