Phormidium Improves Seed Germination and Growth Parameters of *Trifolium alexandrinum* in Hexadecane-contaminated Soil

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Abstract

Petroleum is known as the main source of fuel and one of the most important environmental pollutants. Petroleum contaminated soil negatively influences plant growth and human health. Bioremediation of petroleum-contaminated soil could be achieved by adding cyanobacteria and photosynthetic microalgae in the soil. In the current study, the beneficial effects of cyanobacterium *Phormidium* sp. ISC108 treatment on seed germination and plant growth parameters were investigated on berseem clover plants exposed to 1% hexadecane- contaminated soil. For this purpose, after cultivation of berseem clover seeds in soil with 0 and 1% hexadecane, they were irrigated every two days by 3 ml *Phormidium* (OD600 = 0.7) and water for the cyanobacteria treatment and control, respectively. After 30 days, the biodegradability of hexadecane in the soil around the root was measured by gas chromatography mass-spectrometry (GC-MS).The results showed that *Phormidium* treatment accelerated germination of berseem clover seeds in both control and hexadecane- contaminated soil. Improvement of plant growth indices such as leaf area, plant fresh weight and leaf RWC due to cyanobacteria treatment was observed in hexadecane-contaminated soil.Hexadecane levels in the soil around the root of the plants irrigated by cyanobacteria were significantly decreased. In addition, the hexadecane degradation in the soil around the roots of berseem clover plant was increased in both control (water) treatment. In conclusion, due to positive effects of *Phormidium* treatment on seed germination, plant growth of berseem clover and hexadecane degradation, it can be effectively used to enhance plant capacity to cope with hexadecane toxicity in petroleum-contaminated soils.

Keywords: Cyanobacteria, Hexadecane, *Trifolium alexandrinum* L., *Phormidium* sp., Bioremediation.

Introduction

Petroleum pollution caused by oil reservoirs, transportation operations and other activities has initiated environmental problems in aquatic and terrestrial ecosystems (Wang et al., 2011). Among environmental pollutants, polycyclic aromatic hydrocarbons and pesticides have become environmental concerns

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(Wang et al., 2011). Properties of petroleum hydrocarbons such as resistance to decomposition and their diffusion range have made them stable in the environment. On the other hand, these compounds are considered as a hazard for plant growth and human health due to the toxicity of mutagenicity and carcinogenicity (Ma et al., 2012). These compounds also attached to soil particles and sediments, resulting reduced biosorption of these pollutants (Volke et al., 2003; Li et al., 1997).

The dramatically increased accumulation of these pollutants in soil causes sedimentation in aquatic environments (Abdel-Megeed et al., 2010). Overall, there is serious environmental concern about the presence of petroleum hydrocarbons in the environment due to the carcinogenicity of their compounds to animals, the mutation in bacteria, their toxicity to plant growth and the possibility of entry into the human and animal food chain (Kastner, 2000).

The major part of petroleum is consist of hydrocarbons. Their decomposition is the least important process for removing petroleum from the environment. In addition, although aromatic and polar compounds are a smaller percentage of crude oil, they are more stable and toxic and require more time to decompose (Johnsen et al., 2005).

Hexadecane is also one of the major constituents of diesel fuels found in the aliphatic portion of crude oils compounds, including moderate carbon chain alkanes and common pollutants of the soils (Setti et al., 1993). The reason for the researchers' choice of hexadecane has been due to its low solubility in water and rapid degradation by many microorganisms (Fernet, 2008).

Plants exhibit different responses to petroleum contamination and their levels of tolerance are vary (Adam and Duncan, 2003). The plant's early responses to drought, salinity and cold stress are very similar (Fernet, 2008). Under these conditions, accumulation of osmolytes, activity of antioxidant systems, as well as increased concentration of abscisic acid (ABA) and altered expression of ABA related genes contribute to the protection of plant cells against the damaging effects of the stress (Ma et al., 2012; Volke et al., 2003; Endo et al., 2008). Some researchers believe that, like other abiotic stresses, petroleum and its hydrocarbons induce the formation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl in plant cells (Ma et al., 2012; Adam and Duncan, 2003). In addition to its direct effects, petroleum also has indirect effects on plants, such as altering the physical and chemical properties of the soil, thereby reducing the amount of water and nutrients and oxygen availability (Baker, 1970; Bossert and Bartha, 1985; De Jong, 1980). In other words, drought is caused by the hydrophobic nature of petroleum compounds around the root (Merkl et al., 2006). This is due to the water repellency of contaminated soil particles through the presence of polar compounds (Morley et al., 2005). Petroleum compounds can directly affect plant growth through stimulating the growth of soil bacteria and consuming essential nutrients and reducing water and oxygen availability in the soil (Schleucher et al., 1994).

Biodegradation is an environmentally friendly method and enables the complete mineralization of petroleum hydrocarbons (El-sheekh et al., 2003; Kuiper et al., 2004; Paul et al., 2005; Wardlaw et al., 2011). Bioremediation is a way to improve contaminated sites by adding specific microorganisms such as fungi, protozoa, algae and microalgae including cyanobacteria or by enhancing soil microorganisms to enhance bioremediation. These microorganisms can break down a wide range of petroleum compounds (Udeani et al., 2009). The general mechanism of biodegradation is that the microorganisms invade the petroleum droplets and then begin to digest and finally produce water and carbon dioxide (Erdogan et al., 2011; Safari et al., 2013).

Microalgae are a group of microorganisms with different applications. These organisms from various branches include members of the eukaryotic and prokaryotic (cyanobacteria) involved in the formation and breakdown of petroleum compounds. These organisms are able to oxidize the aliphatic and aromatic components of the petroleum. Microalgae constitute an important part of the soil ecosystem comprising 27% of the total microbial biomass of the soil (Megharaj et al., 2000). It has been shown that different species of blue-green algae naturally break down both aromatic compounds of hydrocarbons and xenobiotic. cyanobacteria appear to be able to decompose petroleum hydrocarbons by oxidizing them to lower molecular weight components or by changing them to higher polarity components (Gamila et al., 2003; Safari et al., 2013). *Phormidium* sp., belongs to the order *Oscillatoriales* is very similar to *Oscillatoria*, except that there is a thick gelatinous layer around their trichomes that is clearly visible (Marquardt and Palinska, 2007).

Trifolium alexandrinum L. (berseem clover) is one of the most important forage legumes in the Mediterranean and the Middle East (Martiniello and Iannucci, 1998). This plant has the potential to produce a good crop and is one of the best annual plants that can be used to increase soil fertility as well as to provide green fertilizer in alternation cultivation with other crops. Berseem clover fodder is 60% to 80% digestible dry matter for animal feed better than Crimson clover and alfalfa, and produces an average of 3.75 tons per hectare of dry forage (Khoshgoftar, 1992).

In this research, enhanced berseem clover tolerance to hexadecane-contaminated soil has been considered with emphasize on the increasing of hexadecane biodegradation by *Phormidium* sp. The results would be relevant to introduce a practical approach leading to enhancement of tolerance in berseem clover in hexadecane-contaminated soils.

Materials and Methods

Biological materials

Seeds of *Trifolium alexandrim* L .were obtained from Karaj Agricultural Research Institute (Tehran, Iran). *Phormidium* sp. ISC108 was obtained from the Research Institute of Applied Sciences in Shahid Beheshti University branch of ACECR, Tehran, Iran. *Phormidium* was cultured in liquid BG-11 medium (Waterbury and Stanier, 1981) and were kept in a 16/8 h light/dark photoperiod regime under OSRAM Lumilux Cool White light (120 μmol $m^{-2}s^{-1}$) at 23 ± 2°C.

Seed Sterilization

All stages of seeds sterilization were performed under laminar flow condition. Berseem clover seeds were soaked in 70% (v/v) ethanol for 1 min and then washed with sterile distilled water for 5 min. The disinfection process was done with 1% (v/v) sodium hypochlorite solution for 5 min and finally rinsed 3 times with sterile distilled water.

Preparation of culture medium

The soil was prepared from the Iranian Institute of Plant Protection Research contained 20% perlite, 27% sand, 13% clay and 40% silt. The soil was autoclaved twice for soil free of microbial and fungal agents.

Hexadecane contamination

1 ml n-hexadecane was added to 5 ml of acetone which is a solvent to complete dissolution and then distilled water was added to obtain a final volume 100 ml. The solution added thoroughly to 100 g autoclave soil until obtaining homogenized 1% hexadecane-contaminated soil. Treated medium were incubated for at least 24 h at 23 ± 2°C to evaporate the acetone from the soil. Then the treated soil was distributed in culture trays including buckets of 5 cm in depth for seeds or seedlings cultivation. 100 g soil soaked with 100 ml distilled water were used as control.

Cyanobacterial (*Phormidium*) *treatment*

Sterilized seeds for germination or seedlings were cultured in soil with and without hexadecane contamination. During the growth period, all treatments were irrigated each day with 5 ml of tap water. In cyanobacterial treatments, in addition to irrigation, 3 ml of *Phormidium* (OD600: 0.6–0.8) per seed were applied 3 times a week.

All cultures were kept in a 16/8 h light/dark photoperiod regime under OSRAM Lumilux Cool White light (120 μmol $m^{-2}s^{-1}$) at 23 ± 2°C.

Seed germination percentage

The number of germinated seeds was counted 3 days after seed sowing when they started to emerge. Seed was considered as germinated when the root was emerged from the seed coat. The seed germination percentage was calculated by the following equation (Maguire, 1962):

Seed germination percentage = (germinated seed number/ total seed number) × 100.

Measurements of plant growth parameters

Thirty days after seedling culture, plant growth parameters such as root length and leaf area were measured by analyses of the scanned images using Image J software (version 1.44P; US National Institutes of Health, Bethesda, Maryland, USA) (Collins, 2007). Fresh weight (FW) of different parts of root and aerial part (shoot + leaf) were recorded on the same day.

Relative water content (RWC)

For measuring RWC, 10 leaf discs (1 cm diameter) were made from the leaves and the fresh weight of the samples was measured immediately. The leaf discs were placed into the tubes containing 10 ml distilled water and kept at room temperature for 24 h. The turgid leaf discs weight was measured and samples were placed in an oven at 70° C for 48 hours to measure their dry weight. The RWC of the samples was calculated according to the following equation (Ahmad et al., 2018).

RWC = [(fresh weight –dry weight) / (fully turgid weight - dry weight)] × 100

Degradation of hexadecane

Biodegradation of hexadecane was measured by a Shimadzu GC-15A, an instrument with a flame ionization detector (FID), using a capillary column Rtx-5 MS (30 meters, 0.25 mm ID). The analytical program was done according to Al-Hasan et al. (1998) protocol.

Fe and Zn concentration

Aerial parts of the plants after 30 days of seedling cultured dried at 70°C for 24 h. The dried tissueswere weighted and placed in an oven at 700-500°C for 4h and subsequently digested with 5ml nitric acid for 18 h (Woodis et al., 1997). The solutions were filtered using Whatman filter paper, and distilled water was added to obtain a final volume of 30 ml. Fe and Zn absorption was measured by atomic absorption/flame emission spectrophotometer (Shimadzu, Japan). Fe or Zn concentration (mg/l) of each sample was calculated using the standard curve prepared with a range of known concentrations. Fe or Zn content (mg/kg dry weight) was calculated by the following equation.

Fe or Zn content (mg/kg) = [Cd concentration (mg/l) × final volume of sample (l)] / dry weight of tissue (kg)

Statistical analysis

All experiments were performed in three biological replicates and a minimum of three technical replicates. The data were presented as the mean ± standard error. Statistically significant differences between the mean values were evaluated by one-way analysis of variance (ANOVA), followed by Duncan test ($p<0.05$), using SPSS software (version 22).

Results

Phormidium treatment increaseseed germination percentage

Maximum percentage of germination (85%) occurred in non-hexadecane soil with *Phormidium* treatment (Fig. 1). In general, *hexadecane contamination* media treated with

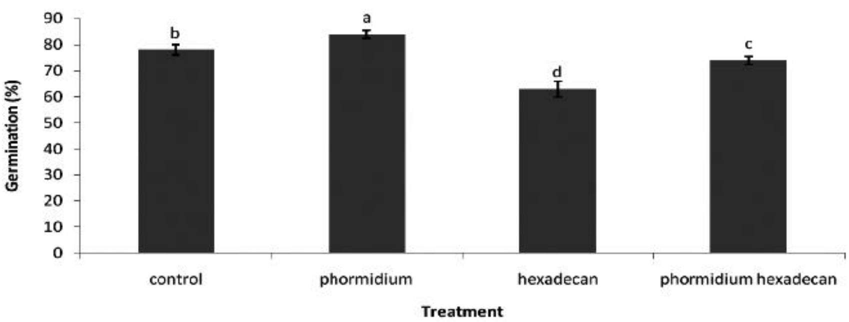


Fig. 1. Effects of *Phormidium* treatment on seed germination percentage of berseem clover under control and hexadecane contaminated soil during 5 days after sowing. Bars indicate mean values of three repetitions ± SEM. Different letters indicate a significant difference between means at the probability level of $P <0.05$.

Phormidium resulted significant increase in germination percentage (Fig. 1).

Phormidium treatment improves plant growth in hexadecane-contaminated soil

Phormidium treatment caused positive effect on root length of both control and hexadecane- exposed plants (Fig. 2). The highest plant growth was recorded in hexadecane-contaminated soil with *Phormidium* treatment (Fig. 2). Maximum leaf area in berseem clover plants was observed in media containing *hexadecane contamination* with *Phormidium* (Fig. 3). However, *Phormidium* treatment in no-hexadecane-soil resulted in 30% reduction in leaf area (Fig. 3). Hexadecane also made it difficult to absorb nutrients from the soil by forming a

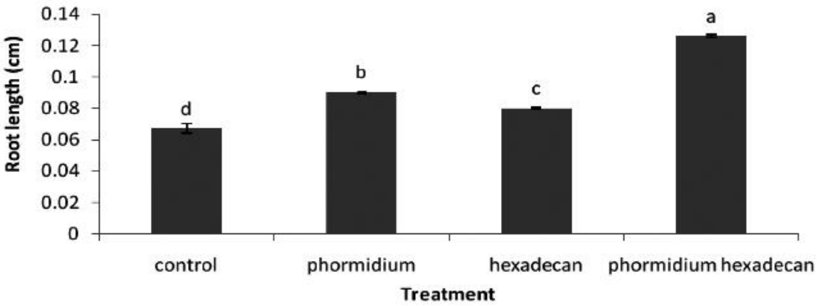


Fig. 2. Effects of *Phormidium* treatment on root length of berseem clover plants undercontrol and hexadecane contaminated soil at 31 days after sowing. The bars are the means of three repelicates ± SEM. Different letters indicate a significant difference between the means at the probability level of $P < 0.05$.

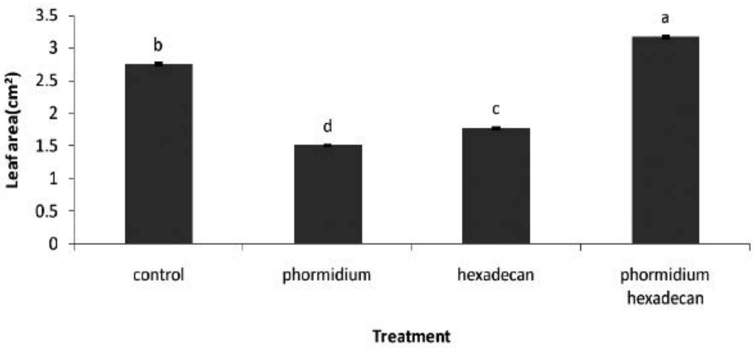


Fig. 3. Effects of *Phormidium* treatment on leaf area of berseem clover plants under control and hexadecane contaminated soil at 31 days after sowing. The bars are the means of three repetitions ± SEM. Different letters indicate a significant difference between the means at the probability level of $P < 0.05$.

layer on the root surface. As a result, hexadecane had a negative effect on the fresh weight of aerial parts. *Phormidium* treatment in berseem clover resulted in a significant increase in aerial fresh weight of both control and hexadecane- exposed plants (Fig. 4). Root fresh weight was decreased by *Phormidium* treatment in hexadecane-contaminated soil (Fig. 5). Maximum value of root fresh weight occurred in hexadecane contaminated soil without *Phormidium*. However, *Phormidium* treatment in control soil resulted in increase of root fresh weight (Fig. 5). *Hexadecane contamination* caused significant decrease in RWC. *Phormidium* treatment caused positive effects on RWC. However, maximum RWC was recorded in plants grown under non-contaminated soil (Fig. 6).

Phormidium treatment enhances Fe and Zn content in plants under hexadecane-contami-

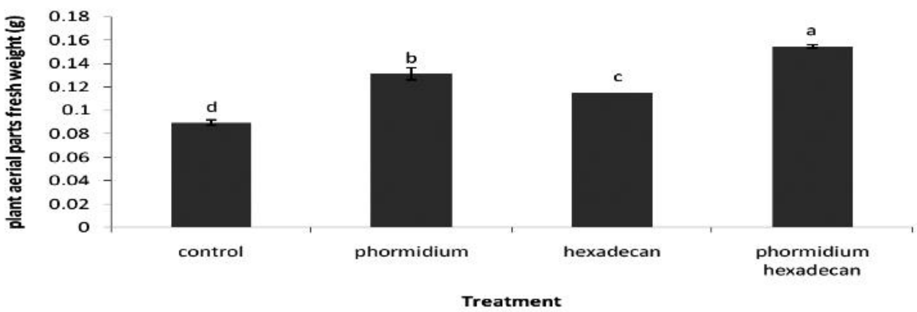


Fig. 4. Effects of *Phormidium* treatment on fresh weights of aerial parts under control and hexadecane contaminated soil in berseem clover plants at 31 days after sowing. The bars are the means of three repetitions ± SEM. Different letters indicate a significant difference between the means at the probability level of $P < 0.05$.

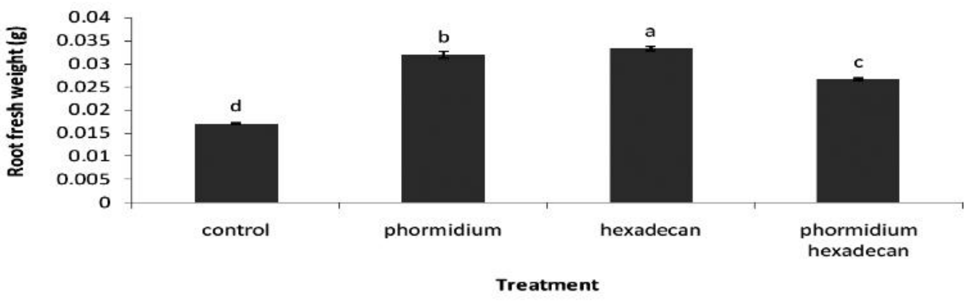


Fig. 5. Effects of *Phormidium* treatment on root fresh weight under control and hexadecane contaminated soil in berseem clover plants at 31 days after sowing. The bars are the means of three repetitions ± SEM. Different letters indicate a significant difference between the means at the probability level of $P < 0.05$.

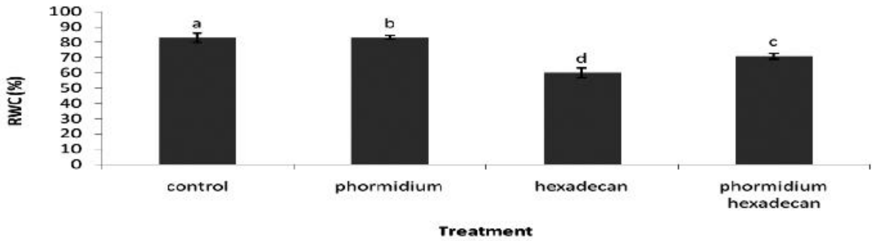
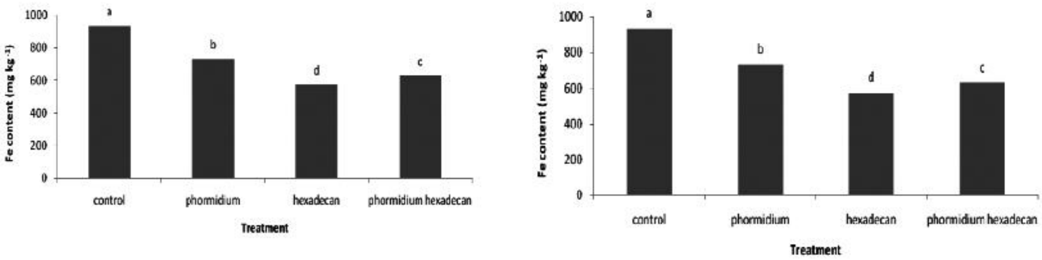


Fig. 6. Effects of *Phormidium* treatment on relative water content (RWC) percentage of berseem clover leaves under control and hexadecane contaminated soil at 31 days after sowing. The bars are the means of three repetitions ± SEM. Different letters indicate a significant difference between the means at the probability level of P <0.05.



a b

Fig. 7. Effects of *Phormidium* treatment on Fe (A) and Zn (B) content of berseem clover plants under control and hexadecane contaminated soil at 31 days after sowing. The bars are the means of three repetitions ± SEM. Different letters indicate a significant difference between the means at the probability level of P <0.05.

nated soil

Maximum content of Fe and Zn occurred in grown plants in control soil (Fig. 7A and 7B). Hexadecane resulted in reduced Fe and Zn content in plants. However, *Phormidium* treatment caused positive effects on Fe and Zn content in plants cultivated in hexadecane-contaminated soil (Fig. 7 a and b).

Phormidium treatment and berseem clover cultivation degraded hexadecane

Hexadecane significantly decreased in soil which berseem clover was grown. Remarkable *degradation of hexadecane* was occurred in the soil which berseem clover was grown and treated with *Phormidium* (Table 1).

Discussion

In the current study, seed germination of berseem clover decreased in hexadecane-contaminated soil compared to the control soil. Seed germination is an important indicator for assessing crop tolerance to petroleum contamination (Muratova et al., 2008). Most studies indicates the negative effect of petro-

Table 1. Effects of *Phormidium* treatment and berseem clover plants on hexadecane degradation in contaminated soil.

	Hexadecane in soil
initial soil before berseem clover cultivation	110521
30 days after berseem clovercultivation	32948
30 days after berseem clover cultivation with *Phormidium* treatment	2658

leum compounds or their derivatives in soil on seed germination (Atagana, 2011; Afzal et al., 2011; Wang and Oyaizu, 2009).

Adam and Duncan (2002) attributed the decrease in seed germination to the toxicity caused by petroleum hydrocarbons or the inhibition of these substances by the entry of water and oxygen into the seed. Some low molecular weight petroleum compounds may pass easily through the membrane, causing toxicity to the seed and ultimately leading to embryo death (Adam and Duncan, 2002). *Phormidium* treatment acceleratedseed germination in both control and hexadecane-contaminated soils. Hypothetically, the cause of this increase may be related to the photosynthetic activity of cyanobacteria. As photosynthesis increases, the amount of oxygen produced in the soil also enhances and therefore, the availability of water and oxygen for the plant increases. *Phormidium* is also a petroleum- degrading cyanobacteria, so part of the hexadecane is degraded by the cyanobacteria.

The toxic effect of petroleum contaminants can alter root morphology and this change directly affects water and nutrient uptake and thus affects plant growth (Reynoso-Cuevas et al., 2008). Petroleum materials cause drought stress for the plant by altering the permeability and structure of the plant plasma membrane (Pena-castro et al., 2006) and soil physico-chemical properties (Baker, 1970). Drought stress is a limiting factor in the early stages of plant growth and stabilization, which influences plant growth and development (Anjum, 2014). Root length usually increases under drought stress (El-Sharkawial et al., 1989). The present findings revealed that hexadecane infestation significantly increase the root length in berseem clover. Whereas, leaf area under *hexadecane contamination* decreased significantly. Increasing root to shoot ratio is a usual plant response to drought stress. The root system is main organ in water absorption. Therefore, wider and deeper roots are able to absorb more water from the lower layers of the soil. In addition, increasing abscisic acid (ABA) at low water levels affects on root and shoot growth, as it stops growth of shoots, but root growth continues (Creelman et al., 1990; Setayesh Mehr and Ganjali, 2013). In this study, shoot fresh weight increased in *Phormidium* treatment. The presence of cyanobacteria in the soil has resulted in oxygen production and improved rhizosphere conditions. So that by decreasing the negative effect of hexadecane on photosynthesis and materialization, the shoot fresh weight increased, in plants which were grown in hexadecane-contaminated soil and treated by *Phormidium*. Gao et al. (2006) examined the effect of hydrocarbons-degrading bacteria on rice plant growth and showed that root and shoot dry weight was decreased in the non-bacterial treatment. The cause of this decrease was attributed to the toxic effects of polycyclic aromatic compounds on plant root and shoot.

Leaf *relative water content (RWC)* is important for plants to maintain sufficient water content in their tissues under stress conditions (Li et al., 2008). Higher RWC can maintain stomatal conductance resulting in higher transpiration and photosynthesis of the plant (Medrano et al., 2002). RWC significantly decreased with increasing drought intensity. Drought stress caused by hexadecane infestation also decreased leaf RWC in berseem clover plants. Treatment by cyanobacteria increases leaf RWC in berseem clover under *hexadecane contamination*. Cyanobacteria by creating water retention by their gelatinous sheath actually counteract the negative effects of hexadecane, which can potentially affect the absorption of available water, effective nutrient mobility, and oxygen transfer.

The reason for decreasing the amount of petroleum hydrocarbons in the soil may be due to the activity of soil microorganisms or rhizosphere discharge (Besalatpour et al., 2008). Root secretions contain enzymes such as lactases, peroxidases and dihalogenases, which can alter the pattern of degradation of organic compounds in the rhizosphere (Besalatpour et al., 2008). The root of the plant can absorb organic substances such as hydrophils and lipophiles (aliphatic, aromatic compounds, alcohols, phenols and amines). It is also capable absorbing very low solubility compounds such as cyclic hydrocarbons (Kvesitadze et al., 2006).

There is increasing evidence indicating the role of photosynthetic organisms, especially cyanobacteria, in the oxidation and degradation of hydrocarbons (Al-Hasan et al., 2001; Raghukumar et al., 2001). Cyanobacteria are able to photosynthesize, consume, and oxidize n-alkanes, which are processed by oxidizing them to lower molecular weight components or by altering them to more polarized components. Algae naturally break down both aromatic and aliphatic hydrocarbon compounds. Foroughi Nia et al. (2013) identified two cyanobacteria Anabaena ISC55 and *Phormidium* ISC24 as best examples for biodegradation of petroleum. In the current study, the soil around the rhizosphere of *Phormidium* treated plants showed a large reduction of soil hexadecane. This can demonstrate an effective role of the cyanobacteria and berseem clover cultivation on hexadecane degradation.

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