

[Research]

Physiological responses and phytoremediation ability of Eastern Coneflower (*Echinacea purpurea*) for crude oil contaminated soil

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ABSTRACT

One of the most important anthropogenic pollution types in countries with oil production is soil and water contamination by petroleum. Phytoremediation is an emerging green technology for cleaning up polluted soil. A greenhouse experiment was conducted to study the effect of oil-contaminated soil on *Echinacea purpurea* with four concentrations of crude oil - contaminated soil: control = 0, 0.5% = 5000, 1% = 10000, and 2% = 20000 mg kg⁻¹. Morphological and physiological traits were evaluated after 90 days. Gas chromatography determined the removal rate percentage of total petroleum hydrocarbons (TPHs) in the soil. The results show that this plant has potential for removing TPHs, up to 45.5% at 1% crude oil contamination, while the removal rate by natural attenuation is only 32%. Data from morphological and flowering indices including shoot and root fresh weights, shoot and root dry weights, flower stem length, flower longevity, flower anthocyanin, and visual stress symptoms show significant differences within treatments. Based on the results, *E. purpurea* can tolerate crude oil concentrations in soil equal to or greater than 5000 and 10000 mg kg⁻¹ (0.5% and 1% w/w). However, flowering was not observed at treatments of 1% and 2% crude oil contamination. As crude oil concentration increased, physiological parameters such as total chlorophyll, protein, and antioxidant capacity significantly decreased, while other parameters including leaf anthocyanin, electrolyte leakage, malondialdehyde, proline, and total carbohydrate all increased. Overall, *E. purpurea* is a widely - spread species that can be effectively used for phytoremediation of ≤10000 mg kg⁻¹ crude oil contaminated soil.

Key words: TPHs, *Echinacea purpurea*, Environmental pollution, Growth, Flowering.

INTRODUCTION

By increasing industrial activities, dramatically more organic contaminants are naturally or by human intervention released into soil and water. One of the most important anthropogenic pollution types in countries with oil and gas production is oil contaminant pollution (Wang *et al.* 2011a; Balasubramaniam 2015). Petroleum is one of the most important energy sources, and it contributes to a country's economic and social development (Segal & Sen 2011; Wang *et al.* 2011b). During its exploration, transition and processing, petroleum has caused major contamination. So,

many countries and regions experience serious soil and water contamination problems as well as ecological risks from petroleum hydrocarbons (Das & Chandran *et al.* 2011; Ite *et al.* 2013). Previous investigations demonstrate the occurrence of crude oil pollution in the Caspian Sea and its littoral states, one of the world's oil hubs (Tolosa *et al.* 2004; Khoshbavar Rostami *et al.* 2012; Eghtesadi Araghi *et al.* 2014; Khoshbavar Rostami & Soltani 2016). The main sources of pollution near the Caspian Sea are offshore oil production and land-based sources (Karpinsky 1992). Concern has centered on the Apsheron

peninsula of Azerbaijan, where over a century of oil production and pipeline construction has left >10,000 ha of land heavily contaminated. In addition, oil refineries in Baku are major sources of land-based pollution (Tolosa *et al.* 2004; Mashroofeh *et al.* 2015). Iran has not currently established oil or natural gas production in the Caspian region, but has significant reserves and also acts as a transit center for the oil and natural gas exports from the other littoral states (Effimoff 2000). The effect of petroleum hydrocarbons on the soil is not only harmful to human health, but also poses a negative impact on plant growth and development (Muratova *et al.* 2008). This impact includes reducing photosynthesis pigments, shortening roots and aerial organs, dissolving biological membranes, reducing antioxidant activity, and changing protective solutes (Shirdam *et al.* 2008; Ashraf *et al.* 2010; Liao *et al.* 2015).

Phytoremediation is an emerging, practical, cost-effective technology that uses specialized plants to clean up polluted soil and water (Suresh & Ravishankar 2004; Soleimani *et al.* 2009). Thus far, phytoremediation has been used for treating many types of contaminants including heavy metals, radionuclides, chlorinated solvents, pesticides, explosives and landfill leachates (Mackova *et al.* 2006; Ahmad *et al.* 2015). Total petroleum hydrocarbons (TPHs) are among the most common groups of persistent organic contaminants (Huang *et al.* 2005). TPHs contain volatile monoaromatic compounds such as benzene, toluene, ethylbenzene, xylene (BTEX), polycyclic aromatic hydrocarbons (PAHs) and aliphatic compounds (Cook & Hesterberg 2013). Several studies show the successful application of plant species such as ornamental plants, grasses (*Poaceae*) and legumes (*Leguminosae*) in remediating oil-contaminated soil (Muratova *et al.* 2008; Ikeura *et al.* 2015). Few studies have applied ornamental flowering plants for phytoremediating crude oil-contaminated soils, which can remedy the contaminated environment and beautify it at the same time. Liao *et al.* (2015) propose the maize, *Zea mays* L.,

for crude oil remediation of contaminated soil with concentrations of 0, 1500, 2500, 5000, and 1000 mg kg⁻¹ dry soil. The total petroleum hydrocarbon reduced in the planted soil was 52.21% to 72.84%. Razmjoo & Adavi (2012) suggested that bermudagrass, *Cynodon dactylon*, is an efficient species for the phytoremediation of petroleum-contaminated soil mixed with different rates of oil sludge containing 0, 2, 4, 6, and 8% TPHs. Liu *et al.* (2010) compared the effectiveness of five plant species in the remediation of oily sludge. Alfalfa, *Medicago sativa*, tall fescue, *Festuca arundinacea*, and soybean, *Glycine max* increased the removal rate of oil-contaminated soil, and the soybean treatment showed the highest removal rate of 34.2%. Peng *et al.* (2009) used *Mirabilis jalapa* L. for remediating petroleum-contaminated soil with three crude-oil concentrations (5000, 10000, and 20000 mg kg⁻¹) and the corresponding controls. They indicated that *M. jalapa* has a peculiar tolerance to petroleum contamination and can effectively promote the degradation of TPHs at ≤10,000 mg kg⁻¹. Balseiro-Romero *et al.* (2016) demonstrated that yellow lupine, *Lupinus luteus*, with its deep and branched root system by a plant-bacteria partnership can improve rhizodegradation of diesel in the rhizosphere of soil samples spiked with diesel at approximately 125000 mg kg⁻¹. The fibrous roots provide a larger surface than a taproot for soil microorganisms to colonize and allow for greater interaction between microorganisms and the contaminants. (Anderson *et al.* 1993; Schwab & Banks, 1994). One perennial plant belonging to the Asteraceae family is the *Echinacea* genus, with nine species native to North America that can be cultivated in different regions of the world for cut flowers, landscape perennials, and pharmaceutical aspects (McKeown 1999; Sabra *et al.* 2012). A single tap root is the main characteristic of all species except *E. purpurea*, which has a fibrous root system (McKeown 1999). *Echinacea* cultivates in wet places with full sunlight and in soils with loamy texture, rich in humic compounds (Dol & Wilkins, 1999). Therefore, the increasing demand from

the pharmaceutical industry as well as the plant's use in beautifying green spaces and its adaptability to different climates and soil conditions have prompted *Echinacea's* cultivation to increase throughout the world and especially in Iran. (Stanisavijevic et al. 2009; Sabra et al. 2012).

Sabra et al. (2012) examined three *Echinacea* species under different salt treatments by measuring different growth indices and antioxidant enzyme activity. They demonstrated that *E. purpurea* can tolerate saline soil more than *E. angustifolia* and *E. pallida*. Asadi-Sanam et al. (2015a) indicate that the *E. purpurea* species can tolerate freezing conditions by improving protective solutes and the antioxidant enzyme activities. Chapman & Auge (1994) measured the water relationship parameters and osmotic adjustment among four different ornamental perennials under drought conditions and concluded the presence of limited drought tolerance in *E. purpurea*. Liu et al. (2014) reported that *E. purpurea* can remediate polycyclic aromatic hydrocarbons (PAHs) from oil-contaminated soil by improving soil enzyme activities. Therefore, it can be concluded that *E. purpurea* can tolerate various soil conditions and environmental stresses such as humidity, cold, drought, and saline. Based on the above literature review, *E. purpurea* is a promising species for phytoremediating oil-contaminated soil. Therefore, the aim of this study was to investigate the physiological and biochemical responses of coneflower to crude oil pollutants in the soil as well as to measure the tolerance level and phytoremediation ability of this plant against various contamination levels.

MATERIALS AND METHODS

Soil preparation

The soil in this experiment was collected from a field near the Oil Refinery of Tehran, Iran, and had no previous history of exposure to crude oil and other organic contaminants. The chemical and physical characteristics of the soil before contamination are presented in Table 1. Prior to contamination, we air-dried the soil

and sieved it through a sieve with mesh size of 2 mm to ensure soil homogeneity. The crude oil was purchased from the Oil Refinery of Tehran, Iran. The crude oil concentrations were prepared according to Peng et al. (2009) and evenly sprayed on the soil as 5000, 10000, and 20000 mg kg⁻¹ (0.5%, 1.0%, and 2.0% W_{oil}/W_{dry soil}). We also considered the corresponding control without planting *Echinacea*. The soil (2 kg dry weight per pot) was then blended completely and packed to plastic pots (17 cm diameter by 20 cm height) and also aired in experiment conditions (that mentioned in Table 2) for two weeks before application to ensure the evaporation of the unstable composition in crude oil and better homogeneity. The measured total petroleum hydrocarbon (TPH) levels before transplanting were 0, 1199, 3115, and 5805, mg kg⁻¹, respectively. An amount of 0.5 g kg⁻¹ solid macro complex fertilizer Nitrogen (N): Phosphorus (P): Potassium (K) = 12%:11%:18%, Yaramila™) was added to the soil as the base fertilizer.

Greenhouse pot experiment

This experiment used seedlings from the *E. purpurea*, transplanted at the four-leaf stage with similar biomass. It was carried out in greenhouse conditions of the Municipality of Tehran Research, Training, and Consulting Center of Ornamental Plants, Tehran, Iran, (35.77N and 51.42E), with an altitude of 1520 m above sea level during the spring and summer in 2016. Pots were placed under natural sunlight with a light:dark cycle of approximately 16:8 h. Table 2 shows the climate condition of the greenhouse during the experiment.

This experiment was conducted in completely randomized design with three replications. In each replication, three plants were placed as each plant in one pot (nine experimental units per treatment). The experiment was arranged in four different treatments of crude oil contamination (Control = 0, 0.5% = 5000, 1% = 10000, 2% = 20000 mg kg⁻¹) and three corresponding controls for each treatment without planting. Soil moisture was maintained at 75% field capacity level by daily

watering during the experiment for both planted and unplanted pots (corresponding controls). Plants exposed to these treatments for 90 days. Then we measured physiological and biochemical characteristics. Additionally, TPHs (total petroleum hydrocarbons) of the soil's rhizospheres were measured before and after the experiment, except those of the

controls. The TPHs removal rate was determined using the following equation:

$$\text{Removal rate (\%)} = [\text{TPH}_0 - \text{TPH}_{90} / \text{TPH}_0] \times 100$$

Where TPH_0 is the total petroleum hydrocarbons on day 0 and TPH_{90} is the total petroleum hydrocarbons after 90 days of experiment.

Table 1. Some chemical and physical characteristics of the soil before contamination.

| Parameter | |
|--|------------|
| Ph | 8.3 |
| EC (ds m ⁻¹) | 2.28 |
| Chemical analyses | |
| N (%) | 0.03 |
| P (mg kg ⁻¹) | 10.9 |
| K (mg kg ⁻¹) | 155 |
| Fe (mg kg ⁻¹) | 6.7 |
| Zn (mg kg ⁻¹) | 0.9 |
| Mn (mg kg ⁻¹) | 5.9 |
| Cu (mg kg ⁻¹) | 0.3 |
| Total neutralizing value (TNV) CaCO ₃ (%) | 12.2 |
| Organic carbon (%) | 0.16 |
| Physical analyses | |
| Sand (%) | 62 |
| Silt (%) | 20 |
| Clay (%) | 18 |
| Soil texture | Sandy Loam |

Table 2. The climate condition of the greenhouse during the experiment.

| | |
|----------------------------|--------|
| Maximum temperature (°C) | 27 ± 2 |
| Minimum temperature (°C) | 18 ± 2 |
| Average temperature (°C) | 24 ± 2 |
| Mean relative humidity (%) | 30 |

Total petroleum hydrocarbons (TPHs) analyses

The total petroleum hydrocarbons were determined according to USA EPA 3550 (1986). Soil sampling were performed from the rhizosphere area (root penetration zone, at the distance of 5 cm from the crown of plant, with 10 cm depth, uniformly).

Five grams of air-dried soil sample was dissolved in 10 ml of hexane, kept in an ultrasonic device for 30 minutes, and then centrifuged (Hettich-D7200, Tuttlingen; Germany) at 3,000 rpm for 15 minutes followed by extracting the supernatants. All of these

operations were repeated three times. The obtained extracts were concentrated to 2 ml under a gentle stream of nitrogen gas and then 2 µL of the sample splitless was injected into an AGILENT 6890N gas chromatograph equipped with a flame ionization detector (FID).

The column used for analysis was HP-5 with 30 m length, 32 mm diameters, and 0.25 µm film thickness. The injector temperatures were adjusted at 270°C. The initial column temperature was adjusted at 90°C for 2 minutes, then increased to 300°C with 8°C min⁻¹ slope and remained at 300°C for 10 minutes.

Growth and flowering

Measuring morphological indices including shoot weight, root weight, shoot dry weight, root dry weight and flowering indices such as flower stem length, flower anthocyanin were carried out after 90 days of experiment. Flower longevity (number of day from blooming till the flowers wilting) measured during flowering period for each treatment.

Photosynthesis Pigments

Total chlorophyll (Chl_{a+b}) was determined spectrophotometrically (Itt T80+ UV/VIS; PG Instruments, Leicestershire, UK) according to the method of Arnon (1967). So that, 20 ml acetone 80% was added to 0.5 g of the samples and the mixture was pulverized properly. The resulting extract was centrifuged (Eppendorf Centrifuge 5417R) at 6,000 rpm for 10 minutes. The absorbance reading was taken at 663 nm for chlorophyll a and 645 nm for chlorophyll b. The content of chlorophyll was expressed as mg g⁻¹ Fw⁻¹.

Total anthocyanin content was determined according to pH differential method of Wrolstad (1976) as modified by Lee *et al.* (2005) based on the reversible changes of anthocyanin at pH 1.0 and pH 4.5. For its extraction, methanol containing 1% (v/v) HCl was used. Absorbance was measured at 520 and 700 nm and expressed as cyanidin-3-glycoside (molar extinction coefficient of 26,900 L mol⁻¹ cm⁻¹ and molecular weight of 449.2 g mol⁻¹) equivalents, as mg L⁻¹.

Electrolyte leakage and Lipid peroxidation (MDA content)

For electrolyte leakage (EL), leaf samples were rinsed with distilled water and immersed in 10 mL of distilled water for 12h. The conductivity of the solution (R₁) was determined using a conductivity meter (Jenway 4010 Conductivity meter, Staffordshire, UK). Then samples were heated in boiling water for 20 min and then cooled to room temperature. The conductivity of killed tissues (R₂) was again measured. EL was calculated as the percentage of R₁ to R₂ (Shaoyun *et al.* 2009).

Lipid peroxidation in samples were determined by estimation of malondialdehyde (MDA) content according to the method of Heath and Parker (1968). Extract of MDA was determined using 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. MDA content was calculated using the difference between spectrophotometric absorption ratios at 532 and 600 nm wavelengths and 155 mM cm⁻¹ extinction coefficient.

Total sugars and Proline

The total sugars were estimated by a modified method of Somogyi (1952). Two milliliters of copper sulfate were added to the test tubes containing 2 ml of sample extraction incubated in a boiling water bath then suddenly cooled. Two milliliters of phosphomolybdic reagent were added to the test tubes. The mixture diluted to 10 ml and absorbance was measured by a spectrophotometer at 600 nm. Total sugar content was estimated using a standard curve prepared with glucose and expressed as mg g⁻¹ Fw⁻¹.

The ninhydrin method determined proline concentration according to Bates *et al.* (1973) with some modifications. Three hundred milligrams of frozen leaf material were homogenized in 1.5 mL of sulfosalicylic acid and centrifuged at 12,000 rpm for 10 min. Afterward, 1 mL of supernatant with 1 mL acid ninhydrin (1.25 ninhydrin warmed in 30 mL glacial acetic acid and 20 mL 6 molar phosphoric acid until dissolved) was combined with 1 mL glacial acetic acid in a test tube for one hour in a 100°C water bath, and the reaction terminated in an ice bath. After cooling, the reaction mixture was extracted with 2 mL toluene, mixed vigorously with a test tube stirrer for 15-20 second. The absorbance of the fraction with toluene aspirated from the liquid phase was read at 520 nm by the spectrophotometer and toluene as a blank. Proline concentration was calculated using a calibration curve and expressed as μmol proline per g⁻¹ Fw⁻¹.

Protein content and Antioxidant capacity

Protein content of the samples was extracted using the method developed by Bradford (1976). So that, 0.5 g of the frozen samples was poured into a mortar, then 1 ml of 50 mM phosphate buffer (extraction buffer, pH 7) containing 0.5 mM Na₂-EDTA and 2% polyvinylpyrrolidone (PVPP) were added. The mixture was centrifuged for 20 min at 14,000 rpm at 4 ± 1°C. The obtained extract was analyzed to measure protein content. Fifty microliters of extraction was added and completely mixed with 2.5 mL Bradford solution for 15 min. The mixture's absorption ratio was recorded at 595 nm wavelength by spectrophotometer. BSA calibration curve was used for the determined amount of protein content and expressed as mg g⁻¹ Fw⁻¹.

The antioxidant activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Brand-Williams *et al.* 1995). The sample's absorbance was measured at 517 nm by spectrophotometer. Rate (%) of inhibition of DPPH was measured according to the following formula:

Inhibition of DPPH (%) =

$$[(A_{517\text{Control}} - A_{517\text{Sample}}) / A_{517\text{Control}}] \times 100$$

where $A_{517\text{Control}}$ is the absorbance of the control, and $A_{517\text{Sample}}$ is the absorbance of the sample.

Statistical analysis

The effect of treatments was determined by analysis of variance using SAS software (SAS Institute Inc., Cary, NC, USA v.9.4). Values presented in the text indicate mean values of three replicates. Mean comparisons were calculated using Tukey's test at %95 probability ($p < 0.05$), and the charts were plotted using Excel software.

RESULTS AND DISCUSSION

Total petroleum hydrocarbons (TPHs) removal

Fig. 1 shows the average rate of TPHs removal in *E. purpurea* planted treatments and unplanted (corresponding controls) with different crude oil concentrations.

According to this figure, there are significant differences between planted treatments and corresponding controls in TPHs removal rate. The phytoremediation ability of *E. purpurea* depends also on crude oil concentration in the soil. Maximum TPHs removal rates appeared in 0.5% and 1% treatments (with 43.5% and 45.5% removal rates) respectively and these treatments were classified as one group. TPHs removal rates in 0.5% and 1% corresponding controls were 32% and 31.6%, respectively. Thus, the maximum rate of TPHs removal from soil occurred in 1% treatment with net removal efficiency of 13.84%. The minimum rate of TPH removal obtained from soil in 2% treatment (with 39.5% removal rate).

The difference between TPHs removal efficiencies in treatments with plants and without plants (corresponding controls) represents the soil's natural attenuation process. The process of TPHs removal in soil is based on many factors, including physical, chemical, and biological ones. Peng *et al.* (2008) reported that TPHs removal in unplanted soils is caused by volatilization, eluviation and photolysis as natural removal factors. In planted treatments, beside natural attenuation, root system and its amount significantly affect TPHs removal process. Plants with fibrous root systems have better phytoremediation ability compared to plants with a tap root system due to their wider surface for remediation. Wide root surfaces lead to better activity of the soil's heterotrophic microbes when degrading oil pollution, confirmed when different plants including *Leguminosae* and *Poacea* are used (Muratova *et al.* 2008).

The role of roots in phytoremediation of oil-contaminated soil can be attributed to the following factors: 1) Dead root cells are considered as nutrients, activating soil microorganisms; 2) Substances released from plant roots (organic acids, amino acids, sugars, etc.) are sources of some nutrients, stimulating the growth of microorganisms; 3) Root penetration in the soil forms cavities that improve ventilation and permeability, as well as increase the interaction between degrading

microorganisms and insoluble oil pollutants (Ikeura et al. 2016). The genus *Echinacea* has nine species, eight of those have a tap root system, while *E. purpurea* has a fibrous one (McKeown 1999). *E. purpurea*, with its great and fibrous root system, can remove TPHs (43.5% to 45.5%) from crude oil- contaminated soil less than 1,000 mg per kg dry soil (TPHs concentration = 3115 mg kg⁻¹). Results obtained from this study are comparable with Liu et al. (2014) who attribute the phytoremediation efficiency of *E.*

purpurea to the plant's rhizosphere enzymes activity, as measured in one concentration of soil contamination (PAHs: 122.46 mg kg⁻¹). Another feature of the *E. purpurea* that increases the ability of this plant to remediate TPH from soil, is that it can endure flooding beside aerobic respiration (Asadi-Sanam et al. 2015b). Aerobic respiration releases oxygen from the roots to the rhizosphere and provides required oxygen for microorganisms involved in remediation (Razmjoo & Adavi 2012).

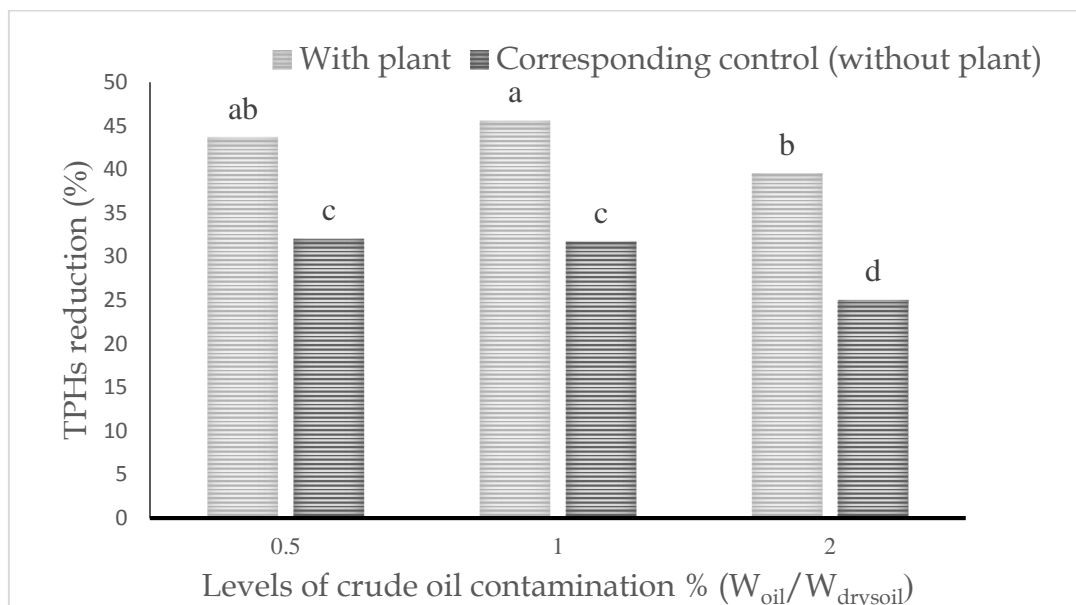


Fig 1. Remediation of TPHs in *E. purpurea*-planted soil and unplanted controls under three crude oil levels, 0.5% = 5000, 1% = 10000, and 2% = 20000 mg kg⁻¹. Different letters are for significantly different groups according to Tukey's test ($p < 0.05$).

Growth and flowering indices

According to data variance analysis of different treatments, the effect of different crude oil concentrations on growth and flowering indices are significant. Table 3 shows the mean comparison of *E. purpurea*'s growth and flowering indices in different contamination levels. The control and 0.5% treatments showed high shoot weights (8 g and 9 g) and shoot dry weights (2.3 g and 2.63 g) respectively, while 2% treatment displayed the lowest shoot weight (2.33 g) and shoot dry weight (0.8 g). The maximum root weight and root dry weight derived from the control, whereas the root weight decreased by increasing oil concentration in soil. The minimum root weight occurred in 2% treatment (0.86 g).

Decreasing the plant growth and biomass is the first and most important plant response to crude oil pollution (Lin et al. 2002). The toxicity of petroleum pollutants is due to the low molecular weight of hydrocarbons. Smaller hydrocarbons enter more easily into the cellular structure of the plants, leading to cell death and also decreased growth of the root system and aerial parts (Ertekin et al. 2015; Zand et al. 2010). In addition, hydrocarbons have a hydrophobic structure that creates an impenetrable layer on the plant's root, leading to the less water and nutrition absorption, thus exposing the plant to stress conditions (Merkel et al. 2004). In all plants, "Reactive Oxygen Species" (ROS), including hydrogen peroxide (H₂O₂), superoxide (O₂⁻) and hydrogen radical (HO) are both signal molecules and toxic by-products. These

compounds are produced during the natural process of photosynthesis in electron transfer chain of chloroplasts and mitochondria and are also found in peroxisome as a toxic by-product. ROS causes the chemical destruction of proteins, carbohydrates and fatty acids. Plants use different methods to counteract and neutralize these compounds (Moller *et al.* 2007). When plants are exposed to abnormal conditions such as oil pollutants, the amount of ROS significantly increases, leading to cellular damage and reduction as well as disruption in the plant's growth and physiological characteristics (Wang *et al.* 2011b).

In this study, the biomass of *E. purpurea* decreased by increasing the amount of oil pollution. This decrement was insignificant in 0.5% treatment (TPHs concentration = 1199 mg kg⁻¹) compared to the control, while a significant reduction in plant growth indices was observed in 1% treatment (TPHs concentration = 3115 mg kg⁻¹) and higher levels, indicating the plant resistance up to the former concentration.

The wet and dry weights of the *E. purpurea* root decreased by elevating the crude oil concentration in the soil, so the 2% treatment produced the minimum weight. Therefore, it can be concluded that its high concentrations in the soil, reduced the growth of different parts of the plant, with such reducing mostly observed in the root, due to decreased cellular activities including cellular division and growth, as well as vital activities such as water and food absorption. Balseiro-Romero *et al.* (2016) studied the effect of oil pollution on the *Lupinus luteus* shoot and root growth indices. They found that shoots and especially root growth, decrease significantly by arising oil concentration. They also reported that *L. luteus* successfully remediates diesel (oil) pollution of soil with a concentration of 125,000 mg kg⁻¹ to 150,000 mg kg⁻¹, which is consistent with our study regarding the resistance of *E. purpurea* and its ability to remediate soil contaminated by 1% crude oil.

In another study, increasing oil pollution up to an initial concentration of 2147 mg kg⁻¹ did not

affect maize, *Zea mays* growth and biomass. This refers to an increased activity of soil bacteria due to oil pollutants, although the activity of these bacteria decreased in high concentrations of oil pollutant (TPHs concentration = 6373 mg kg⁻¹) (Liao *et al.* 2015). Our study on *E. purpurea* is consistent with the above-mentioned results, and its growth rate did not decrease in the initial concentration of the pollutant (0.5%, TPHs concentration = 1199 mg kg⁻¹ TPHs), although the growth rates decreased by arising oil pollution in 2% treatment. Peng *et al.* (2009) also observed *Mirabilis jalapa* maximum phytoremediation efficiency at 1% w/w crude oil concentration, but the growth indices decreased at 1% and 2%. In our study, *E. purpurea* showed adaptation with 0.5% and 1% treatments (TPHs concentration = 1199 and 3115 mg kg⁻¹), while reduced growth indices at 2% treatment (TPHs concentration = 5805 mg kg⁻¹).

According to flowering indices, results showed that the highest flower height occurred in 0.5% oil concentration, while no flowering occurred in 1% and 2%. The highest flower longevity was an average of 35 days, observed in control, while the oil pollutant affected the flowering longevity. The control also produced the highest amount of flower anthocyanin (35.6 mg L⁻¹). Likewise, the oil led to the reducing in flower anthocyanin (Table 3).

One aspect of *Echinacea* application is green spaces because of the flowers' longevity and color variety (Dol & Wilkins 1999). According to results regarding its flowering indices under different oil concentrations, no flowering occurred in levels higher than 0.5% w/w., although in 5% w/w did not stop flowering, yet flower quality decreased. Other researchers also reported that stress negatively affect the growth and flowering indices of plants (Chylinski *et al.* 2007; Ikeura *et al.* 2016).

Oil pollution of soil, as an environmental stress, influences the growth and flowering of plants and also prevents absorption of water and nutrients by covering absorption surfaces on the root (Kaimi *et al.* 2007). Comparable results are observed in the flowering of other plants

affected by oil pollution as an environmental stress.

Ikeura *et al.* (2016) studied the effect of oil contamination on four ornamental plants over 180 days. Their results showed that except

Gazania (*Gazania rigens*), three other species including *Mimosa* (*Mimosa pudica*), *Zinnia* (*Zinnia profusion*), and Cypress vine (*Ipomoea quamoclit*) were destroyed due to decreasing growth indices and no flowering was observed.

Table 3. Changes of growth and flowering indices of *E. purpurea* under four levels of oil contaminated soil, 0 (control), 0.5%, 1% and 2% (w/w).

| Treatment % (w/w) | Shoot weight (g) | Shoot Dry weight (g) | Root weight (g) | Root Dry weight (g) | Flower stem Length(cm) | Flower longevity (day) | Flower anthocyanin (mg.L ⁻¹) |
|-------------------|------------------|----------------------|-----------------|---------------------|------------------------|------------------------|--|
| 0 (control) | 8a | 2.3b | 13.33a | 2.86a | 47b | 35 a | 35.63a |
| 0.5 | 9a | 2.63a | 6.66b | 2.16b | 65.66a | 27b | 19.71b |
| 1 | 6b | 1.33c | 3.66c | 1.16c | 0c | 0c | 0c |
| 2 | 2.33c | 0.8d | 2.33d | 0.86d | 0c | 0c | 0c |

Variation in Shoot weight, Shoot dry weight, Root weight, Root dry weight, Flower stem length, Flower longevity, Flower anthocyanin; Means comparisons based on Tukey's test. Different letters in rows are for significantly different groups ($p < 0.05$).

Physiological and biochemical Indices

Table 4 indicates the mean comparison of various levels of crude oil pollution on physiological and biochemical indices of *E. purpurea*. The highest leaf anthocyanin content was obtained by 2% contamination, while the lowest by control. The amount of leaf anthocyanin in 2% and 1% were found to be 3.2 and 2.9 times higher than the control, respectively. The highest total chlorophyll content was obtained at 0.5% and control. The chlorophyll content decreased by elevating the amount of crude oil in the soil, while the lowest mean was obtained at 2%.

Crude oil pollutant leads to the reducing in chlorophyll, so photosynthesis and the green color of leaves decreases, whereas anthocyanin arises as an alternative pigment. Anthocyanin plays a significant role in a plant's non-enzymatic defense system against environmental stressors. High anthocyanin levels lead to resistance against ROS and osmotic adjustment (Kovinich *et al.* 2015). The presence of oil pollutants in the plant's roots decreases water and nitrogen absorption (Adam & Dunkan 2002; Kaimi *et al.* 2007). Decreased nitrogen uptake leads to reduced chlorophyll production and leaf yellowing (Ercoli *et al.* 1993; Tam & Magistad 1935). In addition, decreased water absorption produces abscisic acid and leaf apertures close. So that,

photosynthesis and chlorophyll production are reduced (Arteca 1996; Daszkowska-Golec 2016; Sah *et al.* 2016). Wang *et al.* (2011b) also concludes that by arising oil pollutants, the chlorophyll content of Reed (*Phragmites australis*) decreases, consistent with the results obtained in the present study. According to our results, the highest (38.3%) and lowest (9.66%) electrolyte leakage were obtained by 2% oil treatment and control, respectively. Also, the highest (5.42 nmol g⁻¹ Fw⁻¹) and lowest (1.61 nmol g⁻¹ Fw⁻¹) MDA contents, as a cell membrane injury biomarker, were obtained by 2% and control, respectively (Table 4).

Electrolyte leakage percentage is one of the indicator of plant cell membrane degradation under the influence of environmental stress conditions (Liu *et al.* 2011). Oil pollutants in the soil reduce the roots' ability to absorb water and minerals from the soil (Kaimi *et al.* 2007). Decreased water absorption leads to destruction of plant cell membrane and increased electrolyte leakage of the cells (Liu *et al.* 2011). Destruction of the membrane leads to structural materials of the cell membrane being released, such as lipids. The MDA content shows peroxidation of cell membrane lipids (Smirnoff 1993). Researchers report that crude oil pollution in soil, like other kinds of stressors, increases ROS production, leading to membrane peroxidation (Azevedo *et al.* 2009;

Guo *et al.* 2006; Tatari *et al.* 2018; Wang *et al.* 2011b). This is proven in the case of oil pollution of other plants such as maize (Liao *et al.* 2015). The 2% treatment and control showed the highest (5.35 mg g⁻¹ Fw⁻¹) and lowest (2.31 mg g⁻¹ Fw⁻¹) total sugar, respectively. Furthermore, these two treatments showed the highest (25.78 μmol g⁻¹ Fw⁻¹) and lowest (6.3 μmol g⁻¹ Fw⁻¹) total proline, respectively (Table 4). Osmotic adjustment is the proline amino acid's most significant role against environmental stresses (Slabbert & Kruger 2014; Dezhban *et al.* 2014). Proline content increased by elevating oil concentration in the soil. Increased absorption of water by the roots leads to osmotic adjustment of plants. Also, proline can protect proteins against oxidative stress and remove ROSs from cells (Kaul *et al.* 2008; Samuel *et al.* 2000).

Proline plays a role in cell viability as a signal molecule when plants are exposed to environmental stressors (Liang *et al.* 2013). The accumulation of soluble sugar in plant's leaves also behaves like proline in the osmotic regulation of plants, leading to osmotic modification and increased environmental stress tolerance (Liu *et al.* 2011). Soluble sugar content increases when oil pollutants in the soil arises, which can be considered the plant's response to the lack of water absorption in oil-contaminated soils. Watanbe *et al.* (2000) also confirm the role of sugar accumulation in the osmotic modification of *populous* species in contracting drought stress. Another role that

can be attributed to the accumulation of sugar in plants is the function of sugars as a signal molecule in the face of environmental stressors and the plant's hemostasis regulation (Rosa *et al.* 2009).

Oil pollutant led to reduced protein accumulation. So that, the highest (5.63 mg g⁻¹ Fw) and lowest (3.08 mg g⁻¹ Fw) protein content were obtained by control and 2% treatment, respectively. It was also true for antioxidant capacity. So, the highest (61.75%) and lowest (7.85%) antioxidant capacity were also obtained by control and 2% treatment, respectively (Table 4). Environmental stressors such as oil pollutant by producing ROSs, destroyed the plant's proteins (Cao *et al.* 2017; Liao *et al.* 2015; Wang *et al.* 2011b). In addition, increasing the oil pollutants in the soil, reduces nitrogen uptake (Adam & Dunkan 2002). Nitrogen is one of the most important components of protein production. So, reducing in its absorption, induces decrease in protein production of plants (Novoa & Loomis 1981). Antioxidant capacity is vital to a plant's resistance to environmental stressors by neutralizing ROSs (Lee *et al.* 2007; Moller *et al.* 2007). Contrary to our results, Hernández-Ortega *et al.* (2011) reported that the antioxidant capacity of the *Melilotus albus* increases by elevating oil concentration. However, in our study, the antioxidant capacity of *E. purpurea* decreased because the antioxidant system of the plants was ruined by severe or prolonged stress (Lin *et al.* 2006).

Table 4. Changes of physiological and biochemical indices of *E. purpurea* under four levels of oil contaminated soil, 0 (control), 0.5%, 1% and 2% (w/w).

| Treatment % (w/w) | Leaf anthocyanin (mg L ⁻¹) | Ch _{a+b} (mg g ⁻¹ Fw) | EL (%) | MDA (nmol g ⁻¹ Fw ⁻¹) | Total Sugar (mg g ⁻¹ Fw) | Proline (μmol g ⁻¹ Fw ⁻¹) | Protein (mg g ⁻¹ Fw ⁻¹) | Antioxidant Capacity (%) |
|-------------------|--|---|--------|--|-------------------------------------|--|--|--------------------------|
| 0 (control) | 3.56c | 1.81a | 9.66d | 1.61c | 2.31c | 6.30c | 5.63a | 61.75a |
| 0.5 | 4.06c | 1.82a | 16.66c | 4.63b | 3.97b | 14.22b | 4b | 51.67b |
| 1 | 10.35b | 1.66b | 32b | 5.56a | 4.67a | 25.98a | 3.48bc | 18.94c |
| 2 | 11.58a | 1.33c | 38.33a | 5.42a | 5.35a | 25.78a | 3.08c | 7.85d |

Variation in Leaf anthocyanin, Ch_{a+b}: Total chlorophyll, EL: Electrolyte leakage, MAD: Malondialdehyde, Proline, Protein, Total sugars, Antioxidant capacity; Means comparisons based on tukey's test. Different letters in rows are for significantly different groups (p<0.05).

CONCLUSION

According to the growth, physiological, and biochemical properties of this experiment, at 2% contamination, the phytoremediation ability of *E. purpurea* reduced. At 1% treatment, this plant showed acceptable phytoremediation ability. For this plant, the 0.5% treatment is the best tolerable level of crude oil pollutant. In addition to the phytoremediation ability, *E. purpurea* showed its highest growth and flowering stages at 0.5% contamination. So, it is possible to use *E. purpurea* as an ornamental plant in agricultural fields and green spaces, and it can also tolerate and refine soil contaminated by crude oil.

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پاسخ های فیزیولوژیک و توانایی سرخارگل (*Echinacea purpurea*) در گیاه پالایی خاک آلوده به نفت خام

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چکیده

یکی از مهمترین انواع آلودگی‌ها که انسان در ایجاد آن دخالت دارد، آلودگی آب و خاک به وسیله نفت در کشورهای تولید کننده است. گیاه پالایی فناوری پاک، سریع و سازگار با طبیعت برای پاکسازی خاک آلوده است. به منظور مطالعه اثر خاک آلوده به نفت بر گیاه سرخارگل گونه پورپورا، آزمایشی در شرایط گلخانه با چهار غلظت صفر، ۰/۵ درصد = ۵۰۰۰ میلی گرم در کیلوگرم، ۱ درصد = ۱۰۰۰۰ میلی گرم در کیلوگرم، و ۲ درصد = ۲۰۰۰۰ میلی گرم در کیلوگرم آلاینده نفت خام طراحی شد. صفات مورفولوژیک و فیزیولوژیک بعد از نود روز مورد ارزیابی قرار گرفت. درصد حذف مجموع هیدروکربن های نفتی خاک (TPHs) به وسیله دستگاه کروماتوگرافی گازی تعیین شد. نتایج نشان داد که این گیاه توانایی حذف ۴۵/۵٪ TPHs خاک را در تیمار ۱٪ آلاینده دارد، در حالی که در این تیمار میزان حذف طبیعی تنها ۳۲٪ بود. نتایج به دست آمده از صفات مورفولوژیکی و گلدهی شامل وزن تر اندام هوایی و ریشه، وزن خشک اندام هوایی و ریشه، طول ساقه گل، ماندگاری گل، آنتوسیانین گل و نشانه‌های ظاهری تنش تفاوت معنی داری را بین تیمارها نشان داد. براساس نتایج به دست آمده سرخارگل می تواند غلظت آلاینده نفت خام را در غلظت های ۰/۵ درصد = ۵۰۰۰ میلی گرم در کیلوگرم و ۱ درصد = ۱۰۰۰۰ میلی گرم در کیلوگرم تحمل نماید، ولی در غلظت‌های ۱ درصد و ۲ درصد گلدهی مشاهده نشد. با افزایش غلظت آلاینده‌های نفت خام شاخص‌های فیزیولوژیکی مانند کلروفیل کل، پروتئین کل و ظرفیت آنتی اکسیدانی کاهش یافت، در حالی که دیگر شاخص‌ها مانند آنتوسیانین برگ، نشت یونی، مالون دآلدئید، پرولین و قند کل افزایش نشان داد. بنابراین، از سرخارگل با قابلیت کشت گسترده می توان برای گیاه پالایی خاک‌های آلوده به نفت خام با غلظت مساوی یا کمتر از ۱۰۰۰۰ میلی‌گرم در کیلوگرم خاک استفاده کرد.

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