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Leucanthemum vulgare lam. crude oil phytoremediation

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ABSTRACT

Sites with crude oil pollution have been successfully treated using phytoremediation, but expanding the range of plants that can be used and understanding how exposure impacts the plants are two areas of study that are important to continue. *Leucanthemum vulgare* has been shown to grow well under a variety of stressful conditions. To examine *L. vulgare*'s ability to both survive crude oil exposure and to reduce crude oil concentrations in soil, plants were placed in soil containing 0, 2.5, 5, 7.5, or 10% w/w crude oil. Total petroleum hydrocarbons (TPH) concentration, peroxidase and catalase activity, proline and phenol content in roots and leaves were determined at the start of planting and every 2 months for 6 months. *L. vulgare* roots were successfully colonized with mycorrhizae under all conditions. Results showed positive correlation between antioxidant compound concentration and crude oil contamination. Also, a significant reduction occurred in TPH content of soil over time in planted pots as compared to controls. The lowest TPH content was recorded after 6 months under all treatments. Results showed *L. vulgare* could survive crude oil exposure and enhance reducing of crude oil from soil.

KEYWORDS

Antioxidant; petroleum; *Leucanthemum vulgare*; mycorrhizae colonization; phytoremediation

Introduction

Oil extraction and oil products cause major environmental problems worldwide, particularly in oil producing regions. Crude oil pollution includes total petroleum hydrocarbons (TPH), which is a complex mixture composed of a variety of materials, mainly saturated hydrocarbons, aromatic compounds, asphaltenes, resin, and associated metals. After crude oil release into the environment, there are many ways to remediate the polluted soils, including soil burning, soil washing, and bioremediation (Mohsenzadeh et al. 2010). Bioremediation is the use of any biological system to treat environmental pollution, although it typically denotes bacterial or microbial remediation; phytoremediation specifically looks at the role of plants in treating contamination. Phytoremediation methods tend to cost less than conventional or mechanical methods, and can be more environmentally friendly. Many factors, such as bioavailability of pollutants, environmental conditions (Tang et al. 2012), soil physicochemical properties, population of rhizosphere microorganisms, and plant species affect the rate of phytoremediation (Khoiy and Noori 2012). Efficacy in phytoremediation processes relies on selection of appropriate plants, and criteria must include plant tolerance to the pollutant (Debiane et al. 2011).

Crude oil as an environmental stressor covers the surface of roots and prevents nutrient and water uptake, and as such, can increase the production of reactive oxygen species (ROS) and therefore, oxidative stress in plants (Sun and Zhou 2008). Crude oil can increase ROS production by both direct and indirect mechanisms, including redox reactions with O_2 and ROS, enzyme induction and depletion of antioxidant defenses.

Plant defense mechanism can improve their tolerance against crude oil. Plant antioxidant systems, including several enzymes such as catalase (CAT), peroxidase (POX), glutathione peroxidase, superoxide dismutase, and non-enzymatic antioxidants, such as proline and phenol, prevent oxidative stress and serve as biomarkers of oxidative stress (Pourcel et al. 2007; Ke et al. 2011). These metabolites not only act as antioxidants but also improve degradation processes.

All organisms, including plants, generate ROS, such as hydrogen peroxidase and hydroxyl radicals, as a signal of abiotic and biotic stresses. During phytoremediation, plant compounds such as POX and H_2O_2 are released which can enhance degradation of organic compounds in the soil (Alkorta and Garbisu 2001). The release of enzymes from plants into the soil is not fully understood and needs more investigation. Some organic exudates of plant roots such as phenols, proteins, and organic acids are a source of carbon and nitrogen for microorganisms, which are then able to degrade the organic pollutants (Alkorta and Garbisu 2001; Okoh 2006). Microorganisms can metabolize petroleum hydrocarbons into nontoxic forms, which can then be used by the organisms as a carbon source (Cunningham et al. 1996).

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Mycorrhizal fungi have also been reported to be capable of degrading specific petroleum hydrocarbons (Biro et al. 2005; Tang et al. 2009). It has also been reported that mycorrhizal colonized plants are more resistant to oxidative stresses such as polycyclic aromatic hydrocarbons exposure compared to non-mycorrhizal plants (Debiane et al. 2009).

In a phytoremediation project, selecting the appropriate plant is a key point. However, there is little information about using ornamental plants for phytoremediation. These plants may be of use on sites with lower levels of contamination, and a goal for site reuse during remediation activities. *Leucanthemum vulgare* (formerly known as *Chrysanthemum leucanthemum*) is a perennial, ornamental plant which can grow in disturbed areas. While Ficko et al. (2010) looked at *L. vulgare*'s ability to take up polycyclic biphenyls from polluted soil, there is no report about its ability to impact petroleum contamination. Our previous work showed the ability of *L. vulgare*'s seeds to germinate and grow in petroleum polluted soil (Noori et al. 2014).

While acute toxicity from contact with TPH has been demonstrated with many plants, the principal objective of this research was to determine changes in biochemical properties of *L. vulgare* following exposure. These include changes in POX and CAT activity, and concentration changes of phenol and proline in roots and leaves. Additionally, we studied the ability of the *L. vulgare* to reduce TPH concentrations in soil. Extent of root mycorrhizal penetration and soil mycorrhizal spore population were also examined to determine effects of this plant/fungal symbiosis in the degradation of TPH in soil.

Materials and methods

Soil properties and plant cultivation

Soil was collected from Tarbiat Modares University (TMU) campus at a depth of 0–30 cm. The soil pH was 7.9 and its texture was classified as sandy loam. Soil samples were sieved through a 2 mm mesh. Triplicate plastic pots for each concentration and each time point, containing approximately 1 kg soil, were mixed thoroughly with crude oil (obtained from Tehran Refinery) to obtain concentrations within ±0.01 of 2.5, 5, 7.5, and 10% (w:w), pots of soil with no oil served as controls and pots with no plants served as blanks. The concentration was selected based on *L. vulgare*'s tolerance and oil concentrations typically found in the environment. Our preliminary study showed that *L. vulgare* cannot tolerate concentrations of 12.5–15% crude oil (Data not shown).

One-year-old *L. vulgare* plants were obtained from the TMU greenhouse. Plant roots were washed thoroughly in distilled water and individual plants were placed in each pot. Plants were harvested every 2 months for 6 months including T_0 (start), T_2 (end of second month), T_4 (fourth month) and T_6 (sixth month). The pots without plants (Blanks) were analyzed for TPH content at T_6. The experiment started in February (T_0) and ended in August (T_6).

Examining mycorrhizal extension

Soil naturally contains mycorrhizal spores, therefore, unsterilized soil was used to grow plants and measure extent of mycorrhizal colonization in their roots in the presence of crude oil. Arbuscular Mycorrhizal fungal (AMF) spores were extracted from soil using the wet-sieving and decanting technique of McKenney and Lindsey (1987). AMF spores were isolated from 20 g air dried soil samples by wet-sieving with distilled water through 106, 63 and 38 μm sieves, followed by centrifugation at 1800 rpm/5 min, using a BECKMAN J2-MI (GMI, Ramsey, MN, USA) high speed Centrifuge, Rotor No: 14. This was repeated with the pellet suspended in 10 ml of 40% sucrose. After centrifugation, spores were transferred into Petri dishes and counted in three replications using a stereomicroscope at 100× magnification. Spore abundance was expressed as the number of AM spores per gram soil (Noori et al. 2014).

To measure mycorrhizal colonization, roots were collected at each time point and cleared in 90% KOH, acidified in 10% HCl, and stained with acidic fuschin (Phillips and Hayman 1970). Extent of root mycorrhizal penetration and colonization was determined by the line intersect method (Giovannetti and Mosse 1980) using optical microscopy, briefly described as the intersect of mycorrhizal roots with each line counted as 1 colonization (N), roots without colonization counted as (N_0) and root mycorrhizal frequency (RMF) percentage was calculated as follows:

$$\text{RMF } \% = (N - N_0)/N \times 100$$

Enzyme extraction and assay

All extractions and essays were performed on ice to preserve enzyme activity. For protein and enzyme extractions, 0.2 g root or leaf samples were homogenized with 3 mL potassium phosphate buffer (pH 6.8). Samples were centrifuged at 10,000 rpm for 15 min (BECKMAN J2-MI high speed Centrifuge, Rotor No: 14), and supernatants were used for determining of protein content and enzyme activity. Total soluble protein content of extracts was determined in accordance with Bradford (1976). All spectrophotometric analyses were conducted using a UV/visible spectrophotometer Spekol 2000 (Analytickagena, Germany). The spectrophotometer was calibrated with blanks for each analysis.

CAT (EC 1.11.1.6) activity in root and leaf was estimated according to Cakmak and Horst (1991), by measuring the reduction of H_2O_2 at 240 nm. The reaction mixture was 25 mM Potassium phosphate buffer and 10 mM H_2O_2. The reduction in absorption was followed for 180 sec using the spectrophotometer. The CAT activity did not show significant change after 60 sec, therefore CAT activity differences between 0 and 60 sec were used to determine CAT activity based on protein content.

Peroxidase (POX; EC 1.11.1.7) activity was measured based on the method described by Ghanati et al. (2002), by measuring the increase of H_2O_2 at 470 nm. The reaction mixture consisted of 20 mM Potassium phosphate buffer (pH: 6.8), 28 mM guayacol and 5 mM H_2O_2. The increase in

absorption was followed using a spectrophotometer for 120 sec; POX activity differences between 0 and 60 sec were used to determine its activity based on its protein content.

Determination of proline content

Proline was determined following Bates et al. (1973). Root and leaf tissues (0.5 g of each, separately) were suspended in 10 ml of 3% sulfosalicylic acid and centrifuged at 10,000 rpm for 10 min (system as above). The supernatant was collected, and 2 ml was added to 2 mL of Ninhydrin and 2 ml glacial acetic acid; the mixture was incubated at 100 °C for 1 h. The mixture was extracted with 2 ml of toluene, and absorbance of the toluene phase was measured at 520 nm. The spectrophotometer was calibrated with toluene blank for each analysis. The content of proline was determined using a standard curve.

Determining of total phenol content

Total phenol of leaf and root samples were determined using Folin–Ciocalteu reagent as described by Maizura et al. (2011). Ground root and leaf tissues (0.1 g of each, separately) were placed into test tubes and mixed thoroughly with 5 ml of 10% Folin–Ciocalteu reagent. After 5 min, 4 ml of 7.5% sodium carbonate (Na_2CO_3) was added and the mixture was incubated for 2 h at room temperature. The supernatant was removed, with care to avoid taking up plant tissue, and the absorbance of the total mixture was measured at 765 nm. The spectrophotometer was calibrated with blank for each analysis. The concentration of the total phenol was determined using a standard curve of gallic acid solution (10, 20, 40, 60, 80, and 100 mg/L).

Soil TPH analysis

Soil in each pot was mixed thoroughly and three 100 g samples were collected randomly and mixed together. Sub-samples of this mixture were prepared and analyzed in accordance with U.S. Environmental Protection Agency (EPA) procedures and methods SM-5520D and 5520F to determine TPH. Briefly, 5 g of soil was mixed with 100 mL *n*-hexane. The mixture was extracted in a soxhlet apparatus, at a rate of 20 cycles/h for 4 h. EPA method 5520F was applied to separate petroleum hydrocarbons from the plant's natural oils; extracted oil re-dissolved with 100 mL *n*-hexane with 3.0 g silica gel/100 mg oil added to the solution. The mixture was stirred for 5 min., filtered through Whatman #1 filter paper and the solvent vaporized in a tare dish for gravimetric measurement of petroleum hydrocarbons. TPH was reported as milligram per gram soil.

Statistical methods

Experiments were conducted with three replicates per time point. Data were subjected to one way ANOVA. When statistical difference between the means of the treatments existed, least significant difference test at the 5% level was applied using Statistical Package for the Social Sciences (SPSS; IBM, Armonk, NY, USA) version 19.

Results and discussion

Plant life cycle

Experiments started with plants in their vegetative phase in February (T_0); after 4 months (T_4) when plants were in their reproductive phase (flowering) and after 6 months (T_6) had returned to their vegetative phase. The potential impact of this cycling of the vegetative/reproductive phase is discussed later.

Determining of spore count and extent of mycorrhizal colonization

Germination of spores of mycorrhizal fungi starts when they find appropriate conditions in the soil, which in turn leads to increases in spore numbers. Comparing each treatment at the four-time points showed that at T_2 abundances of spores increased significantly over T_0 with a 33% increase in unexposed soils compared to a 75% in soils exposed to 10% crude oil, with this increase followed by a reduced over time (Figure 1A). Spore population also increased with exposure to crude oil at each time point. The highest spore counts

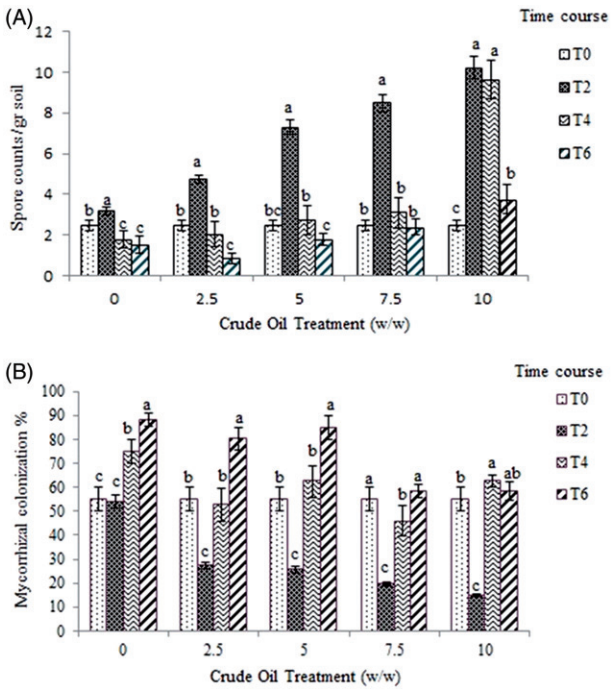

Figure 1. Spore counts in soil (A) and mycorrhizal colonization % in roots of *L. vulgare* (B) exposed to 2.5–10% (w/w) crude oil contamination and 0% as control. One series of samples was used for T_0 as time control. Experiments were repeated every 2 months. Each column represents the average of three repetitions ± standard deviation. The mean difference is significant at 0.05 levels. Means followed by the same letter are not significantly different based on the Duncan test ($p < 0.05$). T_0: at the beginning of experiment; T_2: second month; T_4: fourth month; T_6: sixth month. (A) T_2 had higher number of spores compare to other time records in all concentration. The highest number of spores was counted in 10% crude oil contamination at all the time points. (B) Mycorrhizal colonization was observed in all crude oil treatments. The highest percentage of colonization was observed in T_6 in all treatments.

were seen with 10% (w/w) crude oil exposure in all the time points.

RMF was less at T_2 compared to T_0, potentially due to initial exposure to crude oil, replanting in new soil or changes in ambient conditions between TMU larger general greenhouse where plants were grown to one year old and smaller greenhouse used for these experiments. After 4 months, mycorrhizal colonization recovered gradually and at 6 months, its RMF percentage went back up in all treatments. However, higher concentrations of crude oil (7.5–10%) limited mycorrhizal colonization and it only went back up to initial levels, whereas with control plants and plants exposed to up to 5% (w/w) oil, RMF percentage increased beyond starting levels (Figure 1B). Comparing spore count (Figure 1A) and mycorrhizal colonization percentage (Figure 1B) in second month showed while spores count rose, mycorrhizal colonization dropped. As mentioned earlier, this could be related to root inhibition due to new environmental conditions and the fact that the mycorrhizal fungi had undergone their spore producing phase in the absence of plant roots in the soil, and subsequently, it took time for fungal colonization of new roots under control and crude oil-treated soil to occur, thus the lower colonization percentage was recorded. .

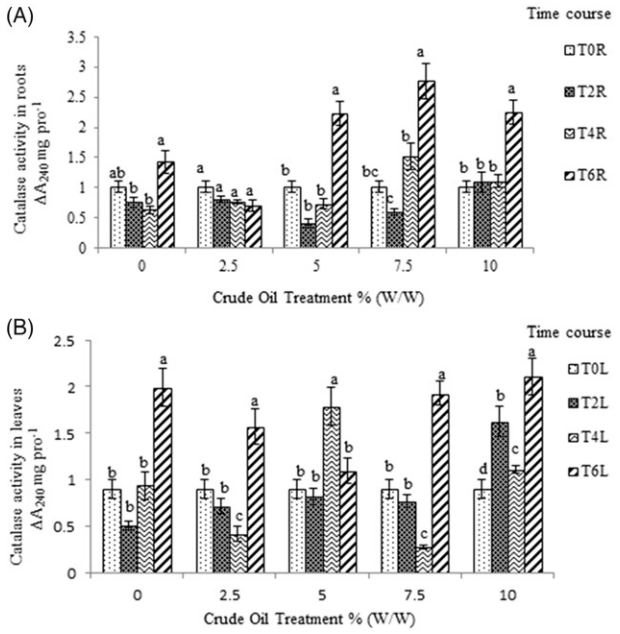

Figure 2. CAT activity in roots (A) and leaves (B) of *L. vulgare* exposed to 2.5–10% (w/w) crude oil contamination and 0% as crude oil control (Figure 1). (A) CAT activity in roots increased after 6 months exposure to crude oil compared to other time points. (B) The highest activity of CAT in leaves was measured after 6 months exposure especially in 10% crude oil.

Enzymatic antioxidant activity

In general, root and leaf CAT activity in plants exposed to crude oil rose significantly ($p < 0.05$) over time. Root CAT activity increased 55, 63, and 55% in 5, 7.5, and 10% crude oil exposure, respectively at T_6 as compare to T_0 (Figure 2A). However, CAT activity in soil exposed to 2.5% crude oil at T_6 did not show significant difference compared to either control plants or T_2 and T_4 plants. Leaf CAT activity improved over time and greater activity was observed in leaves of plants exposed to crude oil as compared to control plants at T_6. Interestingly, there was 42, 63, and 31% reduction of CAT activity in plants exposed to 2.5, 7.5, and 10% (w/w) crude oil at T_4 compared to T_2. CAT activity reduction at T_4 may be related to the life cycle of the plants, as they were in flowering stage at T_4 (Figure 2B). As mentioned earlier, plants spend their energy resources on flowering when in the reproductive phase and thus, potentially produce less antioxidant enzymes.

Although higher root POX activity differed significantly ($p < 0.05$) under all concentrations of crude oil in T_6, there was no significant enhancing in POX activity in roots of plants exposed to 5–7.5% (w/w) crude oil compared to control plants at T_2 and T_4. This indicated that there was a lag time (tolerance period) of about 6 months between exposure time and the plants response to the presence of stressor compounds by increasing its enzymatic antioxidant activity (reaction period). It was also seen that extent of mycorrhizal colonization was greater after 6 months. Interestingly, the mycorrhizal colonization could itself enhanced peroxidase activity in roots (Figure 3A). Leaf POX activity showed significant increase (60–70%) over time, with the highest activity observed at T_6 under all crude oil concentrations

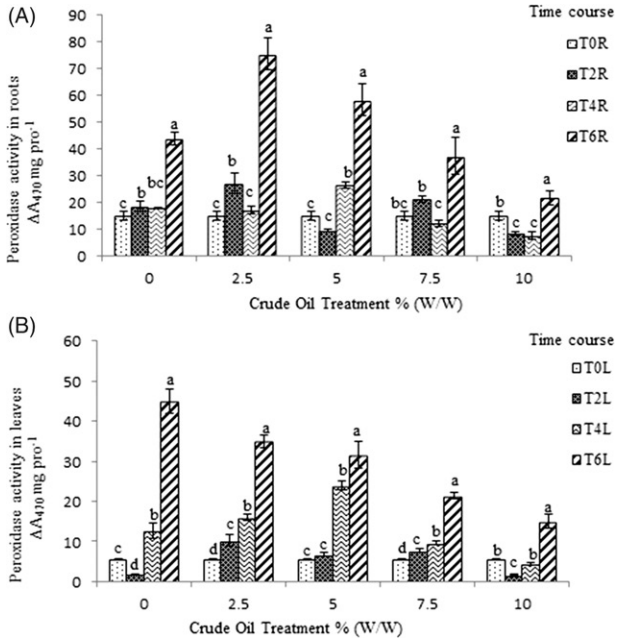

Figure 3. Peroxidase activity in roots (A) and leaves (B) of *L. vulgare* exposed to 2.5–10% (w/w) crude oil contamination and 0% as crude oil control (Figure 1). (A) Peroxidase activity in roots was higher in T_6 in all crude oil treatments. However, its highest activity was in 2.5% crude oil and it reduced by increasing the percentage of crude oil. (B) The result of peroxidase activity in leaves had similar pattern to its activity in roots. Its activity increased during time and the highest activity was at T_6.

compared to POX activity at T_0. Peroxidases are a group of stress-related enzymes that display multifunctional roles and are produced under different physiological circumstances. For example, duration of exposure as discussed earlier for crude oil, the highest activity occurred after 6 months. Root interactions such as mycorrhizal colonizing, though

symbiotic in habit, still a cause of stress more or less as shown in (Figure 3A) and plant age, in this study at the age of 18 months (1-year-old plants exposed for 6 months). Moreover, since POX has other roles in plants, such as in signal transduction as well as its antioxidant activity, sources of change in peroxidase activity (and content) could not be specifically delineated (Figure 3B) and merits a separate investigation.

Proline content

Proline, as a non-enzymatic antioxidant, improves plant tolerance and resistance to stress conditions including biotic or abiotic stresses such as salinity, drought, cold, and osmotic stressors (Rejeb et al. 2014). Root proline content showed significant ($p < 0.05$) increase (50–60%) at 6 months under all treatments and in the control plants, but not at 2 (T_2) or 4 (T_4) months (Figure 4A). Leaf proline content increased 33–42% in T_6 compared to T_0, but not as steady rate. Proline content was lower during the plant reproductive/flowering period (T_4) than that of both vegetative periods (T_2 and T_6). Plant reproduction (flowering) phase is critical for the maintaining of future generations and species survival over time and space, and since flowers are more sensitive to environmental stresses during phase shifts from vegetative to reproductive, plants devote significant amounts of resources, for example proline, toward this effort, leaving less or fewer resources for response to lesser metabolic stress (Figure 4B).

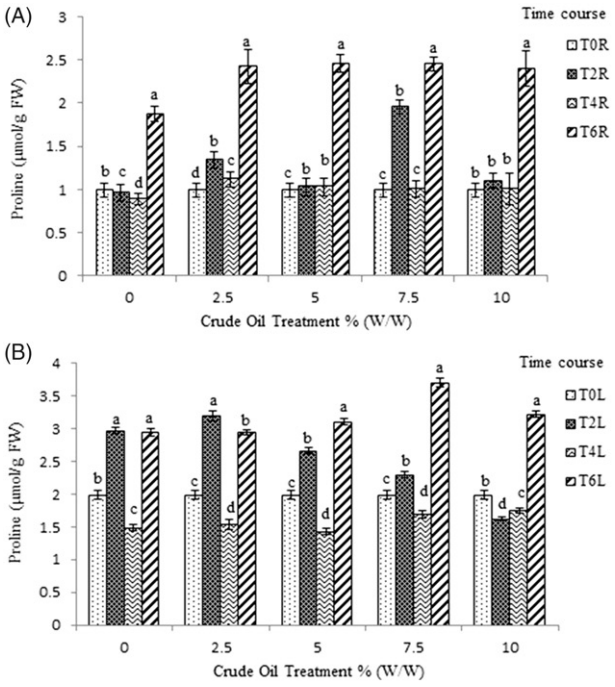

Figure 4. Proline content in roots (A) and leaves (B) of *L. vulgare* exposed to 2.5–10% (w/w) crude oil contamination and 0% as crude oil control (Figure 1). (A) Proline content in roots improved over time and the highest concentration detected was at T_6. (B) Proline content in leaves rose over time in all crude oil concentration. However, there was a significant reduction at T_4 probably due to reproductive phase.

Total phenol content

Phenol is a secondary metabolite which plays a non-enzymatic antioxidant role under stress as well as playing multiple roles in plant metabolism. Root phenol content decreased (20–28%) at T_2 compared to T_0, but recovered after 6 months in 7.5 and 10% (w/w) crude oil treatments by 13 and 4%, respectively. Root phenol content was significantly greater under 7.5 and 10% (w/w) crude oil at T_6 than that of either T_0, T_2, or T_4 (Figure 5A). This emphasizes the role of phenols as antioxidants and their potential to improve root tolerance to exposure to crude oil, particularly at different stages of root or plant development. For example, flavonoids and phenol which are exuded from roots into rhizosphere chemotactically enhance mycorrhizal spore germination followed by initiating symbiosis with roots and subsequent colonization of root tissues. Therefore, phenol reduction at T_2 could be due to mycorrhizal colonization and release of phenol into the rhizosphere prior to, during or subsequent to the impact of colonization. Mycorrhizal colonization usually starts within 2 months after plant cultivation or inoculation, which is concurrent with T_2. Similarly, lowering of phenol content at T_4 in 2.5–7.5% (w/w) crude oil treatments may be because of the plants entering into their reproductive phase. Conversely, plants in 10% (w/w) crude oil treatment showed greater phenol content, and since we did not quantify nor correlate weight of reproductive parts nor seed production with phenol content in any of treatments, it is postulated that plants may have needed more phenol in their roots to remove effects of oil toxicity for survival under higher oil concentration (*e.g.*, 10%). This requires additional investigation to elucidate cause and affect relationships.

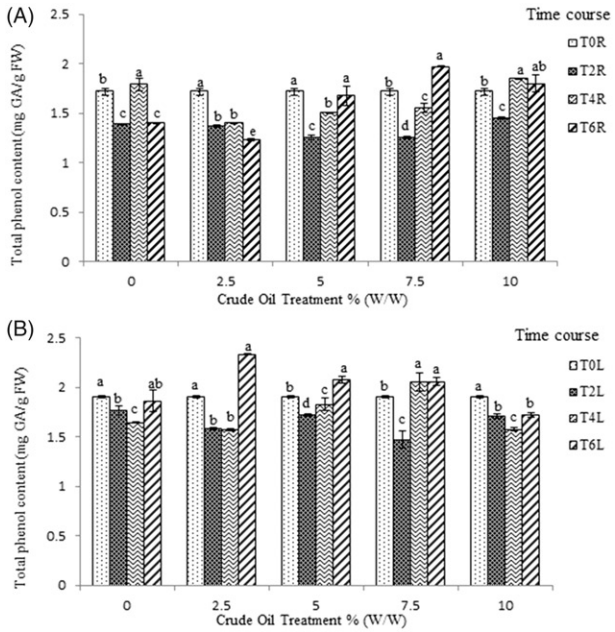

Figure 5. Total Phenol content in roots (A) and leaves (B) of *L. vulgare* exposed to 2.5–10% (w/w) crude oil contamination and 0% as crude oil control (Figure 1). (A) Total phenol content in roots improved in higher concentrations of crude oil over time. (B) Total phenol content in leaves improved in all treatments over time, except in 10% crude oil.

Leaf phenol contents showed similar patterns with increasing (13–48%) at T_6 following reductions at T_2 and T_4. As mentioned earlier, reduction in phenol content in roots in T_2 may be due to phenol releases for mycorrhizal initiation and subsequent penetration and colonization of roots; this loss of phenol from the roots may be replaced with more phenol from the leaves. Regardless, leaf phenol content remains higher compared with root phenol content at all sampling times and in both control and crude oil-treatment plants (Figure 5B). Subsequent studies of phenol synthesis rates in leaves may elucidate the differences in concentration patterns over extended time.

TPH content in soil

Soil TPH content lessened significantly and gradually in all treatments over time. More than half of soil TPH content was removed within 6 months (as much as 13, 34, and 56% at T_2, T_4 [data not shown], and T_6, respectively) while it reduced only 4% in unplanted controls after 6 months (Figure 6). Assuming equal weathering of oil components in planted and unplanted containers, this reduction in TPH content in planted containers as compared to unplanted, showed that *L. vulgare* was able to enhance the decontamination of crude oil over time as much as 50%. Soil microorganism and plant roots remove TPH continuously using excreted enzymes and exudates. This is evident from continuous lowering of TPH in soil even at T_4, which is the plant's reproductive phase, with corresponding reduction in antioxidant synthesis. It seems that mycorrhizal colonization improved this process, while fungi as part of rhizosphere microbial populations have also shown the ability to degrade oil to some extent.

Exploring, extraction and transfer of crude oil and its products lead to its entering into aquatic and terrestrial environments. Crude oil acts as an abiotic stressor to which plants respond in a variety of ways. Some plants are sensitive to petroleum while others, such as *Helianthus annuus*, may tolerate moderate concentrations of crude oil and its

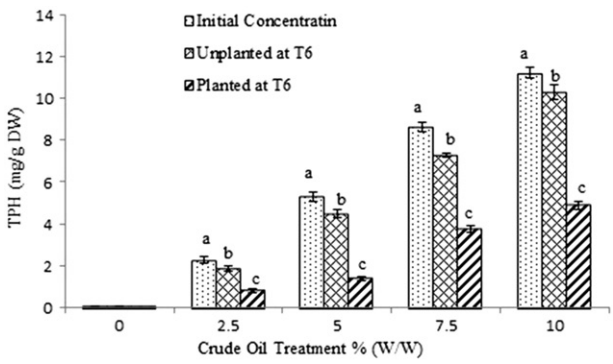

Figure 6. TPH in soils exposed to 2.5–10% (w/w) crude oil contamination and 0% as control in the presence of *L. vulgare* except of Blank pots which did not have any plants. TPH measured at the beginning of experiment, 6 months later in planted pots and unplanted pots. Each column represents the average of three repetitions ± standard deviation. The mean difference is significant at 0.05 levels. Means followed by the same letter are not significantly different based on the Duncan test ($p < 0.05$). TPH reduced significantly in planted pots after 6 months compared to unplanted pots and initial concentration.

products (Frick et al. 1999). In this study, *L. vulgare* was studied to record changes in its physiological parameters under crude oil exposure as well as its ability to enhance removal of crude oil contamination. Nedunuri et al. (2010) study on some native grasses in Ohio, such as Indian Grass (*Sorghastrum nutans*) and Indian Wood Oats (*Unolia latifolia*), showed that vegetated plots had greater TPH removal capacity than un-vegetated control plots. Torres et al. (2005) reported that TPH removal from soil increased over time. This study compared TPH concentrations in pots with plants to those without plants and found that while unplanted pots did lose some TPH over time, those pots with *L. vulgare* had a significantly greater loss of TPH. This shows that *L. vulgare* is able to facilitate the degradation of crude oil pollution from the soil. TPH removal over time in pots with *L. vulgare* ranged from 13% at T_2 to 56% at T_6. At this time, the mechanism of removal is unknown, but could be the result of plant enzymatic and biochemical activities as well as enhancement of the microbial community activity.

RMF percent decreased at T_2 compared to other times while spore counts actually rose. This may be due to crude oil stress slowing down mycorrhizal interaction with roots and instead, encouraging mycorrhizal fungi to primarily undergo spore production and interact later with *L. vulgare* plants. An increase in spore population meant crude oil contamination up to 7.5% did not have negative impact on spore germination and propagation. This is further supported by the fact that at T_4 and T_6, mycorrhizal colonization increased, which demonstrates the fungal tolerance to crude oil. Mycorrhizal fungi, because of their symbiotic behavior, tend to have relatively limited tolerance of contamination. The percentage of mycorrhizal colonization in 10% crude oil after 6 months showed that although mycorrhizal fungal species did tolerate the crude oil exposure, they did not gain tolerance ability over time to high concentrations of crude oil and thus, had lower levels of colonization as compared to lower crude oil concentrations. However, negative correlation between RMF percentage and TPH content in soil showed mycorrhizal colonization is increasing while crude oil degradation is ongoing (Table 1). Mycorrhizal fungi may be able to use carbon from degraded crude oil as a carbon source, therefore reducing crude oil in soil and increasing mycorrhizal colonization as a result of access to increased carbon sources.

Increases in spore counts at T_2 and its subsequent decreasing at T_4 and T_6 compared to T_0 may be due to physiological impacts of long term exposure to crude oil. Ortega-Larrocea (2001) reported increasing periods of exposure to contamination decreased spore populations in soil. Spore propagation is dependent to temperature and season as well, the highest population of mycorrhizal spores is reported to occur in spring and fall (Ghasriani et al. 2007; Noori et al. 2010) with reduced numbers in summer. Sample collection times of T_4 and T_6 were in June and August, where the soil temperature was higher, which probably impacted spore population numbers. Although Zare-Maivan (2013) indicated a role for AM fungi in degrading

Table 1. Correlation between mycorrhizal colonization with reduction in TPH concentration and total phenol concentration in roots and correlation between proline and TPH concentration.

Initial percentage of crude oil in soil	Correlation coefficient of mycorrhizal colonization compared to final TPH concentration in soil	Correlation coefficient of root phenol content (mg/g) compared to percentage of mycorrhizal root colonization	Correlation coefficient of root proline concentration compared to final TPH concentration in soil
0	−0.533	−0.086	−0.291
2.5	−0.587[a]	−0.234	−0.739[b]
5	−0.609[a]	0.771[b]	−0.685[a]
7.5	−0.108	0.905[b]	−0.650[a]
10	−0.435	0.938[b]	−0.785[b]

Correlation between mycorrhizal colonization with TPH concentration and root phenol content and correlation between root proline content and soil TPH concentration in plants exposed to 0, 2.5, 5, 7.5, and 10% crude oil in soil.
[a]Correlation is significant at the 0.05 level (2-tailed).
[b]Correlation is significant at the 0.01 level (2-tailed) with Pearson coefficient correlation.

and adsorbing soil pollutants, additional study is needed to know the role of these fungi under crude oil contamination which would serve to protect the plant roots and organisms against oxidative damage, enhance pollutant tolerance or even improve pollutant degradation.

As ROS are continuously generated in plant cells exposed to stressful environmental conditions, antioxidant defense systems have co-evolved with aerobic metabolism to counteract their oxidative consequences. As such, CAT and POX trigger the conversion of H_2O_2 (a ROS) to H_2O and O_2 (Jeffrey 2002; Debiane et al. 2008). H_2O_2 increases in plant cells under abiotic stress, activating cellular antioxidant enzymes to reduce H_2O_2 damage in cells; those which are not able to reduce H_2O_2 suffer membrane damage, photosynthesis inactivation and finally, death. CAT and POX activity improved in the current study over time, which explains *L. vulgare*' s ability to activate its antioxidant system under conditions of crude oil exposure to minimize oxidative stress and protect the plant cell membrane against ROS. Although, it has been reported that CAT is more active than POX under environmental stresses (Shikanai et al. 1998), both enzymes showed an increase in roots in plants exposed to 5–10% crude oil exposure over the 6-month time period. Such hike in antioxidant enzymatic activity has also been reported in Mangrove species exposed to petroleum compounds (Moreira et al. 2011).

Ke et al. (2011) reported positive correlation between crude oil concentration and enzymatic antioxidant activity which is in agreement with our findings. A negative correlation exists between the levels of CAT and POX activity and TPH concentration over time, (Data not shown) suggesting that enzyme activity increases result in enhanced degradation of TPH.

Proline which is an amino acid known to confer osmotic tolerance during stress conditions (Kavi Kishor et al. 2005). Proline synthesis is dependent on multiple factors, one of which is the carbohydrate pool in the plant cells. Increases in plant carbohydrate levels results in increased proline synthesis, which in turn produces $NADP^+$ for the pentose phosphate pathway. This plays a critical role in the Shikimate pathway, phenol synthesis, photosynthesis, and respiration (Delauney and Verma 1993; Kavi Kishor et al. 2005). Proline content in the plants increased significantly under all treatments at T_2 and T_6 compared to T_0, which supports its role in oxidative stress response. Proline content decreased at T_4 which may be due to the plants being in their reproductive phase at that time point. Plants devote significant resources to ensure successful seed production, therefore it is normal to limit their activity against oxidative stress. Negative correlation between proline and TPH content in the 2.5–10% crude oil exposed plants support this finding that proline protects plant against crude oil contamination (Table 1).

It has been proposed that phenolic compounds also serve as a ROS detoxification system and a backup defense mechanism of vascular plants (Sakihama et al. 2002). Total phenol increased at T_6, especially in roots of plants exposed to 5–10% crude oil and in leaves of plants exposed to 2.5–7.5% crude oil, which supports the theory that phenol compounds increase in plants under oxidative stress. On the other hand, phenolic compounds have been known for their role in mycorrhizal symbiosis signaling (Ponce et al. 2009; Noori et al. 2014) which is supported with findings of positive correlation between total phenol content in roots and their mycorrhizal colonization in 5–10% crude oil treatments (Table 1).

Conclusion

This study showed that *L. vulgare* can tolerate up to 10% crude oil contamination while mycorrhizal fungi colonization became limited at crude oil concentrations over 7.5% (w/w). Plant metabolism changed under crude oil exposure, which caused difference in enzymatic antioxidant activity and non-enzymatic antioxidant compound concentration in roots and leaves. These changes improved the plant's ability to tolerate crude oil exposure and remove oil in collaboration with AM fungi. The conclusion is that *L. vulgare* may be a candidate for phytoremediation of crude oil contaminated soils. Further research is needed to elucidate the exact mechanisms of degradation in the natural environment.

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