

Understanding Biochemical Defense of *Leucas Aspera* in Crude Oil Polluted Habitat and Changes in Soil Properties

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Abstract

The response of an indigenous medicinal herb *Leucas aspera* in crude oil polluted habitat was studied. The productivity, antioxidants, phytochemical and functional group profiles of the plant species in stress conditions were investigated. Besides, changes in enzymes, beneficial bacterial populations and physico-chemical conditions and total oil and grease (TOG) contents in the contaminated soils were also studied. The results showed improvement in physico-chemical conditions, increase in beneficial bacterial population (4.1-5.4 folds) and decrease in TOG (31.3%) level of the contaminated soils by end of the experimental trials. The activities of dehydrogenase, urease, alkaline phosphatase, catalase, amylase and cellulase have increased in the range of 23.6-174.4% after introduction of *L. aspera* in the contaminated soils. Further, there were significant variations in leaf area index, chlorophyll and biomass contents of the experimental plant as against the initial level and control. Besides, the results also revealed significant deviations in the antioxidant and phytochemical profiles of *L. aspera* suggesting the enzymatic defense of the plant species in the crude oil contaminated soils. The fourier-transform infrared (FT-IR) analysis confirmed the uptake and metabolism of some hydrocarbon components by the experimental plant from the contaminated soils.

1. Introduction

Crude oil pollution is a burning and perennial problem amongst the oil producing nations around the globe. The release of crude oil creates an extreme hydrophobic condition in the contaminated land and ultimately causes deterioration in physical, chemical and biological properties of soil. It has been heavily reported that microbial diversity and plant's productivity decreases very rapidly in the oil contaminated sites (Wang et al. 2011, Jiao et al. 2018). The concerns about crude oil pollution has increased considerably during last few decades as the problem is also associated with the contamination of agricultural lands/ecosystems. Crude oil contamination in the agricultural fields negatively affects the crop production (Basumatary et al. 2013) and even severe contamination makes the land unsuitable for further cultivation (Croat et al. 2020). Thus, land degradation due to crude oil contamination has become a global problem (Jiao et al. 2018) and therefore, restorations strategies are necessary in order to overcome the crisis and to enhance the productive land.

Phytoremediation has long been considered as a suitable, eco-friendly, cost effective approach for remediation of crude oil contaminated habitats. The effective use of several species of grasses, legumes, sedges and others has been reported against crude oil pollutants by several workers from time to time (Basumatary et al. 2013, Asemoloye et al. 2017). For example, the plant species such as *Lolium multiflorum*, *Medicago sativa*, *Lotus corniculatus*, *Cynodon dactylon*, *Bidens cernua*, *Zea mays*, *Leptochloa fusca*, *Brachiaria mutica* and others have been used for remediation of hydrocarbons contaminated soil (Tara et al. 2014, Fatima et al. 2018). Nevertheless, selection of suitable plant species is very crucial for successful phytoremediation. Several factors such as resistance to the contaminants or pollutants, tolerance to the adverse environment and low nutrients condition, ability to support diverse microbial population, ability/adaptability for diverse soil types/contaminants, low bioaccumulation and

trophic transfer potential are associated with the selection of suitable plants for phytoremediation trials (Cook and Hesterberg 2013). Nevertheless, better adaptability, robust growth after establishment, presence of fibrous and extensive root systems (Cook and Hesterberg 2013) confirms the suitability of herbs and grasses against the crude oil pollutants when compared with trees. There are numbers of report regarding the better adaptive response of grasses/herbs in crude oil polluted soil as against the tree seedlings or saplings (Chakravarty and Deka 2021, Ali et al. 2013, Basumatary et al. 2012a,b) and accordingly several workers have established them as the suitable candidates for phytoremediation practices (Adesodun et al. 2010, Salazar and Pignata 2014).

Nonetheless, the very success of any phytotechniques depends on the survivability or adaptability of the plants to the local conditions. Crude oil pollutants usually create an oxidative stress in the plants and to overcome the stress plants utilize their antioxidant defense systems (Han et al. 2016, Odukoya et al. 2019). The increasing antioxidant activity of the plants in the crude oil contaminated environment may trigger the cellular defense systems that can be crucial for the plants to cope up with the pollutants and to protect the cells from internal injuries (Ruslan et al. 2018). Therefore, understanding the plant's enzymatic defense is a very crucial step for selection of suitable candidates for phytoremediation trial against crude oil contaminations.

The herb *Leucas aspera* is found to grow abundantly in some of the crude oil polluted sites of India. The reports on phytoremediation potentiality of this herb are very scanty. Here, it is assumed that *Leucas aspera* poses some adaptive advantage to survive in the oil polluted soil which could be related to the antioxidant defense mechanisms of the plant. Hence, in the present investigation enzymatic defense of *Leucas aspera* in the crude oil polluted soil has been studied. In addition, phytoremediation potential of the herb was investigated in terms of dissipation in total oil and grease contents, changes in soil physicochemical and enzyme profiles and beneficial bacterial population. Further, the shift in functional group profiles of the herb species has also been investigated during the study for better understanding in uptake as well as metabolism of oil pollutants.

2. Materials And Methods

2.1 Site description and collection of soil samples and the experimental plant

The Lakowa oil field (latitude 21.0166° and longitude 94.8333°) of Assam, India has been selected for collection of contaminated soil samples. About 200 kg of surface soil at a depth of 0-15cm were collected from the agricultural fields that were heavily contaminated with crude oil. The soil samples were air dried in shade, lumps were broken, debris were discarded and finally sieved (mesh size 2mm) for use in the experimental trials.

The medicinal herb, *Leucas aspera* was selected as the experimental plant as it was found abundantly in the polluted sites of the Lakowa oil field. However, the seeds of this plant were collected locally from

Gauhati University campus, Guwahati, Assam India and grown in well prepared soil bed for use in the experiment.

2.2 Experimental Setup

The pot experiment was carried out in net house under natural condition with two treatment trials. In case of first treatment, *Leucas aspera* was introduced in the contaminated soil whereas in the second treatment the experimental plants were grown in non-contaminated soil to compare the different plant growth parameters in contaminated and normal soil conditions. A control set up without plants was also continued in the contaminated soil. In each case three replicas were maintained for statistical comparison of the results. The details of the treatments of 60 days durational experiment have been presented in the Sect. 2.2.1. Each experimental pot was filled with 200g of processed soil, added with distilled water and finally kept in shade for three days. After three days, seedlings of *Leucas aspera* of average size of 9.03 cm height containing 5 numbers of leaves were introduced to the pots. Each pot was planted with a single plant. The physico-chemical properties, beneficial bacterial population and enzyme profiles of the contaminated soil were analyzed and compared with initial level. After completion of the experiment, productivity parameters and antioxidant properties of the harvested plant samples were also analyzed.

2.2.1 Treatments details of the experiment

T1 = Crude oil contaminated soil (Initial), T2 = Crude oil contaminated soil minus *Leucas aspera*

T3 = Crude oil contaminated soil + *Leucas aspera* T4 = Non-contaminated soil + *Leucas aspera*

2.3 Analysis of soil samples

2.3.1 Physico chemical and total oil and grease (TOG) analysis

The physicochemical characteristics includes the analysis of pH, conductivity, water holding capacity (WHC), total organic carbon (TOC), total kjeldhal nitrogen (TKN), available phosphorus (AP), total potassium (TK) and C/N ratio. For analysis of pH and conductivity 1:5 (w/v) soil and water suspension was prepared and values were obtained in digital pH (Universal 6331) and conductivity meter (Systronics 2485) respectively. Water holding capacity of the samples was determined by using the Keen-Raczkowki's boxes (Piper 1944). Walkey and Black titration method as outlined by Jackson (1975) was used for the estimation of total organic carbon (TOC) values of the samples. Similarly, micro kjeldhal method was employed for estimation of total nitrogen contents in the samples (Jackson 1975). Available phosphorus of the samples were evaluated by using the spectrophotometer (Shimadzu UV 1601) following the stannous chloride method (APHA 1998). Likewise, total potassium contents in the samples were determined by acid digestion method using flame photometer with standard solution (APHA 1998). After calculating the total organic carbon and total kjeldhal nitrogen, the C/N ration of the respective samples

were determined. Again, total oil and grease (TOG) was measured by gravimetric method using dichloromethane (DCM) as the solvent in the soxhlet extractor (Basumatary et al. 2012b, Patowary et al. 2016). The extracted solution was transferred into 100 ml beaker and kept to evaporate until the constant weight and finally the amount of the extracted TOG was calculated.

2.3.2 Determination of enzyme activity and beneficial bacterial population

Soil enzymes such as urease, dehydrogenase, polyphenol oxidase, peroxidase, catalase, cellulase, amylase and alkaline phosphatase activities were analyzed by adopting standard methods. The urease activity was determined by the method of Hoffman and Teicher (1961). Method as outlined by Casida et al. (1964) was used to analyze dehydrogenase activity Cellulase and amylase activities were determined by employing the methods of Pancholy and Rice (1973) and Cole (1977) respectively. Again, methods of Johnson and Temple (1964) were used to measure the activity of catalase enzyme. Alkaline Phosphatase activities were analyzed by employing the method of Tabatabai and Bremner (1969) whereas polyphenol oxidase and peroxidase were measured by the standard protocols of Bach et al. (2013) and German et al. (2011). The total population of nitrogen fixing, phosphate and potassium solubilizing bacteria in the soil samples were enumerated in Jensen's medium, Pycovskaya's and Aleksandrow agar respectively following serial dilutions technique as outlined by Chakravarty and Deka (2021).

2.4 Study of plant samples

The study of plant samples take account of the antioxidant assays, phytochemical profile and productivity parameters. The antioxidant study includes the analysis of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, hydrogen peroxide (H₂O₂) radical scavenging and reducing power activities whereas phytochemical profiling includes the investigation on total phenol and flavonoid contents of the plant samples. The method as outlined by Brand-Williams et al. (1995) was used for analysis of DPPH radical scavenging activities of the plant extracts. For determination of H₂O₂ radical scavenging activities the method given by Ruch et al. (1989) has been employed. The method as outlined by Oyaizu (1986) was used for analysis of reducing power activities of the plant samples. The methods of Hagerman (2002) and Jay et al. (1975) were employed respectively for estimation of total phenol and flavonoid contents of the plant extracts. The productivity parameters of *L. aspera* were analyzed before and completion of the experimental trials in terms of Leaf area index (LAI), estimation of chlorophyll and dry biomass employing the methods outlined by Chakravarty and Deka (2021).

2.5 Fourier Transform Infrared spectroscopy (FT-IR) analysis

The Fourier transform infrared (FT-IR) spectrophotometer (Nicolet 6700) was used to obtain the spectra of the experimental plant samples. The protocol as described by Haghollahi et al. (2016) was followed for preparation of plant samples. In brief, the powdered plant samples (1 mg of each) were mixed with 100 mg KBr (Potassium bromide) in a ratio of 1:100, then it was homogenized in an agate mortar and

pressed and finally pellets were prepared. The spectra of the samples were obtained in the mid-infrared area within the range of $4000-400\text{ cm}^{-1}$.

2.6 Statistical Analysis

The statistical comparisons were done in SPSS software (2018 version). The differences in the values of both soil and plant samples for the studied parameters were evaluated by paired *t-test*, one way ANOVA analysis and LSD *test*.

3. Results And Discussions

3.1 Changes in the soil characteristics

3.1.1 Changes in physico-chemical parameters

The physicochemical properties of the crude oil-contaminated soil samples were analyzed at the beginning and by end of the experimental trials and the results are presented in Table 1. Initially, the pH level in the contaminated soil was found to be 4.235 ± 0.067 which had shifted to 5.734 ± 0.065 in T3 after 60 days of treatment with *Leucas aspera*. However, such denoting change in pH value was not evident in T2 (Table 1) which was kept as control. The shifting in pH towards less acidic condition after plant's treatment could be attributed to the mineralization of oil components in soil. It has been suggested that introduction of plants in contaminated soil enhances the microbial activities which further accelerate the mineralization process in soil (Chakravarty and Deka 2021). The present finding on pH deviation agrees the report of Ukaegbu-Obi and Omeh (2014) who have pointed out that mineralization of crude oil components is responsible for reduction in soil acidity thereby confirming the biodegradation of hydrocarbons in the contaminated soil. Similarly, the soil conductivity and water holding capacity values have also increased significantly in T3 when compared with the initial level (T1). Thus, the increase in conductivity level was found to be 224.051% in T3 as against the minimal aberration in the values in T2 (Table 1). The changes in conductivity might be associated with the anaerobic metabolism of oil pollutants in presence of electron acceptors such as nitrate ions (Osuji et al. 2007). Conductivity is an important parameter of soil as it provide useful information on soil texture and water content which further helps in crop selection and identification of potential areas for improved irrigation and drainage management, and finally provide a means of monitoring spatio-temporal changes in soil properties that potentially influence crop production (Corwin and Lesch 2003, Arshad and Martin 2002). The conductivity values within the range of $0.11-0.57\text{ mS/cm}$ is reported as satisfactory for agricultural practices (Chakravarty and Deka 2021). In T3, there were 4.9 folds increases in water holding capacity (WHC) value when compared with the initial level as found in T1. No remarkable changes were observed in WHC value in T2 which was kept unvegetated. Crude oil is a nonpolar liquid which usually absorb moisture from soil and decreases the WHC level in soil (Devatha et al. 2019). The enhanced values of WHC in T3 could be attributed to the plant-microbe interactions which helps in degradation of oily layer and/or release of ions in soil system and thus improves the water retention capacity of the soil (Chakravarty and Deka 2021).

The TOC was reduced by 11.7% in T3 and 4.8% in T2 from its initial level as found in T1. The greater reduction in TOC in T3 indicates higher and rapid mineralization of organic carbon fraction in presence of plants (Edwin-Wosu 2013). Moreover, the reduction in TOC could also be attributed to either utilization of organic carbon by microorganisms or the release of carbon in the form of CO₂ during microbial respiration (Mrayyan and Battikhi 2005) and this process usually accelerated in presence of plants. As against the increase in available P and total K, the total N content had decreased in both T3 and T2 samples when compared with T1. The decrease of total N was found marginal in T2 but it was 1.703 folds in T3. Reduction in total nitrogen along with carbon could be related to carbon mineralization and hydrocarbon immobilization resulting in excessive microbial activities using carbon materials as an energy source and its attendant demand for more nitrogen (Devatha et al. 2019). Moreover, the observed increment in urease levels might be the reason of potential of microorganisms to use ammonium as a nitrogen source for biodegradation of crude oil pollutants (Dindar et al. 2015). The increase in available P and total K was recorded as 1.8 folds and 1.5 folds respectively in T3 samples. Similarly, T2 samples showed 1.2 folds increase in available P and total K over T1. Different physiological and enzymatic process in soil system helps the plants to uptake the nutrients. The increase in available P and total K may be because of two reasons. First one is the poor uptake of these nutrients by the *Leucas aspera* and the second one is increasing number of P and K solubilizing microorganisms in both T2 and T3. There were marginal changes (6.02%) for C/N ratio in T2 after 60 days of experimental periods (Table 1). The higher decrease in C/N ratio (43.948%) of the oil contaminated soil treated with *Leucas aspera* indicates that plants and/or rhizosphere microbes utilizes both the elements for growth and development (Basumatary et al. 2012a).

3.1.2 Changes in total oil and grease (TOG) levels

The TOG contents of the contaminated soil samples have been presented in Table 1. The results revealed that TOG dissipation was found to be 31.3% in T3 (i.e. vegetated treatment) over the initial level (T1) after 60 days of experimental periods. There was no significant reduction in TOG level in T2 when compared with the initial value of T1. Thus, results showed that the experimental herb *Leucas aspera* has the ability for removal of TOG from the crude oil contaminated soil. The reduction in TOG in T3 samples may be due to the activities of rhizospheric microorganisms and/or uptake the oil components by the experimental plant. The present finding corroborated with the previous findings of Peng et al. (2009) who have reported that about 63.2% dissipation in TOG is possible by employing *Mirabilis Jalapa* in the crude oil contaminated soil. Similarly, Muratova et al. (2008) and Razmjoo and Adavi (2012) have been reported upto 52% and 40% of TOG reduction using rye cultivation over 3 years and Bermuda grass for 6 months of experimental trials respectively. In another study, Akram and Deka (2021) reported about 60–74% reduction in TOG contents by employing *Ageratum conyzoides*, *Polygonum hydropiper* and *Xanthium strumarium*. The less dissipation in TOG (31.3%) as revealed in this study could be related with the time duration (60 days) of the experiment. It has been suggested that both quantitative and qualitative alteration of plant exudates is possible with plant's age. This affects the population and activity of hydrocarbon degrading microorganisms in the rhizosphere which ultimately influence the TOG dissipation rate in the contaminated soil (Zand et al. 2010).

3.2 Changes in soil enzyme activity and beneficial bacterial population

Soil enzymes are reliable indicators used for evaluation of eco-environmental qualities after phytoremediation trial (Liu et al. 2017). The soil enzymes play an important role in improving soil fertility by catalyzing several vital reactions necessary for organic waste decomposition, organic matter oxidation and accelerates the growth of physiologically active microorganisms and thus provide correlative information on biological activities and microbial populations in soil systems (Dindar et al. 2015). The results of soil enzyme activities obtained from the experimental trials are presented graphically in Fig. 1a, 1b and 1c. The results showed increase in the activities of soil dehydrogenase, urease, alkaline phosphatase, catalase, cellulase and amylase but decrease in peroxidase and polyphenol oxidase levels in the T3 samples by end of the 60 days. In case of T2 samples there were marginal changes in all the studied enzymes. The enhancement were found to be 32.3%, 102.8%, 174.4%, 68.5%, 76.16% and 23.6% respectively for soil dehydrogenase, urease, alkaline phosphatase, catalase, amylase and cellulase activities in the T3 samples over the initial levels as obtained in T1 (Fig. 1a, 1b, 1c). Similarly, the reduction in peroxidase and polyphenol oxidase activities was 27.4% and 83.43% (Fig. 1c) when compared with initial level. The present findings are in accordance with the previous works (Tu et al. 2011, Yenn et al. 2014, Liu et al. 2014) where enhanced levels of dehydrogenase, catalase, urease and alkaline phosphatase have been reported after phytoremediation trials of crude oil contaminated soil. The enhanced level of enzymes in T3 samples could be attributed to higher level of oxygen in the rhizosphere zone that results a higher population of aerobic microbes (Wang et al. 2008). Besides, it has also been suggested that root exudation alter the microbial community structure in the rhizosphere which in turn enhance the enzyme activities in the vegetated treatments (Falchini et al. 2003, Baudoin et al. 2003). Usually plant's roots releases large numbers of compounds such as polysaccharides, aromatic compounds, and esters that can serve as sources of substrates and provide energy and elements for enzyme synthesis in soil (Sun et al. 2013). Further, root exudation process is also strengthened and accelerated successively when plants are exposed to abiotic stress such as pollutants (Chen et al. 2017, Zeng et al. 2018). As a whole, introduction of *Leucas aspera* may gradually reduce the toxicity of pollutants on soil enzymes (Duan et al. 2018) and accelerate enzyme synthesis process by enhancing microbial activities. On the other hand, the decline in peroxidase and polyphenol oxidase could be due to two reasons; firstly, formation of intermediate compounds during the remediation process that restricts the growth of specific microbial group responsible for producing these two enzymes and secondly, the activities of both the enzymes perhaps determined by the experimental plant (Panchenko et al. 2017). Nevertheless, further planned study is still needed to explore the proper reasons.

The results of beneficial bacterial population that includes total numbers of N fixers, P and K solubilizer are presented in Table 2. The results are expressed as colony-forming unit per gram of soil (CFU g⁻¹ soil). The results revealed that enhancement in total population of N₂ fixing bacteria were 4.3 folds in T3 as against 1.2 folds in T2 samples by the end of the experimental period. Similarly, for P and K solubilizing bacterial population the increase was 5.4 folds and 4.1 folds respectively in T3 treatment. In T2 samples,

increase was recorded 1.1 fold for both P and K solubilizer population by 60 days of experimental trials. The present finding shows the conformity with the earlier works (Basumatary et al. 2012a) where it was reported that about 6.1 folds increase in beneficial microbial population is possible in the plant's treated contaminated soil. As mentioned above, several compounds such as monosaccharide, amino acids, enzymes and other organic acids are released by roots that stimulate the growth of specific microbial communities (Crarela et al. 2000; Zeng et al. 2018). Besides, it can be hypothesized that introduction of *L. aspera* in the contaminated soil may initiated hydrocarbon mineralization/degradation and had generated several intermediates/metabolites which further perhaps acts as the nutrients for the growth of specific group of bacteria and enhanced their population by end of the phytoremediation trial.

3.3 Changes in plant characteristics

3.3.1 Changes in plant productivity parameters

The results of plant productivity parameters that include leaf area index (LAI), chlorophyll and biomass contents are shown in Table 3. The results showed significant changes in LAI, chlorophyll and biomass contents of the experimental plant in both T3 and T4 samples than the initial levels. The LAI values were enhanced significantly and recorded to be 1.7 folds in T3 and 2.4 folds in T4 samples. While, there was a significant reduction for chlorophyll a, b and total chlorophyll values by 1.2, 1.04 and 1.3 folds in T3 samples; in case of T4 plant samples, these values were enhanced by 1.2, 1.4 and 1.2 folds respectively by end of 60 days. The result for dry biomass of shoots and roots also showed a significant increase in both the samples of T3 and T4 when compared with the initial levels. The increase was recorded to be 3.8 folds and 3.1 folds in T4 and 2.5 folds and 2.8 folds in T3 for shoot and root samples. The lower values of biomass, LAI and chlorophyll in T3 plant samples could be related with the reduced plants growth in the oil contaminated soils. Crude oil contamination alters physico-chemicals conditions, water and nutrients availabilities in soil and adversely affects plants productivity (Chakravarty and Deka 2021). The poor growth of *L. aspera* in T3 decreases leaf length and width which in turn reduces the surface area of leaf available for photosynthesis (Augustina et al. 2015). It has been suggested that water and nutrients deficiency conditions of crude oil contaminated soil cause reduction in LAI and chlorophyll contents in *L. aspera* and brings difficulties in transpiration as well as photosynthesis and finally reduces growth and biomass (Akapo et al. 2011, Njoku 2012). The present findings are in accordance with various previous works where it was reported that decrease in chlorophyll levels are associated with photosynthetic functioning, transpiration and retarded growth in plants and all these processes evidently accelerated in the crude oil contaminated habitats (Han et al. 2016, Hussain et al. 2019).

3.3.2 Changes in plant in vitro antioxidant activities and phytochemical contents

The *in vitro* antioxidant profiles of *L. aspera* that includes DPPH and H₂O₂ free radical scavenging activities and reducing power assay are graphically represented in Fig. 2. The butylated hydroxytoluene (BHT), was taken as the reference compound for comparison of *in vitro* antioxidant results. On the other hand, the results of total polyphenol and flavonoid contents of the experimental plant are shown in

Table 3. A concentration dependent scavenging activity against DPPH and H₂O₂ radicals were observed in the sample extracts of the experimental plant. The plant extract from T3 showed a lower IC₅₀ value of 67.89 ± 2.08 µg/ml for DPPH scavenging when compared with the T4 (89.34 ± 0.963 µg/ml) samples. The IC₅₀ value of the standard solution of BHT was recorded as 41.39 ± 0.652 µg/ml. A similar trend of result was also found for H₂O₂ free radical scavenging activity. The lower IC₅₀ value (35.27 ± 3.081 µg/ml) was noticed in case of T3 sample as against its counterpart growing in T4 (48.6 ± 1.703 µg/ml). The standard BHT showed IC₅₀ value of 15.564 ± 1.02 µg/ml. A direct correlation between IC₅₀ value and free radical-scavenging activity of the plant extract has already been established by earlier researchers. It was suggested that the lower IC₅₀ values for antioxidant under stress conditions indicate higher free radical-scavenging activity of the plants (Okoh et al. 2014, Bouterfas et al. 2016). The increase in DPPH free radical scavenging activity is an index of inhibition in lipid peroxidation which indicates the oxidative stress condition in plants. Further, for the reducing power potential, the absorbance value was found to be increased with increasing concentration of methanolic plant extracts. The order of the result was found as- T4 < T3 < BHT (Fig. 2) which indicates the defense mechanism of the experimental plant during the stress condition exerted by crude oil contaminations. The present findings corroborated with the earlier works (Basu et al. 2010) where it was reported that increase in reducing power of plant species are associated with extreme stress condition. It has been suggested that interactions of antioxidants such as reducing power, DPPH, H₂O₂ free radicals with reactive oxygen species (ROS) or other high level free radicals restrict oxidative stress (OS) in plants by inhibiting lipid peroxidation and improve strong resistance against potential cell injuries (Gupta and Sinha 2009, Chakravarty and Deka 2021, Boruah et al. 2020). Therefore, elevated antioxidant level of *Leucas aspera* in T3 samples as revealed in the present study is a clear indication of strong defense of the plant in stress condition to prevent lipid peroxidation. Further, enhanced level of phenol and flavonoid concentrations of the experimental in T3 over T4 samples could be related with the higher antioxidant activities (Gheldof and Engeseth 2002, Holasova et al. 2002). It has already been established that phenolic compounds are linked with protection of living cells from the cytotoxic effects of H₂O₂ (Nakayama 1994). Moreover, phenolic compounds also possess free radical scavenging properties and flavonoids are reported as superoxide (.O₂⁻) and hydroxyl (.OH) radical scavengers (Tremel and Smejkal 2016, Govindan and Muthukrishnan 2013). Hence, it can be concluded that higher phenolic and flavonoid contents in the plant samples of T3 may perhaps involved in the removal of free radicals from the plant parts.

3.3.3 FT-IR analysis

The main absorbance bands and peaks of FTIR spectra of T3 and T4 plant samples have been presented in Table 4. It showed various common bands and peaks in both T3 and T4 plant samples that represents different functional groups. However, there were differences in terms of peaks/bands sharpness as well as intensities between T3 and T4 samples. The probable functional groups represented by peaks/bands have been confirmed on the basis of earlier reports by several researchers (Devatha et al. 2019, Dominguez-Rosado and Pichtel 2004, Bobby et al. 2012). The broad band in between 3630–3100 cm⁻¹ indicating O-H stretching of flavonoids/phenolic compounds was found in both T3 and T4 samples.

However, this region was comparatively more constrict and intense in T3 plant samples than T4 (Table 4). Similarly, the bands at 2925 cm^{-1} and 2853 cm^{-1} confirming C-H stretching of methylene were also found to be intense in T3 when compared with T4 sample. The peak at 1738 cm^{-1} due to C = O stretching of aldehydes were more clear and intense in T3 than T4 samples. Again, a peak at 1637 cm^{-1} indicating C = C stretching of alkenes more likely to be cyclohexane was apparent and distinct in T3 sample. The peak related to N = O symmetric stretching of 1-nitrohexane appearing at 1385 cm^{-1} was found in both T3 and T4 samples although intensities was marginally higher in T3. Another two peaks at 1321 cm^{-1} and 1262 cm^{-1} indicating C-F stretching of fluoride/polyflouroalkanes were found with marginal higher intensity in T3 when compared with T4 samples. Again peaks and bands at 1109 cm^{-1} , 1078 cm^{-1} , and 1025 cm^{-1} from T3 and 1109 cm^{-1} , 1058 cm^{-1} and 1034 cm^{-1} has indicated C-O stretching of alcohols/ethers/esters/carboxylic acids. Here, the peaks of 1058 cm^{-1} and 1034 cm^{-1} in T4 may be shifted towards 1078 cm^{-1} and 1025 cm^{-1} in T3. The three more peaks at 782 cm^{-1} (representing C-Cl stretching of aliphatic chlorides); 616 cm^{-1} and 520 cm^{-1} (representing C-Br stretching of bromides) were seen in both T3 and T4 samples although intensities were found slightly higher in T3 samples. Thus, the results revealed variations in peaks/bands intensities and sharpness in the samples of T3 and T4 treatments. The high intensities of peaks/bands in T3 as against T4 that confirms the presence of phenols, flavonoids, aliphatic hydrocarbons, aldehydes, alkyl halides, nitro compounds and others could corresponds the presence of petroleum hydrocarbons in the samples (Devatha et al. 2019, Dominguez-Rosado and Pichtel 2004). It was reported that stress imposed due to hydrocarbons pollution can cause increase and/or decrease as well as shifting in bands/peaks when plants uptake and metabolize the hydrocarbon components from soil (Liu et al. 2012, Devatha et al. 2019). Besides, the high intense peaks for C-H stretching of methylene (-CH₂-) in T3 samples also confirm the uptake and metabolism of hydrocarbons (Dominguez-Rosado and Pichtel 2004) by the experimental plant. Further, clear band for C = C stretching of cyclohexane in T3 sample, which is an intermediate compound produced due to partial hydrogenation of benzene also validate about the direct uptake and /or metabolism of hydrocarbon compound from the contaminated soil by the experimental plant. Moreover, the presence of carbonyl compounds such as aldehydes (C = O stretching) may be associated with microbial oxidation process of used oil in the rhizosphere (Dominguez-Rosado and Pichtel 2004). Again, the presence of 1-nitrohexane of N = O symmetric stretching could correspond to uptake of this petroleum component from contaminated soil by the plant. Finally, the presence of alcohols, alkyl halides and in both the samples but with a little bit high intensity spectra in T4 treatment justify that these compounds are building blocks of plants (Bobby et al. 2012).

4. Conclusion

The *Leucas aspera* showed significant shift in antioxidant and phytochemical profiles in order to adapt/survive in crude oil contaminated soils. The physico-chemicals and biological properties have been improved on introduction of *L. aspera* in the oil contaminated soils after 60 days trial. The species have potential to lessen the total oil and grease concentrations and could uptake/metabolize hydrocarbon components from the oil contaminated soil. As a whole, the species have ability to counter the stress

imposed due to hydrocarbon contaminations. Thus, this study confirms the application potential of *Leucas aspera* for long term remediation programmes of crude oil polluted soil.

Declarations

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Consent to publish: Not applicable

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Competing Interests: Nothing to declare

Availability of data and materials: All the data generated during experimental works has been presented in the MS.

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Tables

Table 1: Physico-chemicals and TOG contents of the crude oil contaminated soil at the beginning and after treatment by *Leucas aspera*

Parameters	Treatments		
	T1	T2	T3
pH	4.235±0.067 ^a	4.241 ±0.024 ^a	5.734 ±0.065 ^c
Conductivity (mS/cm)	0.079±0.004 ^b	0.083±0.007 ^b	0.256±0.013 ^c
Water holding capacity (%)	9.903±0.067 ^a	9.911±0.326 ^a	48.941±0.886 ^b
Total Organic Carbon (%)	17.943±1.12 ^p	13.124±0.642 ^q	6.265±1.009 ^r
Total Nitrogen (mg/kg)	903.452±3.037 ^k	887.046±2.062 ^k	530.45±2.048 ⁿ
Available Phosphorus (mg/kg)	31.053±0.842 ^a	38.23±0.645 ^b	54.957±0.364 ^d
Total Potassium (mg/kg)	272.933±7.345 ^a	348.268±5.874 ^p	414.362±5.092 ^d
TOG (g/kg)*	114.067±5.758 ^b	112.95±3.533 ^b	78.333±6.454 ^c
C/N Ratio*	210.719±4.587 ^p	198.04± 6.985 ^p	118.113±5.534 ^r

Mean value ± SD, n=3; the different letters within the same row represents the significant differences of the values (ANOVA, LSD test, $P \leq 0.05$). *TOG=Total oil and grease, *C/N=carbon-nitrogen ratio.

Table 2: Showing the total population of N fixers, P and K solubilizer in the crude oil contaminated soil at the beginning and after 60 days of introduction of *Leucas aspera*.

Treatments	N fixer	P solubilizer	K solubilizer
		($\times 10^7$ CFU/g soil)	
T1	16.667±0.126 ^p	2.333±0.037 ^a	5.667±0.585 ^m
T2	20.667±0.263 ^p	2.667±0.097 ^a	6.333±0.008 ^m
T3	72.333±0.456 ^q	12.667±0.294 ^b	23.333±0.489 ⁿ

Mean value ± SD, n=3; the different letters within the same column represents the significant differences of the values (ANOVA, LSD test, $P < 0.05$).

Table 3: Showing the changes in productivity parameters and phytochemical contents of *Lucas aspera*

Parameters	Treatments		
	Initial	T4	T3
Productivity			
Leaf Area Index (LAI)	0.009±0.005 ^a	0.022±0.021 ^b	0.015±0.016 ^c
Dry Biomass (g)			
Shoot	0.096±0.09 ^m	0.369±0.207 ⁿ	0.237± 0.111 ^h
Root	0.035±0.093 ^p	0.108 ± 0.035 ^r	0.097±0.055 ^r
Chlorophyll (µgml⁻¹)			
*Chl-a	3.815±0.061 ^h	4.731±0.009 ^k	3.149±0.012 ^m
*Chl-b	0.404±0.003 ^a	0.554±0.008 ^b	0.390±0.005 ^c
Total chlorophyll	10.459±1.013 ^a	12.497±3.004 ^g	8.361±0.084 ^h
Phytochemical contents			
i) Phenol (mg gallic acid/g of extract)	-----	0.952±0.103 ^a	1.453±0.432 ^b
ii) Flavonoid (mg quercitin/g of extract)	-----	16.074±1.005 ^p	17.943±1.092 ^q

Mean value ± SD, n=3; the different letters within the same row represents the significant differences of the values (ANOVA, LSD test, Paired t-test, P<0.05). *chl-a=Chlorophyll-a, *chl-b=chlorophyll-b.

Table 4: Showing the main bands and peaks of IR spectra along with their assignments in the tested plant samples grown on T3 and T4 treatments

Bands and Peaks (cm ⁻¹)	Assignments	Samples
3630-3100	O-H stretching broad band of flavonoids/phenolic compounds	Present in both the samples, more intense in T3 sample
2925, 2853	C-H stretching representing methylene	Present in both the samples, intense in T3 sample
1738	C=O stretching of aldehydes	Distinct and marginal sharp in T3 sample than T4
1637	C=C stretching of alkenes such as cyclohexane	Present in both the samples, slightly intense in T3 samples
1385	N=O Symmetric stretching of 1-nitrohexane	Marginal intense in sample from T3
1321, 1262	C-F stretching of fluoride/polyfluoroalkanes	Marginally intense in T3 sample than T4
1109, 1078, 1058, 1034, 1025	C-O stretching of alcohols/ethers/esters/carboxylic acids	Sharp and intense peak in case of T4 sample
782	C-Cl stretching of aliphatic chlorides	Present in both the samples, more sharp in T3 sample
616, 520	C-Br stretching of bromides	Marginally intense in T3 sample

T3= Crude oil contaminated soil +*Leucas aspera*; T4= Non-contaminated soil+ *Leucas aspera*

Figures

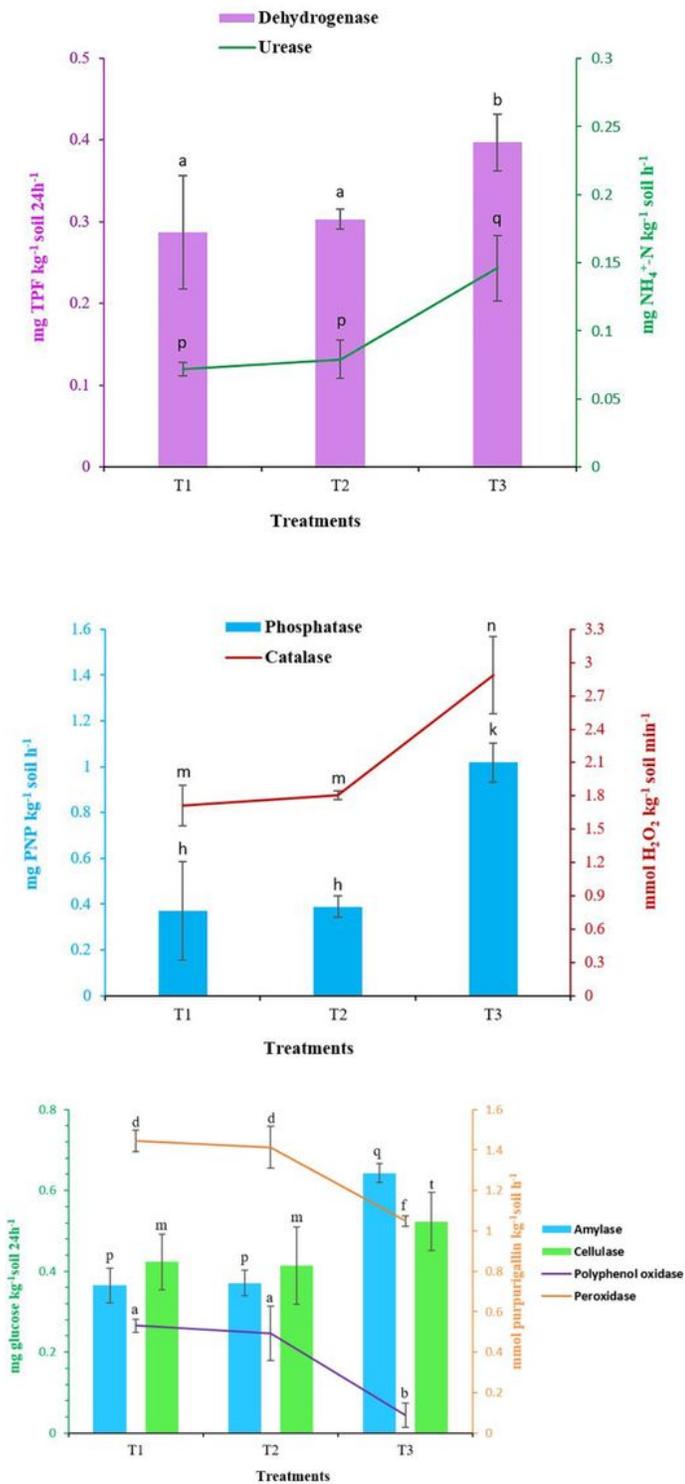


Figure 1

a: Showing changes in dehydrogenase and urease activities in crude oil contaminated soil at the beginning and by the end of the experimental trials. Values are mean, n=3, error bars indicate SD. Different letters above error bars stand for significant differences of the values for a particular enzyme among the different treatments (ANOVA, LSD test, $P \leq 0.05$). b: Showing changes in alkaline phosphatase and catalase activities in crude oil contaminated soil at the beginning and by the end of the experimental

trials. Values are mean, n=3, error bars indicate SD. Different letters above error bars stand for significant differences of the values for a particular enzyme among the different treatments (ANOVA, LSD test, $P \leq 0.05$). c: Showing changes in amylase, cellulase, polyphenol oxidase and peroxidase activities in crude oil contaminated soil at the beginning and by the end of the experimental trials. Values are mean, n=3, error bars indicate SD. Different letters above error bars stand for significant differences of the values for a particular enzyme among the different treatments (ANOVA, LSD test, $P \leq 0.05$).

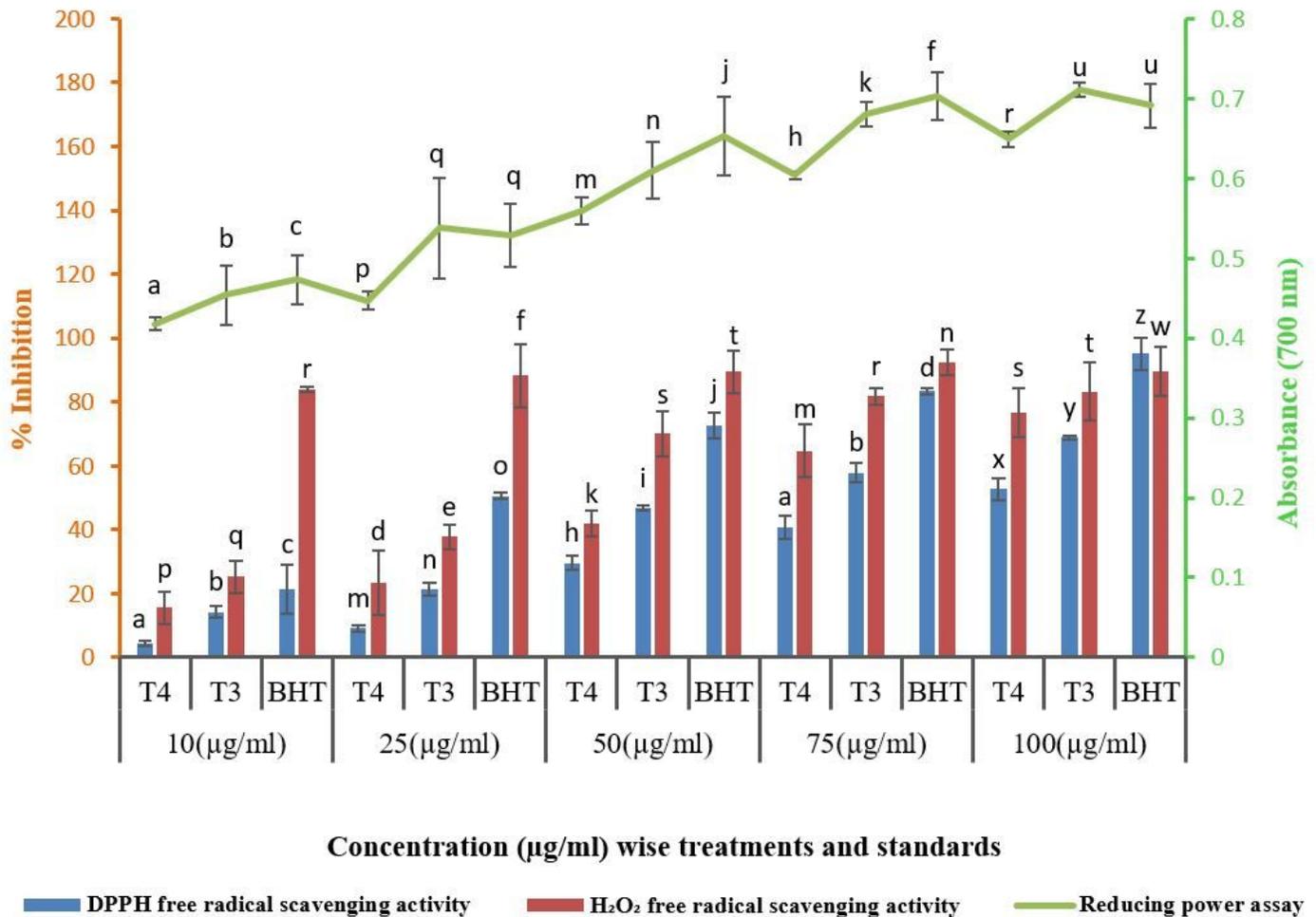


Figure 2

Showing changes in DPPH free radical scavenging activity, H₂O₂ free radical scavenging activity and reducing power assay of *L. aspera* extract grown in crude oil contaminated soil and non-contaminated soil. Values are mean, n=3, error bars stand for SD. Significant differences of the values of different treatments and standards in a particular concentration are indicated by different letters (ANOVA, LSD test, $P \leq 0.05$)

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