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*Research Article*

# Higher Remediation of Petroleum Hydrocarbons by *Enterobacter* sp. MN17 is Coupled with Improved Wheat Growth in Contaminated Soil

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## Abstract

The use of petroleum products has an increasing trend in our current mechanized society, but their toxic impacts on environmental quality are also inevitable. Total petroleum hydrocarbons (TPHs) pose serious threats to the soil-plant-human system. Bioremediation of TPHs contaminated soil can be an eco-friendly and viable option on a large-scale. Here, we investigated the possible potential of co-application of wheat and *Enterobacter* sp. MN17 in the degradation of TPHs under diesel oil-contaminated soil. Sterilized and non-sterilized soils were manually spiked with commercially available diesel oil (10,000 mg $kg^{-1}$ soil), and seeds of wheat genotype Ujala-15 were inoculated with *Enterobacter* sp. MN17. Results showed that wheat seedlings grown under spiked soil were significantly short in height (46-91%) with lower biomass accumulation and reduced photosynthetic activity including net photosynthetic rate (~82%), transpiration rate (~71%), stomatal conductance (~63%), and chlorophyll contents (32-41%). The availability of diesel oil led to altering the mineral uptake (6-52%) by wheat seedlings. However, inoculation of *Enterobacter* sp. MN17, up to some extent, reversed the adverse impacts of diesel oil on plant morphological and physiological traits. Importantly, PHA 100 plus detected up to 26% lower residual TPHs in bacteria inoculated soil with or without wheat grown, under both non-sterilized and sterilized conditions. Together, our study provides valuable scientific basis that the use of oil-degrading microorganisms such as *Enterobacter* sp. MN17 can be an effective tool in the remediation of TPHs-contaminated soils. The findings of this study can be helpful to minimize the undesirable effects of crude oil on plant performance, environmental quality, and human health.

**Keywords:** Hydrocarbon-contamination; Environmental pollution; Phytoremediation; Plant-soil interaction; Plant physiology; Food security

## 1. Introduction

In the present era, the petroleum-based industry has an increasing trend to achieve the energy requirements of a mechanized society. The increase in the use of motor oil poses serious threats to plant, animal, and human health due to the higher release of total petroleum hydrocarbon (TPHs) by various kinds of automobile and machinery vehicles (Ahmed and Fakhruddin, 2018). For instance, TPHs can be one of the reasons for cancers and mutation in the immune system of humans (Kuppusamy *et al*., 2016; Ossai et al., 2019). In plants, TPHs toxicity can alter physiological activity such as elevated levels of soluble protein content, and SOD activity suggested plant resistance to TPHs-induced stress (Odukoya et al., 2019). Similarly, the aesthetic quality of the environment is being deteriorated and the death of living biota is also being caused by oil spills (Edwin-Wosu and Albert, 2010; Ahmed and Fakhruddin, 2018). Nevertheless, the toxicity of these TPHs to soil-plant and human systems may vary according to the toxic fraction present in their products (Abha and Cameotra, 2012).

There are different pathways by which hydrocarbons enter into the environment such as they may leak during their exploration, maintenance, transportation, and storage. The unintended release of TPHs, i.e., accidental leakage, oil spills, waste of industries, or by-products of industries lead to serious ecological impacts (Koltowski et al., 2016; Ossai et al., 2019; Hung et al., 2020). It was estimated that about 1.7–8.8 million metric tonnes of TPHs are being released annually into the marine environment globally with 90% attributed to accidents due to human failures (Zhu et al., 2001; Dadrasnia et al., 2013). Further, ~0.5-10% TPHs are detected in contaminated soils which led to an increase TPHs concentration in different plant tissues by 0.5 to 16,300 mg $kg^{-1}$ (Hunt et al., 2018). Further, in September 2009 a major oil spill of 18 tankers had occurred due to the collision of

two luggage-carrying trains in Sindh province of Pakistan. However, it is unrealistic to predict the exact amounts of leaked TPHs in our environment due to their unintentional release.

Soil is the ultimate destination of leaked hydrocarbons (Balseiro-Romero et al., 2018). The released refinery products can later the physiochemical characteristics of soils, and ultimately affect plant performance (Hung et al., 2020; Grifoni et al., 2020). For instance, the presence TPHs in the soil negatively impact the germination, growth, and development of sugar sorghum and white mustard (Hawrot-Paw et al., 2020). In paddy soils, 400 mg $kg^{-1}$ of TPHs resulted in lower plant growth, chlorophyll content, water potential, and dry biomass of rice by 24, 29, 54, and 25% respectively, compared to control (Li et al. 2008). At the molecular level, the expression levels of *SOD*, *CAT*, *PRX*, *PPO*s, *GST*s, and *NAP*2 changed significantly in the *Avicennia marina* grown in oil-contaminated soil (Moradi et al., 2020). Therefore, it is very important to remove TPHs from the soil system to improve the quality of the environment and human health.

Many different approaches are being widely used to treat or reduce the TPHs from the soil system. These techniques include physical removal, chemical treatment, and biological remediation (Ossai et al., 2019; Haider et al., 2021). Among them, biological methods such as bioremediation, bioaugmentation, phytoremediation, or rhizo-remediation are considered cost-effective, aesthetically pleasant, environmentally friendly, and applicable over a large area (Bhatnagar and Kumari, 2013). The process of bioremediation largely depends on the metabolic activity of the microbes. Since TPHs are a mixture of complex aliphatic (alkane, alkene, hexane, etc.) and aromatic (PAHs) compounds, therefore, sometimes *in situ* remediations by microorganisms become difficult (Ossai et al., 2019). Similarly, plants can uptake TPHs via the root system or absorb TPHs by leaf from the atmosphere (Haider et al., 2021), and phytoremediation can be an effective option in the removal TPHs from contaminated soils (Xu et al., 2019; Ma et al., 2020).

The selection of appropriate microorganisms is important in the remediation of contaminated soil. In earlier studies, *Azospirillum brasilense* strain SR80 (Muratova et al., 2005), and *Microbacterium* sp. F10a (Sheng et al., 2009) were screened and characterized for the biodegradation of PHs in contaminated soils. Further, *Enterobacter* sp. MN17 has been successfully tested in the removal or tolerance to heavy metals such as cadmium (Saeed et al., 2019; Naveed et al., 2020; Sabir et al., 2020), and the solubilization of residual zinc in soil (Ullah et al., 2019; Ullah et al., 2020). Recently, *Enterobacter* sp. was isolated from naturally hydrocarbon-contaminated soils (Ejaz et al., 2021), suggesting that these bacterial isolates comprising *Enterobacter* sp. MN17 may be useful in the remediation of TPHs-contaminated soils. Accordingly, this study was designed with the objective to investigate the possible potential of bioremediation of diesel oil-contaminated soil. Herein, wheat genotype Ujala-15 inoculated with *Enterobacter* sp. MN17 was grown in diesel oil-contaminated soil under both sterilized and non-sterilized conditions. Overall, wheat biomass and photosynthetic activity improved, and nutrient uptake was altered by seed inoculation with *Enterobacter* sp. MN17. To confirm the remediation of TPHs, we designed another experiment in which higher residual TPHs were detected in control where no crop was grown, or bacteria was inoculated. The findings of this study further our understanding of the bioremediation of TPHs-contaminated soils, which not only improve environmental quality but also the performance of crop plants.

## 2. Materials and Methods

### 2.1. Plant growth conditions and treatment plan

This study was conducted in a rain-protected greenhouse with a light/dark regime of 14/10 h, relative humidity of 50–60%, air temperature of 28 °C/18 °C, and natural daylight. The treatment plan is shown in Figure 1, and each treatment was replicated three times according to a completely randomized design (CRD). Additionally, the present study was planned under both sterilized and non-sterilized soil conditions. The soil was collected in bulk from the local research field of the Institute of Soil and Environmental Sciences (ISES), University of Agriculture Faisalabad (UAF), Pakistan (31.439052˚ N, 73.069335˚ E). The sample preparation method was similar as described in our previous study (Ishfaq et al., 2021; Wakeel and Ishfaq, 2022). The physicochemical properties of soil were determined following standard protocols (Pansu and Gautheyrou, 2007), and detail is given in Table 1.

**Table 1.** Physicochemical properties of soil used for experiments

| Characteristics of soil | Units | Values |
|---|---|---|
| **pH*s*** | --- | 7.95 |
| **EC*e*** | dS $m^{-1}$ | 1.30 |
| **Sand** | % | 48 |
| **Silt** | % | 30 |
| **Clay** | % | 22 |
| **Textural class** | --- | Sandy clay loam |
| **Total N** | % | 0.07 |
| **Available P** | mg $kg^{-1}$ | 9.8 |
| **Available K** | mg $kg^{-1}$ | 129 |
| **SOM** | g $kg^{-1}$ | 4.3 |
| **CEC** | C $mol_c$ $kg^{-1}$ | 14.25 |
| **SP** | % | 32 |

*Where: pHs, the negative logarithm of the hydrogen ion activity of saturated soil paste; ECe, the electrical conductivity of soil extract; N, nitrogen; P, phosphorus*

*(using Olsen method); K, potassium ($NH_4$-acetate extractable); SOM, soil organic matter; CEC, cation exchange capacity, and SP, saturation percentage*

### 2.2. Culture media preparation and spiking of soil with diesel oil

The strain of *Enterobacter* sp. MN17, previously isolated and identified (accession number KT375575), was kindly provided by Dr. Muhammad Naveed (Environmental Microbiology Laboratory, ISES, UAF). The culture media for the growth of bacteria was prepared by placing a loopful of inoculum into sterilized tryptic soy broth medium at 28 ± 2°C and 180 rpm for 72 h, as described (Naveed et al., 2020). After thorough shaking on an orbital shaker (Firstek Scientific, Tokyo, Japan), the Erlenmeyer flask was placed at room temperature to cool down. Followed by, media was placed in the incubator for 48 hours for growth of *Enterobacter*. After completion of the incubation period, the optical density was tested to ensure the growth of bacteria in the range of $10^8$–$10^9$ CFU $mL^{-1}$.

Before spiking of soil with diesel oil, half soil was autoclaved at 121°C temperature and 15 psi pressure for sterilization purposes. The soil was spiked with commercially available diesel oil as reported previously (Brinch et al., 2002). In brief, 10,000 mg diesel oil and 20 mL acetone were thoroughly mixed manually in 25% (250 g) of soil. The container was closed with aluminum foil for 5 minutes to let the solvent disperse. Thereafter the solvent was evaporated for 16 h, and the remaining 75% (750 g) of the soil sample was added and thoroughly mixed. The non-sterilized soil was also spiked with the same concentration of diesel oil and acetone. Followed by, spiked soil (both sterilized and non-sterilized) was incubated at room temperature for 15 days to complete the aging process (Ali et al., 2020). The required concentration of diesel oil was guided by our preliminary experiments, and was computed in mg $kg^{-1}$ by the following formula:

$$Density\ of\ diesel\ oil\ = \left(\frac{Mass}{Volume}\right)$$

$$Required\ mass\ = density\ \times volume$$

### 2.3. Experimental setup

The 2 kg prepared soil (spiked or non-spiked) was filled in the earthen pot. Before sowing, seeds were inoculated with prepared media of *Enterobacter* sp. MN17 as described previously (Saeed et al., 2019). Followed by, six seeds of wheat genotype Ujala-15 were sown in each experimental unit. A basic dose of NPK was added at the time of seed sowing as recommendations provided for wheat by the Punjab Agriculture Department for major crops of Pakistan (http://dai.agripunjab.gov.pk/croptechnologies). After seedling establishment (one week of germination), three plants were maintained in each pot. Seedlings were irrigated regularly according to the 60% of field capacity level of the soil. The crop was harvested after 65 days of sowing.

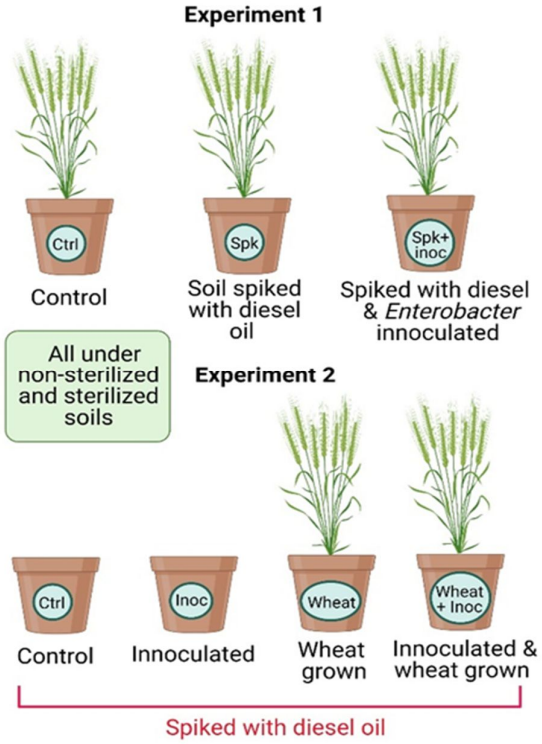


**Figure 1.** Diagrammatic representation of treatment plan

### 2.4. Plant growth measurements

Plant growth measurements were recorded at the time of harvesting. Root length and plant height were measured with the help of a ruler from top-to-bottom. Fresh biomass of excised root and shoot were noted by weighing on a digital balance. Later on, root and shoot samples were oven-dried at 65±5 °C till constant weight for dry biomass, and further elemental analysis. The mean length/weight value of all plants from the individual pot was considered as an independent biological replicate.

### 2.5. Leaf gas exchange and chlorophyll contents determination

Leaf gas exchange and spectral plant analysis diagnostic (SPAD) values which represent chlorophyll contents ($Chl_{SPAD}$), were determined non-destructively by the infrared gas analyzer (IRGA) (LCi Bio Scientific Ltd., Herts, UK) and chlorophyll meter (SPAD-502, Konica Minolta Sensing Inc., Japan), respectively. The youngest fully expanded leaf was analysed three times from top, middle, and bottom at 9:30–11:30 a.m on the day of harvesting. The readings were recorded from all plants, and the average value from each pot was considered as one independent biological replicate.

### 2.6. Elemental analysis in wheat tissue

Over-dried and fine ground plant samples were wet

digested for the determination of minerals as described previously (Ishfaq et al., 2018; Kiran et al., 2021; Mubarak et al., 2022). Briefly, 0.5g sample was weighted in Pyrex digestion flask and 15mL of concentrated $H_2SO_4$ was poured in it. Keep the prepared mixture in the fume hood overnight, and the next day 7.5 mL of $H_2O_2$ were added before digestion at 350ºC. The solution was heated until the colour of the sample turns into colourless and let cool down at room temperature. In the second step, the solution was diluted with the help of distilled water and stored for NPK analysis. The K concentration was detected with the help of a flame photometer (FP7, Jenway, Essex, UK), and P was determined by the Olsen method (Olsen *et al*., 1954) by running samples at 420 nm wavelength (T60 UV-Visible Spectrophotometer-PG Instruments Limited, Leicestershire, UK). Meanwhile, a well-established Kjeldahl method was used for the determination of total N in the collected aliquot, as described (Harborne, 1998).

### 2.7. Quantification of total petroleum hydrocarbons (TPHs) in soil

To confirm the degradation of TPHs with and without inoculation of *Enterobacter* sp. MN17, soil samples were collected after crop harvesting and quantified by using PHA 100 plus - Portable Hydrocarbon Analyzer (PHA-100 plus, PETROSENSE, San Diego, CA, USA). For this purpose, the instrument was first pre-conditioned by placing the probe in $C_2$ standard solution for 15 minutes. Later on, the probe was washed with distilled water and gently dried with tissue paper to remove droplets of water. Subsequently, the instrument was calibrated by sequentially placing the probe in given standards (blank, $C_1$, and $C_2$ solutions). Followed by, the readings of collected soil samples were recorded by following the manufacturer's protocol (: https://www.equipcoservices.com/pdf/manuals/petrosense_pha-100.pdf)

### 2.8. Data analysis

All collected data from each experimental unit were analysed by Microsoft Excel-2019. The analysis of variance (ANOVA) combined with the Tukey honest significant difference (HSD) test was performed by Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) to analyse the statistical difference ($p < 0.05$) across treatments. Principal component analysis (PCA) and correlation matrix were plotted by XLSTAT-2019 (Addisoft, USA).

## 3. Results

### 3.1. Plant growth attributed regulated by seed inoculation in diesel oil-contaminated soil

Results show that plant biomass significantly ($p < 0.05$) reduced with mixing of diesel oil in both non-sterilized and sterilized soils, compared to control treatment (Figure 2). In non-sterilized soil, plant height and root length were reduced by 91% and 52% without inoculation of *Enterobacter* sp. MN17, and 79% and 22%, respectively with inoculation of bacteria (Figure 2 a & b). Similarly, shoot fresh weight, root fresh weight, shoot dry weight and root dry weight reduced by 68%, 83%, 47%, and 97% respectively in wheat seedling grown in spiked soil without seed inoculation, and 39%, 53%, 90%, and 66%, respectively with inoculation of *Enterobacter* (Figure 2 c-f). Suggesting that seed inoculation with *Enterobacter* sp. MN17 reduced the toxicity induced by diesel oil on the wheat seedling.

The same trend is observed in sterilized soil. In brief, plant height and root length reduced by 46% and 17% without inoculation, and 35% and 7%, respectively with inoculation of *Enterobacter* (Figure 2 a & b). Similarly, 74%, 83%, 82%, and 91% lower in shoot fresh weight, root fresh weight, shoot dry weight and root dry weight respectively observed in wheat seedling grown in sole spiked soil, and 35%, 50%, 43%, and 67%, respectively with inoculation of *Enterobacter* (Figure 2 c-f). Suggesting that in most cases a higher reduction in growth is found in non-sterilized, compared to sterilized soil.

### 3.2. Photosynthetic activity with and without seed inoculation under diesel oil contamination

Leaf gas exchange measurements and chlorophyll contents significantly ($p < 0.05$) reduced in diesel oil-contaminated soil under both non-sterilized and sterilized conditions, compared to control (Figure 3). In non-sterilized soil, net photosynthetic rate (µmol $m^{-2}$ $s^{-1}$), transpiration rate (mmol $m^{-2}$ $s^{-1}$), and stomatal conductance (mmol $m^{-2}$ $s^{-1}$) were reduced by 82%, 71%, and 63% respectively in spiked soil without seed inoculation, and 50%, 34%, and 30% respectively with inoculation of *Enterobacter* (Figure 3 a & c). In the case of sterilized soil, net photosynthetic rate reduced by 83% and 37%, transpiration rate reduced by 80% and 23%, and stomatal conductance decreased by 51% and 29% respectively without and with inoculation of bacteria (Figure 3 a & c). Suggesting that seed inoculation with *Enterobacter* reverse the diesel oil toxicity up to some extent, and comparatively more reduction in gas exchange measurement observed in non-sterilized soil.

The chlorophyll contents (*Chl*SPAD) of wheat seedlings were also recorded non-destructively at the time of harvesting. The results shown in Figure 3d indicate that diesel oil contamination led to reduced chlorophyll contents by 32%, meanwhile, seed inoculation in spiked soil reduced chlorophyll contents by 24% in non-sterilized soil. In the case of sterilized soil, 41% reduction in chlorophyll contents was recorded in diesel oil spiked soil, and 25% in seed inoculated treatment, compared to control (Figure 3 d).

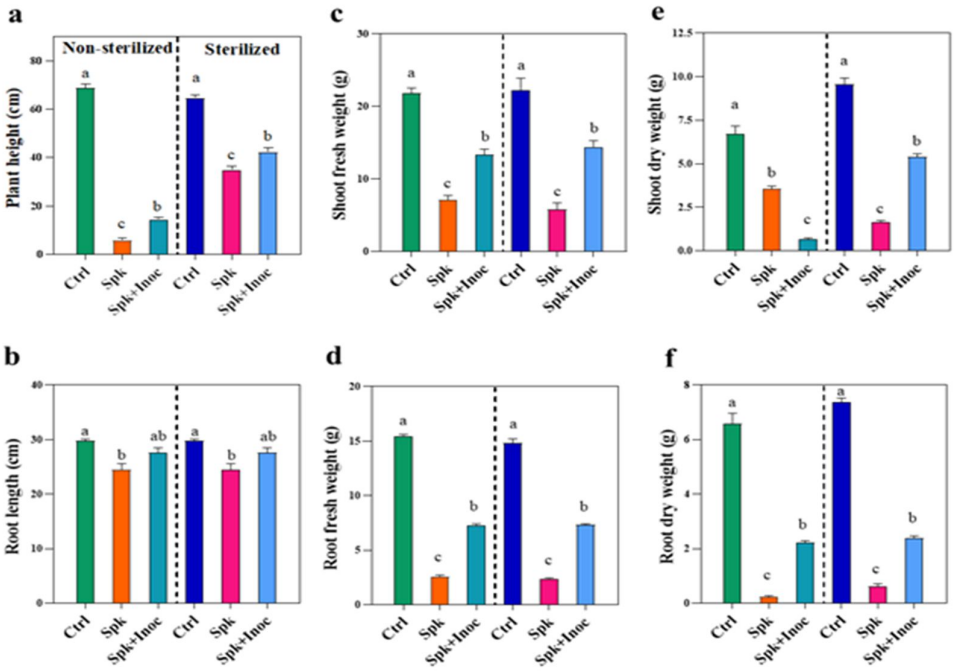


**Figure 2.** Wheat growth traits regulated with and without inoculation of *Enterobacter* sp. MN17 in diesel oil-contaminated soil. (a) plant height (cm), (b) root length (cm), (c) shoot fresh weight (g), (d) root fresh weight (g), (e) shoot dry weight (g), and (f) root dry weight (g). Measurements were taken after 65 days of seed germination. The bar graph shows the mean value while whiskers represent the maximum/minimum values of 3 independent biological replicates. Letters indicate significant differences at $*p < 0.05$ according to Tukey's HSD test. (Ctrl, control; Spk, spiked with diesel oil; Spk+Inoc, spiked with diesel oil and seed inoculated *Enterobacter* sp. MN17)

### 3.3. Elemental concentrations in shoot mediated by inoculation of bacteria in the presence of diesel oil

Spiked soil with diesel oil altered the elemental concentrations in wheat seedlings (Figure 3 a & b). In non-sterilized soil, soil contamination with diesel oil reduced nitrogen (N) concentration in the wheat shoot by 46% without inoculation, and 18% with inoculation of *Enterobacter*, compared to control. Meanwhile, phosphorus (P) concentration increased by 48% in inoculated treatment, and it was detected 18% higher in spiked soil without inoculation of bacteria, compared to control treatment. In the case of potassium (K), spiked soil increased K concentration in the shoot by 12%, meanwhile, K was found minimum in inoculated soil which was 11% lesser than control treatment (Figure 3 a).

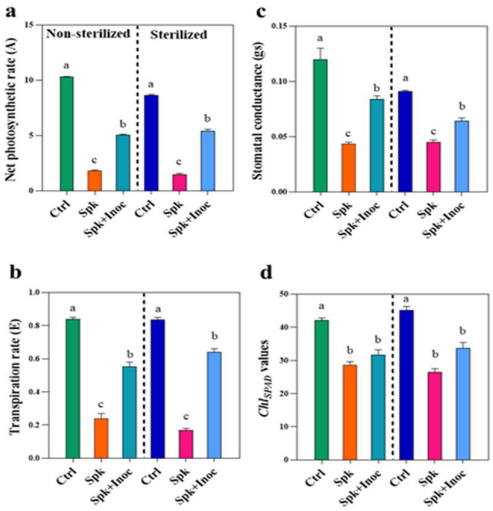


**Figure 3.** Photosynthetic activity of wheat seedling regulated by with and without inoculation of *Enterobacter* sp. MN17 in diesel oil-contaminated soil. (a) net photosynthetic rate (µmol $m^{-2}$ $s^{-1}$), (b) transpiration rate (mmol $m^{-2}$ $s^{-1}$), (c) stomatal conductance (mmol $m^{-2}$ $s^{-1}$), and (d) $Chl_{SPAD}$ values which represent chlorophyll contents. Measurements were taken after 65 days of seed germination. The bar graph shows the mean value while whiskers represent the maximum/minimum values of 3 independent biological replicates. Letters indicate significant differences at $*p < 0.05$ according to Tukey's HSD test. (Ctrl, control; Spk, spiked with diesel oil; Spk+Inoc, spiked with diesel oil and seed inoculated *Enterobacter* sp. MN17)

In the case of sterilized soil, different patterns of P and K uptake were observed (Figure 3 b). Briefly, soil contamination with inoculation reduced N concentration in the shoot by 52% without inoculation and 31% with inoculation of bacteria. Spiked soil improved P concentration by 6%, meanwhile, it was found 11% lesser in inoculated soil compared to control treatment. The maximum K concentration in sterilized soil was detected in inoculated soil which was 12% higher than control. However, 10% higher K concentration was detected in spiked soil without inoculation of *Enterobacter*, compared to control treatment.

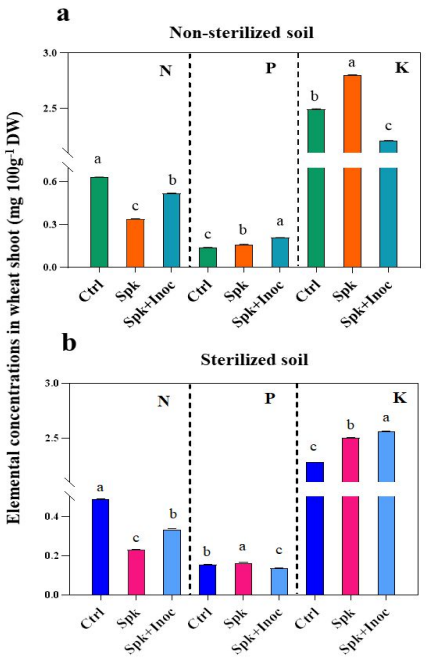


**Figure 4.** Elemental concentrations in wheat shoot mediated by with and without inoculation of *Enterobacter* sp. MN17 in diesel oil-contaminated soil. (a) nitrogen, phosphorus, and potassium concentrations (mg 100g$^{-1}$ DW) under non-sterilized condition, (b) nitrogen, phosphorus, and potassium concentrations (mg 100g$^{-1}$ DW) under sterilized condition. The bar graph shows the mean value while whiskers represent the maximum/minimum values of 3 independent biological replicates. Letters indicate significant differences at *$p < 0.05$ according to Tukey's HSD test. (Ctrl, control; Spk, spiked with diesel oil; Spk+Inoc, spiked with diesel oil and seed inoculated *Enterobacter* sp. MN17; N, nitrogen; P, phosphorus; and K, potassium)

### 3.4. Residual TPHs concentrations in the soil after crop harvesting and/or Enterobacter inoculation

To confirm the degradation of residual TPHs in inoculated soil with *Enterobacter* and/or wheat grown, all collected samples were subjected to PHA 100 plus - Portable Hydrocarbon Analyzer. In non-sterilized soil, maximum TPHs concentration (6083 mg kg$^{-1}$ soil) was detected in control treatment where no biological amendment(s) was applied to remove contamination. Meanwhile, TPHs concentration significantly ($p < 0.05$) reduced by 22% in bacteria inoculated soil and 17% in the soil where wheat seedling was grown, compared to control. Notably, 26% lower TPHs concentration (4502 mg kg$^{-1}$ soil) was detected with co-application of *Enterobacter* and wheat (Figure 5 a).

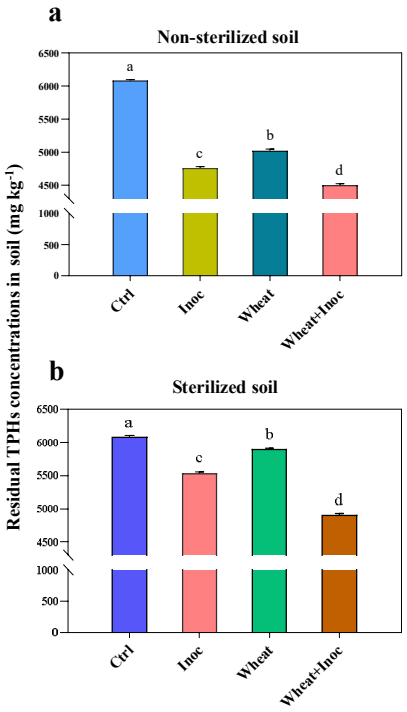


**Figure 5.** Remediation of TPHs in soil mediated by sole or combined application of *Enterobacter* sp. MN17 and wheat in diesel oil-contaminated soil. (a) residual TPHs concentrations in non-sterilized soil (mg kg$^{-1}$), (b) residual TPHs concentrations in sterilized soil (mg kg$^{-1}$). Soil samples were collected after harvesting the crop (65 days after seed germination). The bar graph shows the mean value while whiskers represent the maximum/minimum values of 3 independent biological replicates. Letters indicate significant differences at *$p < 0.05$ according to Tukey's HSD test. (Ctrl, control; Inoc, inoculated *Enterobacter* sp. MN17; wheat, wheat grown without inoculation; wheat+Inoc, wheat grown with inoculation of *Enterobacter* sp. MN17)

The *Enterobacter* and/or wheat also significantly ($p < 0.05$) improve the removal of TPHs in sterilized soil spiked with diesel oil. The maximum TPHs concentration (6088 mg kg$^{-1}$ soil) was detected without the application of biological amendments. However, TPHs concentration reduced by 9% in bacteria inoculated soil and 3% in wheat grown soil, compared to control. Furthermore, the co-application application of *Enterobacter* and wheat reduced TPHs concentration by 19% in diesel oil-contaminated soil (Figure 5 b).

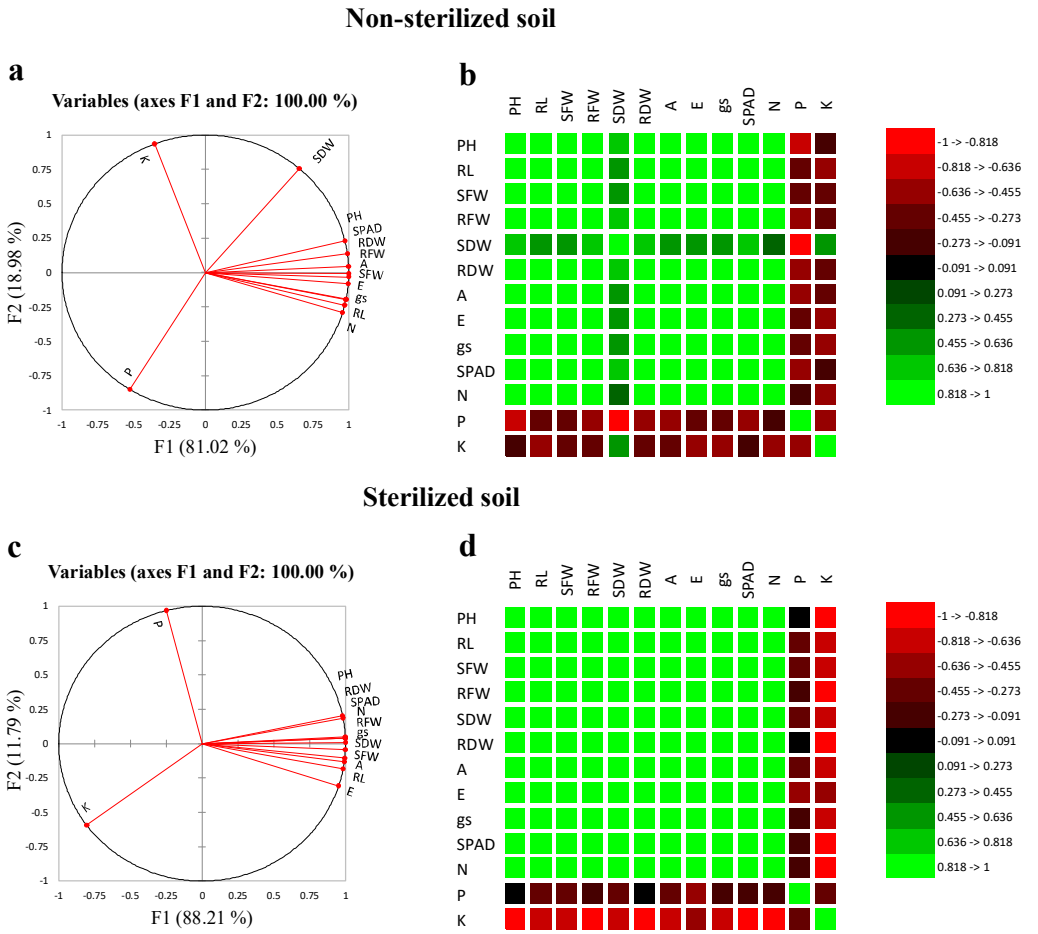


**Figure 6:** Principal component analysis (PCA) (a & c), and correlation matrix (b & d) of wheat growth, photosynthetic activity, and mineral concentrations under non-sterilized and sterilized soil conditions. In PCA, the lines originating from the central point indicate the positive or negative correlations of different variables, and their closeness represents correlation strength with a particular treatment. The variables including P and K are negatively correlated, meanwhile, the remaining are positively correlated. Where; PH, plant height; RL, root length; SFW, shoot fresh weight; RFW, root fresh weight; SDW, shoot dry weight; RDW, root dry weight; A, net photosynthetic activity; E, transpiration rate; $g_s$, stomatal conductance; SPAD, chlorophyll contents ($Chl_{SPAD}$); N, nitrogen; P, phosphorus; and K, potassium

### 3.5. Principal component analysis (PCA) and correlation matrix

The correlation-based principal component analysis (PCA) was performed to determine the response pattern of wheat seedling to inoculation with *Enterobacter* sp. MN17 under TPHs contaminated soil. Accordingly, *"one PCA on merged groups"* was mapped under both non-sterilized and sterilized conditions. The loading plots of PCA are given in Figure 6. In non-sterilized soil, out of the 100% of total variance represented by the two components, 81.02% variance participated by PC1 and 18.98% by PC2. In sterilized soil, out of the 100% of total variance represented by the two components, 88.2% variance participated by PC1 and 11.79% by PC2 (Figure 6 a & c). Further, the correlation matrix confirmed a strong and positive correlation among all observed variables except for K and P concentrations in wheat seedlings which were negatively correlated under both non-sterilized and sterilized conditions (Figure 6 b & d).

## 4. Discussion

### 4.1. Seed inoculation with Enterobacter sp. MN17 reversed the toxic impacts of diesel oil on wheat performance

The results presented in this study indicate that the performance of wheat seedlings was reduced by spiking soil with diesel oil. However, the inoculation

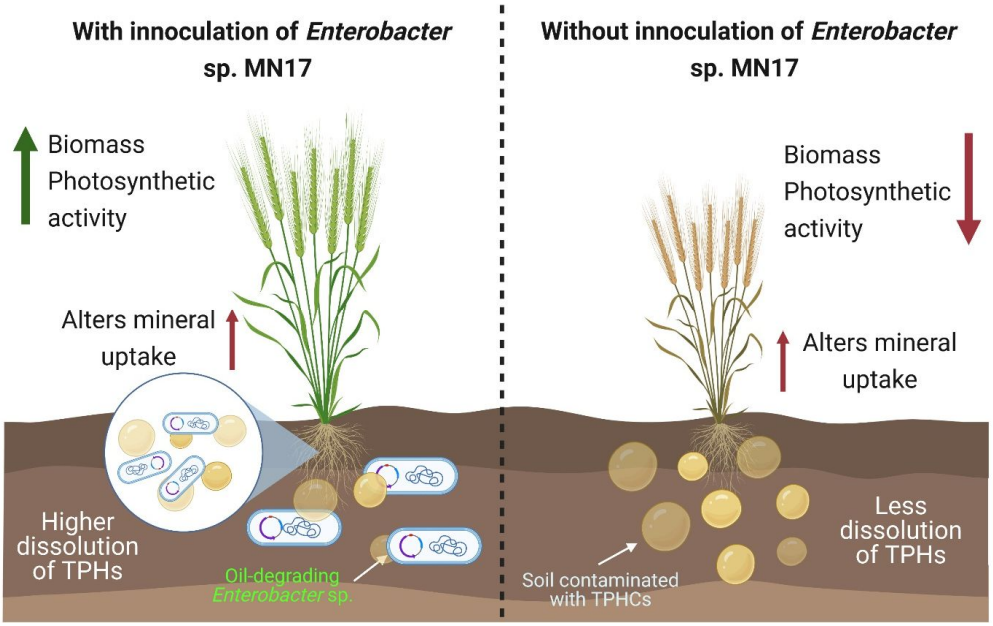


**Figure 7:** Scheme depicting the comparative effects of wheat growth, nutrient uptake, and degradation of TPHs with and without application of *Enterobacter* sp. MN17 in contaminated soil. *Enterobacter* degrades the TPHs in contaminated soils which led to reverse the toxic impacts of TPHs on wheat performance.

of *Enterobacter* sp. MN17, up to some extent, reversed the negative impacts of diesel oil on wheat growth and photosynthetic activity, and altered mineral uptake (as shown in Figures 2, 3 & 4). Since the elevated level of TPHs induced severe phytotoxicity, and multifaceted changes can be observed in plants grown under the high level of TPHs (Odukoya et al., 2019; Ossai et al., 2019; Haider et al., 2021). For instance, due to the higher accumulation of hydrophobic TPHs in thylakoid membranes (Duxbury et al., 1997; Arellano *et al*., 2015), TPHs bring structural changes and can disturb the smooth electrons transport in photosynthetic apparatus (Kummerova et al., 2008; Tomar et al., 2015). Further, due to higher oxidative stress and reduction in chlorophyll contents (Babu et al., 2005), lower leaf gas exchange measurements are recorded in our study, which ultimately reduced the wheat growth in diesel oil spiked soil. In the present study, we also observed a delay in seed germination in diesel oil-contaminated soil (data is not shown), this might be due to the higher toxicity of hydrocarbon. From a broader perspective, the higher reduction in growth and photosynthetic activity observed in non-sterilized diesel oil-contaminated soil, which may be due to the higher availability of competing microorganisms in non-sterilized soil. However, still further studies are needed to understand the underlying mechanism(s).

The toxicity of TPHs can considerably alter the physicochemical properties of soil, for instance, TPHs can modify the composition of organic matter, soil structure, pH, electrical conductivity (EC), and soil microbial population (Wang et al., 2013; Odukoya et al., 2019; Ambaye et al., 2022). They all noticeably modify the mineral uptake by crop plants. In our study, a lower concentration of N in the wheat shoot is recorded grown on diesel oil-contaminated soil. However, P and K are negatively correlated with other variable, as shown by plotting principal component analysis (PCA) and correlation matrix (Figure 6 a-d). Such different behaviour of P and K suggest that further research studies are required in this regard. On other treatment, the inoculation of *Enterobacter* reversed the adverse effects of diesel oil on wheat performance. In earlier studies, consistent findings have been observed, suggesting that application of *Enterobacter* sp. MN17 can improve plant growth and physicochemical traits by improving stress tolerance (Saeed et al., 2019; Naveed et al., 2020; Sabir et al., 2020), or increasing the solubility of soil minerals such as zinc (Ullah et al., 2019; Ullah et al., 2020). However, still bona fide mechanism(s) of plant stress tolerance regulated by *Enterobacter* sp. MN17 is so far unknown.

### 4.2. Enterobacter sp. MN17 and/or wheat enhanced the degradation of TPHs in contaminated soils

The question that was aimed to answer in this study was to understand the possible potential of bioremediation of diesel oil spiked soil. To confirm the removal of TPHs, the collected soil samples from each experimental unit were analysed by PHA 100 plus. The present study revealed that the residual TPHs significantly reduced with the application of biological amendment(s), namely, bacteria and/or wheat.

Meanwhile, soil having only contamination (control) showed less removal of TPHs, as shown in Figure 5. Our results suggest that the co-application of *Enterobacter* sp. MN17 with plant or their sole application has potential in the remediation of TPHs-contaminated soil. Nearly the same trend is observed in both non-sterilized and sterilized soil conditions. These findings are consistent with earlier studies. For instance, the co-application application of *Enterobacter* and biochar can reduce the residual TPHs in contaminated soil (Ali et al., 2020). This might be due to the application of bacteria breakdown organic pollutants into harmless products such as water, $CO_2$, and fatty acids (Wang et al., 2011; Qiao et al., 2013), which may reverse the toxic effects of TPHs on the plant (as schematically shown in Figure 7).

Since *Enterobacter* is a beneficial microorganism (Yousaf *et al.,* 2013), and such higher degradation of TPHs might be due to the specific catabolic activity of formation of organic acids, chelation, protonation, or chemical transformation in soil (Sessitsch et al., 2013; Poi, 2017; Ossai et al., 2019; Ambaye et al., 2022). Similarly, plants can accumulate organic pollutants in their roots, and the biological interaction of plant-microorganism may alter the composition of organic pollutants in the rhizosphere (Blain et al., 2017; Iqbal, 2018). Recently, *Enterobacter* sp. are screened in naturally hydrocarbon-contaminated sites (Ejaz et al., 2021), and our study strengthens the evidence about the involvement of *Enterobacter* sp. MN17 in the degradation of TPHs. However, still, further research studies are needed to identify the effective bacterial consortium for large-scale biodegradation of hydrocarbon-contaminated soils.

## 5. Conclusions

The work presented in this study narrates the potential use of *Enterobacter* sp. MN17 in the remediation of TPHs-contaminated soil and improving wheat tolerance to crude oil toxicity. Since diesel oil considerably reduced biomass accumulation (35-97%), altered mineral uptake (6 to 52%), and decreased photosynthetic activity (32-82%) of the wheat seedlings. Meanwhile, inoculation of *Enterobacter*, up to some extent, reversed the adverse impacts of diesel oil on wheat health. Notably, up to 26% lower residual TPHs in soil inoculated with bacteria further strengthen the evidence of higher degradation of TPHs by *Enterobacter* sp. MN17. Together, our study provides a useful scientific basis in the remediation of crude oil contaminated soils with the application of *Enterobacter* sp. MN17, and to close the yield gaps of crop plants cultivated in TPHs-contaminated soils. In the future, long-term field experiments are required to identify the effectiveness of closely related bacterial consortium(s) for the productive utilization of hydrocarbon-contaminated soils.

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## Author Contributions

Conceptualization, A.W. and M.I.K.; methodology, A.W. and S.A.; investigation, S.A.; resources, A.W. and M.I.K.; data curation, S.A. and M.I.; writing—original draft preparation, M.I., A.W. and S.A.; writing—review and editing, A.W., M.I. and M.I.K.; project administration, A.W.; funding acquisition, A.W. All authors have read and agreed to the published version of the manuscript.

## Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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