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Microbe-Assisted Rhizoremediation of Hydrocarbons and Growth Promotion of Chickpea Plants in Petroleum Hydrocarbons-Contaminated Soil

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Abstract: The present work aimed to develop and investigate microbial consortia for petroleum hydrocarbons (PHCs) detoxification and plant growth improvement in hydrocarbons-contaminated soil. Here, we isolated several bacteria from PHCs-contaminated soils to make bacterial consortia and two of the best consortia were tested in a pot experiment to evaluate their potential for PHCs removal and chickpea growth promotion in PHCs-contaminated soil. Results demonstrated that the PHCs exerted considerable phytotoxic effects on chickpea growth and physiology by causing a 13–29% and a 12–43% reduction in agronomic and physiological traits, respectively. However, in the presence of bacterial consortia, the phytotoxicity of PHCs to chickpea plants was minimized, resulting in a 7.0–24% and a 6.0–35% increase in agronomic and physiological traits, respectively over un-inoculated controls. Bacterial consortia also boosted nutrient uptake and the antioxidant mechanism of the chickpea. In addition, chickpea plants alone phytoremediated 52% of initial PHCs concentration. The addition of bacterial consortia in the presence of chickpea plants could remove 74–80% of the initial PHCs concentration in soil. Based on our research findings, we suggest that the use of multi-trait bacterial consortia could be a sustainable and environmentally friendly strategy for PHCs remediation and plant growth promotion in hydrocarbons in contaminated soil.

Keywords: petroleum hydrocarbons; phytotoxicity; bacterial consortium; phytoremediation; chickpea

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1. Introduction

The world population is growing at a faster rate, necessitating the production of more food on currently available and/or polluted land as well as pollution cleanup by more desirable, sustainable and cost-effective means [1–3]. Among the most pervasive environmental toxins, petroleum hydrocarbons (PHCs) pose a significant risk to all forms of life, ranging from plants to humans [1]. PHCs deteriorate the soil's physicochemical attributes (water holding capacity, porosity, ion exchange capacity and pH), rendering the soil undesirable for crop stand [4]. The PHCs toxicity in plants increases the biosynthesis of reactive oxygen species (ROS), ethylene, and hydrogen peroxide, which causes leaf deformation, decreased plant biomass, and productivity [5]. A stressed plant produces a higher amount of ROS that disturbs the metabolic processes and degrades its proteins, nucleic acids, and cell membrane [3]. Additionally, it is shown that soil microbiota is declining in PHCs-contaminated soils, notably as a result of the lack of easily accessible carbon and macronutrients [6,7]. Therefore, it is essential to remove PHCs from polluted environments to protect ecosystems and ensure sustainable crop production.

Among the available remediation options, microbes-assisted rhizoremediation could be a viable alternative for removing PHCs and alleviating the abiotic stressors on plants

caused by PHCs pollution [8,9]. Compared to traditional methods, microbe-assisted phytoremediation has gained popularity due to its low cost, higher removal efficiency and lack of toxic by-product formations [10,11]. Because of their fast proliferation potential and metabolic flexibility, bacteria are recognized as the main PHCs degraders in soil, especially in the plant rhizosphere where their activity is sustained by the release of root exudates [12,13]. Several bacterial isolates particularly belonging to genera *Azobacter*, *Arthrobacter*, *Achromobacter*, *Acinetobacter*, *Actinobacteria*, *Alcaligenes*, *Bacillus*, *Cellulomonas*, *Stenotrophomonas* and *Pseudomonas* are known to have the ability to remove or use hydrocarbons as a source of energy for their growth and reproduction [11,14–16] (Table S1). However, in the field studies, successful reports on the use of single bacterial strains for the detoxification of PHCs and their intermediate products remained limited [17]. Single bacterial species have a limited ability to breakdown all PHCs fractions, particularly due to the absence of a specialized set of enzymes for the complete mineralization of PHCs [8,18]. Differently, the mixed populations or bacterial consortia (BC), with relatively higher synergistic activities and enzymatic diversity, cause higher hydrocarbons mobility, bioavailability and pollutant exposure to remove higher amounts of PHCs from the soil without exerting deleterious impacts on terrestrial biota or growing plants [19,20].

It has been reported that the PHCs removal rates can be enhanced by modifying the rhizosphere of the plants [21,22]. Further, microbial processes including nitrogen fixation, ACC-deaminase activity, siderophores synthesis and phosphorus solubilization could be helpful in improving a plant's health under PHCs stress [23,24]. Plant roots, in turn, secrete massive volumes of hormones, vitamins, amino acids, organic acids, sugars, mucilage, and other chemicals that promote microbial activity in the rhizosphere [24,25]. Since most of the root exudates and many aromatic fractions of PHCs have structural similarities, microbial communities utilize them as a carbon and energy source to increase their biomass. This results in the removal of PHCs from the soil [26,27]. Furthermore, plants coupled with multi-traits bacterial culture have been shown to demonstrate improved physical and physiological responses (such as seedling emergence, root/shoot biomasses, root/shoot length, photosynthetic pigments, and stomatal performance) and accelerate the decomposition of PHCs [24,28].

The chickpea (*Cicer arietinum* L.) is one of the important food crops in developing countries [29,30]. Pakistan is ranked as the second leading producer of chickpeas in Asia and accounts for approximately 7.0% of the world's cultivation [31,32]. Another reason behind the selection of chickpeas as a test crop was its nitrogen fixing ability that restores the soil health and fertility status and helps the plants to grow in nutrient deficient soils [14,33]. Additionally, chickpea plants could produce a higher level of carboxylates that decrease the pH of the rhizosphere and thus, enhance the nutrient availability for plant growth, even in stressful conditions [34]. In legumes, the accumulation and degradation of PHCs are more frequent in the roots rather than the aerial parts [34–37]. Moreover, the PHCs accumulation in leaves and grains is attributed to aerial deposition rather than plant intake from the root zone [35,37]. It has been demonstrated that legumes frequently favor microbial communities that can break down hydrocarbons [36,38]. In the phytoremediation process, the harmful hydrophobic compounds such as PHCs, with octanol-water partition coefficients (logKow) values higher than three tend to get tightly bound to the root surface. As a result, plants are unable to uptake the pollutants through phytoaccumulation [34,39]. Hence, the chickpea could be a suitable test plant to be cultivated on PHCs- contaminated soil.

Overall, the production of high-quality food, as well as the removal of contaminants from the polluted soil are equally important. For this purpose, using the interactions of plants and microorganisms (particularly bacterial consortia) for the removal of pollutants could be an efficient and eco-friendly solution. Thus, the main objective of this research study was to assess the phytotoxic effects of PHCs on chickpea plants and to investigate the impact of two newly developed bacterial consortia on the remediation of PHCs and the growth promotion of chickpea plants in PHCs-contaminated soil.

2. Materials and Methods

2.1. Soil Samples Collection and Lab Analysis

The PHCs-contaminated soil (mixed contamination of crude oil, diesel, petroleum, etc.) was collected from the PHCs-contaminated sites (30°10′03.7″ N, 70°56′44.1″ E field area adjacent to PARCO, Muzaffargarh, Pakistan and 34°11′59.7″ N, 73°14′29.9″ E service stations around various hotspots of Abbottabad, Pakistan) and brought to the research area of the University of Agriculture, Faisalabad, Pakistan (UAF). A range of analytical techniques was used to assess the physicochemical characteristics of PHCs-contaminated soil before and after the execution of the experiment [40,41] (see results in Tables 1 and S2). After standardization of the instrument with known concentrations of xylene and water-mixed diesel, the TPH analyzer (PHA-100 plus, PETROSENSE, Dallas, TX, USA) was used to determine PHCs in soil. Briefly, a 50 g of soil sample was added to the glass jar and mixed with deionized water until a small layer was observed on the top of the soil. Finally, the TPH analyzer's probe was inserted into the jar to take a measurement. After that, the probe was permitted to rest in room air for five minutes prior to the next measurement. The precision and accuracy of readings is ±10%.

Table 1. Physicochemical properties of the soil used in this study.

Parameters	Uncontaminated Soil	Contaminated Soil
Texture	Sandy loam (sand 53%, silt 25% and clay 22%)	Sandy loam (sand 73%, silt 08% and clay 19%)
pH	7.95 ± 0.17	8.2 ± 0.24
EC (dS m^{-1})	1.07 ± 0.28	1.45 ± 0.21
CEC (mmolc L^{-1})	16.3 ± 1.43	14.3 ± 1.73
Organic matter (%)	0.73 ± 0.05	0.47 ± 0.03
Total N (mg kg^{-1})	179.8 ± 3.20	91.8 ± 8.42
Available P (mg kg^{-1})	86.7 ± 1.09	32.7 ± 3.49
Available K (mg kg^{-1})	58.4 ± 2.35	39.5 ± 2.05
PHCs (%)	ND	1.2 ± 0.04

Values represent means (n = 3) ± standard errors; EC, electrical conductivity; CEC, cation exchange capacity; PHCs, Petroleum hydrocarbons; ND, not detectable.

Both of the soil samples were employed further to isolate, characterize and identify the PHCs degraders and plant growth promoters. Soil from the field area close to PARCO, Muzaffargarh, Pakistan was selected and further used for the rhizoremediation experiment. The soil was prepared by cleaning, air-drying and sieving through a 2 mm sieve to assure its uniformity [42].

2.2. Prescreening and Enrichment of PHCs Degrading Microbial Cultures in Liquid Media

Soil samples (collected from two different contaminated sites) were used as inocula for the enrichment of bacterial consortia. For this purpose, a carbon-free mineral salt medium (MSM) was prepared by following the protocol of Suja et al. [43]. The medium was autoclaved twice to eliminate any microbial life, and then 10,000 mg L^{-1} or 1% filter sterilized diesel was added to the autoclaved medium as a sole carbon source and the medium was kept on a rotary shaker for 2 days for thoroughly mixing of the diesel with MSM. Hereafter, 10 g soil (collected from contaminated sites) was added (as inoculum) to each autoclaved microcosm flask containing 90 mL autoclaved MSM under a laminar flow hood to avoid any microbial contamination. Each flask was covered with autoclaved aluminum foil and then incubated in a rotary shaker at 150 rpm for 7 days at 25 ± 3 °C in the dark. Triplicate flasks were prepared for each site. After a successful first enrichment experiment, sequential subculturing was performed up to four times to enrich and initially screen the PHCs-degrading microbial culture.

2.3. Isolation of PHCs-Degrading Bacterial Strains from the Enriched Culture by Agar Plate Method

Following the enrichment and preliminary screening, the carbon-free MSM with agar was properly sterilized and transferred to agar plates for solidification. Thereafter, a 100 µL filter-sterilized diesel (as a source of carbon) was poured over each plate. Similarly, on each agar plate, 1 µL of previously enhanced culture was dispersed. The plates were then incubated at 25 ± 3 °C for 3 days, and colony formation on the plates was observed. Only bacteria that can tolerate, degrade or thrive on PHCs were grown under these specific conditions [44]. Following the same procedure, the purified bacterial colonies were cultivated individually on freshly prepared agar plates.

2.4. Characterization of PHCs-Degrading Bacterial Strains

Based on their morphological traits, ten bacterial isolates from each location were initially selected. Thereafter, the four best isolates from Abbottabad, Pakistan and five isolates from Muzaffargarh, Pakistan were further chosen based on their plant growth promoting traits (i.e., auxin, exopolysaccharides, oxidase, ACC-deaminase activity, siderophores production, hydrogen cyanide production and phosphate solubilization (see results in Table S3). All the mentioned characteristics were evaluated by following the guidelines of Rafique et al. [25].

2.5. Identification of Bacterial Strains and Preparation of Bacterial Consortia

The particular bacterial isolates, previously screened and characterized on the basis of their ability to stimulate plant growth, were sent to Macrogen, South Korea for 16S rRNA gene Sanger sequencing for the identification of these strains. The four bacterial strains MH-2, MH-3, MH-6 and MH-9 (accession number ON7114529-ON714532), identified as *Alcaligenes faecalis*, *Alcaligenes* sp., *Achromobacter denitrificans* and *Sphingobacterium spiritivorum*, respectively, were mixed in the same proportion to make bacterial consortium 1 (BC1). The other five strains, MH-10, MH-13, MH-18, MH-22 and MH-24 (accession number ON7114533-ON714537), identified as *Sphingobacterium spiritivorum*, *Achromobacter xylosoxidans*, *Stenotrophomonas* sp., *Alcaligenes faecalis*, and *Stenotrophomonas rhizophila*, respectively, were mixed to develop bacterial consortium 2 (BC2). These two developed bacterial consortia were used for further rhizoremediation experiments.

2.6. Experimental Setup

In the UAF research facility, a pot experiment was conducted with nine distinct treatments (with different combinations of two selected bacterial consortia and chickpea plants) and each treatment was replicated three times (see treatment detail in Table 2). The treatment pots were laid out in a completely randomized design (CRD). Six surface sterilized seeds of chickpea (*Cicer arietinum* L., NIAB-CH-2016) inoculated or un-inoculated with bacterial cultures were planted in corresponding pots. Each pot consisted of 7 kg uncontaminated or naturally PHCs-contaminated (i.e., 12,000 mg kg^{-1}) soil (details provided in Section 2.1). The suggested amounts of N, P and K fertilizers (i.e., 20 kg N ha^{-1} as urea, 60 kg P ha^{-1} as single super-phosphate and 60 kg K ha^{-1} as sulfate of potash) were applied to each pot [45,46].

2.7. Seedling Emergence and Growth Attributes of Chickpea Plants

Seedling emergence was monitored from the 2nd day to 7th day after sowing, and the thinning of plants was conducted after 20 days of sowing to maintain three plants in each pot. The chickpea plants were harvested at maturity (after 120 days). The growth and yield attributes of the chickpeas such as shoot and root lengths, fresh and dry biomasses, number of branches, number of nodules and number of pods per chickpea plant were recorded. For the determination of dry biomasses, plant shoots and roots were dried at 65 °C till constant weights [42].

Table 2. Detail of treatments used in this study.

Treatments	Detail
Control	Uncontaminated soil with chickpea plants but without bacterial consortium
PHCs	Hydrocarbons contaminated soil without chickpea plants and microbes
PHCs + CP	Hydrocarbons contaminated soil with chickpea plants but without bacterial consortium
CP + BC1	Uncontaminated soil with chickpea plants and bacterial consortium 1
PHCs + BC1	Hydrocarbons contaminated soil with bacterial consortium 1 but without chickpea plants
PHCs + CP + BC1	Hydrocarbons contaminated soil with chickpea plants and bacterial consortium 1
CP + BC2	Uncontaminated soil with chickpea plants and bacterial consortium 2
PHCs + BC2	Hydrocarbons contaminated soil with bacterial consortium 2 but without chickpea plants
PHCs + CP + BC2	Hydrocarbons contaminated soil with chickpea plants and bacterial consortium 2

BC1, bacterial consortium of selected bacterial strains-1; BC2, bacterial consortium of selected bacterial strains-2; PHCs, petroleum hydrocarbons; CP, Chickpea plants.

2.8. Photosynthetic Pigments and Physiological Attributes of Plants

Chlorophyll contents such as chlorophyll a, chlorophyll b, total chlorophylls and carotenoids were determined following the standard procedures [47]. Briefly, 0.5 g freshly harvested leaves were ground in 5 mL of 80% methanol and centrifuged at 5000 rpm for 10 min at 20 °C. Then the supernatant was collected and run through a spectrophotometer (Thermo Electron Corporation, Evolution-300 LC, Warwickshire, UK). Optical density (OD) was measured at different wavelengths such as 480 nm, 645 nm and 663 nm. Similarly, the SPAD values (relative chlorophyll contents) of leaves were taken by the SPAD meter (SPAD-502 Konica, Minolta). Fluorescence yield (Ft), photosynthetically active radiation (PAR), electron transport rates (ETR) and quantum yields (YII) were measured by a photosynthetic yield analyzer (MINI-PAM-II) (WALZ Mess und Regeltechnik, Effeltrich, Germany) by following the standard protocols [41].

2.9. Membrane Stability Index and Relative Water Contents of Plants

The membrane stability index (MSI) was determined by following the methods of Sairam et al. [48] where leachates (ions) extracted from plant tissues were collected in distilled water and used for the estimation of MSI. Briefly, 0.2 g fresh leaf samples were taken in test tubes and filled with doubled distilled water in two separate sets. One set, containing the same material, was kept at 40 °C in a water bath for 30 min, then its EC1 was recorded by using an EC meter. The second set was kept at 100 °C for 15 min and after that, EC2 was determined by the EC meter and then MSI was calculated by using Equation (1):

$$MSI = [1 - (EC1/EC2)] \times 100 \quad (1)$$

The relative water contents (RWC) were also determined by following the protocol described by Sairam et al. [48]. Briefly, 0.5 g fresh leaves of chickpea plants were hydrated immediately by dipping them in a distilled water for approximately 4 h to gain maximum turgidity. Then the leaf samples were taken out and dried immediately using a tissue paper. The turgid weight (TW) of samples was recorded and samples were kept in an oven for two days at 65 °C to get dry weight (DW). Finally, the RWC of leaves were determined by using Equation (2):

$$RWC = [(FW - DW)/(TW - DW)] \quad (2)$$

2.10. Proline and Antioxidant Contents of Plants

The total proline contents of plant leaves were determined by following the protocols of Bates et al. [49]. Briefly, 2.5 g of ninhydrin was added in 30 mL phosphoric acid (6 M) and

45 mL of glacial acetic acid (100%). This mixture was kept at 4 °C for 24 h. Then 0.2 g freshly harvested leaf sample was mixed with 4 mL of sulfosalicylic acid (3%) and centrifuged at 4000 rpm for 20 min. The mixture was kept in a water bath at 100 °C for 60 min and then placed in an ice bath for instant cooling. Later, the 400 µL of ninhydrin solution was mixed with the same amount of leaf extract (supernatant) and the same amount of glacial acetic acid. The 800 µL of toluene was vigorously mixed in the solution and we waited until the distinctive phases were separated. The red color supernatant was separated, and absorbance was checked at 520 nm on a spectrophotometer.

The procedures described by Ahmad et al. [3] were used to estimate the catalase (CAT) activity. For this purpose, a 10.4 mL reaction mixture was used containing 200 µL enzyme extract, 200 µL H_2O_2 (0.3 M) and 10 mL phosphate buffer (0.05 M) with EDTA (0.002 M) at pH 7.0. The induced reduction in absorbance was observed at 240 nm using a spectrophotometer, after the disappearance of H_2O_2. Similarly, the superoxide dismutase (SOD) activity was observed through the developed methodology of Beauchamp and Fridovich [50] by checking the reduction in absorbance of nitroblue tetrazolium chloride at 560 nm. For peroxidase (POD) activity, the method developed by Angelini et al. [51] was employed to examine the transformation of guaiacol to tetra-guaiacol by observing the absorbance at 436 nm.

2.11. Nitrogen, Phosphorus and Potassium in Plants and Residual PHCs in Soil

After the digestion of plant samples [40], N, P and K contents in plant samples were determined through the Kjeldahl apparatus, spectrophotometer and flame photometer by following the standard procedures described by Afzal et al. [42]. Similarly, the remaining PHCs contents in soil were determined using a TPH analyzer (PHA-100 plus, PETROSENSE, Dallas, TX, USA) following the detailed methodology described above in Section 2.1.

2.12. Statistical Analysis

The normal distribution of the data was evaluated by the Shapiro–Wilk Normality Test (with $p \leq 0.05$) and presented in Table S4. Afterward, the F-test was applied to the obtained data to check the significant or non-significant differences among the different treatment means. Estimated variations from mean values (n = 3) were presented by the standard error. The analysis of variance (ANOVA) was performed on the given data by following the two-factor factorial under a completely randomized design (CRD) by using Statistix 8.1 software [52]. The least significant difference (LSD) test was used to investigate the level of significance ($p \leq 0.05$).

3. Results

3.1. Seedling Emergence and Growth Attributes of Chickpea Plants

The findings of the current study demonstrated that PHCs contamination significantly decreased seedling emergence (Table 3). With a few exceptions, most of the seedlings began to sprout within two days after seeding, regardless of treatments. While comparing the emergence of chickpea seedlings with and without PHCs, the addition of BC revealed a substantial improvement in seedling emergence. When compared to uncontaminated pots, there was a 33.2% decrease in seedling emergence in PHCs-contaminated pots. In uncontaminated soil, the application of BC1 and BC2 resulted in a small increase in seedling emergence than the un-inoculated control (Table 3). However, compared to the un-inoculated contaminated control, the treatments containing PHCs inoculated with BC1 caused a 23.1% increase in seedling emergence. However, BC2 in the presence of PHCs caused a significant increase of 28.6% in seedling emergence, indicating the vital role of microorganisms in the seed germination of chickpea.

The PHCs contamination resulted in a considerable reduction (12.7% to 25.0%) in the agronomic attributes i.e., shoot length, root length, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight as compared to the uncontaminated control (Table 4 and Figure 1). Similar reductions in the number of nodules per plant (28.1%), number of

pods per plant (26.2%), number of stems (20.0%) and number of compound leaves per plant (28.8%) were also noticed with PHCs contamination compared to the uncontaminated control (Table 2). While in uncontaminated soil, the treatments containing bacterial cultures (i.e., BC1 and BC2) produced a significantly higher shoot length (23.2% and 29.2%), root length (18.9% and 31.2%), shoot fresh weight (14.8% and 18.8%), shoot dry weight (22.9% and 29.1%), root fresh weight (5.05% and 12.8%), root dry weight (10.4% and 13.5%), number of nodules per plant (13.5% and 25.6%), number of pods per plant (11.6% and 20.8%), number of stems per plant (9.09% and 23.1%) and number of compound leaves per plant (17.6% and 29.3%) as compared to the un-inoculated control. Furthermore, the treatments containing PHCs along with BC1 and BC2 induced a higher shoot length (11.4% and 17.3), root length (7.69% and 14.3%), shoot fresh weight (21.4% and 24.2%), shoot dry weight (13.8% and 24.7%), root fresh weight (10.2% and 13.3%), root dry weight (8.59% and 10.8%), number of nodules per plant (11.5% and 17.9%), number of pods per plant (8.16% and 13.5%), number of stems per plant (11.1% and 20.0%) and number of compound leaves per plant (11.0% and 18.2%) as compared to un-inoculated contaminated controls (Table 4 and Figure 1), indicating the significant contribution of microbes in growth improvement of chickpea plants.

Table 3. Effect of bacterial consortia addition on seedling emergence of chickpeas under petroleum hydrocarbons stress.

Treatments	Seedling Emergence (%)				
	3 DAS	4 DAS	5 DAS	6 DAS	7 DAS
Control	44.4 ± 5.57 ab	66.7 ± 9.62 ab	77.8 ± 5.54 ab	83.3 ± 9.62 ab	83.3 ± 9.62 ab
PHCs + CP	11.1 ± 5.57 c	38.9 ± 5.57 c	55.6 ± 5.57 c	55.6 ± 5.57 c	55.6 ± 5.57 c
CP + BC1	50.1 ± 9.59 a	72.2 ± 5.53 a	83.3 ± 9.62 a	88.9 ± 5.57 ab	88.9 ± 5.57 ab
PHCs + CP + BC1	22.2 ± 5.53 c	44.4 ± 5.57 c	61.1 ± 5.57 bc	72.2 ± 5.54 bc	72.2 ± 5.54 bc
CP + BC2	55.6 ± 5.57 a	72.2 ± 5.54 a	88.9 ± 5.57 a	94.4 ± 5.57 a	94.4 ± 5.57 a
PHCs + CP + BC2	27.8 ± 5.53 bc	50.0 ± 9.65 bc	72.2 ± 5.54 abc	77.8 ± 5.54 ab	77.8 ± 5.54 ab

Seedling emergence in all treatments was noted on daily basis (from 3rd DAS to 7th DAS). Values are the means of three replicates ± standard errors. Means with different letters are significantly different at $p \leq 0.05$ according to LSD test. BC1, bacterial consortium of selected bacterial strains-1; BC2, bacterial consortium of selected bacterial strains-2; PHCs, petroleum hydrocarbons; CP, chickpea plant; DAS, days after sowing.

Table 4. Effect of bacterial consortia on morphological attributes of chickpea plants grown under petroleum hydrocarbons stress.

Treatments	SL (cm)	RL (cm)	NN	NP	NS	NCL
Control	36.3 ± 0.88 b	21.3 ± 0.88 bc	10.7 ± 0.73 c	20.33 ± 0.88 b	3.33 ± 0.33 ab	71.7 ± 4.92 c
PHCs + CP	28.7 ± 1.20 c	16.0 ± 0.58 d	7.67 ± 0.60 e	15.0 ± 0.58 c	2.67 ± 0.33 b	51.0 ± 4.17 d
CP + BC1	47.3 ± 2.03 a	26.3 ± 2.61 ab	12.3 ± 0.33 b	23.0 ± 1.16 ab	3.67 ± 0.33 ab	87.0 ± 5.29 b
PHCs + CP + BC1	32.3 ± 0.88 bc	17.3 ± 0.33 cd	8.67 ± 0.29 de	16.3 ± 0.68 bc	3.00 ± 0.58 b	57.3 ± 2.97 d
CP + BC2	51.3 ± 3.93 a	31.0 ± 2.31 a	14.3 ± 0.33 a	25.7 ± 0.88 a	4.33 ± 0.33 a	101 ± 3.85 a
PHCs + CP + BC2	34.7 ± 0.88 bc	18.7 ± 1.20 cd	9.33 ± 0.33 cd	17.3 ± 0.33 c	3.33 ± 0.33 ab	62.3 ± 1.77 cd

These parameters were determined in all treatments at the time of harvesting on the same day (i.e., 120 DAS). Values are the means of three replicates ± standard errors. Means with different letters are significantly different at $p \leq 0.05$ according to LSD test: SL, Shoot length; RL, root length; NN, number of nodules; NP, number of pods per plant; NS, number of stems per plant; and NCL, number of compound leaves per plant; BC1, bacterial consortium of selected bacterial strains-1; BC2, bacterial consortium of selected bacterial strains-2; PHCs, petroleum hydrocarbons; CP, chickpea plant.

3.2. Photosynthetic Pigments and Physiological Attributes of Plants

In this study, the physiological attributes of chickpea plants were also disturbed due to hydrocarbons stress. Around a 31.4%, 19.9%, 26.5% and 43.3% reduction in Chl a, Chl b, total Chl and carotenoids, respectively, was observed under PHCs contamination, as compared to the uncontaminated control treatments (Figure 2). Similarly, a significant decrease (up to 26.3%) in physiological attributes, i.e., SPAD value, Ft, PAR, YII, ETR, RWC

and MSI, was observed under the PHCs contamination, as compared to uncontaminated controls (Table 5 and Figure 2). While in uncontaminated soil, the addition of bacterial cultures (i.e., BC1 and BC2) produced significantly higher Chl a, Chl b, total Chl, carotenoids, SPAD value, Ft, PAR, YII, ETR, RWC and MSI, showing up to a 40.6% increase over the un-inoculated controls (Table 5 and Figure 2). Similarly, the inoculation of BC1 and BC2 in PHCs-contaminated soil also produced higher Chl a, Chl b, total Chl, carotenoids, SPAD value, Ft, PAR, YII, ETR, RWC, and MSI inducing up to a 35.4% increase over un-inoculated contaminated controls (Table 5 and Figure 2) suggesting the important role of microbes in improving the overall physiology of chickpea plants under PHCs stress.

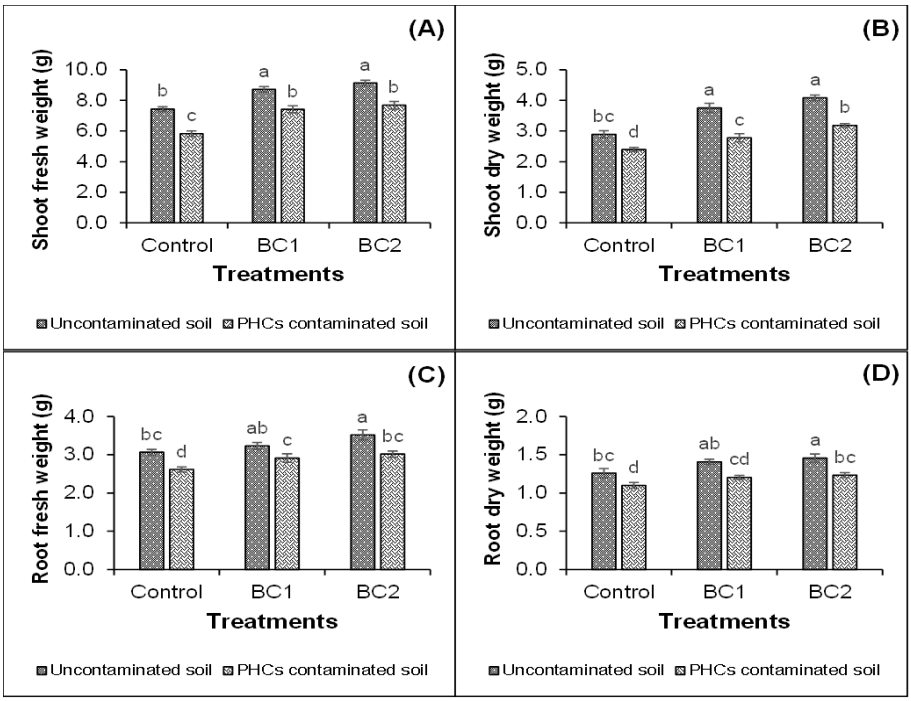

Figure 1. Effect of bacterial consortia of selected bacterial strains on the shoot fresh (**A**) and dry (**B**) weights, and root fresh (**C**) and dry (**D**) weights of chickpeas grown under hydrocarbons contamination. Columns represent the means of triplicate. Standard errors are represented by bars and small letters above columns denote the statistical variation among different means. PHCs, petroleum hydrocarbons; BC1, bacterial consortium of selected bacterial strains-1; BC2, bacterial consortium of selected bacterial strains-2.

Table 5. Effect of bacterial consortia on physiological attributes of chickpea plants grown under petroleum hydrocarbons stress.

Treatments	CC (SPAD Value)	Ft ($\mu molm^{-2}$ s^{-1})	PAR ($\mu molm^{-2}$ s^{-1})	YII ($\mu molm^{-2}$ s^{-1})	ETR ($\mu molm^{-2}$ s^{-1})
Control	41.3 ± 0.89 b	232.7 ± 23.1 cd	746 ± 20.1 a	0.74 ± 0.04 bc	50.1 ± 2.98 c
PHCs + CP	31.7 ± 1.41 c	173.0 ± 22.3 d	550 ± 22.4 c	0.59 ± 0.03 d	43.7 ± 2.02 c
CP + BC1	46.4 ± 0.40 ba	320.3 ± 22.3 b	777 ± 18.5 a	0.80 ± 0.01 ab	61.3 ± 4.87 ab
PHCs + CP + BC1	38.5 ± 1.43 b	230.3 ± 10.4 cd	618 ± 13.9 b	0.68 ± 0.02 cd	48.1 ± 1.95 c
CP + BC2	49.7 ± 1.02 a	392 ± 15.9 a	783 ± 21.2 a	0.88 ± 0.02 a	70.0 ± 2.66 a
PHCs + CP + BC2	40.9 ± 0.84 b	268 ± 8.97 bc	644 ± 11.2 b	0.72 ± 0.01 bc	51.3 ± 2.43 bc

Values are the means of three replicates ± standard errors. Means with different letters are significantly different at $p \leq 0.05$ according to LSD test: CC, chlorophyll contents (SPAD value); Ft, fluorescence yield; PAR, photosynthetically active radiation; YII, photochemical quantum yield; ETR, electron transport rate; BC1, bacterial consortium of selected bacterial strains-1; BC2, bacterial consortium of selected bacterial strains-2; PHCs, petroleum hydrocarbons; CP, chickpea plant.

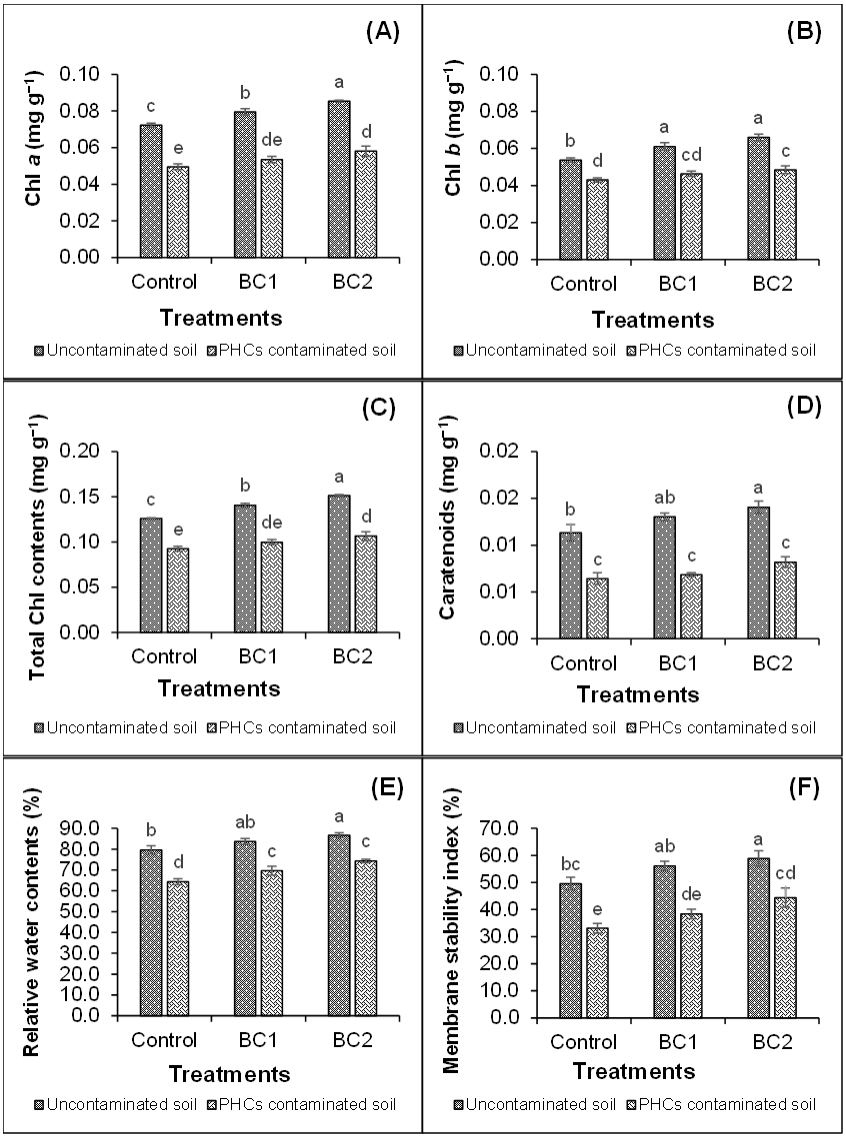

Figure 2. Effect of bacterial consortia on chlorophyll a (**A**), chlorophyll b (**B**), total chlorophylls (**C**), carotenoids (**D**), relative water contents (**E**) and membrane stability index (**F**) of fresh leaves of chickpea grown under hydrocarbons stress. Columns represent the means of triplicate. Standard errors are represented by bars and small letters above columns denote the statistical variation among different means. PHCs, petroleum hydrocarbons; BC1, bacterial consortium of selected bacterial strains-1; BC2, bacterial consortium of selected bacterial strains-2.

3.3. Proline and Antioxidant Contents of Chickpea Plants

Under PHCs contamination, proline and antioxidant contents of chickpea plants were significantly increased, showing 32.2%, 27.1%, 34.1% and 21.3% higher proline, CAT, SOD and POD contents, respectively, than that of uncontaminated control (Table 6). However, in PHCs-contaminated soil, the treatments containing bacterial cultures, i.e., BC1 and BC2, induced lower proline (13.8% and 11.6%), CAT (11.6% and 14.7%), SOD (6.52% and 13.1%) and POD (10.6% and 15.5%) contents as compared to un-inoculated contaminated control (Table 6).

3.4. Nutrient Uptake by Plants and Removal of Phcs from Soil

Approximately, a 28.3%, a 22.1% and a 23.6% reduction in N, P and K contents, respectively, in chickpea plants were observed under PHCs contamination, as compared to uncontaminated control treatments (Table 7), indicating the significant phytotoxic effect of PHCs on nutrient uptake of chickpea plants. However, in uncontaminated soil, the

treatments containing bacterial cultures (BC1 and BC2) showed a significantly higher uptake of N (20.1% and 25.7%), P (9.48% and 14.3%) and K (20.4% and 27.9%) by chickpea plants as compared to their respective un-inoculated counterparts (treatments without BC and PHCs). Similarly, the addition of microbes significantly improved nutrient uptake by chickpea plants in PHCs-contaminated soil (Table 7) indicating the significant role of microbes in plant nutrition.

Table 6. Effect of bacterial consortia on the production of various antioxidants in chickpea plants grown under petroleum hydrocarbons stress.

Treatments	PC (μmole g^{-1} FW)	CAT (U mg^{-1} Protein FW)	SOD (U mg^{-1} Protein FW)	POD (U mg^{-1} Protein FW)
Control	23.7 ± 1.34 d	4.29 ± 0.32 b	7.44 ± 0.27 c	69.3 ± 2.60 c
PHCs + CP	35.0 ± 1.88 a	5.89 ± 0.56 a	11.3 ± 0.56 a	88.1 ± 3.52 a
CP + BC1	25.7 ± 1.59 cd	4.85 ± 0.21 b	7.78 ± 0.58 c	76.7 ± 1.70 bc
PHCs + CP + BC1	30.2 ± 1.71 abc	5.21 ± 0.32 ab	10.6 ± 1.12 ab	78.7 ± 3.33 b
CP + BC2	26.5 ± 2.11 bcd	5.09 ± 0.08 ab	8.58 ± 0.73 bc	77.8 ± 2.32 bc
PHCs + CP + BC2	30.9 ± 1.02 ab	5.29 ± 0.15 ab	9.81 ± 0.20 ab	74.4 ± 1.19 b

Values are the means of three replicates ± standard errors. Means with different letters are significantly different at $p \leq 0.05$ according to LSD test: PC, proline contents; CAT, catalase; SOD, superoxide dismutase; POD, peroxidase; BC1, bacterial consortium of selected bacterial strains-1; BC2, bacterial consortium of selected bacterial strains-2; PHCs, petroleum hydrocarbons; CP, chickpea plant; FW, fresh weight.

Table 7. Effect of bacterial consortia on the uptake of macronutrients in chickpea plants grown under petroleum hydrocarbons stress.

Treatments	Nutrient Uptake (%) by Plants		
	Nitrogen	Phosphorus	Potassium
Control	0.025 ± 0.001 b	0.015 ± 0.001 ab	0.015 ± 0.001 b
PHCs + CP	0.017 ± 0.001 c	0.012 ± 0.001 c	0.012 ± 0.001 c
CP + BC1	0.031 ± 0.001 a	0.017 ± 0.001 ab	0.020 ± 0.001 a
PHCs + CP + BC1	0.021 ± 0.001 c	0.014 ± 0.001 bc	0.015 ± 0.001 b
CP + BC2	0.034 ± 0.001 a	0.017 ± 0.001 a	0.021 ± 0.001 a
PHCs + CP + BC2	0.022 ± 0.001 bc	0.014 ± 0.001 bc	0.016 ± 0.001 b

Values are the means of three replicates ± standard errors. Means with different letters are significantly different at $p \leq 0.05$ according to LSD test. BC1, bacterial consortium of selected bacterial strains-1; BC2, bacterial consortium of selected bacterial strains-2; PHCs, petroleum hydrocarbons; CP, chickpea plant.

The addition of microbes (both BC1 and BC2) showed a significant reduction in remaining PHCs concentration in soil in the presence or absence of chickpea plants (Figure 3). At the end of experiment (i.e., after 120 days), the unplanted and un-inoculated PHCs contaminated soil showed a 16.9% reduction in PHCs contents as compared to the initial concentration. The plants only (without inoculation) treatments showed removal of approximately 52.0% of initial concentrations of PHCs. Regardless of the presence of chickpea plants, the addition of microbes (both BC1 and BC2) resulted in a substantial increase in the removal (36–45%) of PHCs from the soil. However, the PHCs removal from soil was further enhanced in the presence of chickpea plants. Approximately 74% and 80% PHCs removal was observed by the application of BC1 and BC2, respectively, in planted treatments that were significantly higher than that of their respective unplanted controls (Figure 3). In general, among both bacterial cultures, BC2 showed relatively higher hydrocarbons removal, as well as nutrient uptake and growth enhancement of chickpea plants in PHCs-contaminated soil.

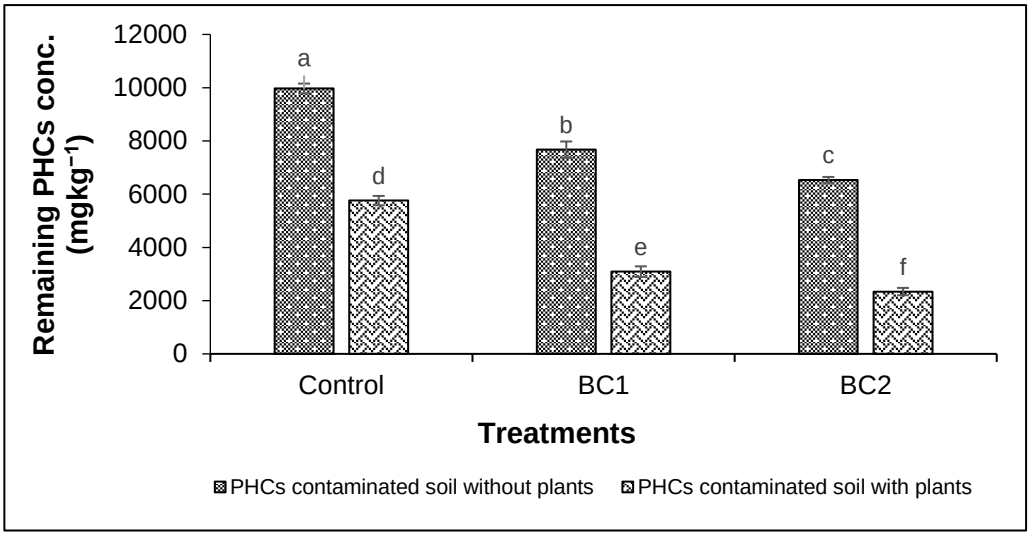

Figure 3. Effect of microbial consortia on PHCs removal from hydrocarbons-contaminated soil in the presence or absence of chickpea plants. Columns represent the means of triplicate. Standard errors are represented by bars and small letters above columns denote the statistical variation among different means. PHCs, petroleum hydrocarbons; BC1, bacterial consortium of selected bacterial strains-1; BC2, bacterial consortium of selected bacterial strains-2.

4. Discussion

In this study, hydrocarbons posed considerable phytotoxicity to chickpea plants by reducing plant growth, physiology, nutrient uptake and yield. However, the presence of bacterial cultures (both BC1 and BC2) greatly alleviated the phytotoxic effects of PHCs on chickpea plants in PHCs-contaminated soil. Further, the addition of bacterial cultures resulted in higher hydrocarbons removal from the soil.

4.1. Seedling Emergence and Growth Attributes of Chickpea Plants

The reduced seedling emergence of chickpea was observed in the present study. This decreased seedling emergence may be due to the volatilized PHCs fractions that penetrate the seed coat, hinder the metabolic processes and/or kill the embryo. Another possible reason could be the hydrophobic nature of PHCs that inhibits water absorption by the seeds and disrupts intercellular activities [8,48]. In soils contaminated with hydrocarbons, the addition of microorganisms could lessen the toxicity of the hydrocarbons by changing the soil pH and CN ratio and providing sufficient moisture to the seeds to cause early sprouting [3,8].

Petroleum hydrocarbons suppress plant growth and induce plants to have shorter shoots and roots [53–55]. The PHCs also prevent oxygen from reaching the soil, leading to anaerobiosis and poor plant growth and development. We also found reduced fresh and dry biomasses of chickpea under the PHCs contamination [41]. This may be due to residual PHCs or their metabolites, which damage cells, limit nutrition and water intake, and thus stunt plant development [56]. The altering of plant biochemical processes by PHCs (such as cell membrane permeability and enzymatic disruptions), which leads to subsequent changes in plant physiology, could be another reason behind the stunted growth of chickpea in PHCs contaminated soil [1].

In the present study, the application of bacterial cultures significantly reduced the phytotoxic effects of hydrocarbons on plants and increased plant growth and development in PHCs contaminated soil. It is possible that bacterial consortia degraded a part of PHCs in the root zone and resulted in the reduction of PHCs concentration in soil which subsequently reduced the phytotoxicity of contaminated soil [2,56]. These findings can be supported and confirmed by plant growth promoting characteristics of microorganisms present in the bacterial consortia (see Table S3). Our results showed that the microbes in both

cultures—particularly those belonging to *Alcaligenes*, *Stenotrophomonas*, *Achromobacter*, and *Sphingobacterium*—have the ability for IAA, exopolysaccharides, siderophore and hydrogen cyanide production as well as phosphate solubilization [57–60]. Additionally, bacteria that secrete growth hormones such as auxin, phosphate and iron solubilizing enzymes, ACC deaminase, and gibberellins help plants to resist the toxic effects of pollutants [58,61]. Another explanation for the growth prompting impact of inoculated microorganisms under PHCs stress may be related to changes in the pH of the root zone and increased nutrient absorption by the plants. Additionally, it has been suggested that microorganisms may enhance plant growth and development by controlling evapotranspiration and the production of pigments used for photosynthetic activity [62,63].

4.2. Photosynthetic Pigments and Physiological Attributes of Plants

Furthermore, PHCs toxicity reduced the chlorophyll contents, carotenoids and anthocyanin contents. Hydrocarbons toxicity lowered the photosynthetic rate by disturbing the chlorophyll synthesis as chlorophyll absorbs photons and begins to accelerate the rate of electron transport to absorb the energy they contain [64]. Here, however, we found that the introduction of bacterial cultures mitigated the deleterious effects of PHCs on photosynthesis and chlorophyll production [3] and this might be due to the increased levels of H_2O_2 that curtails biosynthesis of chlorophyll in stressed conditions [65,66]. The application of BC could have hovered the production of H_2O_2 synthesis. Therefore, it is assumed that the application of BC could have enhanced photosynthetic activity and the formation of chlorophyll pigments by reducing plant oxidative stress [3]. Likewise, the decrease in RWC and MSI of chickpea plants in the present research can be caused by a buildup of PHCs in the cell membrane that led to cell rupture [67]. The PHCs reduced the intake of water by blocking the stomatal openings of the leaves or by producing ROS resulting in stunted plant growth [68,69]. Applying BC was shown to reduce the toxicity of PHCs to chickpea plants and enhance the plant's overall physiology. This could be due to the fact that under PHCs stress, bacterial cultures with ACC-deaminase activity could lower ethylene synthesis by hydrolyzing the ACC [70–72]. The developed bacterial cultures (BC1 and BC2) used in this work were quite effective and successful in alleviating the harmful effects of PHCs on the growth and development of chickpea plants [41,73].

4.3. Proline and Antioxidant Contents of Chickpea Plants

Here, we also found higher levels of proline in chickpea leaves in the presence of PHCs [74,75]. The presence of proline helps to maintain cell structures, synchronize cellular metabolic processes, destroy free radicals, and activate stress-related systems [76]. Thus, the increased proline buildup in the leaf tissues may have aided stressed plants in maintaining their redox potential and controlling their cellular metabolite levels [77]. The proline controls the production of metabolites, which are essential for plant growth [78]. In addition, the plants that have been exposed to hydrocarbons stress produce higher amounts of ROS that could damage the cell membranes and imbalance the ionic homeostasis [79]. In stressed plants, higher proline levels regulate the metabolic processes and reduce ROS generation [80]. This might be due to the fact that proline alleviates stress, regulates mitochondrial activity, cellular proliferation, membrane stability and prevents electrolyte leakage from the plant cells [81]. In addition, here we have noticed that the stress-responsive enzymes including SOD and POD were more active in PHCs stress, while the CAT activity remained relatively lower. The antioxidative mechanism in plants prevents them from being damaged by contaminants [3]. According to Gill et al. [82], the SOD acts as a first defensive mechanism against stress, as opposed to ROS. The SOD transforms superoxide to H_2O_2 and O_2 in the first step, then POD and CAT convert this H_2O_2 to H_2O and O_2 [83]. As a result, plant antioxidative enzymes help in the reduction of abiotic stress [84]. In this case, bacterial cultures under hydrocarbon stress were found very efficient in modulating the activities of CAT, SOD, and POD. The melanin, which is produced by enhanced SOD activity by microbes, could promote antioxidant activity in plants under hydrocarbons stress through

scavenging free radicals [63], since the biochemical pathway for protein synthesis starts on the recovery of superoxides by the SOD activity [3]. The treatments containing PHCs inoculated with bacterial consortia showed relatively lower levels of antioxidants enzymes with respect to the uninoculated PHCs contaminated control. This might be due to the stress relieving and catabolic activities of microbes that reduce the impact and toxicity of hydrocarbons on the plants [72]. Studies reported higher rhizodegradation (by their catabolic enzymes) of PHCs in the presence of microbes that reduces the impact of toxicity and stress on plants [77,81,82]. Similarly, the lower levels of antioxidants in microbes-applied treatments may also be attributed due to higher nutrient uptake causing a reduction in pollutant stress [77].

4.4. Nutrient Uptake by Plants and Removal of PHCs from Soil

In this study, the PHCs reduced nutrient uptake and accumulation in plants. This decrease in N absorption could be related to the pollutants-induced alteration in soil pH and increased CN ratio [85]. This insufficient uptake of N could ultimately result in decreased cellular proliferation and leaf growth in PHCs contamination [86]. Likewise, under PHCs stress, the absorption of P and K by plants was also reduced [87]. Since the P and K control several metabolic processes—such as energy transfer, macro-molecules biosynthesis, signal transduction, respiration and photosynthesis—this results in reduced plant growth and development [88]. However, the application of bacterial cultures could greatly enhance the nutrient absorption by the plants, probably through the synthesis and subsequent release of enzymes increasing nutrient solubility and/or the breakdown of PHCs [89]. The addition of bacterial consortia (carrying K solubilizing bacteria including *Alcaligenes*, *Achromobacter* and *Stenotrophomonas*) enhanced the K uptake in plants by solubilizing the fixed K in the soil. The other possible reason could be the reduction in PHCs level in the rhizosphere due to the degradation of pollutants by the bacterial consortia which resulted in improved nutrient uptake by plants [1,21,26].

Several rhizoremediation studies reported that the microorganisms (due to enzymatic diversity and adaptability) are helpful in improving plant growth and effectively remediating PHCs and related chemicals from hazardous environments [20]. In this study, we developed two BC that were capable of removing significant amounts of PHCs from the soil. The co-existence of PHCs-degrading and plant growth-promoting microorganisms in BC1 and BC2 considerably improved the rhizoremediation process [28,41]. Even though plants confront phytotoxicity in the presence of higher amounts of pollutants, they can phytoremediate a certain amount of organic contaminants from the soil through various mechanisms [90]. Furthermore, the phytoremediation ability of plants was enhanced in the presence of added bacterial cultures. This could be due to the synthesis of organic acids, release of chelators (chelation), protonation, chemical transformation, and phosphate solubilization by the added microbes resulting in greater removal of PHCs from the soil. These processes are also known to increase the availability of PHCs to plants, microbes, and other terrestrial biota [41]. Here, we mixed and used different bacterial strains belonging to various genera (i.e., *Alcaligenes*, *Achromobacter*, *Sphingobacterium*, and *Stenotrophomonas*) to construct bacterial consortia. The *Alclaligene* sp. are capable of producing phosphatase enzymes which can solubilize the fixed phosphate in soil and thus can enhance plant growth [91] and subsequently improve the phytoremediation ability of plants. The *Achromobacter denitrificans* sp., carrying catechol 2,3-dioxygenase and hexadecane monooxygenase, is reported to degrade both aromatics as well as long chain hydrocarbons [92]. More recently, Xiao et al. [93] reported that the *Stenotrophomonas maltophilia* strain W18 was able to degrade PAHs (Table S1). Furthermore, the *Sphingobacterium*, belonging to the phylum Bacteriodetes, is a gram-negative bacterium found in a variety of environments including plant rhizosphere [94] and is known to be involved in the biodegradation of hydrocarbons [95]. The bisurfactants-producing ability of *Sphingobacterium* may increase the bioavailability of hydrocarbons and thus improves the bioremediation process [96].

To the best of our knowledge, this is the first report describing the effects of two newly developed bacterial consortia on the degradation of PHCs and the growth enhancement of chickpeas in PHCs-contaminated soil. The findings of this study enhance our understanding of how plants and microbes interact in PHCs-contaminated soil, provide new insight into how beneficial microbes can be used to improve PHCs remediation and crop productivity in hydrocarbon polluted environments, and unfold new avenues for research into the significance of biological interactions in hydrocarbon-polluted environments.

5. Conclusions

The present study aimed to develop plant growth promoting and PHCs-degrading bacterial consortia. Furthermore, we evaluated their ability to alleviate the phytotoxic effects of PHCs on chickpea plants in diesel-contaminated soil. Results demonstrated that the presence of PHCs caused significant inhibition in the physiology, nutrient uptake, growth and yield of chickpea plants. However, the application of bacterial consortia alleviated the toxicity of PHCs to chickpea plants and increased overall plant growth in PHCs-contaminated soil, probably by improving the plants' antioxidant defense and plant growth-promoting properties. Furthermore, the microbes (with plant growth promoting attributes) in the bacterial cultures were capable of removing substantial amounts of PHCs from the soil and also improved the phytoremediation ability of chickpea plants for PHCs. Overall, the use of both consortia was quite successful; however, BC2 was more effective than BC1 in promoting plant growth and removing PHCs from the soil. Findings from the present study suggest that the novel bacterial consortia are useful for hydrocarbons remediation and plant growth promotion in hydrocarbons contaminated soil and could be promising bioresources for future phytoremediation efforts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15076081/s1, Table S1: Microbial degradation of petroleum hydrocarbons by previous studies and this work; Table S2: The EC and pH values of soil at the end of experiment; Table S3: Characterization of plant growth promoting traits of selected microbial isolates; Table S4: Normal distribution or normality of data (with $p \leq 0.05$). References [15,19,41,43,73,93,97–122] are cited in the Supplementary Materials.

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