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Evaluation of the efficiency of soil bacteria in promoting plant growth in hydrocarbon-contaminated soil

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I. Abstract

Petroleum is a significant source of soil pollution in addition to being a vital energy resource for promoting economic growth. Because petroleum is carcinogenic, its toxicity can have a negative influence on the environment in addition to having severe impacts on humans and animals. As a result, its removal from the environment raises concerns. The impact of rhizobacteria on soybean growth and tolerance in a medium influenced by petroleum hydrocarbons (crude oil) was examined. This study evaluated the effects of introducing plant-growth-promoting rhizobacterial strains into soybean seeds in a crude oil-impacted medium. The rhizobacterial strains included in this investigation were tested according to standard protocols for characteristics that support plant growth, such as phosphate solubilization and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. The modified root elongation assay was used to measure the strains' ability to elongate plant roots and shoots. Using the biochemical test and Vitech system as a confirmed method, the isolates were determined to be *Bacillus sp*., *Rhodospirillum*, and *Pseudomonas*. The findings demonstrated that, in comparison to uninoculated seeds, seeds inoculated with plant growth-promoting rhizobacteria (PGPR) demonstrated notable vegetative development at different concentrations of petroleum crude oil (1, 5, and 10 ml). At a concentration of 1 milliliter of oil, the inoculated seeds' root and shoot lengths were 12.5 and 5.2 cm (*Bacillus*), 6.4 and 1.6 cm (*Rhodospirillum*), 5.6 and 4.4 cm (*Pseudomonas)*, and 0.8 and 1.4 cm (uninoculated). In the oil-impacted medium, the highest Tolerance Index (TI) for PGPR-inoculated soybean was recorded for B. sp. (2.06 to 1.71), *Rhodospirillum* (0.57 to 0.61), and Pseudomonas (0.80 to 0.69). This work suggests that B. sp. could be employed as a crude oil-tolerant rhizobacterium (PGPR) to reduce the detrimental effects of oil on plants and so encourage plant growth in the soil impacted by this pollutant.

Key words: Rhizobacteria, promote plant development, growth parameters, crude oil, impacted medium, and tolerance index.

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II. Introduction

Since the first discovered of crude oil, oil spills have been a worldwide problem. Modern civilization and urbanization have made the ongoing degradation of oil waste in the soil environment with hydrocarbons and refinery products a major problem. Crude oil spills reduce the growth and resistance of plants smothered by the oil's exclusion of air by disrupting the physical, chemical, and biological structure of the soil. According to Arshad and Frankenberger (2005), the depletion of oxygen in the soil reduces microbial activity, which in turn disrupts the plant-soil water relationship. This poses a serious risk to the region's food production (crop yield) and, if left unchecked, could destroy the ecosystems. According to reports, a group of rhizospheric bacteria known as plant growth promoting rhizobacterials (PGPR) can increase plant development and yield in a variety of crops (Radwan *et al*., 2007). A collection of free-living soil bacteria known as PGPR colonizes plant roots after being inoculated onto seeds, promoting plant growth (Kloepper *et al*., 1991).

It's unclear exactly how PGPR increases plant growth and detoxifies pollutants, but it's thought to be through at least one mechanism, either directly, indirectly, or in combination. These mechanisms include: enhancing nutrient acquisition (biofertilizers), such as asymbiotic nitrogen fixation; inhibiting plant diseases (bioprotectants), such as antibiotic production; and generating phytohormones (biostimulants), such as indole acetic acid, cytokinins, etc. (ACC) deaminase prevents plant ethylene levels from becoming growth-inhibitory. (Arshad and Frankenberger, 2005; Boddey and Dobereiner, 2000; Contesto *et al*., 2008). According to research, PGPR is active in the consumption of hydrocarbons and possesses unique properties that can degrade and detoxify pollutants (such as heavy metals) by mobilizing, immobilizing, or leaving them inactive. This increases the plant's resistance to heavy metal ion absorption (Belimov *et al*., 2004; Radwan *et al*., 2007; Glick, Glick, 2010; Roy *et al*., 2013). According to research frontiers, they are used in the bioremediation of crude oil and polycyclic aromatic hydrocarbons (PAHs) (Roy *et al*., 2013; Zhang *et al*., 2007). Using the PGPR-enhanced phytoremediation technology in both lab and field experiments, they have also been shown to significantly lower the levels of salt and petroleum hydrocarbons in soils that are contaminated by salt and petroleum (Gerhardt et al., 2009). Alcaligenes, Enterobacter, Acetobacter, Klebsiella, Bradyrhizobium, and bacteria from the families Azospirillum, Pseudomonas, Xanthomonas, and Rhizobium, among others, have been linked to plant (Poonguzhali *et al*., 2008; Kumar *et al*., 2008; Ahemad and Khan, 2010), and Wani *et al*. (2007), all mention growth-promoting rhizobacteria (PGPR). These results stimulate our interest in examining the effects of PGPR inoculation on the growth response and tolerance of soybean seeds exposed to varying concentrations of crude oil in a laboratory environment.

III. Materials and methods

Samples collection: The rhizospheres of tomato, soybean, pepper, and crude oil-polluted soils from several farms in Baghdad provided the rhizospheres employed in this investigation. We purchased dry soy seeds from the neighborhood market. The source of crude oil was the Al-Dora Petroleum refinery.

Bacterial isolates with PGPR characteristics isolation:

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To generate the suspensions, 90 milliliters of sterile distilled water were thoroughly combined with 10 grams of rhizospheric soil (Cappuccino and Sherman, 2005). Utilizing serial dilution of 0.1 ml and physiological saline, the suspensions were serially diluted (10^{-1} to 10^{-6}) and plated on tryptic soy agar, nutritional agar, and nitrogen-free mannitol salt agar. They were then incubated for 48 hours at 28°C. Using repeated culture, isolated colonies were separated, purified, and kept at 4°C in nutrient broth with 30% glycerol (Kumar et al., 2008).

The solubilization of phosphate

The (Verma *et al*., 2001) method was used to screen all bacterial isolates for inorganic phosphat solubilization. A loopful of fresh bacterial culture was streaked onto Pikovaskaya's medium supplemented with inorganic phosphate, and the plates were then incubated at 28±2°C for four days. Mineral phosphate solubilization is indicated by a distinct halo surrounding the bacterial colonies.

Using the germinating seed bioassay (ACC)-deaminase to measure aminocyclopropane-1-carboxylate activity

The impact of bacterial isolates on root elongation was investigated using the approach outlined by Dey *et al,*. (2004) and Belimov et al., (2004). After sterilizing the S.bean seed surface for three minutes with 20% NaOCl (sodium hypochlorite), the seeds were rinsed three times with sterile deionized water. In 1% water agar plates, all of the seeds were left to germinate for 48 hours at 25°C. Three seeds per Petri dish were placed on moist filter paper after being individually dipped for one hour in 20 milliliters of bacterial cultures that had been cultured for 48 hours in nutrient broth (NB). For every treatment, two replications of the Petri dishes were incubated in the dark at 30±0.1°C. As a control, the seedlings were given uninoculated NB. Following five days of incubation, the seedlings' root length was measured in centimeters.

Development of the inoculum

Each isolate was inoculated into a loopful of freshly prepared agar plates in 15 milliliters of nutrition broth (NB), and the mixture was then cultured for 24 hours at 30 °C. McFarland's solution was used to standardize the cell density in each culture to 10^6–10^7 cfu/ml.

Applying McFarland's grading solution

A standard inoculum was created by standardizing a 50 ml suspension of each bacterial inoculum with a 0.5 M McFarland's solution that had already been prepared. This was accomplished by adding enough sterile distilled water as a suspending fluid to lower the microbial count until the turbidity of the suspension matched that of the McFarland's solution.

Treatment via experimentation

The modified root elongation assay of Belimov *et al*. (2005) was used to assess the isolated PGPR strains' plant root- and shoot-elongation-promoting (PRSEP) activities. S. bean seeds surface sterilized with 10% sodium hypochlorite were immersed in separate PGPR suspensions for one hour before being put in Petri dishes with filter

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papers impregnated with crude oil (0.0, 1.0, 5.0, and 10.0 ml). All treatments were carried out in duplicate and kept in a growth chamber in the dark at 30°C for five days; Isolate A infected soybean seeds plus a concentration of 0.0 ml crude oil (control).

The ratio of the root lengths of seedlings cultivated with and without the particular additional crude oil was used to calculate the tolerance index (TI) (Burd et al., 1998).

R or SLcr / R or SLc = TI.

R or SLcr represents the root or shoot length of seeds cultivated in the presence of crude oil in the absence of rhizobacteria. In contrast, R or SLcrb represents the root or shoot length of seeds cultivated in the presence of both rhizobacteria and crude oil.

Identifying the isolates of PGPR

Upon screening the isolates for the ability to elongate plant roots and shoots, three with a high crude oil tolerance index were identified using standard microbiological techniques and certain biochemical tests, which were conducted according to Bergey's Manual for Determinative Bacteriology (Cappuccino and Sherman, 2005). Using the Vitek system confirmed the identification (Shahab *et al*., 2009). The findings of screening for characteristics that promote plant development were used to choose potential isolates.

Analysis of statistics

The study's data were statistically analyzed at a 0.05 confidence level ($p < 0.05$) using analysis of variance (ANOVA).

IV. Results and discussion

Identification and PGPR characteristics of isolated rhizobacteria

Following the diagnosis of bacterial isolates based on certain biochemical assays (Table 1), a confirmatory test was conducted utilizing the VITECH system method, which identified them as Rhodospirillum *rubrum, Pseudomonas fluorescence and Bacillus subtilis*. Two characteristics that promote plant growth were displayed by each of these organisms: phosphate solubilization and ACC deaminase activity. A free-living plant growth-promoting rhizobacteria (PGPR), P. fluorescence can colonize around plant roots and incorporate free atmospheric molecules into plant-absorbable nutrient forms, along with improved plant growth and crop yield. The PGPR, being more potent chelators, starve the deleterious rhizobacteria of their iron nutrient, thus protecting the plants from the harmful effects of DRB, resulting in better growth and yield (Sakthivel et al., 2009). Iron chelation, antibiotic production, enhanced nutrient uptake, and seedling emergence promotion are promoted by plant phytohormone production.

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Morphological and some biochemical tests of the bacterial isolate			
B.subtilis	***P. fluorescence***		***R. rubrum***
G+ bacilli	**G-rod-shaped bacteria**		**G-spiral-shape.**
Colony morphology Circular, rough, opaque, fuzzy white slightly yellow with jagged edges	**circular, non-pigmented**		**purple-colored**
Catalase (+ve)	**(+ve)**		**(+ve)**
Citrate (+ve)	**Starch hydrolysis**	**(-ve)**	**Fermentation of glucose (+ve)**
Motility (+ve) (Flagellated)	**Gelatin liquefaction**	**(+ve)**	**Lipase (+ve)**
Gas production (-ve)	**Fluorescent pigment**	**(+ve)**	**Protease (+ve)**
Gelatin Hydrolysis (+ve)	**Oxidase test**	**(+ve)**	
Indole(-ve)			
MR (Methyl Red) (-ve)			
Nitrate Reduction (+ve)			

Outcomes of the pilot study growth

S. bean roots and shoots' growth reaction demonstrated that they were vulnerable to all concentrations of crude oil, and that when the concentrations increased, particularly at the 10 ml dosage, this response became more apparent, as seen in Figures 1 and 2.

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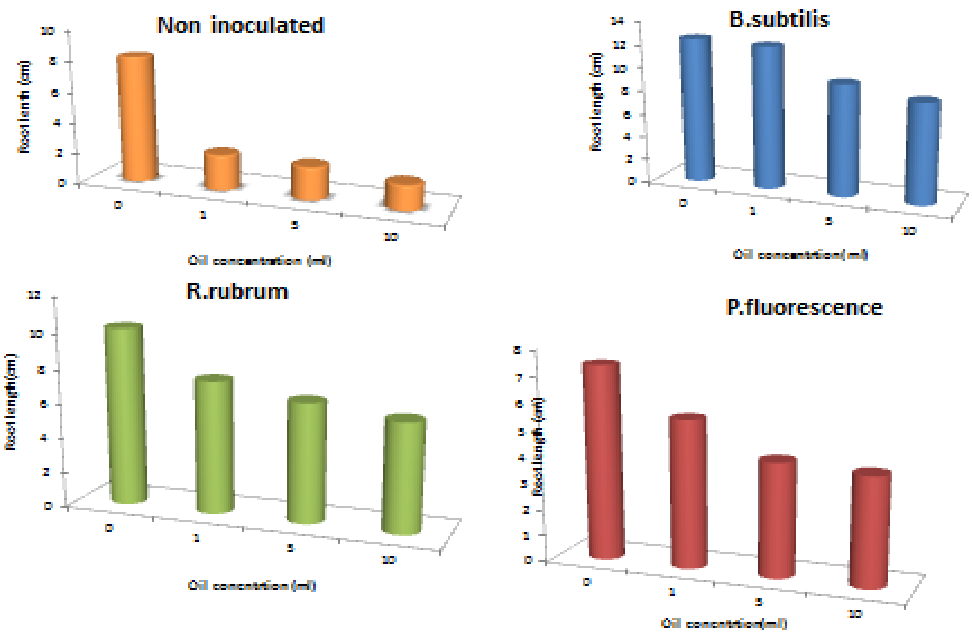

Figure (1) Root lengths of PGPR-inoculated and non-PGPR-inoculated seeds exposed to crude oil and crude oil

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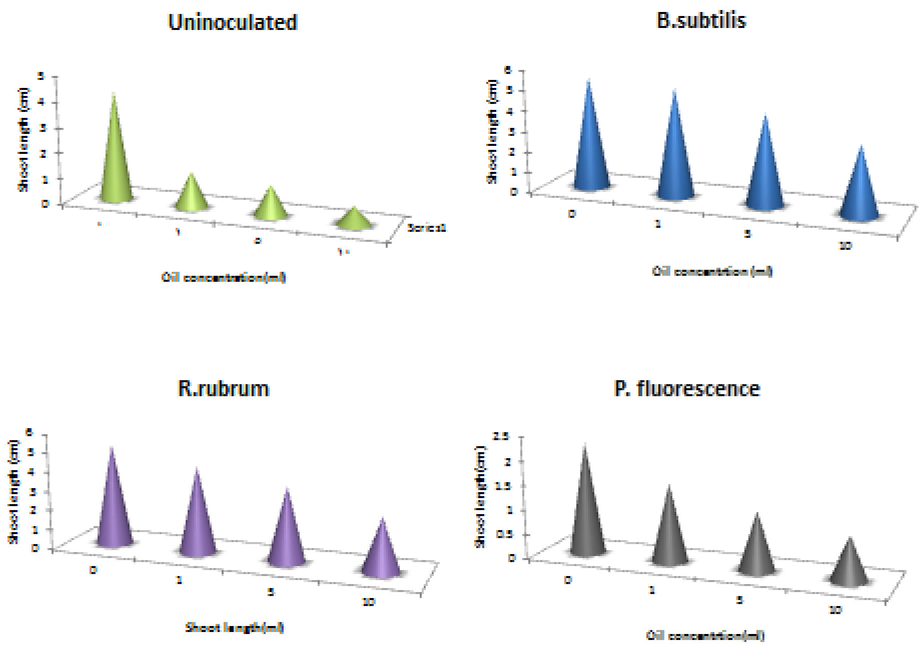

Figure (2) Root lengths of PGPR-inoculated and non-PGPR-inoculated seeds exposed to crude oil and crude oil

As shown in Table 2, *B. subtilis* demonstrated a variety of PGP activities, including ACC deaminase and phosphate solubilization. According to similar research, B. spp. is effective at solubilizing phosphate and has a variety of potentials that stimulate the growth of plants, including the formation of ACC deaminase and phosphate solubilization (Mehta *et al*., 2010 and Ghosh *et al*., 2012).

Table 2: The bacterial isolates' production of PGPR

Bacterial isolates	solubilizing phosphate	ACC deaminase activity
B. subtilis	+	+
R. rubrum	+	+

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P. Fluorescence	-	+

The greatest seedling development seen during the lab trial may have been produced by these characteristics/potentials acting alone or in concert.

With the exception of its capacity to produce plant growth components, *R. rubrum* also demonstrated a variety of activities that PRG. Therefore, there is a chance that the study will significantly improve seedling growth. This is consistent with the results of Plociniczak et al. (2013), who obtained bacterial isolates from a metal-contaminated soil that had the ability to boost plant development and increase *Sinapis alba's* uptake of certain heavy metals. It was also linked by (Jha *et al*., 2011) to the enhancement of plant growth.

Response to growth

The control group's bean seeds were susceptible to oil treatment at all three contamination levels. (1, 5, and 10 ml), as demonstrated by the results of the experimental treatment, which resulted in poor germination. This is comparable to the research done by Ogbuehi and Ezeibekwe (2009), who found that the cassava variety was susceptible to pollution from crude oil and that its growth performance decreased as the concentration of crude oil increased. Even at the highest crude oil concentrations (5 and 10 ml), inoculating bean seeds with PGPR strains increased the growth of the roots and shoots in comparison to the roots and shoots of the seeds cultivated without inoculation (Figures 1 and 2). The combination of the many plants growth-promoting features displayed by PGPR species (phosphate solubilization generation, ACC deaminase activity) is responsible for this growth response.

These species most likely reduced the growth-inhibiting effect of "stress ethylene," which was brought on by the crude oil contamination, while also making the nutrients accessible that the seeds needed. Similar results were reported by Franco-Hernandez et al. (2010), when tomato seeds were placed in a medium compromised with lead and arsenic and two PGPR strains (*Pseudomonas sp.*) identified from two plant species growing in a metal-affected soil.

PGPR inoculation enhanced seed growth, but it had no discernible effect on the development of seeds in the control (0 ml oil with PGPR) in comparison to the control (0 ml oil without PGPR). Since some of the species were isolated from soil contaminated by crude oil, there may not have been a considerable increase in seed growth, which could be explained by the PGPR strains' inability to use oil as a carbon source.

Level of tolerance

In the presence of varying quantities of the petroleum crude oil under investigation, the impact of inoculating with PGPR strains before seed germination was investigated (Tables 3 and 4). Comparing the impact of the various experimental treatments was made simpler by the presentation of the experimental data as TI. According to Franco-Hernandez *et al*. (2010), a TI of 1.0 means that the treatment was not inhibiting, whereas a TI of 0.1 means that the treated plants' growth was only 10% of the control plants' growth.

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According to Tables 3 and 4, respectively, the results indicate that the TI for the infected seeds' roots varied from 2.08 to 0.59, while the TI for the roots of the non-inoculated seeds ranged from 0.06 to 0, and shoots from 0.09 to 0.

Table (3) Tolerance index (TI) of *S. bean* roots and shoots to various crude oil concentrations.

Treatment	***B.subtilis***	***R.rubrum***	***P.fluorescence***
No inoculation			
Root		**Shoot**	
1ml(oil)	**0.06**	**0.09**	
5ml(oil)	**0.03**	**0.05**	
10ml(oil)	**0.0**	**0.0**	

Table (4) Tolerance index (TI) of *S.bean* roots and shoots to various crude oil concentrations after inoculation with PGPR strains

Treatment	***B.subtilis***		***R.rubrum***		***P.fluorescence***		***Control***
	R	***S***	***R***	***S***	***R***	***S***	
1ml(oil)	**2.08**	**0.6**	**1.09**	**0.5**	**2.03**	**0.2**	**0.09**
5ml(oil)	**0.52**	**0. 3**	**0.90**	**0.2**	**1.20**	**0.1**	**0.04**
10ml(oil)	**0.42**	**0.1**	**0.40**	**0.2**	**0.50**	**0.09**	**0.0**

In the presence of varying amounts of the petroleum crude oil tested, the impact of inoculating S. bean seeds with PGPR strains before seed germination was investigated. Because it disrupts the soil structure, the findings of these studies were reported as the Tolerance Index (TI), facilitating the comparison of the results of the various experimental treatments. In the contaminated soil, crude oil contamination poses a major environmental threat because it degrades the soil's structure, lowers the amount of nutrients available to plants, and raises the levels of harmful metals in the soil, all of which cause plants to grow less well or die completely. In order to highlight the

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effectiveness of certain PGPR species in increasing the uptake (tolerance) of oil pollutants by S. bean seeds and subsequently improving the growth of bean seedlings, this study was conducted. This suggested that, in comparison to the non-inoculated seeds, inoculation with PGPR strains increased the beans' immunity. This enhanced their ability to withstand crude oil. The species preserved the seeds' safe development in the presence of oil at higher concentrations, in contrast to the growth of seeds in control 1. The PGPR species' ACC deaminase enzyme activity may be the cause of this reaction. The petroleum crude oil most likely caused the synthesis of "stress ethylene" by activating the plant enzyme ACC synthase, which changes the substance S-adenosyl methionine into ACC. Ethylene's direct precursor in all higher plants. A fraction of the freshly produced ACC was extracted from plant roots or seeds Burd *et al.* ,1998), absorbed by the PGPR, and transformed into easily digestible ammonia and α-ketobutyrate by the enzyme ACC deaminase. The activity of this enzyme directly results in a decrease in the quantity of "stress ethylene" that the plant produces. Therefore, plant ethylene levels were kept from becoming growth inhibitory by ACC deaminase (Dey *et al*., 2004). This is comparable to the findings of (Contesto *et al*., 2008), who investigated how two rhizobacterial strains isolated from soil contaminated with heavy metals affected the growth and heavy metal tolerance of Lens esculenta.

The ability of PGPR strains (*P. aeruginosa*), obtained from an oil-contaminated site, to withstand aliphatic, PAH, and asphaltene fractions in bioremediation research was reported by Roy *et al.* (2013). In comparison to *P. fluorescence* and *R. rubrum*-inoculated seeds, the development of -inoculated seeds was substantial in the various crude oil concentrations, according to the tolerance index of seeds produced for the same experimental treatment.

The fact that *B. subtilis* had both PGPR characteristics, which probably worked together to enhance seed growth more successfully than the other two PGPR isolates, could account for this result. The results of the study also suggest that the seeds grew best when treated with a 1 ml concentration of crude oil. The results of a prior study by Ogbuehi and Ezeibekwe 2009), which demonstrated that cassava growth performance was enhanced at low pollution levels but considerably reduced at high pollution levels, are in agreement with this.

V. Conclusion

In conclusion, (PGPR) species that exhibit a range of plant growth-promoting traits increased the root and shoot lengths of S. bean seedlings in crude oil-polluted medium. Therefore, by giving plants the vital nutrients they need and detoxifying the hydrocarbons (crude oil), PGPR might be created as inoculants to stabilize soil contaminated by crude oil, hence encouraging the growth of plants in such polluted soil. As a result, this study recommends that R. rubrum and B. subtilis be utilized as crude oil-tolerant to treat plants in order to boost plant biomass and ensure plant stability and growth in soils contaminated by crude oil.

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