

Full Length Research Paper

Laboratory study on influence of plant growth promoting rhizobacteria (PGPR) on growth response and tolerance of *Zea mays* to petroleum hydrocarbon

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The influence of rhizobacteria on the growth and tolerance of *Zea mays* (maize) in a petroleum hydrocarbon (crude oil) impacted medium was investigated. This study evaluated the effect of inoculating maize seeds with plant growth promoting rhizobacterial strains in a crude oil impacted medium. The rhizobacterial strains used in this study were tested for plant growth promoting traits [indole-3-acetic acid production, siderophore production, phosphate solubilisation and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity] following standard methods and plant root and shoot elongation activity of these strains were assayed using modified root elongation assay. The isolates were identified as *Bacillus circulans*, *Enterobacter intermedius* and *Staphylococcus carnosus*, by analytical profile index (API). The results showed that seeds inoculated with plant growth promoting rhizobacteria (PGPR) exhibited significant vegetative growth at various petroleum crude oil concentrations (1, 2 and 4 ml) compared with uninoculated seeds. The root and shoot length of inoculated seeds were 10.2 and 4.3 cm (*B. circulans*), 7.2 and 1.7 cm (*E. intermedius*), 5.2 and 3.8 cm (*S. carnosus*) and 0.9 and 1.5 cm (uninoculated), all at 1 ml concentration of petroleum crude oil. Tolerance Index (TI) for PGPR inoculated maize seeds in the crude oil impacted medium was highest with *B. circulans* (2.08 to 1.92), *S. carnosus* (0.69 to 0.59) and *E. intermedius* (0.79 to 0.71). Thus, this study suggests that *B. circulans* could be employed as a crude oil tolerant rhizobacterium (PGPR) for mitigating the toxic effects of crude oil on plants and subsequently enhancing the plant growth in a crude oil-impacted soil.

Key words: Plant growth-promoting rhizobacteria, tolerance index, crude-oil, impacted medium, growth parameters.

INTRODUCTION

Oil spillage is a global issue that has been occurring since the discovery of crude oil. Incessant pollution of the soil environment with petroleum and refinery products in the Niger Delta has become a huge challenge owing to modern civilization and urbanization. Crude oil spills hampers soil physical, chemical and biological structure thus reducing growth and resistance of plants

to biotic and abiotic factors making them more prone to pathogen infestation or/and even complete mortality of plants (Chronopoulos *et al.*, 1997; Pezeshki *et al.*, 2000; Schutzenhubel and Polie, 2002; Roy *et al.*, 2013). Further limitations to the growth of plants and microbes may arise from the severity of a contamination and the heterogeneous nature of soil. Plant on such soil becomes

suffocated due to the exclusion of air by oil. The exhaustion of oxygen in the soil decreases the microbial activity and subsequently interferes with the plant soil water relationship (Esenowo et al., 2006), this is a major threat to food production (crop yield) in the region and this could lead to total destruction of the ecosystems if not effectively checked. Certain rhizospheric bacteria collectively known as plant growth promoting rhizobacterial (PGPR) have been reported to enhance plant growth and yield in different crops (Radwan et al., 2007). PGPR is a group of free-living soil bacteria that colonize the root of plants following inoculation onto seed and enhance plant growth (Kloepper *et al.*, 1991). Although, the exact mechanism by which PGPR enhance plant growth and detoxify contaminants is not fully understood, they are thought to promote growth through at least one mechanism, directly, indirectly or and synergistically viz: suppression of plant diseases {(bioprotectants) e.g. production of antibiotics, etc}, improved nutrient acquisition {(biofertilizers) e.g. asymbiotic nitrogen fixation}, phytohormone production {(biostimulants) e.g. indole acetic acid, cytokinins, etc} or lowering of "stress ethylene" (ethylene that is synthesized as a response to various environmental stresses including extremes of temperature, high light, flooding; drought; the presence of toxic metals and organic pollutants, radiation, wounding, insect predation, high salt) concentration by the production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which prevents plant ethylene levels from becoming growth inhibitory (Arshad and Frankenberger, 2005; Boddey and Dobereiner, 2000; Contesto et al., 2008).

PGPR have also been reported by researchers to be active in hydrocarbon consumption and exhibit differential potentials to degrade and detoxify contaminants (for instance, heavy metals), by either immobilizing, mobilizing or rendering them inactive, thus improving plant's tolerance to the uptake of heavy metals ions (Nies, 1999; Belimov et al., 2004; Radwan et al., 2007; Glick, 2010; Roy et al., 2013). Research frontiers have reported their use in bioremediation of crude oil and PAH (polycyclic aromatic hydrocarbon) contaminated sites. (Zhuang et al., 2007; Roy et al., 2013). They have also been proven to reduce petroleum hydrocarbon and salt levels substantially in petroleum and salt contaminated soils both in laboratory and field studies in PGPR-enhanced phytoremediation system (PEPS) (Gerhardt et al., 2009). Bacteria belonging to the genera *Azospirillum*, *Pseudomonas*, *Xanthomonas* and *Rhizobium* as well as *Alcaligenes*, *Enterobacter*, *Acetobacter*, *Klebsiella* and *Bradyrhizobium* etc, have been implicated as plant

growth promoting rhizobacteria (PGPR) (Wani et al., 2007; Poonguzhali et al., 2008; Kumar et al., 2008; Ahemad and Khan, 2010).

These findings aroused our interest in studying the effect of PGPR inoculation on the growth response and tolerance of *Zea mays* seeds exposed to varying crude oil concentrations under laboratory conditions.

MATERIALS AND METHODS

Sample collection

The rhizospheric soils used in this study were collected from rhizosphere of maize, pepper and crude oil polluted soil from Ogoni land in Rivers State, Nigeria. Dry maize seeds (Bende maize seeds) were obtained from a local market in Port Harcourt. Crude oil was obtained from Shell Petroleum Development Corporation, Port Harcourt (SPDC) following recommendation from Department of Petroleum Resources (DPR), Port Harcourt.

Isolation of bacterial isolates with PGPR traits

Ten grams of rhizospheric soil was thoroughly mixed with 90 ml of sterile distilled water to make suspensions (Cappuccino and Sherman, 1992). Suspensions were diluted serially (10^{-1} to 10^{-6}) using physiological saline and 0.1 ml of individual dilutions plated on nutrient agar, nitrogen-free mannitol salt agar, tryptic soy agar, and incubated at 28°C for 48 h. Discrete colonies were isolated, purified with repeated culturing and maintained in nutrient broth with 30% glycerol at 4°C (Kumar et al., 2008).

Screening of bacterial isolates for PGPR traits Indole-3-acetic acid (IAA)

The production of IAA by the isolates was determined according to the method of Bano and Musarrat (2003). Discs (8 mm diameter) from colonies of the isolates, grown on yeast malt extract (YM) agar and incubated at 30°C for 5 days, were transferred to 5 ml YM broth containing 2 mg/ml L-tryptophan. These cultures were incubated at 30°C with shaking at 125 rpm for 7 days and then harvested by centrifugation at 11,000 × g for 15 min. One millilitre of the supernatant was mixed with 2 ml of Salkowski reagent; the appearance of a pink colour indicated IAA production. Optical density (OD) was read at 530 nm.

Siderophore production

Bacterial culture (2 days old) was streaked on Chrome azural-S (CAS) agar medium. Orange halos surrounding the bacteria colony indicates production of siderophore, as described by Alexander and Zuberer (1991).

Phosphate solubilization

All bacterial isolates were screened for inorganic phosphate

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solubilisation according to the method developed by Verma et al. (2001). A loopful of fresh bacterial culture was streaked onto Pikovaskaya's medium amended with inorganic phosphate and plates were incubated at 28±2°C for 4 days. A clear halo around the bacterial colonies indicates solubilisation of mineral phosphate.

1-Aminocyclopropane-1-carboxylate (ACC)-deaminase activity by germinating seed bioassay

The effect of bacterial isolates on root elongation was studied by germinating seed bioassay as per methodology described by Dey et al. (2004) and Belimov et al. (2004). Seed surface of *Z. mays* were sterilized with 20% NaOCl (sodium hypochlorite) for 3 min and washed three times with sterile de-ionized water. All the seeds were allowed to germinate at 25°C in 1% water agar plates for 48 h. Germinated seeds were individually dipped for 1 h in 20 ml bacterial cultures grown for 48 h in nutrient broth (NB) and transferred to wet filter paper in Petri dishes taking three seeds per dish. The Petri dishes were incubated in dark at 30±0.1°C with two replications for each treatment. The seedlings treated with uninoculated NB served as control. The root length of seedlings was measured in cm after 5 days of incubation.

Inoculum development

A loopful of freshly prepared agar plates of each isolate was inoculated into 15 ml nutrient broth (NB) and incubated at 30°C for 24 h. The cell density in each culture was standardized to 10⁶ to 10⁷ cfu/ml with McFarland's solution.

Standardization with McFarland's solution

In order to obtain a standard inoculum, 50 ml suspension of each bacterial inoculum was standardized with already prepared 0.5 M McFarland's solution by adding sufficient suspending fluid (sterile distilled water) to reduce microbial count until its turbidity matches with that of the McFarland's solution.

Experimental treatment

The plant root and shoot elongation promoting (PRSEP) activity of the isolated PGPR strains were determined using the modified root elongation assay of Belimov et al. (2005). Ten percent (10%) sodium hypochlorite surface sterilized maize seeds were submerged in individual PGPR suspensions for 1 h and placed in Petri dishes with crude oil impregnated filter papers (0.0, 1.0, 2.0 and 4.0 ml). All the treatment were performed in duplicates and maintained at 30°C in a growth chamber in dark for 5 days following these experimental treatments; Isolate A inoculated maize seeds + 0.0 ml crude oil concentration (control 1); Isolate B inoculated maize seeds + 0.0 ml crude oil concentration (control 1) Isolate C inoculated maize seeds + 0.0 ml crude oil concentration (control 1). Maize seeds alone [zero crude oil and zero PGPR] (control 2); Maize seeds + crude oil (control 3); Isolate A inoculated maize seeds + 1 ml crude oil concentration Isolate B inoculated maize seeds + 1 ml crude oil concentration Isolate C inoculated maize seeds + 1 ml crude oil concentration. Isolate A inoculated maize seeds + 2 ml crude oil concentration Isolate B inoculated maize seeds + 2 ml crude oil concentration Isolate C inoculated maize seeds + 2 ml crude oil concentration. Isolate A inoculated maize seeds + 4 ml crude oil concentration Isolate B inoculated maize seeds + 4 ml crude oil concentration Isolate C inoculated maize seeds + 4 ml crude oil concentration. Root and shoot lengths of *Z. mays* were measured with a meter rule after incubation and

tolerance index (TI) was determined as the ratio of the root lengths of seedlings grown in the presence and absence of the specific added crude oil (Burd et al., 1998).

$$TI = R \text{ or } SL_{cr} / R \text{ or } SL_c$$

Where, R or SL_{cr} is the root or shoot length of seeds grown in the presence of crude oil without rhizobacteria and R or SL_{crb} is the root or shoot length of seeds grown in presence of crude oil and rhizobacteria.

Identification of PGPR isolates

Three isolates having high tolerance index to crude oil during screening for plant root and shoot elongation activity of the isolates were characterized following standard microbiological procedures to identify the isolates (Cappuccino and Sherman, 2005). The isolates were further identified to species level based on Analytical Profile Index (API), according to the method of Shahab et al. (2009), at the Department of Biotechnology, Federal Institute of Industrial Research, Oshodi, Lagos State, Nigeria. Potential isolates were selected on the basis of results from screening for plant growth promoting properties.

Statistical analysis

Data obtained from this study were subjected to statistical analysis using analysis of variance ANOVA at 0.05 confidence level (p < 0.05).

RESULTS AND DISCUSSION

Identity and PGPR traits of rhizobacterial isolates

The rhizobacterial isolates were identified following analytical profile index (API) method as *Bacillus circulans*, *Staphylococcus carnosus* and *Enterobacter intermedius*. Each of these organisms exhibited two or four of these plant growth promoting traits (phosphate solubilisation, Indole-3-acetic (IAA) production, siderophore production and ACC deaminase activity).

Results from experimental treatment growth response

The growth response of *Z. mays* roots and shoots to petroleum crude oil showed they were susceptible to all the crude oil concentrations, and this response was more prominent as the crude oil concentrations increased; particularly at 4 ml concentration as shown in Figures 1 and 2.

Tolerance index

The effect of inoculating the *Z. mays* seeds with PGPR strains before the seeds germination, in presence of the different concentrations of the petroleum crude oil assayed was examined (Figure 3). The results of these experiments were presented as Tolerance index (TI), making easier to compare the effects of the different

Table 1. PGPR traits of the bacterial isolates

Bacterial isolates	IAA production	Phosphate solubilisation	Siderophore production	ACC deaminase activity
<i>B. circulans</i>	+	+	+	+
<i>S. carnosus</i>	+	-	-	+
<i>E. intermedius</i>	+	+	-	+

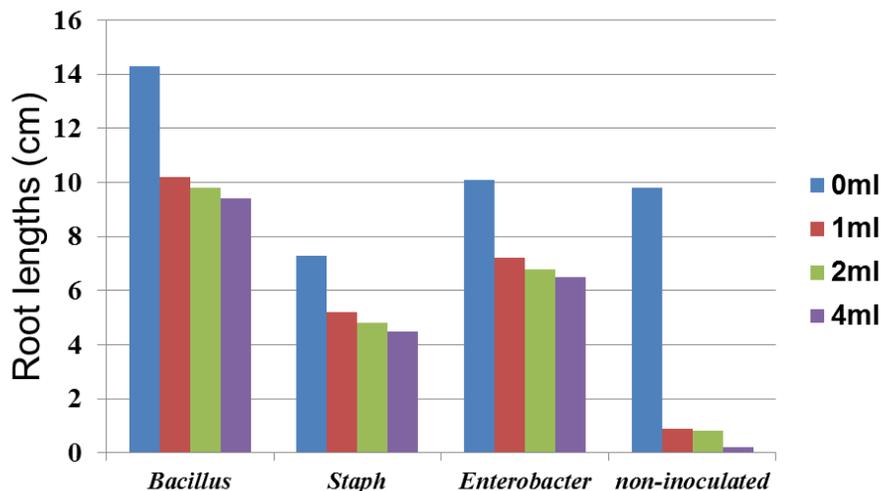


Figure 1. Root lengths of PGPR inoculated and non-PGPR inoculated seeds exposed to crude oil and crude oil free matrix.

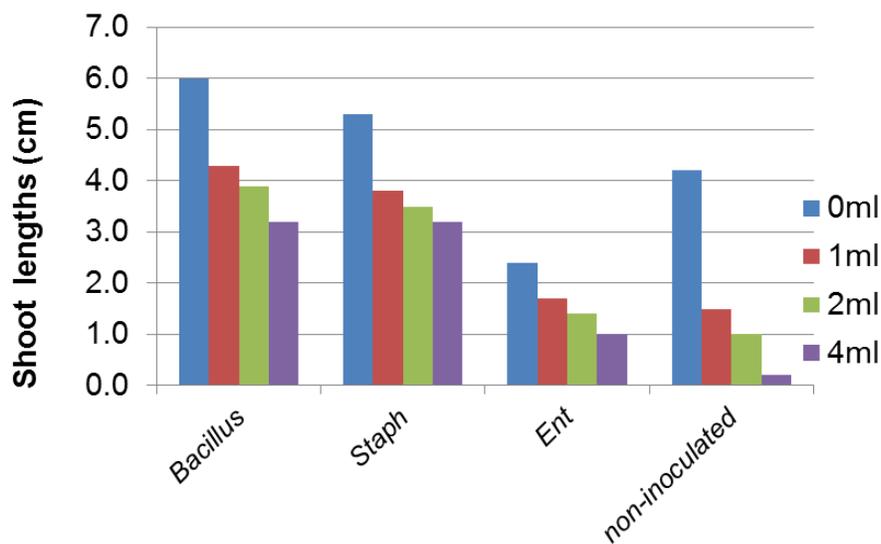


Figure 2. Shoot lengths of PGPR inoculated and non-PGPR inoculated seeds exposed to crude oil and crude oil free matrix.

experimental treatments.

DISCUSSION

Crude oil contamination is a serious environmental

challenge in the Niger-delta and this is because it destroys the soil structure, reduces the available plant nutrients and increases the toxic levels of metals in the soil which consequently leads to impaired plant growth or complete mortality of plants. This study was performed to underscore the performance of some PGPR species

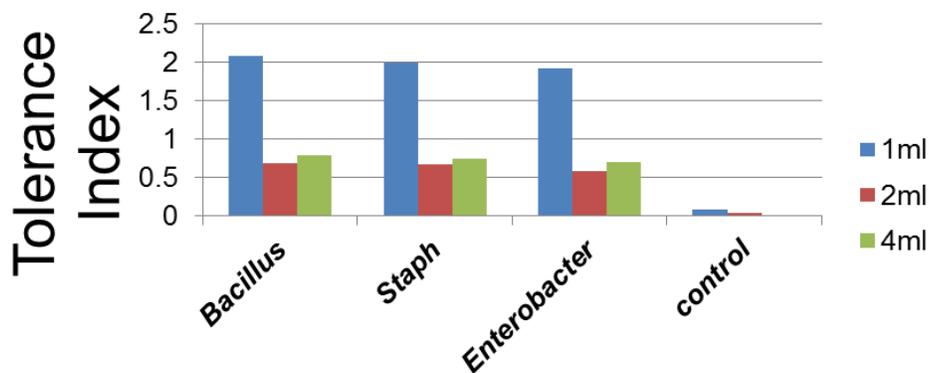


Figure 3. Tolerance index (TI) of *Zea mays* roots to various crude oil concentrations.

in boosting the uptake (tolerance) of petroleum crude oil pollutant by maize seeds and subsequent enhancement of maize seedling growth.

Identity of PGPR isolates

PGPR isolates were identified as *B. circulans*, *S. carnosus* and *E. intermedius* following analytical profile index (API) algorithm. *B. circulans* exhibited multifarious plant growth promoting activities (ACC deaminase, siderophore, phosphate solubilisation and indole acetic acid production) as presented in Table 1. Similar studies have reported *B. circulans* as efficient in phosphate solubilisation and have also been shown to possess multifarious plant growth promoting potentials such as indole-3-acetic acid and siderophore production (Mehta et al., 2010 and Ghosh et al., 2012). It could be inferred that these traits/potentials worked directly or in synergy to produce maximum seedling growth observed during the laboratory study. *E. intermedius* also exhibited multifarious plant growth promoting activities in exception of potential for siderophore production. Thus, it is potential for significant seedling growth enhancement during the study. This is in accordance with the findings of Plociniczak et al. (2013) who isolated *E. intermedius* MH8b with plant growth promoting potentials from a metal-contaminated soil to promote plant growth and enhance the uptake of some heavy metals by *Sinapis alba* L. Jha et al. (2011) also implicated *E. intermedius* as a role player in plant growth promotion.

Growth response

Results from experimental treatment showed that in the control, maize seeds were susceptible to crude oil treatment at the three levels of contamination (1, 2 and 4 ml) evident by impaired germination (Plate 1 A). This is similar to the study carried out by (Ogbuehi and Ezeibekwe, 2009), who observed that cassava variety NR8082 was susceptible to crude oil pollution and

their growth performance reduced with increased concentration of crude oil. Inoculation of maize seeds with PGPR strains enhanced the growth of the roots and shoots even at the highest concentration of the crude oil (2 and 4 ml) compared to the roots and shoots of the seeds grown without inoculation ((Figure 1 and 2, Plate A, B and C). This growth response can be attributed to synergy of the multiple plant growth promoting traits exhibited by PGPR species (indole acetic acid production, and ACC deaminase activity). These species probably made bioavailable the necessary nutrients required by seeds while reducing the growth inhibitory effect of “stress ethylene” induced by the crude oil contamination. Franco-Hernandez et al. (2010), reported similar findings with two PGPR strains (*Pseudomonas* sp.) isolated from two plant species growing in a metal contaminated soil], when applied to tomato seeds in a lead and arsenic contaminated medium. The root lengths of the PGPR treated maize seeds ranged from 10.2 to 4.5 cm compared to the control that ranged from 0.9 to 0.2 cm. The shoot lengths of the PGPR treated maize seeds ranged from 4.3 to 1.0 cm compared to the control that ranged from 1.5 to 0.2 cm.

PGPR inoculation enhanced growth of the seeds but did not show significant increase in seedling growth in control (0 ml crude oil with PGPR) when compared to control (0 ml crude oil without PGPR) as shown in Plate D and E alone. This could be due to lack of carbon source (crude oil) for the PGPR strains to utilize, as some of the species were isolated from crude oil polluted soil hence no significant increase in growth of seeds.

Tolerance index

The effect of inoculating the *Z. mays* seeds with PGPR strains before the seeds germination, in presence of the different concentrations of the petroleum crude oil assayed was examined (Figures 3 and 4). The results of

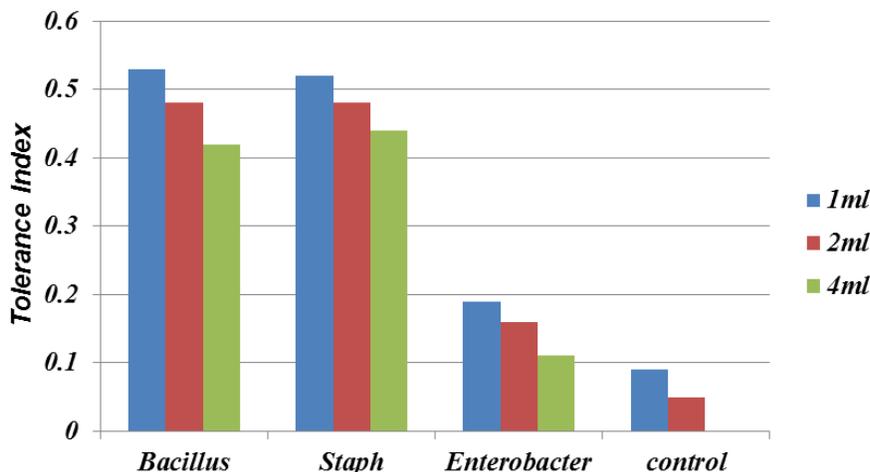


Figure 4. Tolerance index (TI) of Zea mays shoots to various crude oil concentrations.

Table 2. Tolerance index (TI) of Zea mays to various crude oil concentrations.

Treatment	Tolerance index
Non-inoculated seeds roots	
1ml crude oil	0.07
2ml crude oil	0.03
Flooding with crude oil	0
Shoots	
1ml crude oil	0.09
2ml crude oil	0.05
Flooding with crude oil	0

these experiments were presented as TI, making it easier to compare the effects of the different experimental treatments. A TI of 1.0 indicates that the treatment was not inhibitory, while a TI of 0.1 indicates that the growth of treated plants was only 10% of the growth of the control plants (Burd et al., 1998). The results show that TI for the roots of the non-inoculated seeds ranged from 0.07 to 0 and shoots ranged from 0.09 to 0, while TI for the roots of the inoculated seeds ranged from 2.08 to 0.59 as shown in Table 2 and Figure 3, respectively. This inferred that inoculation with PGPR strains boosted the immunity of the maize seeds and subsequently enhanced improved tolerance to the crude oil, compared to the non-inoculated seeds. The species kept the safe development of the seeds in the presence of petroleum crude oil at higher concentrations, compared to the growth of seeds in control 1. This response could be attributed to ACC deaminase enzyme activity in PGPR species. The petroleum crude oil probably induced the production of "stress ethylene" by synthesizing plant enzyme ACC synthase that converts the compound S-adenosyl methionine to ACC, the

immediate precursor of ethylene in all higher plants. A portion of the newly synthesized ACC was excluded from seeds or plant roots (Dey et al., 2004), taken up by the PGPR, and converted by the enzyme ACC deaminase to ammonia and α -ketobutyrate, compounds that are readily assimilated. As a direct consequence of this enzyme's activity, the amount of "stress ethylene" produced by the plant is reduced. Hence, ACC deaminase prevented plant ethylene levels from becoming growth inhibitory (Contesto et al., 2008). This is similar to the report of Franco-Hernandez et al. (2010), who studied the effect of two rhizobacterial strains (isolated from heavy metal contaminated soil) on *Lens esculenta* growth and tolerance to heavy metals.

Report by Roy et al. (2013) informed the ability of PGPR strains (*Pseudomonas aeruginosa* strains AS 03 and NA 108) sourced from a crude oil contaminated site, to tolerate aliphatic, aromatic, asphaltene fractions and PAH in a bioremediation study. Tolerance index of maize seeds obtained for experimental treatment (PGPR+ crude oil) showed that the development of *B. circulans*-inoculated maize seeds was significant in the

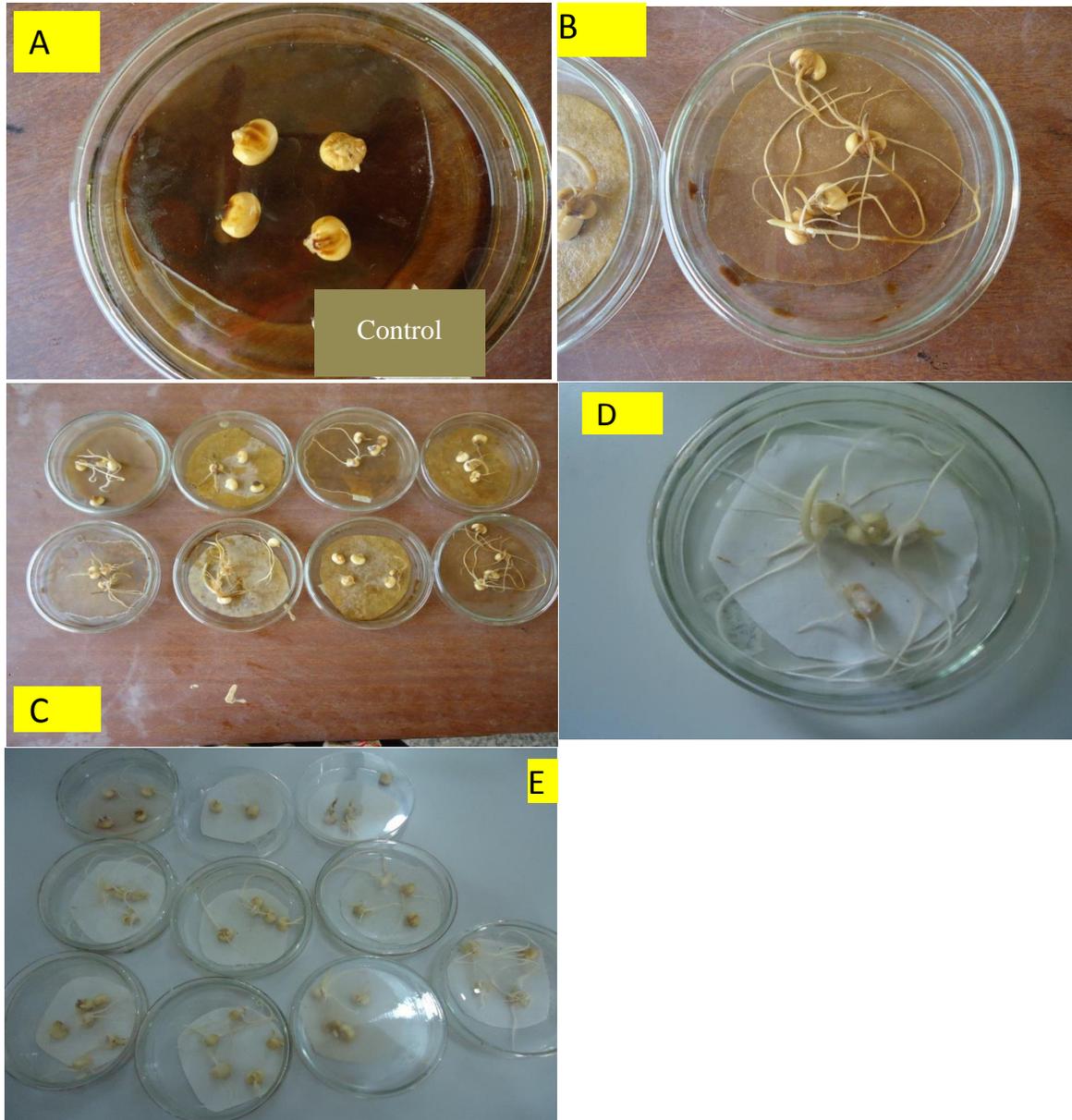


Plate 1. **A:** *Zea mays* seeds exposed to crude oil (4 ml) alone during the laboratory study. **B and C:** *Zea mays* seeds inoculated with PGPR strains and exposed to crude oil (4 ml) during the laboratory study. **D and E:** *Zea mays* seeds inoculated with PGPR alone.

different crude oil concentrations compared to *E. intermedius* and *S. carnosus* – inoculated seeds. This response could be attributed to the fact that *B. circulans* had all four of the PGPR abilities, which probably worked in synergy to enhance growth of the seeds better than the other two PGPR isolates. Results from this study also suggest that growth of the seeds were best at 1 ml concentration of crude oil treatment. This is in line with the results of Ogbuehi and Ezeibekwe (2009) conclusion in a related study, that cassava growth performance was enhanced at low level pollution but

severely reduced by high level of pollution.

Conclusion

In conclusion, plant growth promoting rhizobacterial (PGPR) species exhibiting multiple plant growth promoting traits, enhanced seedling growth (root and shoot lengths) of *Z. mays* (maize) in crude oil polluted medium. Thus, plant growth promoting rhizobacteria could be developed as inoculants to stabilize crude oil polluted

soil, by providing the essential nutrients required by plants and detoxifying the hydrocarbons (crude oil), thus enhancing plant growth in such contaminated soil. Therefore, this study suggests that *B. circulans* and *E. intermedius* can be used as crude oil-tolerant plant growth promoting rhizobacteria (PGPR) and can be recommended to treat plants for the increase of plant biomass for the stability and growth of plants in a crude oil polluted soils.

Conflict of interests

The author(s) did not declare any conflict of interest.

Abbreviations: ACC, 1-Aminocyclopropane-1- carboxylate; API, analytical profile index; PGPR, plant growth promoting rhizobacteria; TI, tolerance index; PAH, polycyclic aromatic hydrocarbon; NB, nutrient broth.

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